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Calorie Restriction Effect on Circadian Clock Gene Expression

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CALORIE RESTRICTION EFFECT ON CIRCADIAN CLOCK GENE EXPRESSION

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DEDICATION

I dedicate my work to my Grandmother, Mother and my Husband. My Grandmother and Mother for all their love and efforts they put in to get me till here. My Husband, Chintan, for his immense support, understanding, patience and care. I am also grateful to him for bearing with all my day-to-day frustrations and stress throughout my PhD. Love you guys.

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CALORIE RESTRICTION EFFECT ON CIRCADIAN CLOCK GENE EXPRESSION

SONAL A. PATEL

ABSTRACT

Calorie Restriction (CR) is a powerful paradigm known to delay aging and thus increase longevity in several organisms, from yeast to non-human primates. Many molecular pathways have been proposed to mediate the beneficial effects of CR, however, the mechanism is still unknown. Circadian clock which is an internal time keeping system is regulated by feeding. Thus our aim was to study the effect of CR on the circadian clock. Here we show that CR significantly affects the expression of circadian clock genes in mice at the mRNA and protein levels, suggesting that CR reprograms the clocks at the transcriptional and post-transcriptional level. CR also affected the circadian output through up- or down-regulation of the expression of several clock-controlled transcriptional factors and the longevity candidate genes. CR-dependent effects on some clock gene expression were impaired in the liver of mice deficient for BMAL1, suggesting importance of this transcriptional factor for the transcriptional reprogramming of the clock, however, BMAL1-independent mechanisms exist too. We have shown that Bmal1 deficient mice develop premature aging phenotype and have a shortened lifespan. We decided to apply 30%CR to these mice and found that CR did not increase the lifespan of these Bmal1 mutants, further suggesting that BMAL1 is necessary for full benefits of CR. We also analyzed the plasma levels of IGF-1 and insulin, which were found to be impaired in Bmal1 deficient mice on 30%CR. We propose that CR recruits biological clocks as a natural

mechanism of metabolic optimization and synchronization of the several downstream pathways under limited nutrient conditions.

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LIST OF ABBREVIATIONS

ADF	Alternate Day Fasting
AMPK	Adenosine Monophosphate Activated Protein Kinase
AL	Ad-libitum
Alas2	5'-Aminolevulinate Synthase 2
ARNTL	Aryl Hydrocarbon Receptor Nuclear Translocator-like Protein
ASPD	Advanced Sleep Phase Disorder
ATP	Adenosine Triphosphate
BMAL1	Brain and Muscle ARNTL 1
bHLH	Basic Helix Loop Helix
BDNF	Brain-derived neurotrophic factor
CCG	Clock Controlled Gene
cDNA	Complementary DNA
CKI	Casein Kinase 1
CLOCK	Circadian Locomotor Output Cycles Kaput
CLD	Cytoplasmic Localization Domain
cm	centimeter
CR	Calorie Restriction

cAMP	cyclic adenosine monophosphate
CREB	cAMP response element binding protein
CRSWD	Circadian Rhythm Sleep-Wake Disorder
CRY	Cryptochrome
Cyp4a12b	cytochrome P450, family 4, subfamily a, polypeptide 12B
DBP	D site of albumin promotor Binding Protein
DEC	Differentially Expressed in Chondrocytes
DNA	Deoxyribonucleic Acid
dNTP	Deoxy Nucleotide Triphosphate
DSPD	Delayed Sleep Phase Disorder
DTT	Dithiothreitol
E-Box	Enhancer Box
ELISA	Enzyme-linked immunosorbent assay
F	Fasting
FAA	Food Anticipatory Activity
FEO	Food Entrainable Oscillator
FEPO	Food Entrainable Peripheral Oscillator
FASPS	Familial Advanced Sleep Phase Disorder

FBXL3	F-box/LRR-Repeat Protein 3
FOXO	Forkhead box O3
FRD	Free running disorder
Fmo3	Flavin monooxygenase 3
GSK3β	Glycogen Synthase Kinase 3β
GPCR	G-protein coupled receptor
HAT	Histone acetyl transferase
HLF	Hepatic Leukamia Factor
HFD	High Fat Diet
IACUC	Institutional Animal Care and Use Committee
IGF-1	Insulin-like Growth Factor 1
IGFBP	Insulin-like Growth Factor Binding Protein
IGF-1R	Insulin-like Growth Factor-1 Receptor
IGFALS	Insulin-Like Growth Factor Binding Protein, Acid Labile Subunit
IF	Intermittent Fasting
ISWD	Irregular Sleep Wake Disorder
mg	milligram
mL	Milliliter

mRNA	Messenger RNA
mTOR	mammalian Target of Rapamycin
MAPK	Mitogen-activated protein kinases
Mup4	major urinary protein 4
NAMO	Nutrient Anticipation Metabolic Oscillator
NAD	Nicotinamide adenine dinucleotide
NAMPT	Nicotinamide phosphoribosyl transferase
NES	Nuclear Export Signal
NFIL3	Nuclear factor, interleukin 3 regulated
NIA	National Institute of Aging
NLS	Nuclear Localization Signal
NPAS2	Neuronal PAS Domain- Containing Protein 2
NRF2	Nuclear factor (erythroid-derived 2)-like 2
PAS	Period Arnt Sim
PARP16	poly ADP ribose polymerase 16
PCR	Polymerase Chain Reaction
PER	Period
PF	Periodic Fasting

PI3K	Phosphoinositide 3-kinase
Pparα	Peroxisome proliferator-activated receptor alpha
PP	Phosphatases
PVDF	Polyvinylidene Difluoride
Rev-Erb	reverse orientation c-erbA
RHT	Retino Hypothalamic Tract
RNA	Ribonucleic Acid
RORE	Retinoic Acid Related Orphan Receptor Element
ROR	Retinoic Acid Related Orphan Receptor
ROS	Reactive Oxygen Species
rRNA	Ribosomal RNA
RTqPCR	Reverse Transcriptase Quantitative PCR
SCN	Suprachiasmatic Nucleus
SD	Standard Deviation
SIRT1	Sirtuin
TR	Time-restricted Feeding
βTrcp1	F-box/WD repeat-containing protein 1A
UV	Ultra Violet

Ub	Ubiquitin
Ube3a	Ubiquitin Protein Ligase E3A
WT	Wild-type
μg	Microgram
μL	Microliter

CHAPTER 1

INTRODUCTION

The circadian clock is an internal time-keeping system generating rhythms with 24h periodicity in physiology, behavior and metabolism(Green et al. 2008). In mammals, coordination of the circadian system across the body involves the regulation of the signals emanating from the central clock in the SCN which is entrained by the light (Reppert & Weaver 2002). Additionally, timed-feeding is another important zeitgeber that entrains the peripheral circadian clocks located in different organs of our body. Together, these clocks (central and peripheral) regulate the physiological and behavioral outputs. Circadian clock disruption is associated with the development of several pathophysiological conditions; moreover, it is also implicated in the control of aging processes in different organisms(Froy & Miskin 2010). Disruption of the circadian clock either by the environmental disturbances or through mutations in circadian clock genes results in reduced longevity (Fu et al. 2002; Kondratov, Kondratova, Gorbacheva, et al. 2006; Dubrovsky et al. 2010).

Calorie restriction (CR), a reduced intake of calorie without malnutrition, is a powerful intervention known for decades to delay aging and increase lifespan across different species including, mammals (McDonald & Ramsey 2010; Piper & Bartke 2008). In the mammals, CR is received in a time-restricted manner. Time-restricted feeding is known to affect the peripheral circadian clocks. It was proposed that the circadian clock may be one of the component of the mechanism driving the beneficial effects of the CR in the mammals. Recent data demonstrate the interaction between the circadian clock and different aging-controlling pathways implicated in CR mechanism such as, the sirtuin (Chang & Guarente 2013; Nakahata et al. 2008), insulin/IGF and mTOR signaling pathways (Rohini V Khapre et al. 2014). However, the effect of CR on the molecular circadian clockwork has not been studied. We, therefore, decided to study the effect of CR on the clock genes expression. Addressing this question will further aid in elucidating the mechanisms of the CR beneficial effects and the chrono nutrition strategy may contribute towards better healthspan and healthy aging in humans.

1.1 Clockwork Physiology

The Earth rotation around its own axis generates daily light and dark cycles that affect the lifestyle of all the organisms from cyanobacteria to humans. The adaptation to the recurring changes in the environment resulted in diurnal activity cycles such as foraging for food, predator activity and temperature, humidity and lighting conditions compatible with an organism's lifestyle. Anticipating these changes rather than just reacting to them confers a selective advantage to an organism for optimizing its physiology and behavior with regard to the external and internal changes. Indeed, organisms from bacteria to mammals have

evolves an internal timing system, known as circadian clocks (Circa = around, Diem = day), that coordinates the behavior, physiology and metabolic processes in an anticipatory fashion. For example, in plants the photosynthetic genes expression increases a few hours before sunrise to optimum photosynthesis(Dodd et al. 2005), while rodents such as mice up regulate the expression of detoxification enzymes before the food consumption activity in order to detoxify the food associated toxins absorbed (Gachon & Firsov 2011; DeBruyne et al. 2014). In humans and most mammals there are roughly 24 hour rhythms in body temperature, blood pressure, hormonal secretions, metabolism, as well as physiological parameters(Aschoff 1983; Green et al. 2008; Eckel-Mahan & Storm 2009). While synchronization is advantageous, asynchrony with the external environment may be detrimental. Indeed, a dysfunctional circadian clock is associated with a number of human disorders and diseases. Oscillatory circadian clock genes drive rhythms in cellular metabolism, endocrine control, and epigenetic regulation (Bellet & Sassone-Corsi 2010). Indeed, microarray studies indicate that circadian genes oscillate differentially in different organs, with roughly 10% of transcripts exhibiting oscillations in gene expression (Storch et al. 2002; Duffield 2003; Miller et al. 2007).

1.2 Circadian Clock

Circadian clock is an entrainable, and self-sustained oscillators that generates internal timing of approximately 24 h in the absence of the external stimuli. Clocks located throughout the body in peripheral organs such as liver, kidney, spleen and muscle are organized into a coherent, hierarchical system by a master clock located in the suprachiasmatic nucleus (SCN)(Ko & Takahashi 2006). The SCN in the hypothalamus

comprises of approximately 15-20,000 neurons that form a highly unified circadian network (Mohawk & Takahashi 2011). The master clock receives the light input from the retina through the retino-hypothalamic tract (RHT) that synchronizes internal clock timing to the external light/dark cycle, which it passes on to the peripheral clock via neuronal and humoral pathways **(Figure 1-1)**. These molecular clocks present in the SCN and throughout the peripheral tissues share the same molecular architecture and generate more or less similar circadian rhythms(Yoo et al. 2004). The clocks in the SCN differ from that in the periphery in the high degree of intercellular coupling among neurons due to which the SCN clocks are less prone to perturbations in phase. While the phase of the peripheral clocks are susceptible to the adjustments based on the synchronization by the master clock through the circulating hormonal and the systemic cues. The peripheral clocks synchronization is essential for generating rhythms in temporally coordinated physiology and metabolism (Saini et al. 2011). Although light is the dominant cue for the SCN clock, the peripheral clocks are known to be driven either indirectly by rhythmic feeding time, sleep/wake cycle or body temperature or directly by the feeding regimen (Damiola et al. 2000).

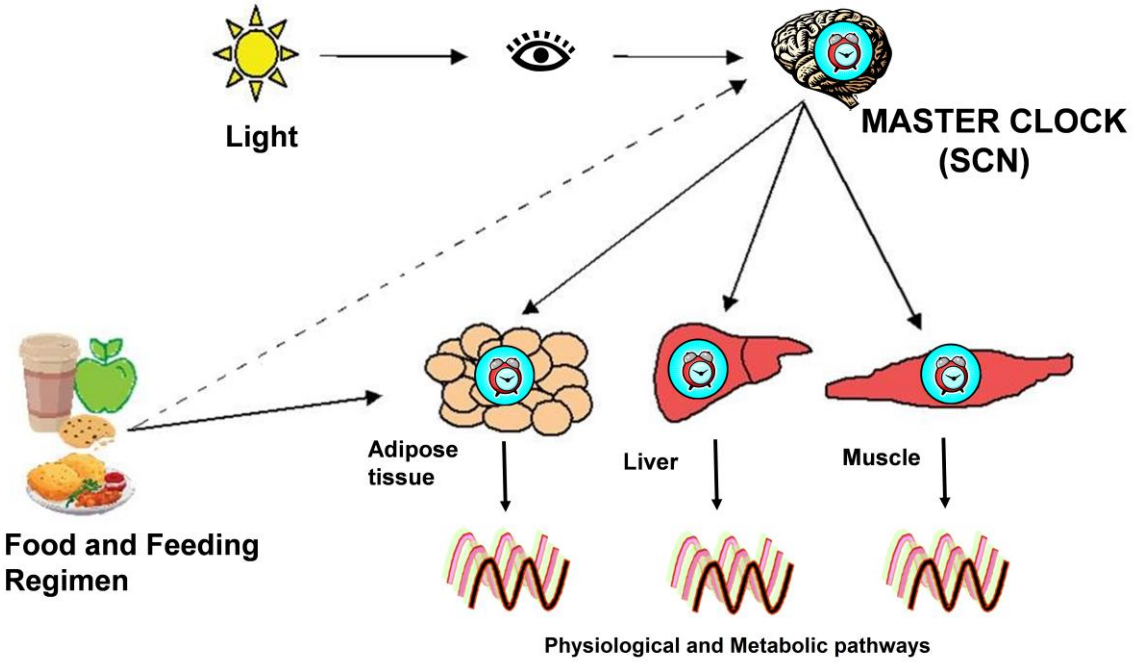

Figure 1-1. Hierarchal Organization of Mammalian Circadian Clock. Resetting signals by Light and Food. Light resets the master clock in SCN which in turn synchronize the peripheral clocks via humoral and neuronal signals, SCN also dictates rhythms in time of feeding, and behavior. Food and feeding regimen can directly reset the peripheral clocks uncoupling it from the SCN clock.

1.3 Molecular Circadian Clocks

The molecular clock in mammals is cell-autonomous and is generated by the interlocking transcription/translation feedback loops that function to produce robust 24 h rhythms of gene expression. The core feedback loop is driven by two transcriptional activators (CLOCK and BMAL1) and circadian repressors (PERs and CRYs) **(Figure 1-2)**. BMAL1 and CLOCK the core circadian transcription factors, share three characteristic regions involved in their function: one BHLH (basic Helix- Loop- Helix) region and two PAS (Per-Arnt-Sim) region which is required for DNA binding and heterodimerization(Lowrey & Takahashi 2011). CLOCK and BMAL1 form a heterodimeric transcription factor complex, which activates transcription of the repressor Pers (Per1, Per2 and Per3) and Crys (Cry1, and Cry2)(Gekakis et al. 1998; Kume et al. 1999; Hogenesch et al. 1998), Rev-Erbs (Rev-Erbα and β) and Rors (Rorα, β and γ) (Sato et al. 2004) as well as other clock-controlled output genes. PERs and CRYs heterodimerize and translocate to the nucleus to interact with CLOCK:BMAL1, inhibiting further transcriptional activity. Additional layer of regulation exist with a second transcription/translation feedback loop which is generated by Rev-Erbs(Preitner et al. 2002) and Rors (Sato et al. 2004), by binding to the ROR-element in the promoter region of BMAL1 and CRY1 either activating or repressing its transcription respectively **(Figure 1-2)**. This regulation drives rhythmic changes in the Bmal1 and Cry1 transcription. It particularly induces delay in Cry1 expression which is crucial for the proper circadian timing(Ukai-Tadenuma et al. 2011). In addition to RORα, PPARα also acts as a positive regulator of *Bmal1* expression by binding to a PPARα response element (PPRE) located in the *Bmal1* promoter. BMAL1, in turn, is an upstream

regulator of *Pparα* gene expression, producing an additional regulatory feedback loop (Canaple et al. 2006).

Another autoregulatory loop is represented by Dec1 and Dec2 (Differentially expressed in Chondrocytes), the clock-controlled bHLH transcription factors. BMAL1:CLOCK complex drives the expression of Decs by binding to the E-box elements in their promoter region. DECs act as repressors of BMAL1: CLOCK induced transactivation of target genes. They do so by either physically binding to *Bmal1* through bHLH region or competing with the BMAL1: CLOCK complex for binding to E-box elements of their target genes (Honma et al. 2002). The PAR leucine zipper transcription factor DBP (albumin gene D-site binding protein) is another circadian protein whose expression is promoted by the BMAL1:CLOCK complex while Dbp itself binds to the promoter of mPer1 to promote its expression (Yamaguchi et al. 2000). These interlocking feedback loops provide robustness against any noise and environmental perturbations for maintaining the accurate circadian timing, and also helps to generate phase delays in circadian output that creates optimally timed rhythms in gene expression for local physiology (Brown et al. 2012). Other clock-controlled genes (CCGs) that BMAL1:CLOCK targets such as thyrotroph embryonic factor (Tef), hepatic leukemia factor (Hlf) and E4BP4 do not exert a direct regulatory role on the feedback loop, but do play an important regulatory role in activating the downstream metabolic target genes through direct binding to D-boxes (Yoshitane et al. 2014).

Though, circadian clock is majorly regulated at the transcriptional level, the translational and post-translational modifications also play an important role in generating

24h rhythms in gene expression. Post-translational modifications that act as the integral part of the circadian clock include phosphorylation, acetylation, SUMOylation, methylation and ubiquitination (Robinson & Reddy 2014). Phosphorylation of several circadian clock proteins is mediated by several kinases (GSK3β, AMPK, CKIα, CKIδ and CKIε) and phosphatases (PP1 and PP5)(Partch et al. 2013) that regulate the phosphorylation and thereby localization and stability of these integral core clock proteins. Additionally, Clock proteins are also targets of ubiquitin ligases and degraded through ubiquitin (Ub)-dependent proteasome pathways. For example, Cryptochromes are targeted for FBXL3 ubiquitin ligase mediated proteasomal degradation by AMPK phosphorylation. Similarly, Periods are targeted for degradation through β-TRCP1/2 ubiquitin ligases by phosphorylation via kinases CK1δ and CK1ε. Recent report uncovered a ubiquitin ligase for BMAL1, UBE3A as an E3 ligase that binds and destabilizes BMAL1. In contrast to ubiquitination, SUMOylation does not directly target clock proteins for degradation but it rather regulates other functions such as protein–protein interactions, nuclear localization, and transcriptional activity and ubiquitination as well. For example, SUMOylation of BMAL1 by conjugation with SUMO2/3 also promotes nuclear localization as well as ubiquitin-dependent degradation (Kondratov et al. 2003; Cardone et al. 2005). In the mouse liver, SUMOylation of BMAL1 displays a circadian rhythm that coincides with its transcriptional activity. Additionally, BMAL1 is acetylated by CLOCK and recruits CRY to the BMAL1:CLOCK complex (Hirayama et al. 2007). Epigenetic control of the circadian clock is another important regulatory mechanism that drives rhythms in the circadian clock gene expression. Indeed, CLOCK was first found to have an intrinsic

histone acetyl transferase (HAT) activity(Doi et al. 2006) that revealed the molecular link between epigenetic control and the circadian clock. Importantly, CLOCK-mediated H3K14 acetylation at the circadian clock genes promoter region follows a circadian rhythmicity. In addition to acetylation, methylation is thought to contribute to regulating circadian transcription. Histone methylation is a critical modification that has been linked to the circadian clock control (Katada & Sassone-Corsi 2010; Valekunja et al. 2013).

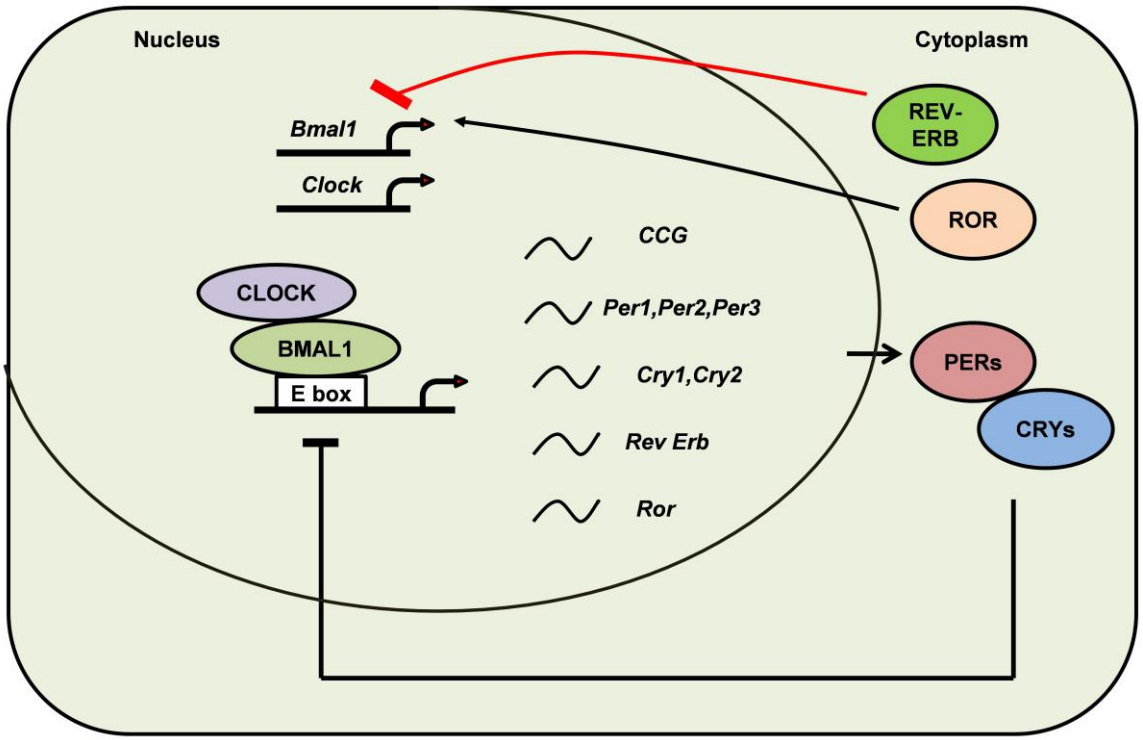

Figure 1-2. Molecular mechanism of the mammalian circadian clock. Transcriptional factors, CLOCK and BMAL1 form a complex and drive the expression of *Pers, Crys, Rev Erbs*, *Rors* and several other clock-controlled genes (CCGs). PERs and CRYs protein interact with each other and down-regulate activity of CLOCK:BMAL1, thus, inhibiting their own expression as well as that of *Reverbs, Rors* and clock controlled genes(CCGs). On the other hand, Rev-Erbs and Rors inhibit or activate the Bmal1 transcription respectively.

1.4 Circadian Clock Proteins

1. CLOCK: BMAL1

The functional domains present in the mammalian CLOCK and BMAL1 are presented in the **Figure 1-3**. Both the protein consists of one basic helix–loop–helix

(bHLH) domain and two PAS domains, that are required for DNA binding and heterodimerization, respectively. The CLOCK protein also consists of C-terminal domain which has a poly-glutamine repeat (amino acids 751-769). This domain is a characteristic of activation domains in many transcription factors. Phosphorylation is what mainly dictates the activity and the subcellular distribution of CLOCK and BMAL1 in the mouse liver and SCN. The phosphorylation of both the proteins is induced by the CLOCK:BMAL1 complex formation which in turn facilitates their nuclear localization. CLOCK and BMAL1 contain highly conserved putative NES (nuclear export signal) and NLS (nuclear localization signal) domains, adjacent to the PAS domain. However, the significance of these domains are yet not determined. It is likely that the association of CLOCK, BMAL1 and/or CRY proteins might mask or unmask these signals, in turn affecting the subcellular localization of CLOCK and BMAL1 proteins. Phosphorylation of BMAL1 protein is regulated by multiple kinases such as CKI, GSK3β and MAPK. Transactivation studies have shown that MAPK-mediated phosphorylation of BMAL1 results in in decrease in BMAL1 transcriptional activity while CKI-induced phosphorylation stimulates it. Finally, the CLOCK and BMAL1 phosphorylation by GSK3β target them for ubiquitin ligase mediated proteasomal degradation(Hirayama & Sassone-Corsi 2005).

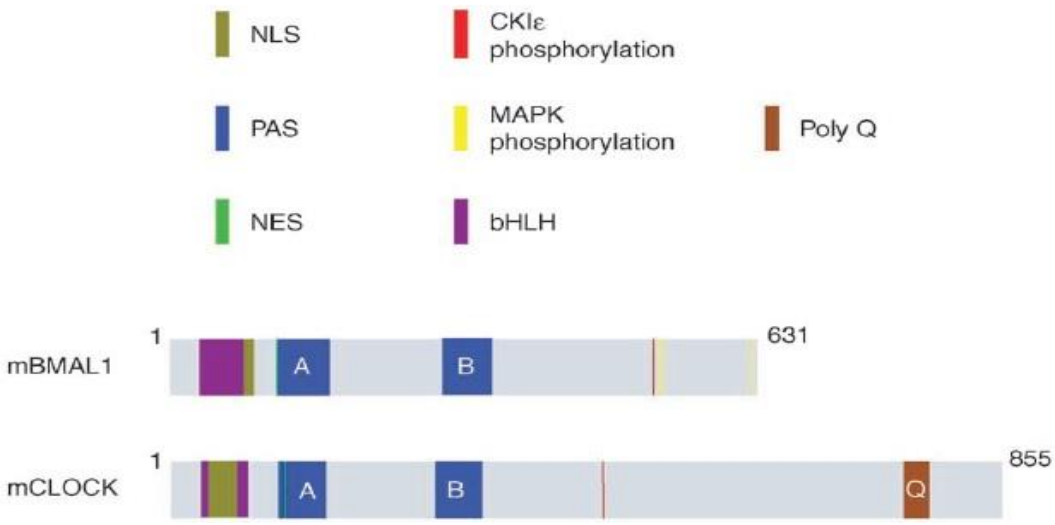

Figure 1-3. Schematic representation of the functional domains present in mouse CLOCK and BMAL1 proteins. Abbreviations: bHLH, basic helix–loop–helix; CKI, casein kinase I; MAPK, mitogen-activated protein kinase; NES, nuclear export signal; NLS, nuclear localization signal; PAS, PER-ARNT-SIM; Poly Q, poly-glutamine repeat. This figure is adapted from http://www.sciencedirect.com/science/article/pii/S0959437X05001292.

2. CRYPTOCHROMES (CRYs)

Mammals consist of two CRY proteins mainly CRY1 and CRY2. CRYs have been designated as key negative regulators of the core circadian clock loop since their discovery. Studies on reporter assays and Cry deficient mice have demonstrated the repressive action of Crys on the CLOCK: BMAL1 transcriptional activity. However, the exact mechanism of how CRYs repress the CLOCK:BMAL1 activity is not yet known. Although there are many theories proposed, earlier it was shown that CRY:PER complexes can interact with CLOCK:BMAL1 creating an early repressive phase. In the mouse liver, CRYs were found to mainly be present in the cytoplasm where they dimerize with the PERs and translocate

to the nucleus to downregulate CLOCK: BMAL1 activity. How exactly does CRY translocate to the nucleus is still unclear. In contrast, transient transfection studies CRYs did not need PERs to repress the CLOCK:BMAL1 activity. Recent biochemical studies have demonstrated the formation of early and late repressive complexes, wherein CRYs are found to be present in the late repressive complex with CLOCK:BMAL1 on the DNA invitro and invivo. Thus, PERs seem to hold the CRYs away from the CLOCK:BMAL1 complex by binding to the CRYs till sufficient accumulation of CRYs occur to form the late repressive complex.

Structurally, CRYs consist of the photolyase homology region (PHR) within N-terminal domain while C-terminal domain harbors the canonical flavin-binding site. The N-terminal is conserved in both the CRY proteins whereas the C-termini is somewhat divergent and dispensable for its repressive activity. Yet the C-termini possesses the phosphorylation and NLS which can modulate the clock protein functions. CRYs are phosphorylated by several kinases such as CKI, MAPK and AMPK. Though CKI phospho acceptor sites have not been identified, a putative consensus motif exists in the C-terminal extension of CRY1. In addition to CKI, MAPK was reported to directly interact with and phosphorylate CRY1 and CRY2. It has been proposed that phosphorylation of CRYs by MAPK and CKI reduces the repressor activity of CRYs. Recently, AMPK mediated phosphorylation was shown to target CRY1 for FBXL3 mediated degradation thus, reducing the CRY1 stability. The CC helix on the CRY structure seems to have an important role in its stability. Because this CC helix engages in the interaction with both the PERs and the FBXL3, an E3 ubiquitin-ligase. This very well explains the stabilization

of CRYs when bound to the PERs which restricts the interaction with the FBXL3(Gustafson & Partch 2015; Hirayama & Sassone-Corsi 2005).

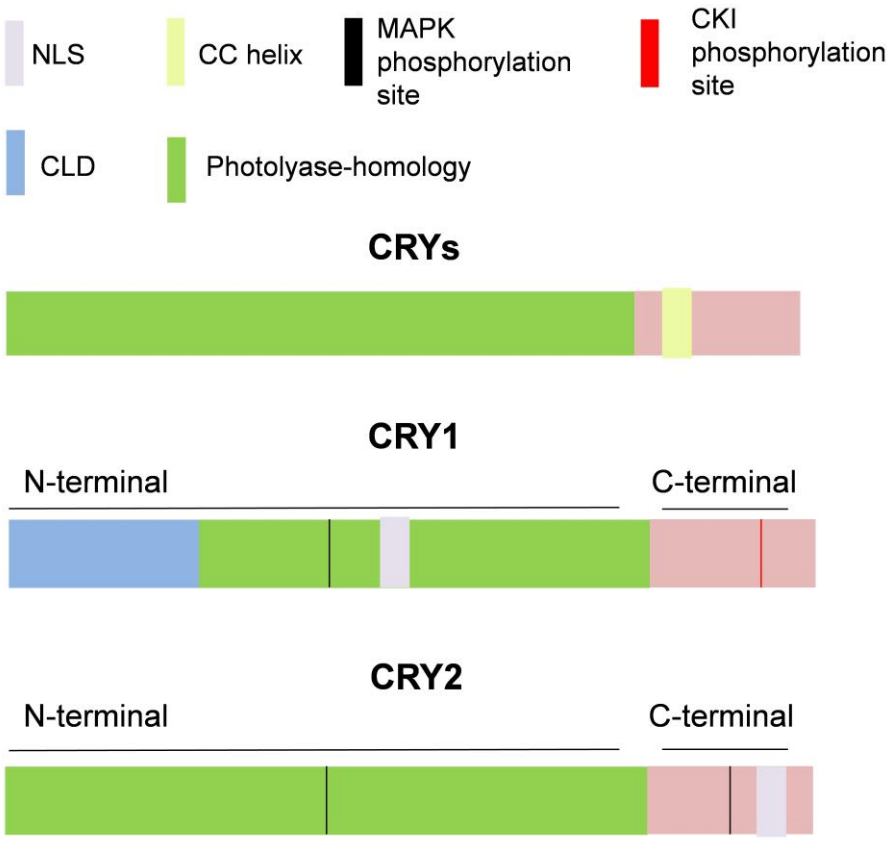

Figure 1-4. Schematic representation of the functional domains present in CRYs. All CRY proteins comprise an N-terminal chromophore-binding domain and a C-terminal extension domain. The percentage of identity between different domains is shown. Abbreviations: CKI, casein kinase I; CLD, cytoplasmic localization domain; MAPK, mitogen-activated protein kinase; NLS, nuclear localization. This figure is adapted from http://www.sciencedirect.com/science/article/pii/S0959437X05001292.

3. PERIODS (PERs)

PER proteins play an important role within the mammalian clock system, as changes in their post-translational modification state dictates the stability and localization of the early PER: CRY repressive complexes. Introduction of PER proteins at different time points in the feedback loop either advances or delays the molecular oscillator by controlling the early repressive complex assembly. Thus, understanding the molecular and biochemical basis of the PER interactions with the other clock proteins will provide key insights into the molecular mechanism of the circadian timing system.

PERIOD proteins are central components of the circadian clock, and with their structure consisting of two tandemly organized PAS (PER–ARNT–SIM) domains, through which they form homo or hetero dimers. PAS domains were first described in Drosophila proteins, Per, Arnt (aryl hydrocarbon receptor nuclear translocator) and Sim (single-minded), was later commonly found in circadian proteins and various transcription factors. The PAS domains of PER is shown to interact with the PAS domains of the CLOCK: BMAL1 complex which is suggested to represent the basis for recruitment of PER:CRY complexes to CLOCK:BMAL1 early in the repressive phase of the feedback circadian loop. Similar to CRYs, PER proteins function is also modulated by phosphorylation. CKIε has also been shown to control the PER protein stability and subcellular localization. It specifically interacts with and phosphorylates PER1, PER2 and PER3 proteins, regulating each of them in a different manner. CKIε phosphorylates and targets PER3 for degradation, additionally it might also phosphorylate the serine residue in the PER2 motif tagging it for β-TrCP mediated proteasomal degradation of PER2.

PERs also contain a functional NLS adjacent to the CKI-binding domain which could be involved in CKI mediated subcellular distribution of PERs. In addition, PERs also have other localization sequences such as NES and cytoplasmic localization domain (CLD), implicated in nucleocytoplasmic shuttling of PERs. The extreme C-terminus of PER1 and PER2 harbor a conserved CRY-binding domain which is required for the clock function. Interaction with the CRYs at this site is a critical step in the circadian clock loop. Hence, the absence of this particular CBD in PER3 explains its non-essential role in the circadian feedback loop(Gustafson & Partch 2015; Hirayama & Sassone-Corsi 2005).

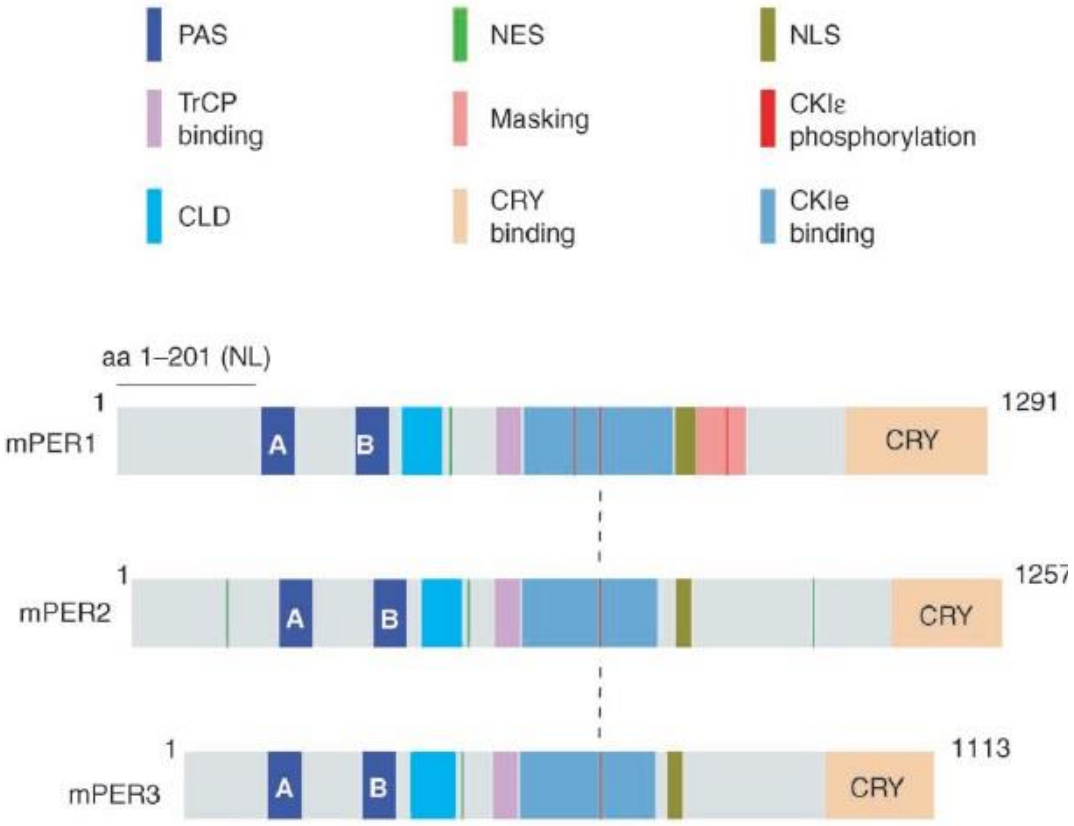

Figure 1-5. Schematic representation of the functional domains present in mouse PER (mPER) proteins. mPER1 N-terminal domain (amino acids 1–201) is required for its nuclear localization (NL) function. The dotted line indicates the well conserved CKI phosphorylation motifs in all three PER proteins. A and B represent the PAS-A and PAS-B motifs, respectively. Abbreviations: CKI, casein kinase I; CLD, cytoplasmic localization domain; CRY, cryptochromes; NES, nuclear export signal; NLS, nuclear localization signal; PAS, PER-ARNT-SIM; PER, period. This figure is adapted from http://www.sciencedirect.com/science/article/pii/S0959437X05001292.

1.5 Circadian Clock and Human Health

The circadian clock regulates various physiological and metabolic processes. Disruption through de synchronization with the environment such as in shift workers or during chronic jetlag has profound effect on the metabolic processes and increases the risk of several metabolic disorders and various types of cancer (Savvidis & Koutsilieris 2012). Detailed studies in understanding the genetic basis of the circadian clock and use of different circadian clock disruption model organisms have defined the functions of circadian clock in pathophysiology of diseases(Harmer et al. 2001; Hastings 2003). Indeed, the abnormal behavior of circadian clock is linked to diverse diseases such as cardiovascular, neurological and metabolic disorders(Takahashi et al. 2008; Savvidis & Koutsilieris 2012)**.** For example, the chances of the occurrence of cardiac events in the morning, with altered circadian gene expression in hypertensive individuals, is high, and epidemiological studies have shown an increased chances of developing diabetes when circadian function is disrupted in humans(Sato et al. 2014; Rakshit et al. 2014). Furthermore, disrupted circadian

clock gene expression mouse models have altered amplitude, period and phase in heart rate and blood pressure, as well as impaired glucose metabolism and hyperglycemia; and mutations in clock genes disrupts the cell growth and proliferation and affects the cell cycle gene expression, DNA damage repair and leads to cancer(Sato et al. 2014; Takahashi et al. 2008).

The master clock predominantly regulates the sleep/wake cycle and feeding time. Modern lifestyle such as late time meals, curtailment of sleep, potentially rotating shift workers and frequent travelling to changing time-zones, results in misalignment between the internal clock and the external light/dark cycle. The disruption of the circadian rhythms by alteration of timing and duration of sleep or constant light exposure or dim light exposure during night time, results in adverse health conditions manifested as the circadian rhythms sleep-wake disorders (CRSWD) (Hida et al. 2012). In addition to the alterations in the lighting conditions, genetic mutations also have an important role in CRSWD manifestation. For example, mutation in Per2 is associated with the familial advanced sleep phase disorder (FASPS), and ck1δ mutation, kinase involved in Per2 degradation, also results in FASPS as a consequence of stabilization of PER2 (Vanselow et al. 2006). While Per3 mutation results in delayed sleep phase disorder (DSPD) where the sleep and wake periods are shifted and a delayed onset of sleep and waking time occurs. Other types of CRSD conditions include advanced sleep phase disorder (ASPD), free-running disorder (FRD), and irregular sleep-wake rhythm (ISWD)(Sack et al. 2007). ASPD is a condition characterized by an advancement in the phase of sleep, with the sleep and wake being shifted to the earlier than usual in affected individuals. Free-running disorder is most

common in blind people and is characterized by the shift in sleep-wake patterns each day, and results from an inability to entrain to daily cues(Gooley 2008; Zhou et al. 2012).

Studies on body temperature and assessment of melatonin concentrations suggests that disruption in circadian rhythms is also observed in individual's suffering from mood disorders. Genetic studies have suggested variations in some of the clock genes to be associated with the mood disorders. One of which is NR1D1 (Rev Erb α), a BMAL1 gene repressor, whose genetic variants have been demonstrated to be associated with bipolar(Partonen 2015; McClung 2007) and depressive disorders (Kishi et al. 2009). Circadian clock repressors such as CRY2 variants are linked with the depressive disorders and bipolar disorders(Sjöholm et al. 2010) while CRY1 variants with depressive disorders (Hua et al. 2014). However, the exact mechanism of how the clock proteins might be contributing factor towards the mood disorders are missing. Additionally, disturbances in circadian rhythm function is also associated with neurological disorders such as Alzheimer's disease and Parkinson's disease. Indeed, the severity of dementia in Alzhemier's disease, and Parkinson's disease in patients is associated with the severity of the circadian clock disruption(Crowley 2011; Campos Costa et al. 2013).

There are very limited number of animal studies available using an actual physical shiftwork protocol. Obviously shiftwork requires shifting of sleep/wake cycle i.e. staying awake during the usual sleep period and sleep during the usual active period of the day. Housing the animals on the rotating wheels to prevent them from falling asleep is one of the way to mimic the shiftwork in humans. In such model groups of animals working during their resting phase, the animals lose their nocturnal urge to eat and voluntarily

consumed their food mostly during their normal resting (i.e., light) phase. Such a forced activity during the resting phase induced increased bodyweight and abdominal fat, impaired glucose tolerance, altered plasma triglyceride diurnal variation, and dampened daily glucose variation.

On the other hand, numerous animal studies with altered food intake and activity mimicking human shiftwork have been used for disrupted clock function. One of the models is based on desynchronization of peripheral clocks from the central clock by the wrong timing of food intake, sleep or activity. The other studies are based on the manipulation of the time of light exposure, including alterations of duration (i.e., continuous light) and timing of light exposure. Shifting timing of food intake disrupts the orchestrated synchrony between peripheral and master clock in the brain, which in turn may lead to metabolic problems since peripheral organs such as liver and muscle are required for maintaining the energy homeostasis. Shifting the time of food intake is indeed an interesting approach since it is associated with development of metabolic disorders such as obesity and shift workers also have changed time of food intake which similarly affect the metabolic processes and thus disturb the energy homeostasis(Gluck et al. 2011; Garaulet et al. 2013; Garaulet M 2014; Hibi et al. 2013).

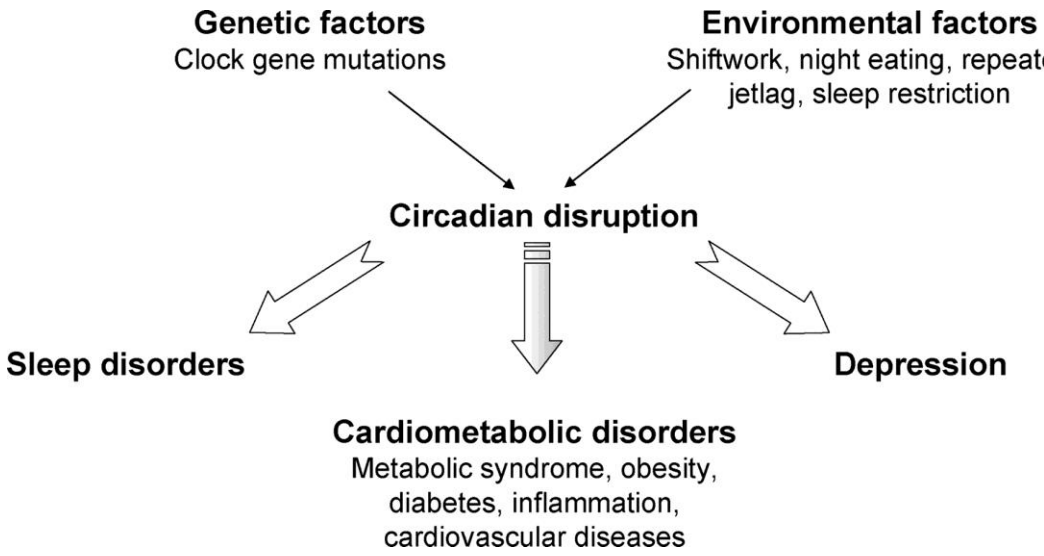

Figure 1-6. Circadian Clock and Human Health. Circadian disruption may arise from genetic (clock gene mutations) or environmental (shift work) factors and contributes to the development of behavioral and cardiometabolic disorders. This figure is adapted from http://atvb.ahajournals.org/content/30/8/1529.full

1.6 Mouse Models of Disrupted Circadian Clock

Years of studies have identified phenotypes and pathological processes involving malfunction of clock genes and the circadian timing system. From mice to human studies presenting altered clock genes expression, shows the importance of circadian timing and its influence on many physiological activities. Additionally, pathological manifestation could significantly alter the clock as a result of maladaptation to the environmental stresses. For instance, recent studies have revealed that genes operating both in positive and negative loops of the molecular clock are important for tumor suppression in vivo, and that the mechanism of clock-controlled tumor suppression is conserved among humans and rodents. A complex biological process as aging is not controlled by the genetics

exclusively, but rather by the interaction between the genetic activity and the environment. Indeed, several circadian properties changes with aging such as 1) reduction in amplitude of circadian rhythms, and 2) phase advances in the rhythms dependent of the SCN clock (Arellanes-Licea et al. 2014). Reciprocally, disruption of some of the circadian clock genes results in aging phenotype. Now, whether the aging phenotype is due to the disruption of the circadian rhythmicity per se or because of the other non-circadian functions of the clock proteins is not yet known. Aging in humans is also linked with the altered rhythms of sleep/wake cycle, hormonal secretions and core body temperature(Hofman & Swaab 2006; Kondratova & Kondratov 2012). Experimental mouse models of circadian clock disruption have been extensively used for studying the importance of the circadian clock genes in pathophysiology. These circadian clock mutants demonstrate different level of changes in the clock and clock controlled genes expression that is responsible for the unique phenotype of each of these clock mutants (Kondratov et al. 2009; Yu & Weaver 2011).

Among the three mouse models, Bmal1-/-, Clock -/- and Clock $^{\Delta 19/\Delta 19}$, the most severe phenotype is observed in the Bmal1-/- mice. Bmal1 is the only gene whose disruption results in a complete loss of circadian rhythmicity in behavior. The most dramatic phenotype observed in these mice is premature aging. We reported previously that Bmal1-/- mice, indistinguishable from WT littermates at birth, start to show signs of growth retardation and premature aging at 16-18 weeks of age. The average lifespan of Bmal1 deficient mice is 37.0 + 12.1 weeks while the average life span of WT mice is approximately 120 weeks (Kondratov, Kondratova, Gorbacheva, et al. 2006). Additional

premature aging related phenotypes of Bmal1 deficient mice includes, greatly reduced lifespan, sarcopenia, age-dependent weight loss, reduced organ weight, cataracts, ectopic calcification of tendons and cartilage, and male and female sterility(Kondratov, Kondratova, Gorbacheva, et al. 2006; Kondratov et al. 2009; Bunger et al. 2005; Alvarez et al. 2008; Boden et al. 2010; Sun et al. 2006; McDearmon et al. 2006). Restoring the expression of BMAL1 in the brain of BMAL1 deficient mice rescued the behavioral arryhthmicity, while rescuing the BMAL1 expression in the muscle does not restore the rhythmicity, but does prevent weight loss (McDearmon et al. 2006). These results suggest that the loss of circadian rhythmicity per se is not the only contributing factor towards the multiple aging-phenotypes observed in BMAL1-/- mice.

CLOCK-deficient mice also have reduced lifespan (Dubrovsky et al. 2010), however, unlike BMAL1-/- mice, these mice do not show age-dependent reductions in body weight or reduced relative organ weights, and are fertile (although reproductive performance is reduced in females). Also, the SCN-driven behavioral rhythms are preserved in CLOCK deficient mice, while peripheral circadian oscillators require functional CLOCK(Debruyne et al. 2006; DeBruyne et al. 2007). CLOCK-deficient mice also appear to differ, phenotypically, from $Clock^{\Delta 19/\Delta 19}$ mutant mice. The $Clock^{\Delta 19/\Delta 19}$ mutant have an intronic splice site point mutation in the Clock gene thus excluding exon 19 from the transcript. This mutant CLOCK protein can bind to BMAL1 but is devoid of transcriptional activity; from biochemical and genetic evidence, the mutant protein appears to function as a dominant negative(Gekakis et al. 1998; King et al. 1997). $Clock^{\Delta 19/\Delta 19}$ mutant mice have a modest aging/cancer phenotype (Antoch et al. 2008). Unlike CLOCK

and BMAL1-/- mice, lifespan of these mutants does not seem to be severely affected. Female Clock$^{\Delta 19/\Delta 19}$ mutant mice are fertile but have reduced fecundity and are prone to dystocia (Kennaway et al. 2004; Dolatshad et al. 2006), in sharp contrast to the sterile BMAL1-/- (Bunger et al. 2000; Alvarez et al. 2008; Boden et al. 2010)and Clock-/-; Npas2m/m double-mutants of both the sexes.

Deficiency of PER1 and PER2 proteins result in loss of circadian rhythms in both behavior and gene expression (Zheng et al. 2001; Bae et al. 2001). Although not as severe as BMAL1-/- mice, PER1 and PER2 knockout mice also show certain features of premature aging such as faster decline in fertility, loss of soft tissues and kyphosis at 12-14 months of age(Zheng et al. 2001; Hua et al. 2006; Hua et al. 2007; Oda et al. 2009). PER1 and PER2 are known to be tumor suppressor genes (Fu & Lee 2003; Wood et al. 2009). PER2 -/- mice has been demonstrated to be more susceptible to genetic or radiation-induced cancer (Wood et al. 2008; Lee et al. 2010). Per1 or Per2 over expression reduces tumor growth in vivo and promotes apoptosis in vitro, whereas their down-regulation promotes cancer cell growth(Yu & Weaver 2011). Similar to PER mutants, CRY1 and CRY2 deficiency also results in arrhythmic behavior. On the contrary, p53-deficient mice that lack both CRY1 and CRY2 proteins are actually resistant to cancer and have increased lifespan, while CRY1/CRY2 deficiency promotes apoptosis upon UV irradiation. Our data indicates that CRY1 deficient mice have reduced lifespan (unpublished). Thus, paradoxically, CRYs and PERs promote and suppress tumorigenesis, respectively. One of the interpretation for the differences in the PERs and CRYs functions is that the disruption of circadian genes indeed has a complex role in aging and cancer with its major

contribution in the circadian clock, along with their non-circadian role in other cellular processes and pathways(Yu & Weaver 2011).

1.7 Circadian Clock and Metabolism

Our understanding of circadian clock influence on the metabolism has significantly advanced with the discovery of clock proteins and their functions. There is an intricate link between the circadian clock and metabolic pathways. Indeed, a properly functioning circadian system has been shown to be important for generation of daily rhythms in energy metabolism and insulin sensitivity. While genetic aberrations in circadian clock machinery can lead to metabolic disorders. Evidences of the circadian clock disruption on the metabolic disorders such as, cardiovascular diseases, increased body mass, elevated plasma glucose and lipid levels, has been observed in night shift and rotating shift workers (Eckel-Mahan & Sassone-Corsi 2013). Genetic models further explain the importance of the interconnectedness between the circadian clock and metabolic disorders such as diabetes and obesity. Whole-body or tissue specific circadian gene knockout mice models often have perturbations in glucose, lipid homeostasis, and reduced insulin sensitivity (Arble et al. 2010; Asher & Sassone-Corsi 2015; Kalsbeek et al. 2014). Bmal1 deficiency leads to severe metabolic phenotype such as dysregulated glucose homeostasis, decreased gluconeogenesis and reduced insulin sensitivity, which further lead to the investigation of other circadian mutant mouse models (Marcheva et al. 2010). Other circadian mutant model like Rev-Erbα and Rev-erbβ double knockout have deregulated lipid metabolism.

Though the master clock in SCN drives circadian variations in glucose uptake and insulin release (La Fleur 2003), peripheral clocks also play an important role in these

processes independent of the SCN (Marcheva et al. 2010; Sadacca et al. 2011; Lamia et al. 2008). Transcriptomic studies have shown that up to 45% of all transcripts in mice display 24 h oscillations (Zhang et al. 2014). Many of these genes are key regulators of glucose and lipid metabolism, as well as of oxidative phosphorylation (Panda et al. 2002; Storch et al. 2002). Metabolic rhythms are not only coordinated by the cell-autonomous circadian clock but also by the feeding/fasting cycle(Asher & Sassone-Corsi 2015; Asher & Schibler 2011; Vollmers et al. 2009). At the cellular level, the circadian clock genes are also in turn regulated by the metabolic transcription factors. For instance, AMP-activated protein kinase (AMPK), a kinase activated upon nutrient deprivation, can phosphorylate CRY1 and targets it for proteasomal degradation. Another protein linking metabolism with the circadian clock is SIRT-1, a NAD+ dependent deacetylase. SIRT-1 directly interacts with CLOCK and deacetylates BMAL1 and PER2 and thus, promotes the BMAL1 transcriptional activity. Also, Bmal1 transcription is inhibited by its transcription repressor, REV-ERB, which is regulated by adipogenesis, while on the other hand, it is activated by a nuclear receptor, ROR, which is involved in lipid homeostasis. Gluconeogenic transcription factor, cAMP-induced CREB, upon activation upregulates Pers expression, again connecting the circadian oscillator to G protein-coupled receptors (GPCRs) pathway via cAMP as a secondary messenger. Hormones such as glucagon which also exhibit circadian oscillation, signal through cAMP pathway. Many other hormones involved in metabolism, such as insulin, glucagon, adiponectin, corticosterone, leptin, and ghrelin, oscillate in a circadian manner and are also largely secreted in a tissue-specific fashion (Froy 2010). Moreover, thermogenesis activated heat-shock factors also are shown to

modulate the transcription of circadian clock components (Zarrinpar et al. 2015). In conclusion, studies circadian and metabolic processes interaction at the cellular levels will help in providing explanation for the growing circadian disruption associated metabolic disorders.

1.8 Circadian Clock and Feeding

Similar to the control of circadian clock on the metabolism, feeding is an important external cue that entrains the peripheral clocks in different organs such as liver, muscle, heart and adipose tissue. The SCN clock responds robustly to light, and is largely unaffected by the feeding cycle, whereas the peripheral organs communicate with the SCN clock via neuronal and hormonal regulation and mainly, feeding rhythms. The diurnal animals, like humans, mainly feed during the day, while the nocturnal animals such as rodents consume the food predominantly at night, when they are mostly active and awake. Feeding in mammals is under homeostatic control and involves humoral factors such as leptin, ghrelin and peptide YY that act on the neurons that ultimately control the urge to eat or to not eat. SCN lesion studies have also identified that SCN ablation destroys the circadian rhythmicity in feeding. Although these lesion studies have been argued for, other studies on circadian mutants indeed demonstrate arrhythmicity in energy intake while on ad-libitum diet. This to some extent suggests that feeding is controlled by the circadian system (Eckel-Mahan & Sassone-Corsi 2013).

Nearly 10% of the hepatic transcriptome oscillations are due to the contributions by the cell-autonomous circadian clock and feeding/fasting cycle. Under the ad-libitum feeding condition, where mice consume most of their food during night time, upto 3000

transcripts have been shown to oscillate daily. While in fasting condition only 350 hepatic transcripts oscillate with daily rhythms. On the other hand, mice fed isocaloric diet during day time show daily rhythms in up to 5000 hepatic transcripts. Importantly, daytime feeding in nocturnal mice leads to change in the phase of oscillation in the clock components and other rhythmic metabolic transcripts (Vollmers et al. 2009). Hence, timing of food intake dictates the phase of the peripheral clock in the liver. Though the feeding rhythms drive majority of the rhythmic transcription, a functional circadian clock is also needed to maintain the rhythms in hepatic transcripts. For example, CRY deficient mice do not have significant hepatic transcript oscillation, and even under isocaloric feeding the oscillations are restored in only 700 transcripts (Vollmers et al. 2009). This indicates that a properly functioning circadian clock, availability of food, and the temporal pattern of feeding play an important role in determining the hepatic circadian transcriptome rhythm.

Several studies have reported the impact of fasting/feeding cycle on the circadian clock by designing experiments of different types of limited food availability with a periodicity of 24h. Restricting food to a certain time of the day has profound effects on the physiology and behavior of animals.

In **time-restricted feeding (TR),** animals receive food ad-libitum or 100% of their daily intake at exactly the same time of the day every day. Within a few days the animals adjust to this new feeding schedule and consume all their food in 2-3 hours of feeding. These animals display food anticipatory activity (FAA) 2-4 h before the food is going to be provided, as indicated by the increased locomotor activity, body temperature, corticosterone secretion and gastrointestinal motility, all of which are very well-known

outputs of circadian system. TR is known to drive the rhythms even in the circadian clock mutants and also the mice with SCN lesions, irrespective of the lighting conditions. TR has been shown to affect and phase shift the clock genes expression in the peripheral organs such as liver, kidney, heart, and pancreas, with no effect on the SCN clock. Thus, TR leads to uncoupling of the SCN clock from the peripheral clocks(Froy & Miskin 2010).

A Food Entrainable Oscillator (FEO) has been proposed to exist that phase shifts the rhythms in the physiological activities, such as body temperature, locomotor activity and heart-rate which are normally regulated by the SCN clock. However, the location of this FEO is still elusive. Although several theories have been proposed of its existence in different areas of the brain yet none have been able to completely account for the oscillation. The existence of the food entrainable peripheral oscillators (FEPOs), situated in the peripheral organs was also suggested, wherein the FEPOs entrain peripheral circadian clocks upon timed feeding(Silver et al. 2011). Yet the anatomical location and the mechanisms of FEO and FEPO is not clearly understood (Mistlberger 2011). Circadian clock transcription factors, CLOCK and BMAL1 were demonstrated to be not necessary for the food anticipatory activity. However, the results for the role of BMAL1 in FAA is controversial. Another clock gene, mPer2 mutants did not exhibit food anticipatory wheel-running activity. Thus, different clock genes mutants have distinct effect for food anticipation(Feillet et al. 2006).

Recently, rhythms have been reported in the mTORC1 activity in several mice tissues. The rhythms were observed under both ad-libitum and TR feeding conditions. The rhythms also persisted in fasted mice for at least three cycles, which further suggested

existence of some internal regulatory mechanism. Interestingly, these rhythms were not disrupted in one of the clock gene, CRY mutants that have a disrupted circadian behavior. This further indicates that a hypothetical nutrient clock is not the same as the light-entrainable clock. This hypothetical clock was named as NAMO (Nutrient Anticipation Metabolic Oscillator)(Rohini V Khapre et al. 2014). Thus the future studies will focus on unveiling the molecular pathways involved in this clock functioning and whether or not the NAMO and the circadian clock interact with each other.

Though the daytime TR has adverse effects on the physiological functions both in humans and animals, TR during the normal physiological time of eating restores the synchronization of the clock oscillations. Indeed, TR during nighttime in nocturnal animals restores the rhythms in circadian clock genes and metabolic processes such as mTOR, CREB, AMPK and other downstream metabolic targets that were dampened by High-Fat Diet (HFD). Furthermore, mice fed HFD in TR manner display significantly less adiposity and improved glucose and lipid metabolism. They also exhibit decreased leptin resistance, hepatic inflammation and steatosis, reduced lipid deposition and improved motor functions(Hatori et al. 2012). Clearly, recent study by Chaix et al has shown that obesogenic diets such not only HFD, but also, high-sucrose and high-fructose diets given in TR manner can also attenuate the severe metabolic consequences of these diets(Chaix et al. 2014). The benefits of TR has also been demonstrated in the circadian clock mutant $Per1^{S714G,}$ where TR prevented the weight gain of these mice on HFD. TR also improved other misalignments of $Per1^{S714G}$ mutant such as feeding phase and oxygen consumption(Liu et al. 2014).

Although there are multiple studies demonstrating the beneficial effects of TR during the active time phase, yet the effect of TR on aging and longevity is not yet addressed. There are studies showing the lifespan improvement upon TR however those studies actually performed a mild calorie restriction which they referred to as the TR group. Hence the effect of TR on lifespan needs to be further addressed in future. Additionally, future studies focused on the exact mechanism driving the benefits of TR on the metabolic rhythms and clear link with the circadian clock could lead to the development of the much needed novel therapies for the metabolic syndrome such as obesity.

Fasting

In humans, fasting refers to ingesting no or minimal amounts of food for the period ranging from 12 hours to three weeks. Many religions including Muslims fast from dawn until dusk during the month of Ramadan, while Christians, Jews, Buddhists and Hindus traditionally fast on designated days of the week or year. Fasting is distinct from calorie restriction (CR) in which the daily calorie intake is reduced in the range of 20–40%, while the 24hr periodicity is maintained. There are different forms of fasting such as Intermittent Fasting (IF) and Periodic Fasting that might provide effective strategies weight reduction, delay aging, and health benefits.

Intermittent Fasting which is also known as Alternate Day Fasting (ADF), is basically the food available ad-libitum every other day. These mice eat almost twice when the food is provided every alternate day. Although, these mice are not calorically restricted, yet they demonstrate some of the features similar to CR mice, such as increased lifespan and better metabolic profile. Some of the metabolic benefits of IF include, improved

glucose metabolism, cardio-protection, neuro protection and reduced risk of cancer and cardiovascular diseases in humans. Though IF has been shown to be as effective as CR, the pathways involved in the beneficial effects of IF are still not known. However, one of the mechanism proposed is through BDNF, Brain derived neurotrophic factor, which is involved in brain development and plasticity, is linked to neuroprotection against any neuronal damage (Froy & Miskin 2010).

Froy et al have shown that when food was introduced during the light period under IF protocol, mice exhibited almost arrhythmicity in clock gene expression in the liver. Whereas nighttime feeding yielded rhythms similar to those generated during ad libitum feeding. Under disruptive lighting conditions, rhythms were maintained even under daytime IF, suggesting that SCN signals were involved in inducing the arrhythmic state in the periphery (Froy et al. 2009). This suggests that IF regimen affects the SCN clock, unlike TR that uncouples the peripheral clock from the SCN. Thus, the synchronization of the SCN clock with the IF regime could drive the beneficial effects on the human health.

Periodic Fasting is another type of fasting which lasts for more than 24 hours. Previous studies on overnight periodic fasting showed that the sharp reduction in the calorie intake affected several clock genes expression in the liver of mice with upregulation in Per1 and downregulation in Per2 mRNA level(Damiola et al. 2000). In our lab we implemented a Fasting (F) protocol in which mice were maintained on nighttime TR for 2 weeks followed by fasting for more than 24 hours on the day of tissue collection. In our study we observed differential effect of Fasting on clock genes and clock controlled genes expression (CCGs) with a moderate increase in the Per1 mRNA levels which well

correlates with the results from previous studies(Patel et al. 2016). In conclusion, circadian clock could act as a major regulator of the fasting effects that can ameliorate disease processes and improve functional outcome in animal models of metabolic disorders and obesity.

1.9 Calorie Restriction

In 1935 McCay et al demonstrated that reduced intake of nutrients without malnutrition (Calorie Restriction, CR) could increase the mean as well as the maximum lifespan of rats(McCay et al. 1935). Since then many studies, in other model systems, such as yeasts, fruit flies, nematodes, and several strains of mice as well as rats, have consistently shown that CR slows aging and increases their lifespan.

CR in Yeast. Short lifespan of these species has tempted the researchers to use yeast for testing the effect of nutrient deprivation and its mechanisms. In yeast, glucose depletion is commonly used to mimic the CR in mammals. Reduction of glucose concentration from 2 to up to 0.01% has been shown to increase the mean and maximum lifespan of these species. Mechanisms proposed in budding yeast S. cerevisiae to mediate lifespan extension include, enhancing Sir2 function through increased NAD/NADH ratio, downregulation of TOR pathway kinases Tor1 and Sch9, and enhancement of mitochondrial function and oxidative stress response pathway (Dang et al. 2014).

CR in C. elegans. CR in nematodes is obtained by diluting the bacteria, mainly E.coli, or reducing the eating capacity of worms. Diluting the bacterial density by 10-fold extends the lifespan in worms by 60%, while higher than that can actually increase the

lifespan of up to 150%. A study by Hansen and co identified genes that could be involved in the lifespan extension responses to CR, such as daf-16 (the FOXO ortholog), sams-1 (encoding S-adenosyl methionine synthetase), rab-10 (encoding a Rab-like GTPase), drr-1 (dietary restriction response, of unknown function), and drr- 2 (encoding a putative RNA-binding protein). Another recent study proposed low energy sensing AMP-activated protein kinase AMPK/Aak-2 and Foxo/Daf-16 are required for the longevity effect of CR (Taormina & Mirisola 2014).

CR in Drosophila melanogaster.

The most common practice for implementing CR in Drosophila is by dilution of the food medium to which the flies have ad libitum access. Drosophila food type consists of an agar-gelled diet of dried autolysed yeast and sucrose. The lack of success of CR in other labs has been interpreted by some that CR does not work in flies. An alternative explanation is that there could be detrimental effect of extended periods of starvation in flies that is probably not found in rodents due to different physiological and metabolic profile. This detrimental effect could possibly be counteracting any beneficial effects of CR protocol and hence fail to extend lifespan. Interestingly, a study by Patridge et al found that when yeast was fed intermittently to flies (every sixth day) with constant access to sugar-water, lifespan was extended by around 30% compared with flies fed yeast and sugar ad-libitum. These results suggest that an excess of carbohydrates may be necessary to rescue the detrimental effect of starvation periods for fruit flies. Thus, depletion or excess of certain nutrients, rather than the reduction in overall calorie intake could be responsible for lifespan extension in flies. At the molecular level, despite studies relating CR to life

span extension, the underpinning molecular mechanisms are poorly understood. Similar to other model organisms, Insulin/IGF-1 signaling pathway is central mechanism for longevity in Drosophila. In addition, Foxo overexpression and reduction in Tor pathway have also been indicated to promote longevity upon food restriction in Drosophila (Piper & Partridge 2007; Taormina & Mirisola 2014).

Calorie Restriction in Mammals

CR in mammals is well-known to lower incidence of most chronic diseases and results in an improved metabolic state thus, extending the lifespan. Rodents are the most common model used to study the effect of CR on lifespan. Several studies have demonstrated CR to extend the lifespan of rodents. CR not only improves the lifespan but also reduces the occurrence of the age-associated pathologies. The amount of calorie reduction and the age at which the CR is started influences the magnitude of the effect on the lifespan (Taormina & Mirisola 2014). However, this relationship between the amount of CR and life extension does not seem to be universal. The effects on lifespan extension are highly dependent on various other factors such as strain and sex. CR extends lifespan in genetically heterogeneous mice created from four inbred strains (BALB/c, C57BL/6, C3H, and DBA2). In some mice strains CR of 40% does not seem to extend the lifespan, while even shortens it, than ad libitum fed control mice. A possible explanation for the failure of 40%CR in these mice strains is that the percent of CR is excessive, and that a lower degree of CR would probably be beneficial. Indeed, in both wild-caught and C57BL/6J mice strain 40% CR shortened the lifespan when started early in life, which suggested that 40% CR is too harsh for these animals. A similar thing could be true for

humans as well with a certain degree of CR that may be optimal for some individuals, could prove to be excessive and harmful in others (Rizza et al. 2014).

Studies in longer-lived mammals such as primates have shown conflicting results (Liao et al. 2010; Mattison, et al. 2012). Two long term studies were initiated in the 1980s for understanding the effect of CR in non-human primates. Both the studies confirmed that CR delays the onset of age-associated diseases, yet the monkeys from NIA study did not demonstrate extended lifespan compared to their control counterparts, in contrast to the Wisconsin study. The reason for this possible discrepancies in the results were attributed to the diet composition and design. The NIA ad libitum controls were actually fed in a time-restricted manner due to which the lifespan of CR monkeys were shown to be not extended. Another possible explanation is that the NIA monkeys was the protein sources and the quality of carbohydrates in both the studies(Solon-Biet et al. 2015)

Several hypotheses have been generated for the CR-induced life extension yet the mechanisms are still not known. The proposed molecular pathways include inflammatory processes, oxidative damage, mitochondrial dysfunction, downregulation of signaling pathways such as TOR, Insulin/IGF-1 and upregulation of Sirtuin activity.

1.10 Molecular effects of Calorie Restriction

Insulin/IGF-1

Insulin-like growth factor-1 (IGF-1) is primarily produced in the liver. Binding of IGF-1 to its specific receptor, the insulin-like growth factor-1 receptor (IGF-1R) leads to activation of TOR pathway which results in cell growth and proliferation. IGF-1 is usually

present in the bound form i.e. IGF binding protein (IGFBP) in the circulation. Increased levels of free IGF-1 in the circulation is associated with the risk of many types of cancer while its reduction lowers the incidence of cancer (Gallagher & LeRoith 2011). We and others have shown that CR reduces the circulating Insulin and IGF-1 levels in mice and thus increases the insulin sensitivity. This is further corroborated by study showing the reversal of CR beneficial effect in preventing progression of bladder cancer upon restoration of IGF-1 levels. The most commonly activated pathway downstream by IGF-1 is phosphoinositide 3-kinase (PI3K)/AKT signaling pathway which has been implicated in different types of cancers. PI3K further interacts with different other signaling molecules downstream, such as mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK), and Sirtuins, that have been suggested to drive the beneficial effects of CR (Ricci 2014).

Mammalian target of rapamycin (mTOR)

mTOR pathway is a key nutrient sensing pathway which is modulated by the nutrient deprivation conditions such as CR. mTOR complex 1 (mTORC1) regulates protein synthesis, promotes cell proliferation, and inhibits autophagy. Upon CR, mTOR signaling is reduced which is speculated to mediate the lifespan extension effects of CR in several organisms(Solon-Biet et al. 2015).

AMP-activated protein kinase (AMPK)

AMPK is a heterotrimeric kinase that is composed of α (α1, α2), β (β1, β2) and γ (γ1, γ2, γ3) subunits. AMPK is a metabolic sensor that is activated upon severe nutrient

deprivation condition. It is a key nutrient sensor that regulates the whole body metabolism. During energy stress conditions, AMPK is activated upon high AMP/ATP ratio, this AMPK activation shuts down the anabolic processes and promotes catabolism. AMPK activation is indicated as one of the mediator of CR effects, however, several studies in mammals have failed to see the activation of AMPK upon CR. These differences in the results calls for additional studies to address this important hypothesis (Cantó & Auwerx 2011).

Sirtuins

Sirtuins are NAD+ dependent deacetylases that are known to regulate the aging process and mediate CR- lifespan extension response in organisms including S. cerevisiae, C. elegans and D. melanogaster (Guarente & Kenyon 2000). Cellular NAD+ levels are elevated in response to CR, as a consequence of reduced energy consumption, thereby activating Sirtuins. Out of the seven SIRT homologs in mammals, SIRT1 remains perhaps the most studied, probably due to its sequence similarity with the yeast Sir2 (Allard et al. 2009; Frye 2000). In contrast to studies in rodents that have shown increased SIRT1 mRNA and protein expression in several tissues, other studies did not observe this effect while some even reported reduction in SIRT levels upon CR. However, it should be noted that the SIRT activity is what drives the benefits of CR and not its expression. Indeed, pharmacological activators of Sirtuin such as resveratrol are known to slow down aging and increase lifespan in mice maintained on high fat diet (Solon-Biet et al. 2015).

1.11 Calorie Restriction and Circadian Clock

Calorie restriction is well known to drive several metabolic changes in animals such as reduced circulating glucose and insulin levels, increased insulin sensitivity, reduced IGF-1 level, decreased activity of mTORC1 signaling pathway, increased resistance to various stresses, increased fat turn over, and increased activity of sirtuins (Piper & Bartke 2008; Brown-Borg 2007; Blagosklonny 2008; Guarente & Picard 2005). In the Chapters following we will discuss how circadian clock can be affected by CR **(Figure 1-7)**.

Insulin is a major regulator of glucose uptake and utilization in insulin-responsive tissues. Circadian clock in pancreas has been shown to regulate insulin production and secretion (Marcheva et al. 2010), indeed, disruption of circadian clock function in Clock or Bmal1 mutants leads to reduced insulin secretion and increased blood glucose level. On the contrary, knock out of Crys or Pers leads to increased insulin level (Zhao et al. 2012; Barclay et al. 2013). Although circadian disruption contributes to a reduced insulin sensitivity, upon high fat diet, many circadian clock mutants paradoxically demonstrate increased insulin sensitivity (Zarrinpar et al. 2015; Rudic et al. 2004). Thus, circadian control of insulin pathway is complicated and warrants further study.

The role of the circadian clocks in the control of glucose homeostasis is well documented (Kalsbeek et al. 2014), and is further well supported by the data from circadian clock disruption mutants (Zarrinpar et al. 2015). CRY proteins, circadian repressors, regulate gluconeogenesis through glucagon signaling, CREB phosphorylation (Zhang et al. 2010), and phosphoenolpyruvate carboxykinase 1 transcription in a glucocorticoid-dependent manner (Lamia et al. 2011). Similarly, PER2 also decreases gluconeogenesis

while promoting glycogen storage by decreasing the activity of glycogen phosphorylase (Zani et al. 2013). The clock transcriptional factor Rev-Erb alpha downregulates the expression of many genes important for hepatic gluconeogenesis while on the contrary gluconeogenesis is reduced upon Bmal1 or Clock mutation (Rudic et al. 2004). Hence, role of the clocks in gluconeogenesis is complex too, and similar to its roles on insulin signaling, its effect on gluconeogenesis is tissue- and clock gene-dependent.

The circadian clocks play an important role in regulation of adipose tissue functions and fat turn over. Circadian clock-regulated lipid metabolism pathways in mammals have been revealed by the large-scale profiling, the regulatory circuits involved in fatty acid metabolism have been identified in the adipose tissue, liver, and muscle (Gooley Joshua J 2014; Kumar Jha et al. 2015). Circadian clock disruption results in abnormal fat storage, lipid transport, triglyceride levels, and a decrease in absorption of dietary lipids. Circadian clock proteins differentially regulate lipid metabolism, for example, the core components of the molecular clock, REV-ERBs and peroxisome proliferator-activated receptors (PPARs) control the cyclic transcriptional repression of multiple genes, including the mRNA expression for several rate-limiting lipolytic enzymes(Gooley Joshua J 2014; Kumar Jha et al. 2015). Thus, multiple studies establish a key role of the molecular circadian clock in regulating lipid homeostasis, this regulation is complicated and how it might contribute to the increased fat turn over upon CR needs to be studied.

An interplay between the Sirtuins, NAD+ dependent histone deacetylases, and circadian clocks has also been reported. NAMPT, a rate limiting enzyme involved NAD+ production through salvage pathway, is also controlled by BMAL/CLOCK complex. The

NAMPT rhythmic expression drives daily oscillations in NAD+ levels (Ramsey et al. 2009; Nakahata et al. 2009). While on the other hand, SIRT1, one of the Sirtuin family protein, deactylates PER and BMAL1 proteins and forms an important part of the feedback regulation in clock (Nakahata et al. 2008; Asher et al. 2008).

Recent studies are focusing on circadian regulation of the mTOR signaling pathway(Rohini V Khapre et al. 2014; Jouffe et al. 2013; Cornu et al. 2014). Rhythmicity in the mTORC1 activity has been observed in the SCN and liver, which in turn, may contribute to the rhythms in protein biosynthesis. Our lab recently demonstrated the important role of BMAL1 as a negative regulator of mTORC1 activity (R. V. Khapre et al. 2014); thus, increased BMAL1 activity may contribute to the reduction of mTORC1 activity in CR, which is a subject of future investigation.

CR increases cell resistance to various stress including, oxidative stress. Circadian clock is also a major regulator of ROS homeostasis(Patel et al. 2014; Hardeland et al. 2003). ROS levels and many products of oxidation have been shown to oscillate in circadian manner in blood and tissues in vivo. At the same time, clock mutants have high levels of ROS and oxidative damage, further supporting the importance of clock in the oxidative stress response (Patel et al. 2014; Hardeland et al. 2003). Melatonin, a clock-controlled hormone, is a well-known antioxidant; the master regulator of antioxidant pathway, Nrf2 is under the direct control of circadian clock transcriptional factor, BMAL1(Pekovic-Vaughan et al. 2014).

Due to significant interlinks between CR and circadian clock and with the large number of metabolic pathways under the control of circadian clock. In this thesis we

studied the effect of CR on circadian clock genes expression and behavior and physiology of one of the clock gene mutant, BMAL1. Based on our results we think that CR requires a significant level of optimization; indeed, an organism has to adapt to the limited resources available. In this limited resources condition, tissues need to synchronize their metabolic activity in order to maintain homeostasis and produce the necessary metabolic enzymes in a timely and effective manner. We believe that CR recruits circadian clock for this metabolic optimization and thus drive the beneficial effects of CR. Indeed, our results on circadian clock mutant support this hypothesis as CR failed to show any improvement on its lifespan and physiology. Finally, the circadian clocks allow for the anticipation of daily events and also confers considerable advantage by efficient usage of energy.

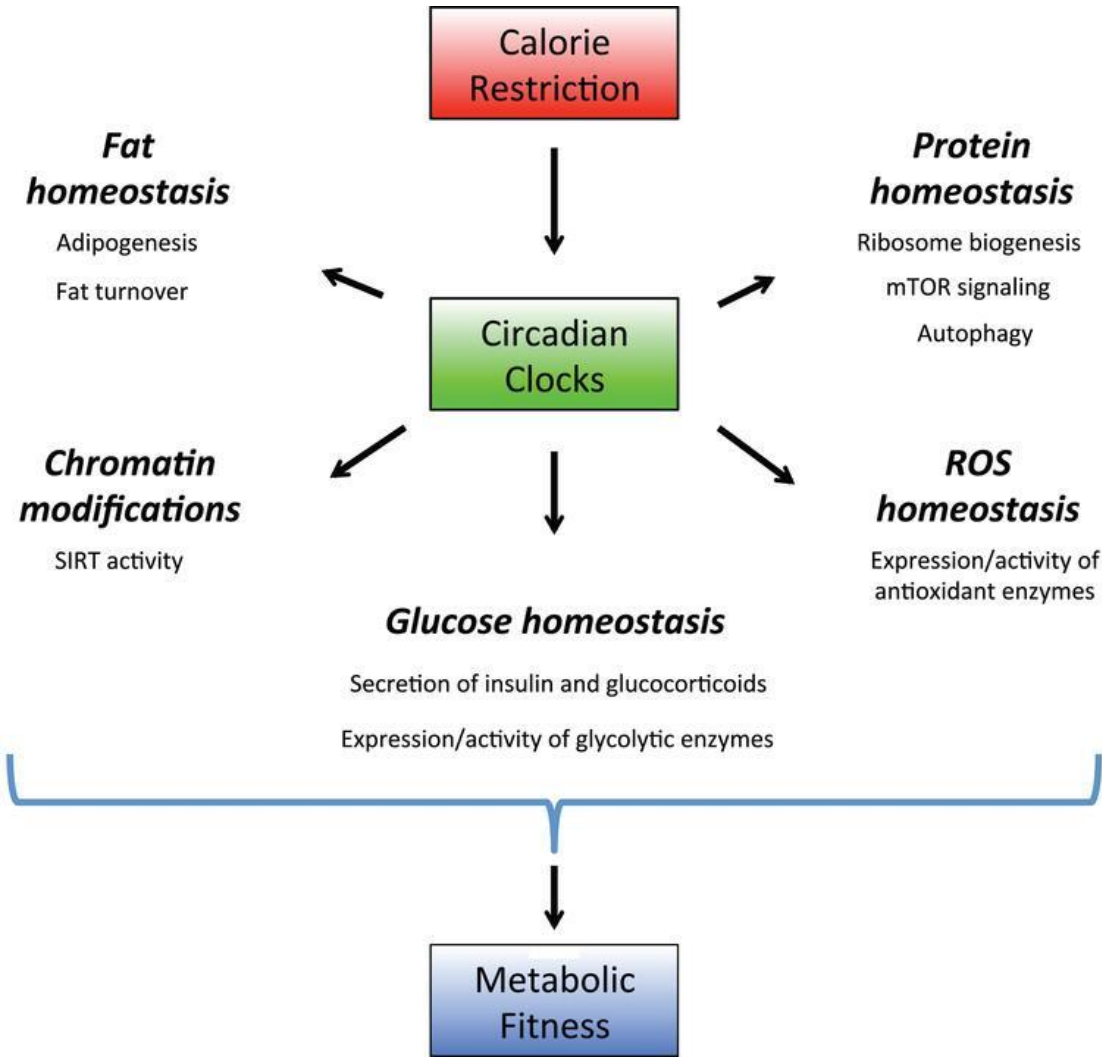

Figure 1-7. Circadian clocks as a part of calorie restriction mechanisms. Circadian clocks regulate expression and activity of the rate-limiting enzymes in multiple signaling pathways. Through these regulations the circadian system controls protein, lipid, amino acids and ROS homeostasis. Calorie restriction recruits the circadian clocks, this recruitment leads to optimization of metabolic processes and increases the organism's fitness, which contributes to calorie restriction-mediated longevity benefits. Adapted from http://content.iospress.com/articles/nutrition-and-healthy-aging/nha160006.

CHAPTER II

MATERIALS AND METHODS

2.1 Experimental Animals

All animal experiments were conducted in accordance with the IACUC regulations at Cleveland State University. All animals were housed under standard conditions of 12 hr light/dark cycle (LD 12:12) with lights turned on at 7:00 am and turned off at 7:00 pm. All animals had free access to food and water unless otherwise stated. All animals were fed regular 18% protein chow diet except breeders which were maintained on 19% protein diet (Harlan). Wild-type (WT) and Bmal1-/- mice were generated as per the Mendelian ratio by breeding mice heterozygous for Bmal1. Mice homozygous for Bmal1 gene (Bmal1-/-) were obtained from Dr. C. Bradfield's laboratory wherein they were generated by backcrossing the C57BL/6J inbred strain (The Jackson Laboratory, Bar Harbor, ME, USA) for 12 generations. PCR-based methods were used for determining the genotypes of mice as previously described (Bunger et. al., 2000). 5 months old wild type (WT) and Bmal1−/−

male mice were used for all the experiments. The ad libitum (AL) group had unlimited access to food throughout the day.

Feeding Regimen

Different feeding regimen schedule used in my thesis work is shown in **Figure 2-1**. Calorie restriction (CR) was started at 3 months of age of mice. For the first week, animals were fed 90% of their daily intake i.e. they were on 10% food restriction, for the second week they were on 20% food restriction and third week onwards mice were maintained on 30% food restriction for 2 months. The CR group received their food once daily at ZT14 (two hours after lights were off). At the end of experiment, tissues were collected at six different time points across the day. The time restricted (TR) feeding group received 100% of their average daily intake every day at ZT14 same as the CR group. TR was started at 4.5 months of age, and continued for 2 weeks before tissue collection. The fasting group (F) was on the above-mentioned TR feeding regime for 2 weeks, but did not receive food for 24 hours or more on the day of tissue collection. All groups had unrestricted access to water. For all experiments three animals of each genotype, feeding regimen and time point were used. Mice were euthanized using CO2 chamber followed by cervical dislocation.

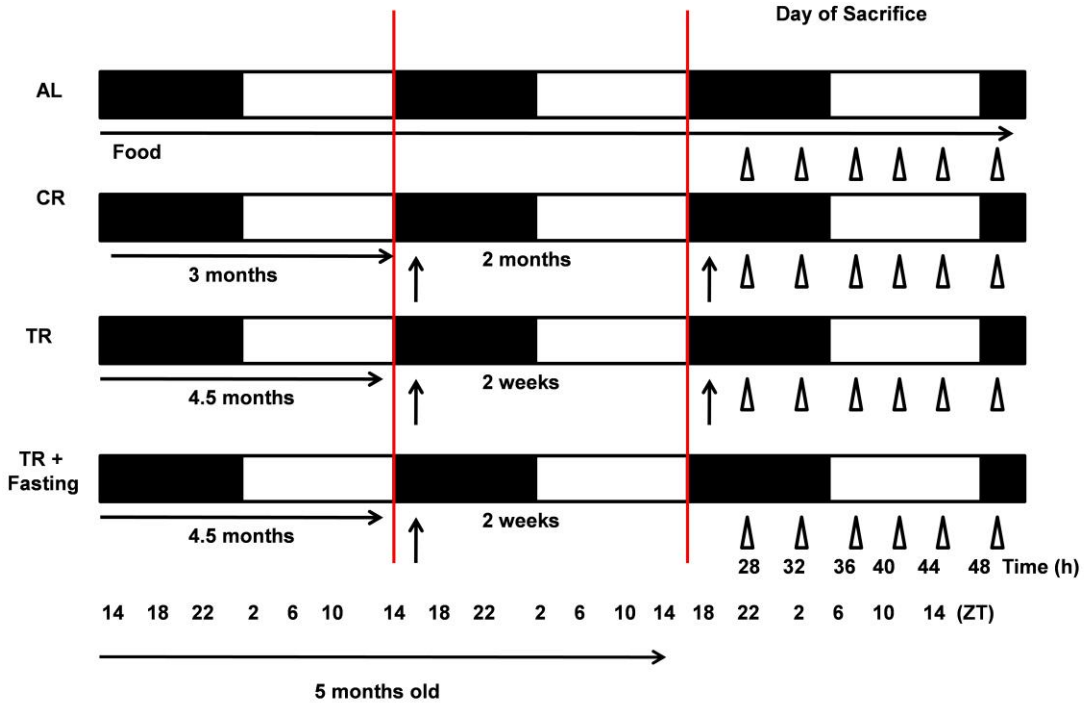

Figure 2-1. Experimental schedule. Arrows represent feeding period while arrow heads indicate times of tissue collection. All mice were maintained under standard animal housing conditions, with free access to food (AL) and water on 12 hours light/dark cycles. For CR experiments, mice had free access to food for first 3 months followed by gradual reduction in calories (90% of daily intake for 1st week; 80% in 2nd week; 70% from week 3 onwards) for 2 months. For TR and Fasting experiments, mice had free access to food for 4.5 months followed by 2 weeks of entrainment to definite amount of food (100% of daily intake) being provided once per day. For CR, TR and Fasting mice, food was provided daily at ZT14 (2 hours after lights are off) indicated by the vertical arrow. For Fasting experiments, food was withheld at ZT14 on the day of sacrifice. These mice were sacrificed at the indicated hours (h) after start of fasting i.e. 28hrs onwards, every 4 hours, over a 24 -hour period.

In-cage Locomotor Activity and Longevity Experiment

In-cage photobeam activity system (San Diego Instruments) was used to measure the in-cage locomotor activity. All mice used in this experiment were 5 months old and were allowed to acclimatize to the conditions before measuring the activity. The locomotor activity for each mouse was recorded for 3 consecutive days. 5 mice of each gender were analyzed for AL and CR feeding. For food anticipatory activity (FAA), CR mice were provided food at ZT2 (2 hours after lights are turned on) and were allowed to adjust to this new feeding time before measuring FAA. Total daily activity for each animal was normalized and set as 24 arbitrary units. The normalization of the total activity is done to make up for the huge difference in the activity of each mouse. For longevity experiment, 36 Bmal1-/- mice (both genders) were on 30% CR, 18 Bmal1-/- mice (both genders) were on AL diet, 27 wild type mice (both genders) were on 30% CR, 41 wild type mice (both genders) were on AL diet. If mice were found in morbid condition or showed signs of severe pathologies, these mice were counted as alive before the day of sacrifice and as missing after the day of the sacrifice. Because CR was started at 3 months of age (90 days), animals which died before the beginning of the CR treatment were excluded from consideration. All animal studies were conducted in accordance with the regulations of the Committee on Animal Care and Use at Cleveland State University.

2.2 RNA isolation and analysis of mRNA expression.

For RNA analysis, liver tissues from 3 male mice on each feeding regimen (AL, CR, TR and Fasting) and for WT and Bmal1-/- were collected every 4 hours throughout the day, and stored at -80°C. Total RNA was isolated from the liver tissue using TriZol

reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's protocol. Briefly, frozen liver piece (~30mg) was minced in 1 ml TriZol reagent with a pestle on ice. RNA samples were centrifuged at 10,000 rpm for 10 mins at 4C. RNA was extracted using 200ul chloroform and shaking vigorously. Following chloroform extraction step, total RNA was precipitated with 500ul isopropanol by centrifugation at 14,000 rpm for 10 mins at 4C and pellet obtained was washed with 1ml 70% Ethanol. RNA pellet was air dried, diluted in 30 µl of RNAse-free water and quantified on Nanodrop. RNA integrity was checked on 1% agarose gel run at 90 V for 30 minutes. 20 µ l of RT mix was prepared using 1 µ g of RNA, 50 ng of 50 uM random hexamer (N8080127, Invitrogen), 10 mM dNTP (DD0058, Biobasic), and 0.1 M DTT. It was then reverse transcribed by reverse transcriptase PCR machine using 200 u/µl of SuperScript® III Reverse Transcriptase (18080- 044, Invitrogen) as per the manufacturer's instructions. Incubation conditions used were: 65°C for 10 minutes followed by incubation on ice for 1 minute; 25°C for 5 minutes; 50°C for 60 minutes; Inactivate the reaction by heating at 70°C for 15 minutes. RNA quantification was performed using qPCR with Universal Syber Green mix (1725125, BioRad). The reaction was carried out in triplicates for the gene of interest and in duplicates for the normalizing control, such as 18s rRNA or Gapdh, using CFX96 qPCR Detection System (BioRad) with 50 ng of cDNA. Thermal cycling conditions used were according to the instructions of SYBR Green mix protocol and relative mRNA abundance was calculated using the comparative delta-Ct method. Melting curve analysis was used for confirming the product specificity. Primer pair was tested for the efficiency that was calculated by standard curve

analysis using standard serial dilutions method. Primers used for the analysis of expression are presented in **Table I.**

Table I: Primers for qPCR

Primers	Forward	Reverse
Bmal1	CAC TGT CCC AGG CAT TCC A	TTC CTC CGC GAT CAT TCG
Clock	GCG ACA GCA GGG ACA CGC CA	CGC GGC GGT AGC GGT GAA TTTT
Per1	AGG TGG CTT TCG TGT TGG	CAA TCG ATG GAT CTG CTC TGA G
Per2	AAT CTT CCA ACA CTC ACC CC	CCT TCA GGG TCC TTA TCA GTTC
Cry1	CGT CTG TTT GTG ATT CGG GG	ATT CAC GCC ACA GGA GTT GC
Cry2	GGC AGA CCG AGA CCC AGT CCA	ATC GAT TGC GCG GGG ACC G
RevErb α	TGG CAT GGT GCT ACT GTG TAA GG	ATA TTC TGT TGG ATG CTC CGG CG
RevErb β	GGA GTT CAT GCT TGT GAA GGC TGT	CAG ACA CTT CTT AAA GCG GCA CTG
Ror α	GGA ATC CAT TAT GGT GTC ATT ACG	GTG GCA TTG CTC TGC TGA CTT

Ror γ	ACT ACG GGG TTA TCA CCT GTG AG	GTG CAG GAG TAG GCC ACA TTA C
E4bp4	ACG GAC CAG GGA GCA GAA C	GGA CTT CAG CCT CTC ATC CAT C
Dbp	CCT GAG GAA CAG AAG GAT GA	ATC TGG TTC TCC TTG AGT CTT
Dec2	ATT GCT TTA CAG AAT GGG GAG CG	AAA GCG CGC GAG GTA TTG CAA GAC
Hlf	CAT CCT GAA GAC GCA TTT A	ATA AGG TGG GTC CCA AG
Fmo3	CACCACCATCCAGACAGATTAC	CCTTGAGAAACAGCCATAGGAG
Cyp4a12b	CTGATGGACGTTCTTTAC	TCAAACACCTCTGGATT
Cyp4a14a	ACGAGCACACAGATGGAGTG	TCTTCTTCCTGGCCTTCTGC
Igfals	GCTCTGAGAACAGGAAG	GATGCTCCAGGATCTGT
Parp16	CATAGCCTTCTTGGCCCGAT	GCGGTCTATTTCCTTGGAATCCTT
Hsd3β5	GTCAATCTGAAAGGTACTC	CCTTGTAGGAATTTGGTC
Hes6	CTGTGTTCTGACCTAGAG	CTGCCTAAGGATGTAGAC
Serpina12	ACCGTGATGATTCTCACAAA	AACATCATGGGTACCTTCAC
Alas2	GTCTTTGGTTCGTCCTCAG	GAACTGGTAGGTTTTAGCCA

2.3 Immunoblot analysis.

For analysis of protein expression tissues from three male mice per time point were used for each feeding regimens and both genotypes (WT and Bmal1 -/-). Western blotting was done on liver samples pooled together from three different mice at each time point for each diet. For tissue lysates preparation, frozen liver pieces were lysed in 300ul of cell signaling lysis buffer (Tris pH 7.5, NaCl, 0.5M EGTA, 0.5 M EDTA, Triton-x 100, Na4P2O7, β-glycerophosphate, 1M Na3VO4). 10ul Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology, Beverly, MA, USA) per ml of lysis buffer was added just before use. Tissues were lysed with sonicator (Fischer Scientific Model 100) for 5-10 seconds on ice. Lysates were centrifuged at 10000 rpm for 12 mins at 4ºC. Supernatant was separated and used for protein concentration determination and lysates were then stored at -80ºC.Protein concentration was determined by Bradford protein assay kit according to the manufacturer's protocol using UV-spectrophotometer. Lysates were then denatured by boiling on dry bath for 10 mins using 2x loading dye (300 mM Tris HCl PH 6.8, 10% SDS, 50% Glycerol, 10% 2-mercaptoethanol, 0.0025% Bromophenol blue) and cell signaling buffer to adjust equal concentration of protein in all the lysates. 45 ug of protein was loaded on 3–8% tris-acetate and 4–12% bis-tris gels (Invitrogen). Proteins were separated initially at 90V and then at 145V. Proteins were transferred electrophoretically on Polyvinylidene Difluoride membrane (Thermo Scientific 88518) (PVDF) at 110 mA for 70 min using transfer buffer containing 20% methanol, Tris Base 3g/L, Glycine 14.4g/L. Ponceau stain was used to check for equal loading of proteins. After transfer, membranes were blocked for non-specific binding in 5% non-fat dry milk in TBS-T buffer (Tris Base 60.57 g/L,

NaCl 87.66 g/L PH adjusted to 7.4 with HCl and 0.1% Tween-20) on shaker for 1 hour at room temperature. Further blots were incubated with specific primary and secondary antibodies as described in the table below (**Table II**). Protein analysis was performed using Clarity TM Western ECL Substrate (BIO-RAD) and quantification was done using Scientific Imaging film and Odyssey FC imaging system (LI-COR).

Table II: Antibodies for Western Blotting

Antibody	Dilutions	Washings	Catalog	Company
BMAL1	1:200 in 5% BSA 4º O/N	3 washings 5 minutes each	sc-8550	Santacruz
PER1	1:200 in 5% BSA 4º O/N	3 washings 5 minutes each	PA1-524	Thermo-Scientific
PER2	1:1000 in 5% BSA 4º O/N	3 washings 5 minutes each	PER21-A	Alpha Diagnostics
CRY1	1:500 in 5% BSA 4º O/N	3 washings 5 minutes each	21414-4	SAB Biosciences
CLOCK	1:5000 in 5% BSA 4º O/N	3 washings 5 minutes each	Generated in lab	Gifted by Dr. Antoch

IGF-1	0.1 µg/ml in 5% BSA 4ºO/N	3 washings 5 minutes each	ab9572	Abcam
β-Actin	1:10000 in 5% BSA 4º O/N	3 washings 5 minutes each	sc-47778	Santacruz
GAPDH	1:5000 in 5% BSA 4º O/N	3 washings 5 minutes each	#2118	Cell Signaling

2.4 Measurement of plasma IGF-1, insulin and glucose levels.

Plasma samples were collected at 6 time points (ZT2, ZT6, ZT10, ZT14, ZT18 and ZT22) from three animals per time point. Plasma IGF-1 level was determined using RayBio Mouse IGF-1 ELISA kit according to manufacturer's protocol. Sandwich ELISA with two different antibodies and streptavidin/biotin HRP system with manufacturer provided TMB substrate was used for detection of both IGF-1 and insulin. The detection limit for IGF-1 measurement is 4 pg/ml and for Insulin is 5 U/ml. Blood Glucose level was measured by True Test result kit by Nipro Diagnostics according to manufacturer's protocol. The detectable range of glucose is 20-600mg/dl.

2.5 Statistical analysis

At least 3 animals for each time points, for each feeding type and for each genotype were used for RNA, and Protein analysis. Two-way ANOVA was performed for analyzing the effect of feeding vs time of the day on mRNA and protein levels. This was followed by

Bonferroni correction for calculating the p value for pairwise comparison in between feeding regimen at each time point for statistically significant difference. Three-way ANOVA analysis was performed for assaying the effect of genotype vs feeding regimen vs time of the day on the mRNA and protein levels. Again Bonferroni correction was applied for pairwise comparison for the statistically significant difference obtained. IBM SPSS Statistics 20 and GraphPad Prism Version 5.04 software packages were used for statistical analysis. $P < 0.05$ was considered as statistically significant difference. Data are shown as average +/- standard deviation (SD). For longevity experiments logrank test was used for analysis. In longevity experiment for analysis of Bmal1-/- CR adjusted group, animals that died during first 40 days after the start of CR were excluded. $P<0.05$ was considered as statistically significant difference.

CHAPTER III

CALORIE RESTRICTION REGULATES CIRCADIAN CLOCK GENES EXPRESSION THROUGH BMAL1 DEPENDENT AND INDEDEPENDENT MECHANISMS[1]

Sonal Patel et al.

3.1 Abstract

Feeding behavior, metabolism and circadian clocks are interlinked. Calorie restriction (CR) is a feeding paradigm known to extend longevity. We found that CR significantly affected the rhythms in the expression of circadian clock genes in mice on the mRNA and protein levels, suggesting that CR reprograms the clocks both transcriptionally and post-transcriptionally. The effect of CR on gene expression was distinct from the effects of time-restricted feeding or fasting. Furthermore, CR affected the circadian output through up- or down-regulation of the expression of several clock controlled transcriptional factors and the longevity candidate genes. CR-dependent effects on some clock gene expression were _____

[1] As it appears in the *Scientific Reports Journal*, DOI: 10.1038/srep25970

impaired in the liver of mice deficient for BMAL1, suggesting importance of this transcriptional factor for the transcriptional reprogramming of the clock, however, BMAL1-independent mechanisms also exist. We propose that CR recruits biological clocks as a natural mechanism of metabolic optimization under conditions of limited energy resources.

3.2 Introduction

Circadian clock is an internal time-keeping system that generates 24 hour rhythms in physiology, behavior and metabolism(Green et al. 2008). In mammals, the master clock located in the SCN of brain is entrained by light that in turn controls the activities of multiple peripheral circadian clocks, which are located in different tissue, such as liver, muscle, adipose tissue, heart etc. These clocks are responsible for the synchronization of various biochemical processes in cells, and thus optimization of metabolism. Circadian clocks disruption is associated with development of various pathological conditions. Further, circadian clocks have also been implicated in control of aging in different organisms (Reppert & Weaver 2002). Indeed, circadian clock activity changes with age which may contribute to the development of age-associated pathologies such as cancer, diabetes and neurodegeneration(Dibner & Schibler 2015; Vinogradova et al. 2009; Videnovic et al. 2014). Disruption caused either through mutations in molecular clock genes (Fu & Lee 2003; Kondratov, Kondratova, Gorbacheva, et al. 2006; Dubrovsky et al. 2010) or by environmental disturbances such as shift of light/dark phase(Gibson et al. 2009; Davidson et al. 2006) results in reduced longevity.

The mammalian circadian clock is made up of the transcriptional translational feedback loops. The major loop involves BHLH (basic-helix-loop-helix) transcription factors CLOCK and BMAL1 that form a heterodimeric complex and regulate the transcription of core clock genes, Periods (Per1, Per2 and Per3) and Cryptochromes (Cry1 and Cry2). Periods and Cryptochrome proteins in turn, inhibit CLOCK:BMAL1 transcriptional activity and their own expression, thus forming a negative feedback mechanism(Sato et al. 2006). Another loop (called the accessory loop) consisting of *Rev Erbs* (α and β) and Retinoic acid receptors (*Ror α* and *Ror γ*) control the Bmal1 transcription by serving as transcription repressors and activators, respectively. Additionally, the CLOCK: BMAL1 complex also regulate transcription of several other clock-controlled transcriptional factors such as *Dec1, Dec2, Dbp, Hlf, Tef and E4bp4* (encoded by Nfil3). The clock and clock-controlled transcription factors function to control several downstream biochemical and metabolic pathways (Sahar & Sassone-Corsi 2012).

Calorie restriction (CR) is reduced consumption of calories or food that does not cause malnutrition. It is a powerful intervention known for decades to reduce occurrence of several devastating diseases, and increases lifespan across different species including mammals (McDonald & Ramsey 2010; Piper & Bartke 2008). Food and feeding regimen are another important cues that entrain the circadian clocks. Indeed, time-restricted feeding has been shown to entrain the circadian clock in the peripheral tissues. In mammals CR is followed in a time-restricted manner and circadian clock has been proposed to be a part of the mechanism facilitating the benefits of CR(Froy & Miskin 2010; Patel et al. 2015; Katewa et al. 2016). Other metabolic pathways implicated in CR such as insulin/IGF,

mTOR signaling pathways(R V Khapre et al. 2014) and sirtuins have recently been shown to interact with circadian clock. However, the effect CR could have on circadian clock has not been addressed yet.

In our study we decided to assay the effects of 30% CR on the circadian clock and clock-controlled genes expression at the mRNA and protein level in the liver, since it is a major metabolic regulator. We applied different feeding regimen (CR, TR, Fasting and AL) to the animals. We observed that CR significantly affected circadian clock system in a manner distinct from TR and fasting. We further analyzed the effects of 30% CR on some of the clock genes expression in the liver of one of the circadian clock mutant i.e. BMAL1 deficient mice.

Lastly, we analyzed the longevity candidate genes that have been reported to be regulated by CR. These longevity candidate genes have been termed so since they were identified to be affected in different studies in long-lived mutants and by several factors, such as CR, diet, age and gender. We assayed the mRNA levels of these candidates in circadian manner in different feeding regimen and showed that the genes were affected in gene- and time-specific manner by CR.

3.3 Material and Methods

Details of the Animal experiment, Feeding Regimen Schedule, mRNA and Protein analysis have been explained in Chapter II.

3.4 Results

Effect of CR on Circadian Clock Transcription Factors.

We employed 30% CR since this is the most commonly used percentage of CR for C57B6 mice and has maximum effect on lifespan (Turturro et al. 1999; Liao et al. 2010). We used 3 different controls in our study i.e. time-restricted feeding (TR), Fasting (F) and commonly used Ad-libitum (AL). CR mice received 70% of their daily AL food intake for two months. Food was provided two hours after the light was turned off (ZT14) since mice are nocturnal, and nighttime is when they are physiologically active. Our first control group, AL had unlimited access to food throughout the day. Second control group, time restricted (TR) feeding, animals were fed 100% of their daily average AL food intake at ZT14 (the same time as CR) for two weeks. Previous studies have shown that this time frame is enough for resetting the circadian clocks in the periphery (Wu et al. 2008; Damiola et al. 2000). We also noticed that the mice on TR and CR regimen consumed all the food within 4-5 hours after food was provided, in agreement with our previous report on TR. The only difference between TR and CR group being the amount of food consumption with the same time of feeding, TR group thus represent as being an appropriate control for timed feeding effect. The third control in our study was Fasting (F) group, this group was maintained on TR schedule for 2 weeks as mentioned above and deprived of food for 24 to 48 hours on the day of tissue collection. Hence this group represents an ideal control for the sharp reduction in the calorie intake without metabolic adaptation as opposed to CR.

We assayed the mRNA expression of the clock transcription factors, Clock and Bmal1 in the liver collected at six different time points from the mice on different feeding regimen mentioned above. The AL group exhibited oscillatory pattern of expression in clock transcription factors as reported in previous studies (**Figure 3-1**). Moreover, we

observed that 30% CR dramatically affected the expression of both Clock and Bmal1 genes. According to the ANOVA analysis, mRNA expression of Bmal1 was significantly increased at several time points across the day upon CR compared to the other controls **(Figure 3-1a**), AL, TR and Fasting. On the other hand, CR not only reduced the expression of Clock gene at different time points but also made it rhythmic (**Figure 3-1b**). TR and Fasting similarly reduced the expression of Clock genes compared with the AL group. Thus, the effect of CR on Bmal1 and Clock is different from TR and Fasting.

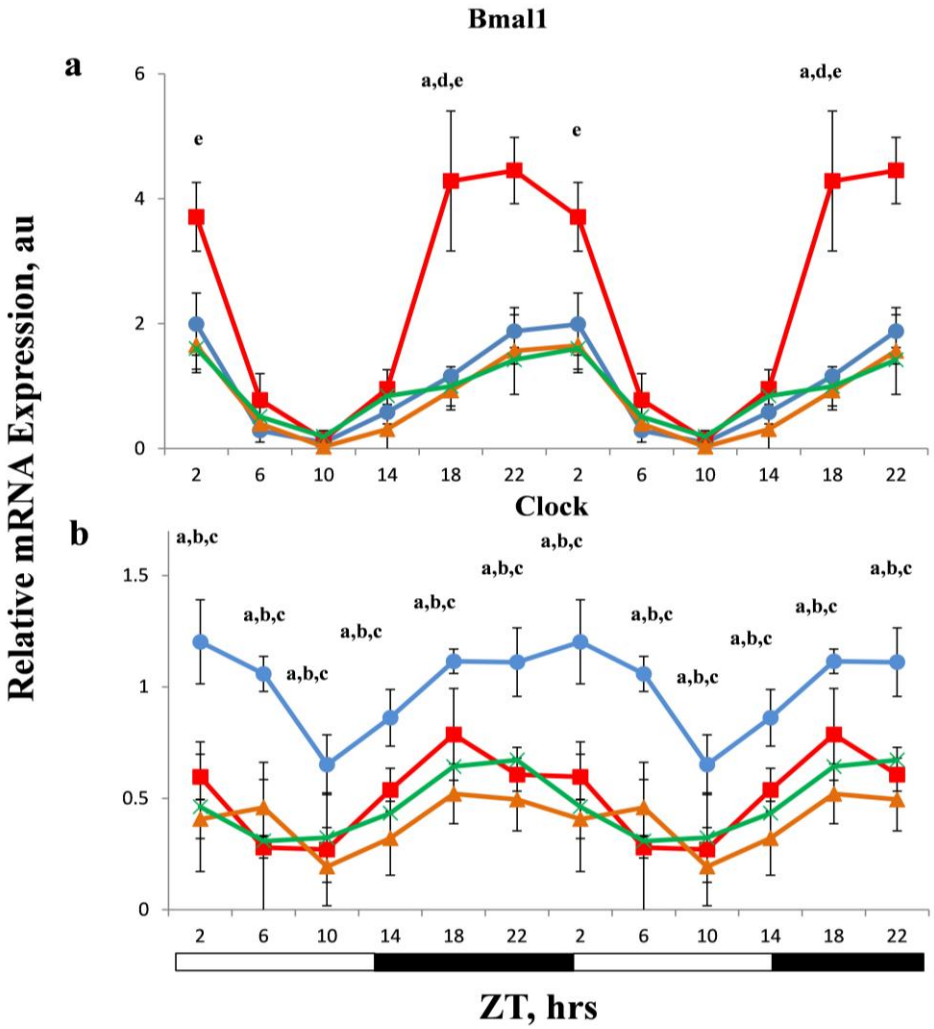

Figure 3-1. Effect of CR on rhythms of circadian clock genes. mRNA expression of core clock genes – (a) Bmal1, and (b) Clock was assayed in the liver of mice (n = 3 per time point) subjected to the following feeding regimens: ad libitum (AL) – blue circles, solid line; 30%CR – red squares and solid lines; TR– orange triangles and solid lines, fasting (F) –green cross and solid lines. For convenience all data are double plotted. Data represents mean ± SD; statistically significant ($p < 0.05$) effects of the feeding (analyzed by two ways ANOVA) at a given time point are indicated by: (a)- between AL and CR groups, (b) – AL and TR, c- AL and fasting, (d)- CR and TR, (e) – CR and Fasting, (f)- TR and Fasting, Light and dark bars at the bottom represent light and dark phase of the day. ZT0 is the time when light is on and ZT12 is the time when light is off.

Effect of CR on circadian clock repressors

We further assayed the effect of CR on the circadian repressors for which we compared the expression of the core circadian genes across the day in the liver of mice subjected to different feeding paradigms i.e.AL, TR, CR and Fasting. According to two-way ANOVA analysis, mRNA expression for Per1, Per2, and Cry2 (**Figure 3-2)** were significantly upregulated at several time points by CR. For Cry1 expression although there was tendency towards upregulation, it was not statistically significant. Circadian rhythms analysis by cosinor wave software shows that most of the feeding regimens did not significantly affect the rhythms or acrophase of most clock genes. However, CR and fasting disrupted the circadian rhythmicity in Per1 expression.

Effect of CR on components of accessory loop.

We additionally assayed the mRNA levels of the Bmal1 transcriptional repressors and activators. Reverbs (Reverb α and Reverb β) and Rors (Ror α, and Ror γ) are well-known players in the accessory loop that compete to either repress or activate Bmal1 transcription respectively. We analyzed the mRNA expression of these clock genes across the day in the liver of mice subjected to different feeding paradigms i.e.AL, TR, CR and Fasting. According to the two-way ANOVA analysis, mRNA expression for Reverb β and Ror γ were significantly increased at several times of the day, while Reverb α and Ror α were not changed upon CR (**Figure 3-3).**

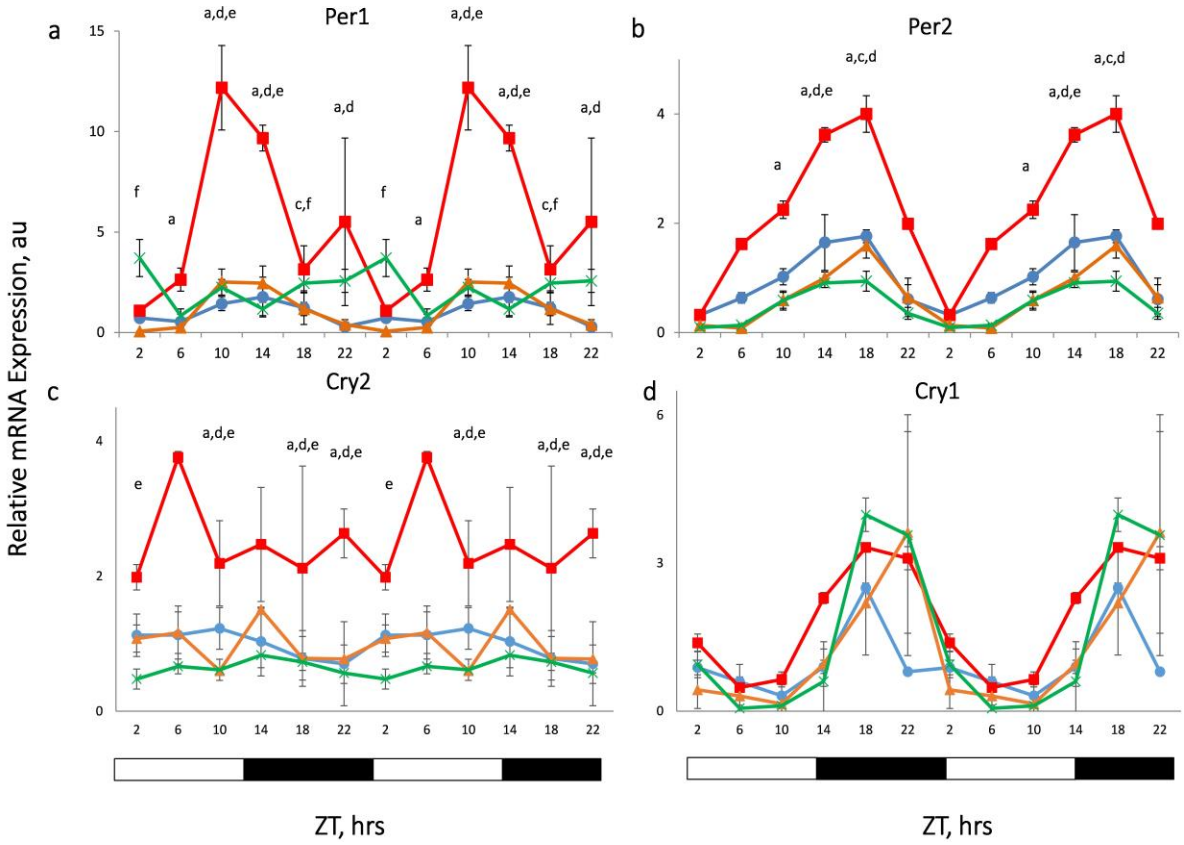

Figure 3-2. Effect of CR on rhythms of circadian repressors. mRNA expression of core clock genes – (a) Per1, (b) Per2, (c) Cry2 and (d) Cry1 was assayed in the liver of mice (n = 3 per time point) subjected to the following feeding regimens: ad libitum (AL) – blue circles, solid line; 30%CR – red squares and solid lines; TR– orange triangles and solid lines, fasting (F) –green cross and solid lines. For convenience all data are double plotted. Data represents mean ± SD; statistically significant ($p < 0.05$) effects of the feeding (analyzed by two ways ANOVA) at a given time point are indicated by: (a)- between AL and CR groups, (b) – AL and TR, c- AL and fasting, (d)- CR and TR, (e) – CR and Fasting, (f)- TR and Fasting, Light and dark bars at the bottom represent light and dark phase of the day. ZT0 is the time when light is on and ZT12 is the time when light is off.

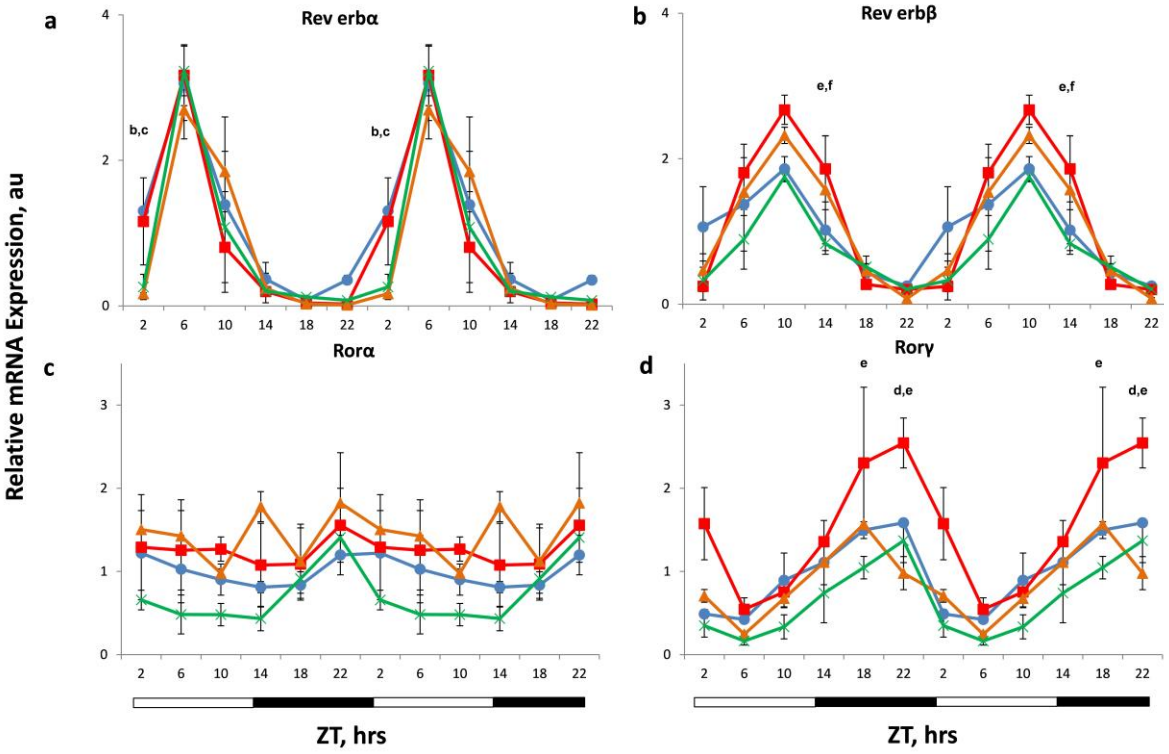

Figure 3-3. Effect of CR on rhythms of Revs and Rors. mRNA expression of core clock genes – (a) Rev Erbα, (b) Rev Erbβ, (c) Rorα and (d) Rorγ was assayed in the liver of mice (n = 3 per time point) subjected to the following feeding regimens: ad libitum (AL) – blue circles, solid line; 30%CR – red squares and solid lines; TR– orange triangles and solid lines, fasting (F) –green cross and solid lines. For convenience all data are double plotted. Data represents mean ± SD; statistically significant ($p < 0.05$) effects of the feeding (analyzed by two ways ANOVA) at a given time point are indicated by: (a)- between AL and CR groups, (b) – AL and TR, c- AL and fasting, (d)- CR and TR, (e) – CR and Fasting, (f)- TR and Fasting, Light and dark bars at the bottom represent light and dark phase of the day. ZT0 is the time when light is on and ZT12 is the time when light is off.

Effect of CR on the clock-controlled genes expression.

Clock-controlled genes including*, Hlf, Tef, Dbp, E4bp4, Dec1, Dec2 and Pparα* are well-known targets of CLOCK/BMAL1 complex. These are known as clock-controlled transcription factors since they are controlled by the CLOCK/BMAL1 transcription factor and in turn control the transcription of the downstream metabolic genes and thus regulate metabolism and physiology. These transcription factors have also been reported to affect the expression of some of the core clock genes by either activating or inhibiting transcription, for example, *Pparα* fine tunes the expression of Bmal1 gene while it is itself a target of BMAL1. The clock-controlled transcription factors have been reported to oscillate robustly in the liver with different phases (Stratmann et al. 2010; Fang et al. 2014). Previous studies have demonstrated these clock-controlled transcription factors mRNA expression to be affected by the feeding/fasting cycle (Kawamoto et al. 2006; Wu et al. 2010). We thus decided to assay the mRNA levels of these transcription factors in the liver of 30% CR, TR, Fasting and AL mice by qPCR. CR induced the expression of Dbp, D box transcription factor while fasting reduced it at ZT6 and ZT10 which is in agreement with the previous study reporting reduction in fasting animals (Kobayashi et al. 2004) **(Figure 3-4a).** Additionally, Hlf and Tef (**Figure 3-4b, and c**) were also upregulated at several time points by CR with Hlf and Dbp also induced by TR whereas Tef was not significantly affected by TR. E4bp4 expression was neither affected by CR nor TR but was instead up regulated by Fasting at the later time points in night compared to AL control **(Figure 3-4d)**.

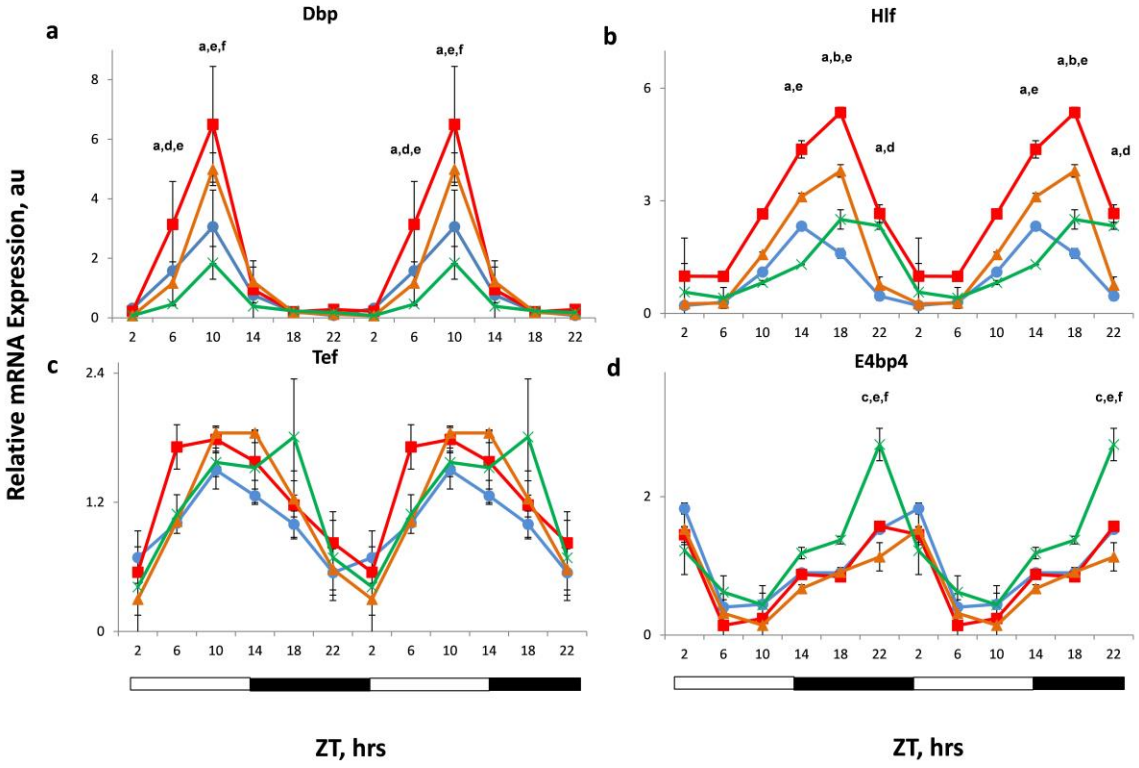

Figure 3-4. Effect of CR on rhythms of clock controlled transcription factors. mRNA expression of core clock genes – (a) Dbp, (b) Hlf, (c) Tef and (d) E4bp4 was assayed in the liver of mice (n = 3 per time point) subjected to the following feeding regimens: ad libitum (AL) – blue circles, solid line; 30%CR – red squares and solid lines; TR– orange triangles and solid lines, fasting (F) –green cross and solid lines. For convenience all data are double plotted. Data represents mean ± SD; statistically significant ($p < 0.05$) effects of the feeding (analyzed by two ways ANOVA) at a given time point are indicated by: (a)- between AL and CR groups, (b) – AL and TR, c- AL and fasting, (d)- CR and TR, (e) – CR and Fasting, (f)- TR and Fasting, Light and dark bars at the bottom represent light and dark phase of the day. ZT0 is the time when light is on and ZT12 is the time when light is off.

Other clock-controlled transcription factors *Dec1* expression was down regulated at Zt22 and up regulated at ZT14 by CR while it was significantly increased by TR (**Figure 3-5a)**. As previously reported, fasting also reduced the expression of *Dec1* at several time points compared to AL. *Dec2* was dramatically down regulated by CR, and on the contrary, was up regulated in TR and Fasting group at most of the time points (**Figure 3-5b**). Inconsistency in the results of the Pparα mRNA expression upon CR have been observed (Fu & Klaassen 2014; Masternak & Bartke 2007). Our results demonstrated Pparα to be not significantly affected by 30%CR or Fasting except ZT 14 where it was up regulated by CR and ZT2 and 6 by Fasting. The expression was also increased at several time points upon TR (**Figure 3-5c**). Overall, these results suggest that CR affects the expression of clock-controlled transcription factors in a way that is significantly different from TR and Fasting.

Effect of CR on transcription complex protein levels.

Next we tested the protein levels of the transcription factors, CLOCK and BMAL1 in the liver of mice from different feeding groups (30% CR, TR, Fasting and AL). Similar rhythmicity in expression was observed in our results as demonstrated previously by others in AL control group (Lee et al. 2001; Ye et al. 2014). Based on the two-way ANOVA analysis, CLOCK and BMAL1 levels were reduced upon CR and Fasting, however, the result was not statistically significant. On the other hand, TR induced the expression of BMAL1 and also affected CLOCK protein level (**Figure 3-6 a, and b**). According to the Cosinor wave analysis, BMAL1 showed rhythmicity, while daily changes in CLOCK was not circadian.

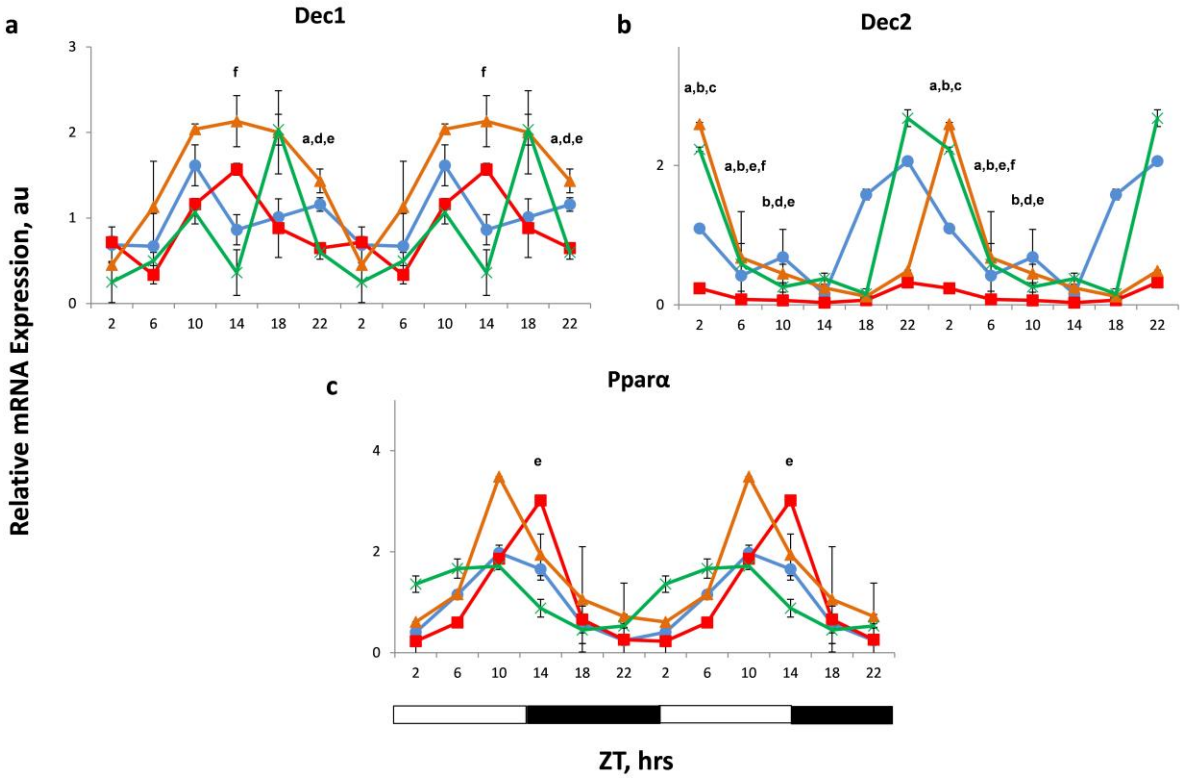

Figure 3-5. Effect of CR on rhythms of clock controlled transcription factors. mRNA expression of core clock genes – (a) Dec1, (b) Dec2, and (c) Pparα was assayed in the liver of mice (n = 3 per time point) subjected to the following feeding regimens: ad libitum (AL) – blue circles, solid line; 30%CR – red squares and solid lines; TR– orange triangles and solid lines, fasting (F) –green cross and solid lines. For convenience all data are double plotted. Data represents mean ± SD; statistically significant ($p < 0.05$) effects of the feeding (analyzed by two ways ANOVA) at a given time point are indicated by: (a)- between AL and CR groups, (b) – AL and TR, c- AL and fasting, (d)- CR and TR, (e) – CR and Fasting, (f)- TR and Fasting. Light and dark bars at the bottom represent light and dark phase of the day. ZT0 is the time when light is on and ZT12 is the time when light is off.

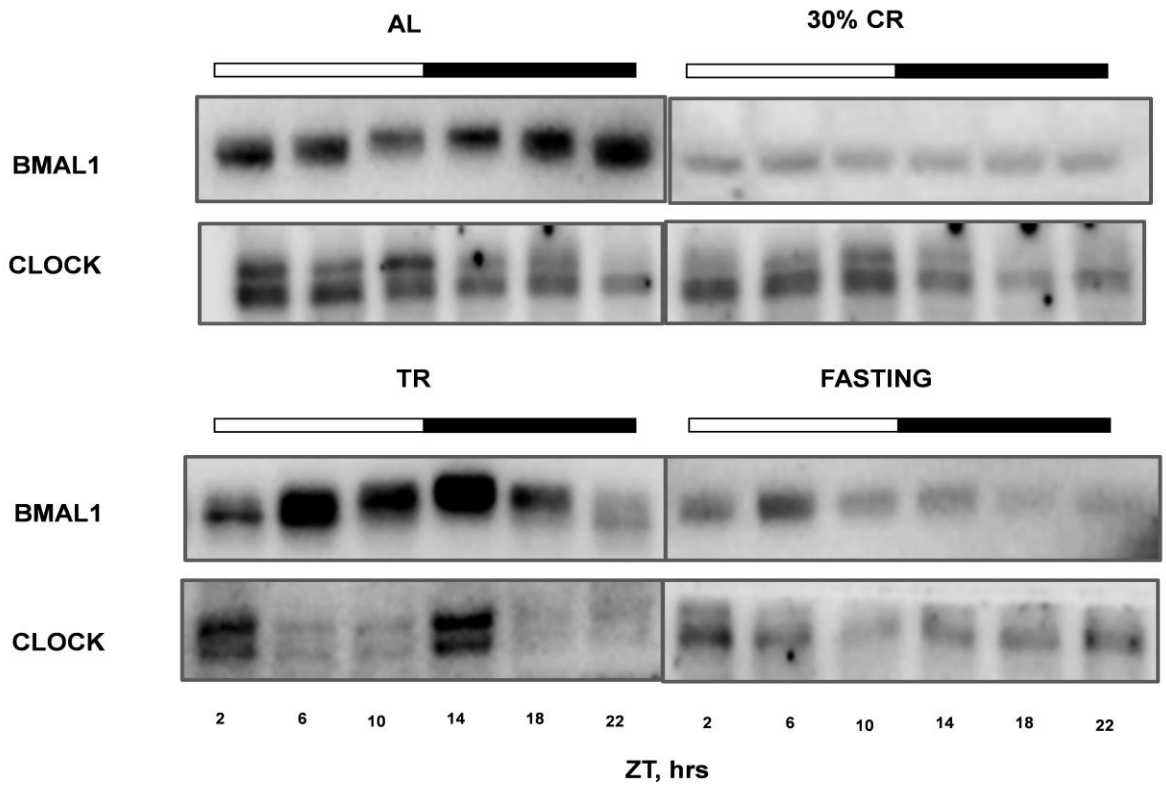

Figure 3-6. Effect of CR on protein levels of clock transcription factors. Representative Western blots for clock proteins; BMAL1, and CLOCK were assayed in the livers of mice (liver samples from three mice were pooled together at each time point) subjected to the following feeding regimens: AL – ad libitum, CR – 30% calorie restriction, TR –time-restricted feeding, F – fasting. Light and dark bars on the top of the figure represent light and dark phase of the day. ZT0 is the time when light is on and ZT12 is the time when light is off.

Effect of CR on core clock protein levels.

Core clock proteins, PER1, PER2 and CRY1 were also tested in liver of different feeding regimen groups, 30% CR, TR, Fasting and AL. These proteins have also been demonstrated to oscillate rhythmically in AL as shown in our study (Lee et al. 2001; Ye et al. 2014). Two-way ANOVA analysis showed CRY1 **(Figure 3-7a)** to be significantly reduced by CR and Fasting that was statistically significant, while on the other hand, though PER1 and PER2 **(Figure 3-7b and c)** were also reduced upon CR it was not statistically significant. Cosinor wave analysis showed PER1 and PER2 to temporally oscillate in AL and TR, with peak around ZT14, while in Fasting the peak was around ZT2 for PER1 and ZT22-2 for PER2. CRY1 expression did not show rhythmicity according to the analysis.

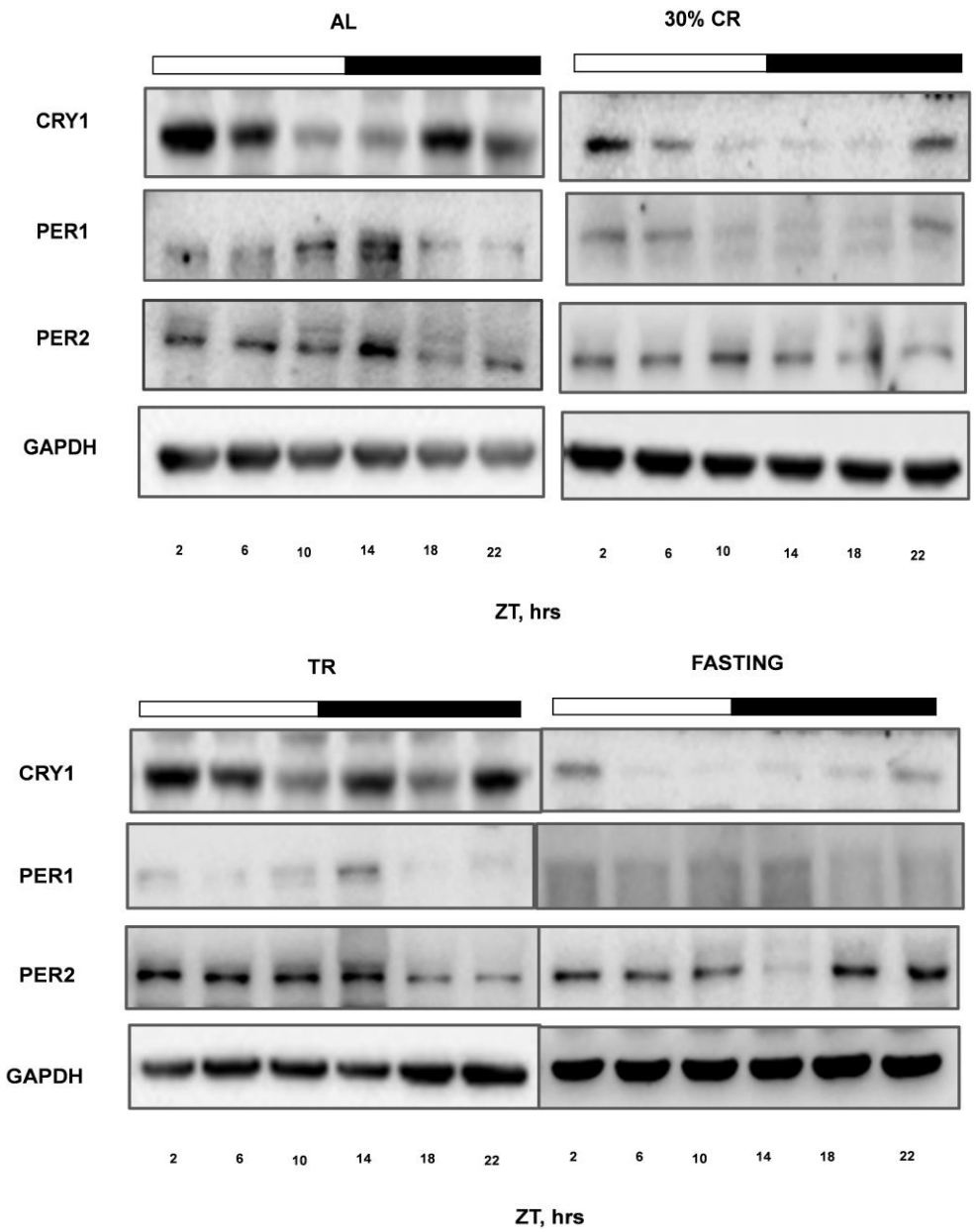

Figure 3-7. Effect of CR on protein levels of circadian clock repressors. Representative Western blots for clock proteins; PER1, PER2, CRY1 AND CRY2 were assayed in the livers of mice (liver samples from three mice were pooled together at each time point) subjected to the following feeding regimens: AL – ad libitum, CR – 30%, TR, and F. Light and dark bars on the top of the figure represent light and dark phase of the day. ZT0 is the time when light is on and ZT12 is the time when light is off.

Effect of CR on core clock genes expression in Bmal1 mutant mice.

Since CR affected the mRNA and protein levels of core clock genes we further decided to study the involvement of BMAL1 in CR effects. For this, we analyzed the expression of some of the core clock genes in liver of Bmal1-/- animals subjected to 30% CR for 2 months. As shown previously, our data too demonstrated arrhythmicity in Per1, Per2 and Cry1 expression in AL fed Bmal1-/- mice. Three-way ANOVA analysis showed that CR affected all three genes expression in wild-type (WT) but in Bmal1-/- the effect was only statistically significant for Cry1 (**Figure 3-8**). Hence, Bmal1 is required for the CR effect on Per1 and Per2 but not for Cry1 expression.

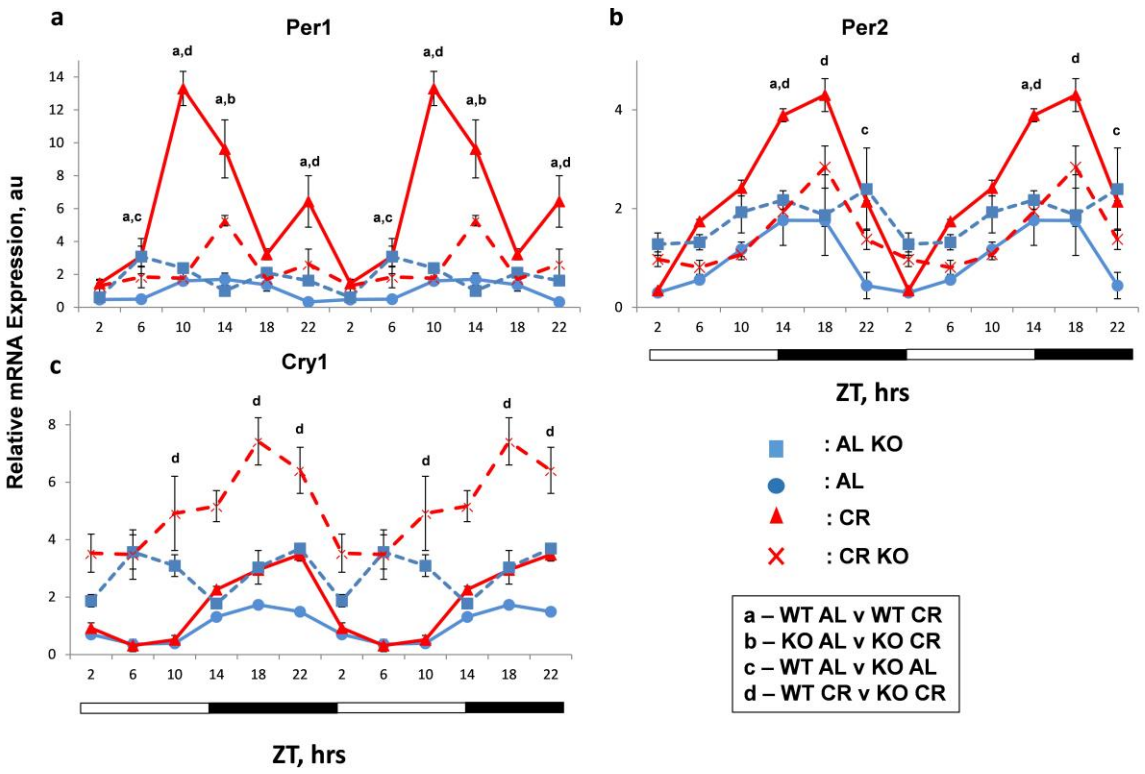

Figure 3-8. Effect of CR on rhythms of clock genes in Bmal1-/- mice. mRNA expression of core clock genes (a) Per1, (b) Per2, and (c) Cry1 were assayed by qPCR in the liver collected at 4 hours interval over period of 24 h from wild type (WT) and Bmal1− /− (KO) mice (n = 3 per time point) subjected to the AL and CR: WT AL controls-blue circles and solid lines; WT CR- red triangles and solid lines; KO AL –blue squares, dashed lines; KO CR- red cross and dashed lines. All the graphs are double-plotted. Data represents mean ± SD; statistically significant ($p < 0.05$) differences between the genotypes and effects of different diets across the time (analyzed by three-way ANOVA) are indicated by: (a)- between WT AL and WT CR groups, (b) – KO AL and KO CR, c- WT AL and KO AL, (d)- WT CR and KO CR. Light and dark bars at the bottom represent light and dark phase of the day. ZT0 is the time when light is on and ZT12 is the time when light is off.

Effect of CR on longevity candidate genes expression.

Microarray data analysis previously showed that CR affected multiple genes in different tissues, such as liver, brain, skeletal muscle of mice, among which were 2 core clock genes, Per1 and Per2. Out of 7 studies reported in liver only 2 studies showed up regulation of Per1 and Per2 while others did not show any change (Swindell 2007; Sun et al. 2013). Another microarray study by Swindell showed genes pooled from different studies that were either changed in long-lived dwarf mice or affected by different diets, gender and age. Based on the expression of these genes in long-lived dwarf mice they were proposed as longevity candidate genes (Swindell 2007). However, one important drawback in these studies is that the tissues were collected at one-time point and was sometimes not even reported, making the comparison difficult. Thus, we decided to study some of these longevity candidate genes in circadian manner, since the effect of CR on Per1 and Per2 gene expression was significant at some while not at other time points. We assayed the mRNA expression of these longevity candidate genes across different times of the day in the liver of mice on different diets as above. Out of the 10 genes we selected expressions of *Fmo3, Cyp4a14, Parp16 and Igfals* have been shown to be up regulated upon CR whereas *Cyp4a12b, Mup4, Hes6, Hsd3β5, Serpina12 and Alas2* expressions were reported to be down regulated in CR samples. In our study, we did not find statistically significant difference in *Igfals* and *Serpina12* genes expression upon CR **(Figure 3-9 a, b).** The expression of flavin monooxygenase gene, *Fmo3*, Cyp450 gene, *Cyp4a14* and Poly ADP-ribosyl polymerase (*Parp16*) were significantly up regulated upon CR at several time

points and showed high amplitude rhythms upon CR compared with AL, TR and Fasting controls **(Figure 3-9 c, d and e)**.

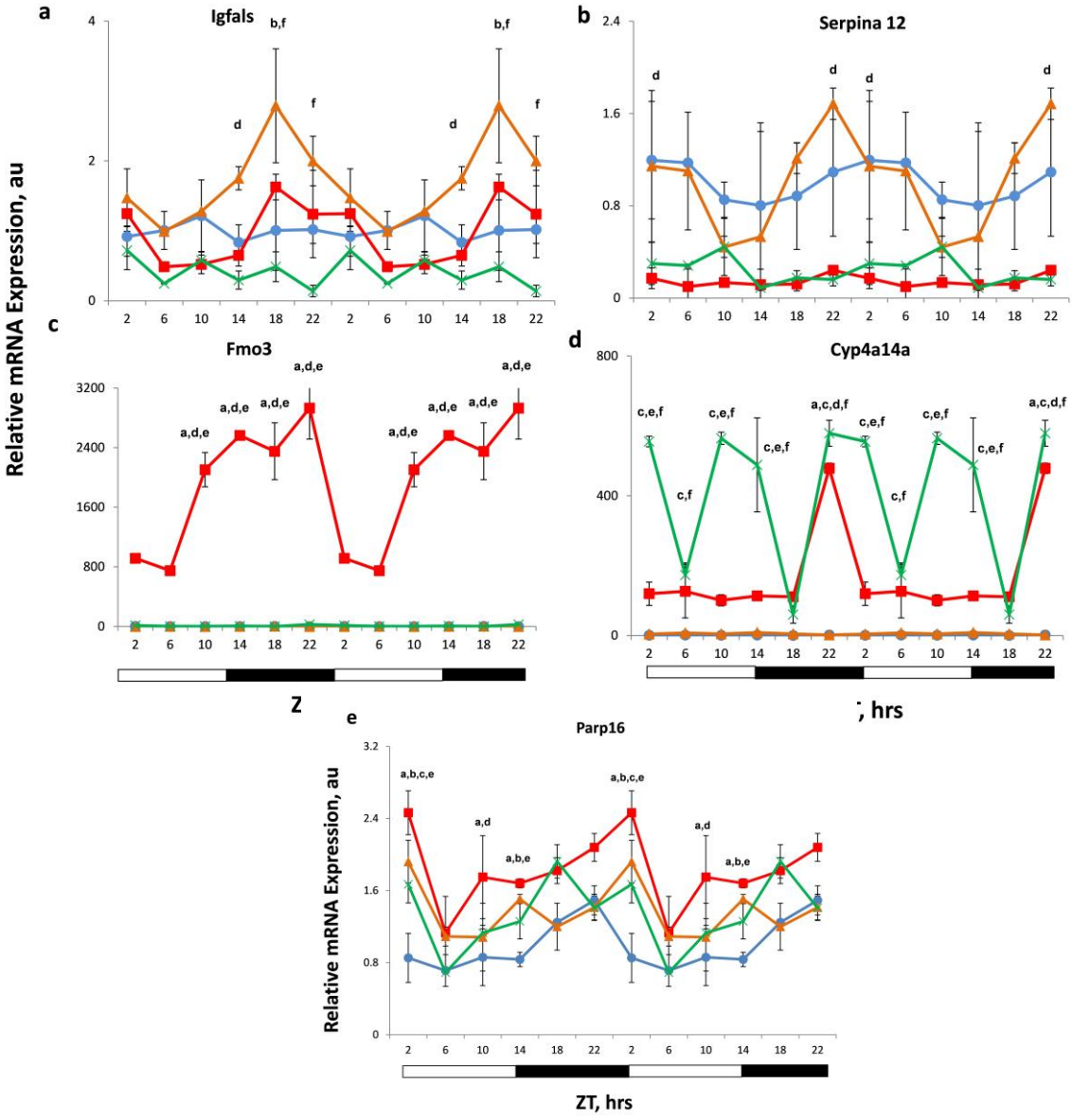

Figure 3-9. Effect of CR on rhythms of longevity candidate genes. mRNA expression of core clock genes (a) Igfals, (b) Serpina12, (c) Fmo3, (d) Cyp4a14a and (e) Parp16 was assayed in the liver of mice (n = 3 per time point) subjected to the following feeding regimens: ad libitum (AL) – blue circles, solid line; 30%CR – red squares and solid lines; TR– orange triangles and solid lines, fasting (F) –green cross and solid lines. For convenience all data are double plotted. Data represents mean ± SD; statistically significant ($p < 0.05$) effects of the feeding

Expression of other Cyp450 gene, *Cyp4a12b*, **(Figure 3-10a)** was dramatically down regulated upon CR, while there was no effect of TR or Fasting. *Mup4* and *Alas2* **(Figure 3-10b, c)** are other two genes whose expressions were also reduced at few but not all time points in CR compared to AL, TR and Fasting. Moreover, expressions of *Hsd3β5 and Hes6* **(Figure 3-10d, e)** were down regulated not only in CR (in agreement with the published microarray data), but also, in Fasting compared to AL and TR controls. Interestingly, some of the genes such as *Hsd3β5* and *Serpina12* exhibited rhythmicity according to the Cosinor wave analysis. These data show that gene expression can significantly vary across the day and demonstrates the importance of the circadian approach for transcriptomic analysis. These results show that CR has a significant effect on *Fmo3, Cyp4a14a, Cyp4a12b, Mup4, Hes6, Parp16,* and *Hsd3B5* gene expression and the effect is different from that of TR and Fasting.

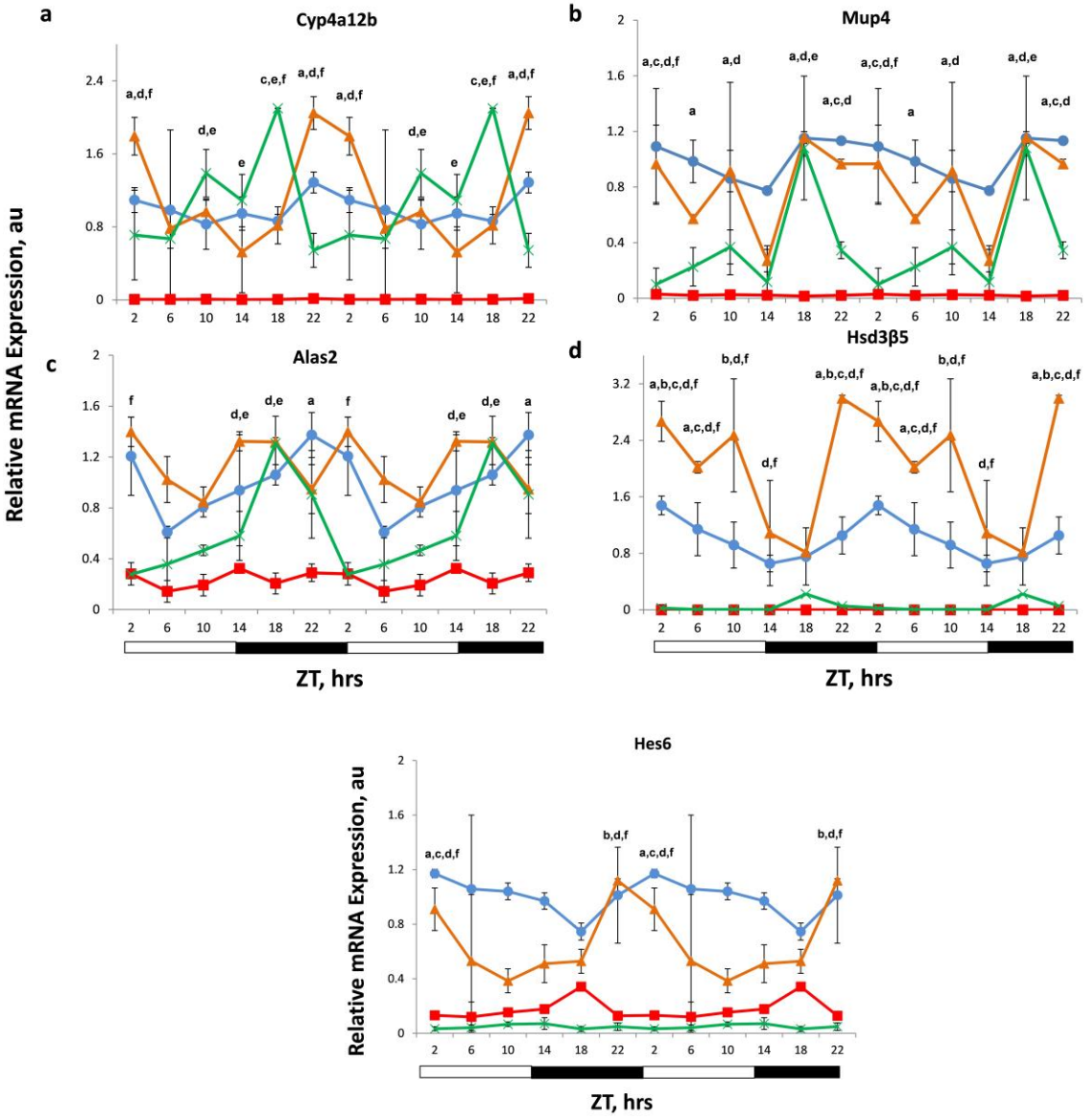

Figure 3-10. Effect of CR on rhythms of longevity candidate genes. mRNA expression of core clock genes (a) Cyp4a12b, (b) Mup4, (c) Alas2, (d) Hsd3β5 and (e) Hes6 was assayed in the liver of mice (n = 3 per time point) subjected to the following feeding regimens: ad libitum (AL) – blue circles, solid line; 30%CR – red squares and solid lines; TR– orange triangles and solid lines, fasting (F) –green cross and solid lines. For convenience all data are double plotted. Data represents mean ± SD; statistically significant ($p < 0.05$) effects of the feeding (analyzed by two

ways ANOVA) at a given time point are indicated by: (a)- between AL and CR groups, (b) – AL and TR, (c)- AL and Fasting, (d)- CR and TR, (e) – CR and Fasting, (f)- TR and Fasting. Light and dark bars at the bottom represent light and dark phase of the day. ZT0 is the time when light is on and ZT12 is the time when light is off.

3.5 Discussion

Circadian Clock and Feeding.

Timed-feeding is an important cue that is known to affect the circadian clock. Indeed it has been reported that time-restricted feeding (TR) affects the circadian clock (Froy & Miskin 2010). Feeding time has been shown to affect the expression of clock genes in the liver: for instance, restricting the intake of food to daytime in the nocturnal animals like rodents leads to the phase shifting in the expression of the clock genes compared with the AL control mice that predominantly eat during the nighttime (i.e. physiological time for rodents) (Damiola et al. 2000; Hara et al. 2001; Bray et al. 2013). In addition to time of feeding, food composition also plays another major role affecting gene expression. In the light of this, high fat diet disrupts the high amplitude rhythms in most of the clock genes expression in the liver (Hatori et al. 2012), however, when given in a time-restricted manner it restores the expressions in the rhythms. In spite of so many studies on clock and nutrients, the detailed mechanisms connecting feeding and nutrients with the clock genes have not been addressed. Clock interacting chromatin modifying enzymes that are responsive to nutrients or energy state have been proposed as being one of the important mediators (Aguilar-Arnal & Sassone-Corsi 2014).

Calorie restriction and clock

Calorie restriction has multiple beneficial effects through physiological and metabolic changes in turn contributing towards delayed aging and longevity (McDonald & Ramsey 2010; Piper & Bartke 2008). There are two major characteristics of CR in

mammals 1) reduced intake of calories and 2) periodicity in the food availability. The periodicity refers to fixed time of feeding during the day which is continued on daily basis as in time-restricted feeding. Since TR is known to affect the circadian clock and CR is kind of TR we decided to study the effect of CR on the circadian clock gene expression. Thus in the study described above we compared the effect of short term 30% CR with other feeding regimen such as TR and Fasting. The time of feeding was same for all the groups i.e. during the beginning of the dark phase. Since the food was provided at the physiological time at which these animals are active, none of the feeding regimen dramatically affected the phase of the clock genes expression.

CR effect on clock genes expression in WT mice.

CR significantly induced the expression of several clock and clock-controlled genes. The effect of CR on the circadian clock genes expression has been assayed previously in the SCN (Mendoza et al. 2005). However, it is difficult to compare the results of Mendoza study with our results directly since different tissues were tested and the time of feeding was different (at ZT6 in Mendoza et al. and at ZT14 at our study). Interestingly, in both the studies CR has significant effect on the expression of several but not all tested clock genes. Another study showed a microarray data analysis of the list of genes pooled from multiple independent studies that are influenced by CR in different tissues in mice (W R Swindell 2008; William R. Swindell 2008). This study identified two clock genes, *Per1* and *Per2*, to be affected by CR among the list of genes whose expression was also affected upon CR in many tissues. Per1 and Per2 expression was up regulated in only two out of the seven liver samples tested(W R Swindell 2008). Our data which also showed

induction of *Per1* and *Per2* expression at different times of the day upon CR indeed resolved the discrepancy in the above study due the different times of the tissue collection. Previously, *Per1* expression was shown to be up regulated upon sharp reduction in calories i.e. fasting (Kawamoto et al. 2006) while Per2 was downregulated (Kobayashi et al. 2004). Our study confirmed this similar up and down regulation in Per1 and Per2 expression upon fasting respectively. Similar to fasting, Per1 expression was also increased in CR but the response was more dramatic while, on the contrary, Per2 expression was also increased significantly in CR. Similar contrasting results were also obtained in *Per3*, *Cry2* and *Ror γ* mRNAs, where fasting reduced their expression compared to CR. Importantly, fasting did not have any effect on Bmal1 expression which was induced by CR and Dec2 that was suppressed by CR.

Our data signifies that CR affects the clock and clock controlled gene expression in time and gene specific manner. Certainly, BMAL1 is not the only transcription factor affecting the expression of these clock and clock controlled genes, several other transcription factors such as CREB, are also known to drive the expression of these genes while Bmal1 expression itself is regulated by several transcription factors such as REVs, RORs, PGCα-1 and PPARα. Thus, it is also possible that CR affects activity of some or all of these different other transcription factors making the effect of CR on gene expression even more complicated. Despite this the effect of CR on the molecular circadian clockwork in the liver could, most likely, be due to metabolic adaptation to CR, as the effect is different from the effects of periodic feeding with no restriction of calories and sharp reduction in calorie intake.

CR effect on clock genes expression in Bmal1 mutant mice.

BMAL1 being one of the major transcription factor affected by CR, we analyzed the effect in Bmal1-/- mice. Some of the clock genes expression was significantly impaired by CR in the liver of Bmal1− /− mice, which supports the involvement of BMAL1. However, CR did not affect all BMAL1 targets such as the induction of Cry1 expression by CR was intact even in Bmal1− /− mice, which further suggested the existence of both BMAL1-dependent and BMAL1-independent mechanisms. Food anticipation rhythms have been demonstrated in the Bmal1-independent manner thus, it is not surprising that the BMAL1-independent mechanisms exists too (Mohawk & Menaker 2009; Pendergast et al. 2009). The circadian clock is regulated at different levels. Though BMAL1 acts at the core of the feedback loop, it is an organization of multiple accessory negative and positive feedback loops: E4BP4 which is itself regulated by BMAL: CLOCK complex, contributes towards the regulation of *Per2, Cry1, Clock* and *Ror γ* genes expression, and transcriptional factors, REV-ERB and ROR family control the expression of *Bmal1* and *E4bp4* (Fang et al. 2014). Furthermore, circadian clock is regulated at post transcriptional and post translational levels (Kondratov, Kondratova, Lee, et al. 2006) hence, simply studying one transcriptional factor would not be sufficient to explain the effect of CR on gene expression.

CR effect on clock protein expression.

At the protein level not much studies have been done to understand the effect of different feeding regimen on clock genes. In our study while we observed increased expression of several clock genes at the mRNA level, we detected significant reduction at

the protein level of some of the clock genes, especially CRY1. BMAL1, CLOCK, PER1 and PER2 showed a tendency towards decrease in 30% CR but was not statistically significant, on the other hand, CRY1 was significantly down regulated upon 30% CR.

Our mRNA and protein results of core clock genes upon CR are contradictory with each other, since, we observed upregulation at the mRNA level while downregulation at the protein level in CR. Upregulation of BMAL1 target genes such as Pers and Crys are an indication of increased transcriptional activity of BMAL1. Our protein data is in somewhat agreement with this increased transcriptional activity of BMAL1, as reduction in BMAL1 and CLOCK protein levels suggests increased degradation of these proteins and this in turn is directly proportional to the activity of BMAL1. It is also worth noting that we have only checked for the total protein levels while not its post translational modifications such as phosphorylation. It is known that BMAL1 activity and stability is regulated by its phosphorylation status, therefore, it is possible the ratio of total: phosphorylated BMAL1 is low and there could actually be increased phosphorylated BMAL1 which would suggest increased BMAL1 activity. Also, CRYs and PERs have repressor function in the feedback loop wherein they inhibit their own transcription through inhibition of BMAL1 activity, thus, reduced CRYs and PERs upon CR again points towards high BMAL1 activity. Another possibility is that there could be reduced production of proteins which means that the effect is at the translation level rather than at the transcription level. Additionally, we cannot rule out the possibility of regulation at the post translational level too. Like BMAL1, CRY1 is phosphorylated by several kinases including Casein kinases (CKε and δ) and AMPK, a serine/threonine kinase. AMPK, particularly, is activated upon treatment with

low glucose conditions. The phosphorylation by AMPK targets CRY1 for proteasome-mediated degradation (Lamia et al. 2009). Also, AMPK phosphorylates CKδ and in turn contributes towards clock regulation. CR and fasting both represent a condition of low blood glucose level (Trepanowski et al. 2011; Anson et al. 2003). Thus, it is possible that AMPK could be activated by CR and in turn could lead to reduced CRY1 protein level because of enhanced degradation. It is worth noting that while CR and fasting represent low glucose level condition, it is relatively moderate reduction as compared to the severe glucose depletion condition in cell culture. Therefore, it is still a matter of debate as to whether this level of reduction in glucose levels is sufficient to activate AMPK in vivo. In such a case other mechanisms also cannot be ignored i.e. reduction in CRY1 translation or increased ubiquitin ligase mediated degradation (Lee et al. 2014; Yoo et al. 2013). Though CR and fasting both dramatically reduce the protein levels, it would be hard to say that the mechanism through which it occurs will be the same for both the feeding regimen. Also, PER1 and PER2 protein levels were reduced in contrast to its mRNA levels that was induced by CR. Studies have shown that PER stability is regulated by its phosphorylation by Casein kinases and complex formation with these kinases and CRYs and thus it is possible that reduction in CRY1 would be associated with reduced complex formation with PERs and hence reduced PER stability and decreased protein levels. Irrespective of the mechanisms affecting circadian clock protein levels, fasting and CR have very different effects on the mRNA expression n. While CR affects the mRNA expression of multiple circadian clock and clock controlled genes, fasting did not significantly demonstrate such an effect on the mRNA expression. Hence though the reduction in CRY1 protein may

contribute to the increased expression of clock genes in CR, absence of such an effect in fasting argues for the existence of CRY-independent mechanisms too.

CR effect on longevity candidate genes expression.

Microarray studies put down several dozens of genes through the transcriptome analysis as being affected by CR and were termed as the longevity candidate genes. Importantly, in most of these studies the analysis was performed at any one-time point that was not well defined. We randomly picked ten such candidate longevity genes and decided to analyze them using circadian approach. The genes selected were known to influence different processes such as xenobiotic metabolism, protein binding and transport, steroid biosynthesis, heme binding and cell proliferation (Swindell 2007). We found that two out of ten selected genes were truly regulated exclusively by CR at all the tested time points, for example, expression of *Cyp4a12b* was suppressed whereas expression of *Fmo3* was induced by CR specifically but not by other feeding regimen. Four other longevity candidate genes (*Parp16, Alas2, Igfals* and *Mup4*) expression were also changed upon CR, but the effect was time of the day-dependent. This time-of-the-day dependence effect of CR could be the reason for the contradiction in the previous studies for some of these genes: for instance, according to our results *Parp16* is significantly up regulated only at ZT2, 10 and 14 but not at ZT6, 18 and 22. Out of the 10 total tested genes 7 genes expression demonstrated changes across the day while only 2 were found to be circadian. Since both CR and Fasting up or down regulated most of the longevity candidate genes, the effect on these genes cannot said to be CR-specific. Not many studies analyze gene expression in circadian manner while our data puts a light on the importance of the gene expression

analysis across the circadian cycle which should be taken into consideration for future studies. Importantly, we only analyzed the transcript level of these different longevity candidates, however, the effect at the protein level has not been studied. Thus, taking into consideration the opposite effect at the clock mRNA and protein level of CR, it would be interesting to see what would be the effect of CR on the protein levels of these different longevity candidates. Though these genes have been shown to be changed dramatically upon CR their importance in aging and lifespan extension has not been studied directly which also needs to be addressed in future. Our results thus argue for taking into account the previous studies with caution.

Because 6 out of the 10 tested longevity-associated candidate genes, and 6 out of 11 tested circadian clock genes in our study were shown to be affected by CR which indicated that clock genes could be considered as longevity-associated candidate genes. This is further corroborated by longevity analysis study among different species that recognized circadian rhythms as one of the candidate target contributing towards the evolution of longevity (Li & De Magalhães 2013) which further strengthens our hypothesis.

Difference between TR and CR effects

Circadian clock in the peripheral tissues is affected by time-restricted feeding (TR) which is shown by studies where food is provided during the inactive period in rodents. While we and others have demonstrated that CR and TR have different effects on circadian clocks: in contrast to CR, which affects the clock in the SCN, TR cannot entrain the SCN clock (Challet 2010). Importantly however metabolic benefits of TR have been

demonstrated in rodents on high fat diet (Hatori et al. 2012), yet the benefits of TR for animals on the regular chow diet have not been assayed. While there is a clear difference between CR and TR in terms of expression of circadian clock, clock-controlled and longevity candidate genes we cannot exclude a potentially beneficial impact of TR. Another point worth noting is while CR extends lifespan, TR does not extend lifespan in rodents. Circadian clock is a major regulator of metabolism (Asher & Sassone-Corsi 2015) and CR also drives the changes in metabolism in different organisms (Piper & Bartke 2008), interestingly our data demonstrates the effect of CR on circadian clock and clock controlled transcription factors. Therefore, beneficial effects of CR on longevity and metabolism correlates well with the effects of CR on the circadian clocks.

Future Directions

Future studies will focus on studying the mechanism of effect of CR especially at the translational and post-translational level such as phosphorylation of different core clock genes. Phosphorylation of clock proteins such as BMAL1, PERs and CRYs by different kinases targets them for degradation. Thus, it would be interesting to study ubiquitination by different ubiquitin ligases such as Fbxl3 and Fbxl21 that are known to target these proteins for proteasome-mediated degradation. Since, AMPK is a kinase which has been demonstrated to be affected by CR and while it is also involved in CRY degradation process, understanding the AMPK pathway regulated CRY degradation would be another important study. Moreover, we just studied the clock genes expression in one circadian clock mutant, Bmal1, what would be the effect in other clock mutants such as Cry mutants also needs to be addressed.

Conclusion

We propose that CR-dependent effect on the circadian clocks is necessary for the metabolic adaptation to CR. According to the existing paradigm, clock controlled transcription factors control the expression of multiple rate-limiting enzymes that are involved in various metabolic processes (Mazzoccoli et al. 2012). Thus circadian clock is required for proper synchronization of the downstream metabolic processes. Probably CR recruits circadian clock for the maintenance of physiological homeostasis under conditions of limited energy supply which is required for the optimization of biochemical and metabolic processes. Thus, circadian clock is acting at the core through which CR produces its beneficial effects on longevity and thus is required for the fitness of the organism. Future studies on the effects of CR in other circadian clock mutants will help to further understand the connections between clock and CR.

CHAPTER IV

CIRCADIAN CLOCKS GOVERN CALORIE RESTRICTION–MEDIATED LIFE SPAN EXTENSION THROUGH BMAL1- AND IGF-1-DEPENDENT MECHANISMS.[2]

Sonal Patel et al.

4.1 Abstract

Calorie restriction (CR) increases longevity in many species by unknown mechanisms. The circadian clock was proposed as a potential mediator of CR. Deficiency of the core component of the circadian clock-transcriptional factor BMAL1 (brain and muscle ARNT [aryl hydrocarbon receptor nuclear translocator]-like protein 1)—results in accelerated aging. Here we investigated the role of BMAL1 in mechanisms of CR. The 30% CR diet increased the life span of wild-type (WT) mice by 20% compared to mice on an ad libitum (AL) diet but failed to increase life span of Bmal1-/- mice. BMAL1 deficiency impaired CR-mediated changes in the plasma levels of IGF-1 and insulin. We

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detected a statistically significantly reduction of IGF-1 in CR vs. AL by 50 to 70% in WT mice at several daily time points tested, while in Bmal1-/- the reduction was not significant. Insulin levels in WT were reduced by 5 to 9%, while Bmal1-/- induced it by 10 to 35% at all time points tested. We propose that BMAL1 is an important mediator of CR, and activation of BMAL1 might link CR mechanisms with biologic clocks.

4.2 Introduction

Calorie restriction (CR) is a powerful intervention that delays aging and increases longevity in different organisms, including mammals (Fontana et al. 2010; Taormina & Mirisola 2014; Ravussin et al. 2015; Redman & ... 2011). The exact molecular mechanisms of CR are not known, though multiple theories have been put forward to show the beneficial effects of CR on health and lifespan. Some of the pathways proposed include mammalian target of rapamycin (mTOR) signaling pathway, the insulin/IGF-1 signaling pathways, and the sirtuin-controlled pathway which are affected by CR in animals and are considered to be some of the potential mediators of CR effect (Gallinetti et al. 2013; Gesing et al. 2014; Wang 2014). NAD dependent protein deacetylase, Sirtuin (silent mating type information regulation 2 homolog) 1 (SIRT1) activation has been shown to be necessary for the complete beneficial effects of CR(Mercken et al. 2014; Nogueira et al. 2012; Kitada et al. 2013; Kennedy et al. 1997). Indeed, CR fails to improve several behavioral and physiologic parameters in SIRT1-null mice, such as these animals do not demonstrate an increase in daily activity upon CR(Chen et al. 2005). Additionally, CR also demonstrates different metabolic changes in WT and SIRT1-null mice (Chen et al. 2008; Boily et al. 2008) and finally, life span of SIRT1-null mice is not increased upon CR (Boily et al. 2008). SIRT1

regulates several transcription factors, one of which is the helix–loop–helix transcription factor BMAL1 (brain and muscle ARNT [aryl hydrocarbon receptor nuclear translocator]-like protein 1) (Nakahata et al. 2008).

BMAL1 is a core transcription factor and an important component of the circadian clock (Bunger et al. 2000). Circadian clock is an internal clock that generates 24 hour rhythms in behavior, physiology, and metabolism (Green et al. 2008; Hunt & Sassone-Corsi 2007; Dibner et al. 2010; Froy 2011). Other functions of BMAL1 that may or may not be linked to its role as a clock component include, antioxidant defense, memory formation, control of metabolism, glucose homeostasis, and immune system (Rudic et al. 2004; Kondratova & Kondratov 2012; Musiek et al. 2013). Indeed, BMAL1 deficiency in mice leads to dramatically shortened life span and development of accelerated aging phenotype. However, this phenotype is unique to BMAL1 mutant mice and is not observed in other circadian clock mutants (Kondratov, Kondratova, Gorbacheva, et al. 2006). BMAL1 and its target transcriptional factors control downstream signaling pathways which are implicated in the CR driven benefits. In connection with that, BMAL1 regulates antioxidant defense mechanism of the organism because absence of BMAL1 is associated with oxidative stress and development of degenerative diseases (Musiek et al. 2013; Pekovic-Vaughan et al. 2014). Moreover, SIRT1 which is involved in CR effect, controls BMAL1 activity while in turn, BMAL1 may regulate SIRT1 activity through transcriptional control of nicotinamide phosphoribosyl transferase (NAMPT), a rate-limiting enzyme in NAD biosynthesis, thus, forming a feedback loop (Chang & Guarente 2013; Ramsey et al. 2009; Nakahata et al. 2009). Furthermore, TOR signaling is also under

regulation by BMAL1 as was shown by the dysregulation of mTOR activity in BMAL1 deficient mice (Jouffe et al. 2013; R V Khapre et al. 2014; Cornu et al. 2014). We thus hypothesized that BMAL1 could be acting as a part of the mechanism required for the beneficial effects of CR in health and longevity. For this we studied the effects of CR on different parameters of Bmal1-/- mice and observed that CR did not improve the condition of these mice nor did it increase the longevity of these animals, suggesting that BMAL1 is an important component of the CR mechanisms required for CR mediated effects on lifespan. Thus, our hypothesis signifies the important link between the biological clocks and CR in mammals.

4.3 Materials and Methods

Details of the Animal experiment, Feeding Regimen Schedule, mRNA and Protein analysis have been explained in Chapter II.

4.4 Results

Behavioral response of Bmal1-/- mice to 30% CR.

Testing the food anticipatory activity is one of the ways to analyze the behavioral activity. Studies in which WT mice subjected to CR have demonstrated robust increase in locomotion around the time of feeding (i.e. food anticipation) (Luby et al. 2012). Locomotor activity is independent of the circadian clock function that has been shown in the Bmal1-/- mice. We therefore, decided to investigate the CR-induced food anticipatory activity in Bmal1-/- mice. For this we monitored in cage locomotion for 3 consecutive days. Results of behavioral activity in male and female mice are presented in **Figure 4-1**

and 4-2 respectively. Three-way ANOVA analysis to demonstrate the interaction of genotype vs diet vs time was found to be statistically significant ($F = 2.688$, $P = 0.015$). For both WT and Bmal1-/- mice, an increase in activity was observed at ZT12 and ZT13 which was statistically significant ($P < 0.05$, post hoc analysis using Bonferroni correction), an indication of food anticipation. This suggests that BMAL1 is not required for the CR-mediated food anticipatory activity.

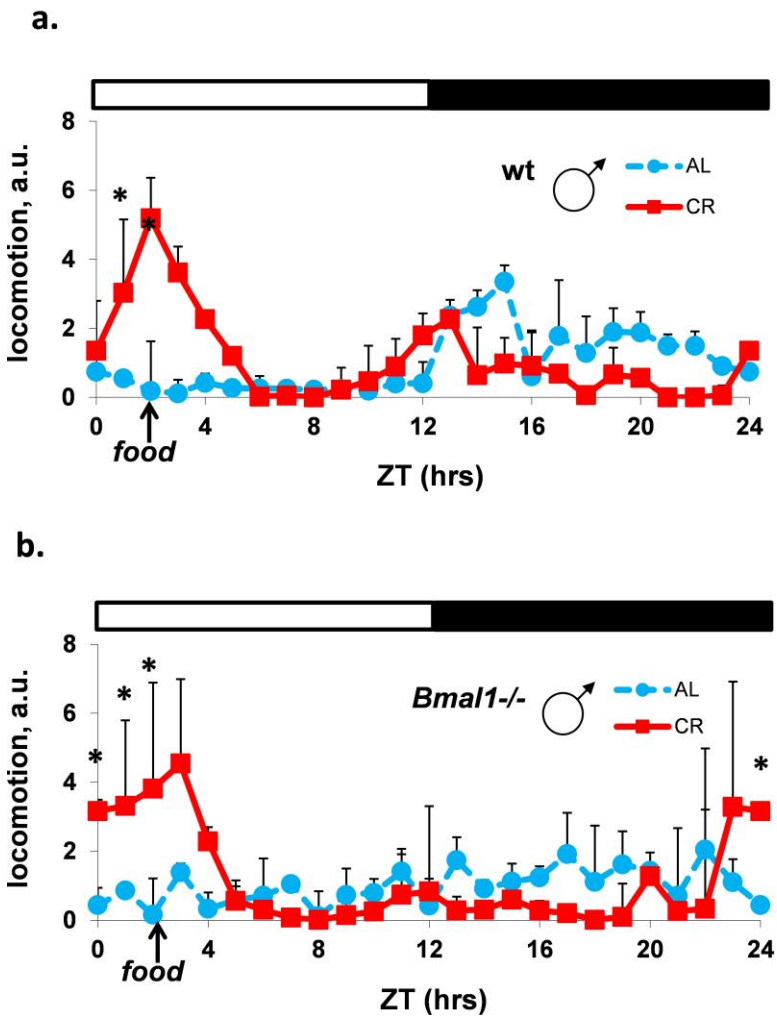


Figure 4-1. CR effect on locomotor activity in WT and Bmal1-/- males. In-cage locomotion of WT (a) and Bmal1-/- (b) male mice. Mice were subjected to following diets: AL, blue circles, blue dotted lines; 30% CR, red squares, solid red lines. Each graph represents average normalized activity per hour and SE (3 consecutive days for every animal, 5 mice per group). a.u., arbitrary units of normalized daily locomotor activity; total daily activity was set as 24 a.u. 0 and 24 h time points were double plotted. *Statistically significant increase in locomotor activity before feeding (food anticipation) for CR group compared to AL group. Light and dark bars represent light and dark phase of day. ZT0 is time when light is on and ZT12 is time when light is off. Food for CR group was provided at ZT14.

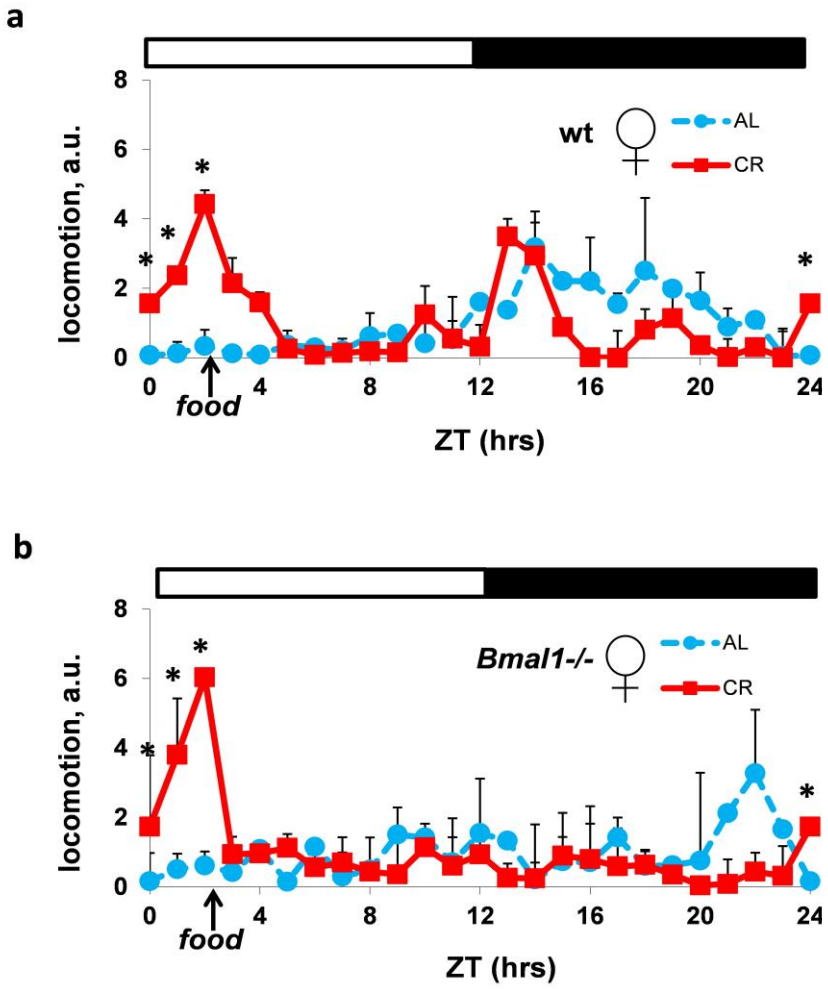
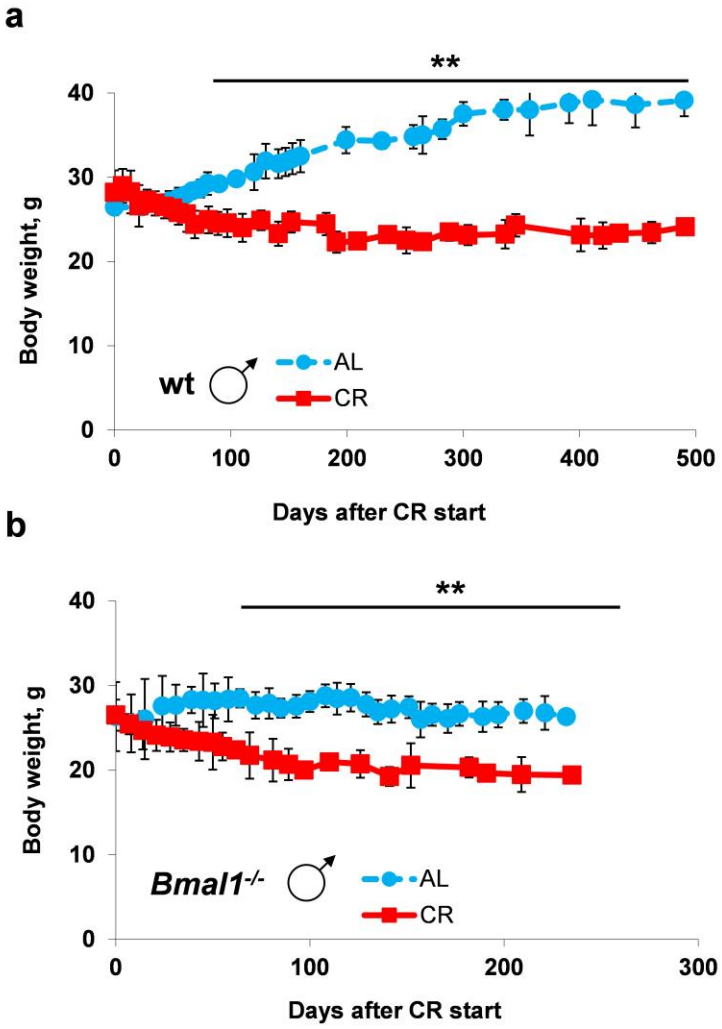


Figure 4-2. CR effect on locomotor activity in WT and Bmal1-/- females. In-cage locomotion of WT (a) and Bmal1-/- (b) female mice. Mice were subjected to following diets: AL, blue circles, blue dotted lines; 30% CR, red squares, solid red lines. Each graph represents average normalized activity per hour and SE (3 consecutive days for every animal, 5 mice per group). a.u., arbitrary units of normalized daily locomotor activity; total daily activity was set as 24 a.u. 0 and 24 h time points were double plotted. *Statistically significant increase in locomotor activity before feeding (food anticipation) for CR group compared to AL group. Light and dark bars represent light and dark phase of day. ZT0 is time when light is on and ZT12 is time when light is off. Food for CR group was provided at ZT14.

Effect of 30% CR on body weights of Bmal1-/- mice.

Next we assayed the body weights of WT and *Bmal1-/-* mice on 30% CR which was started at 3 month of age of mice. At this age the WT and *Bmal1-/-* mice are indistinguishable based on the appearance and body weight. At the beginning of the experiment, average body weights were as follows: WT males, 27.3 ± 2.1 g; *Bmal1-/-* males, 26.4 ± 3.3 g; WT females, 20.7 ± 1.1 g; and *Bmal1-/-* females, 20.6 ± 1.6 g (**Figure 4-3**).



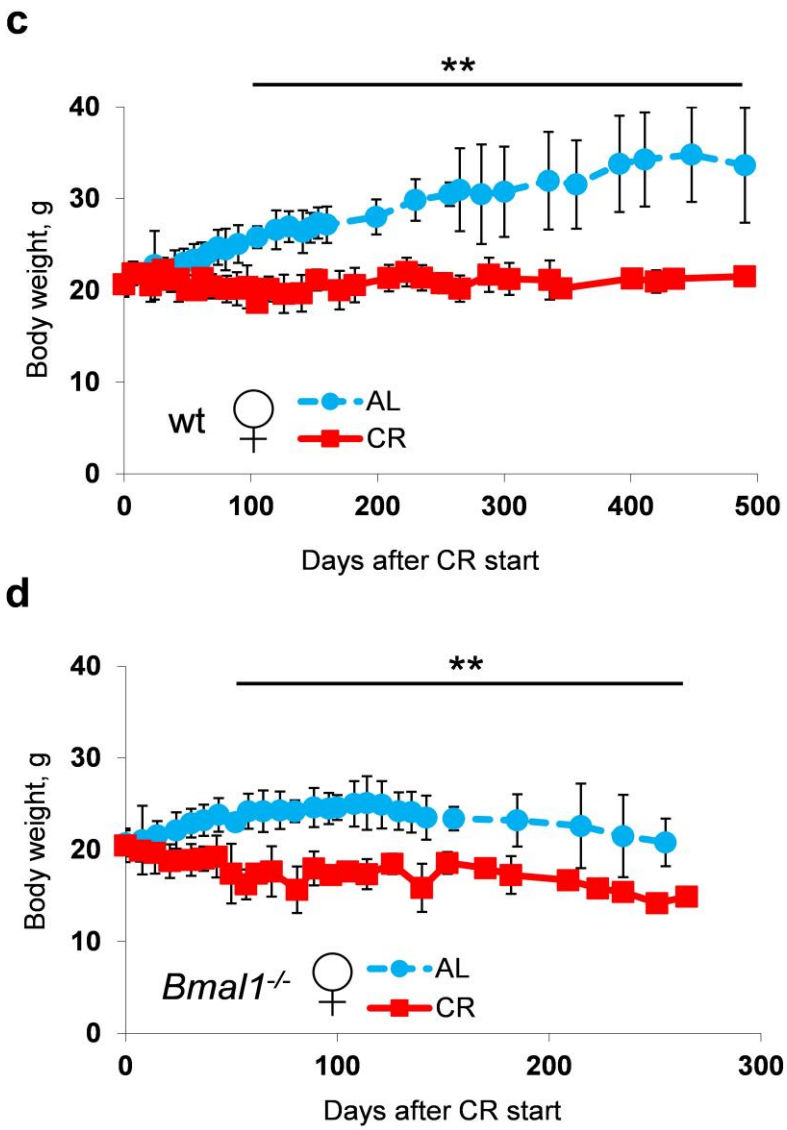


Figure 4-3. Gender based effect of 30% CR on body weight of wild type and Bmal1-/- mice. (a) and (b) Changes in body weight of male wild type (upper panel, N=16) and Bmal1-/- (upper panel, N=13) mice respectively. (c) and (d) Changes in body weight of female wild type (lower panel, N=14) and Bmal1-/- (lower panel, N=19) mice respectively. CR - red squares and solid red lines, AL - blue circles and blue dotted lines. ** - statistically significant difference between AL and CR groups was detected at indicated range of age.

As expected, body weight of both WT and Bmal1-/- mice was significantly affected by 30% CR compared with AL controls (**Figure 4-4).** WT mice showed normal response to the 30% CR as there was some reduction in body weight during the first few weeks of CR, after which their body weight was stabilized and did not change during the rest of the experiment. Although, the stabilized weight was about 85% of their original weight for males and about 90% of the original weight for females. On the contrary, the body weight of *Bmal1-/-* mice demonstrated significant reduction upon 30% CR as compared to the WT mice. While WT mice body weight stabilized, *Bmal1-/-* mice did not demonstrate similar stabilization in the body weight upon 30% CR.

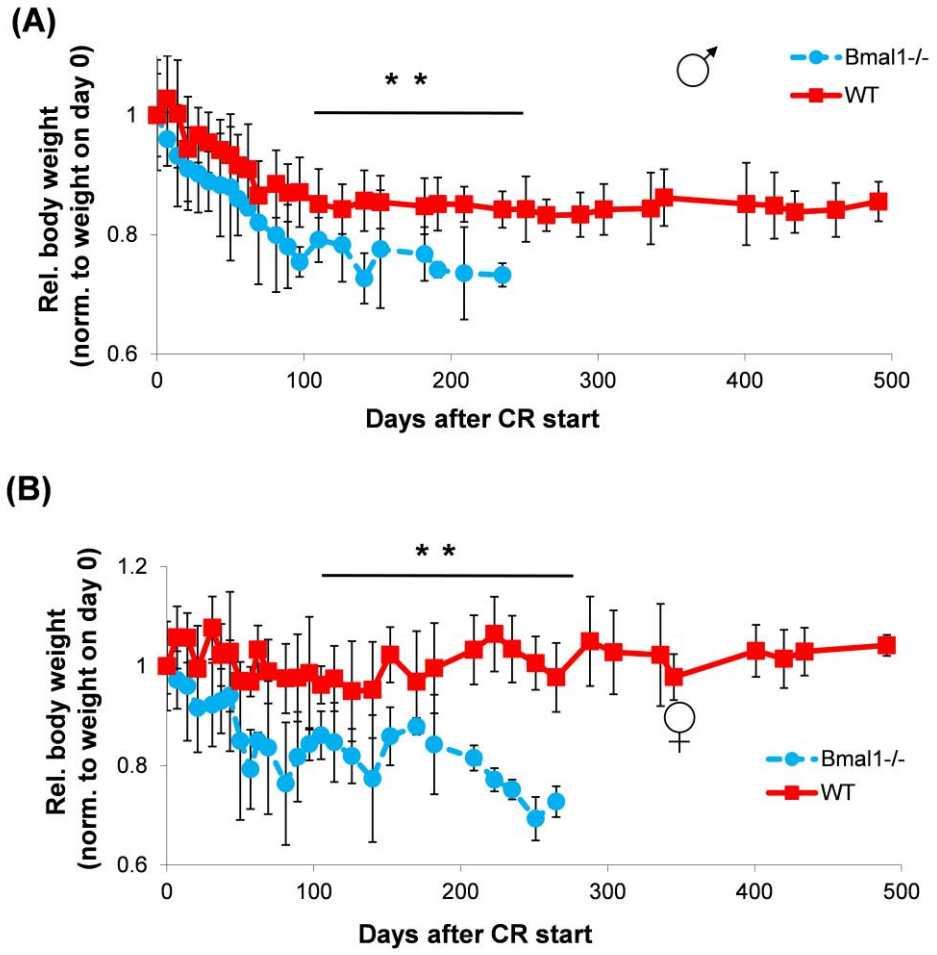


Figure 4-4. Effect of 30%CR on body weights of WT and Bmal1-/- mice. A) Changes in body weights of male WT mice (n = 16, red squares and solid red line) and Bmal1-/- mice (n = 13, blue circles and blue dotted line). B) Changes in body weights for female WT (n = 14, red squares and solid red line) and Bmal1-/- (n = 19, blue circles and blue dotted line) mice. Mouse weights were normalized to weight of animals at start of experiment. **Statistically significant difference between genotypes detected at indicated age range.

Food and water intake of WT and Bmal1-/- mice.

Both WT and *Bmal1-/-* consumed the same amount of food (3.4 ± 0.31 g for males and 3.1 ± 0.23 for females). Although, the actual average food consumption was the same for both genotypes at the start of the experiment, *Bmal1-/-* mice actually consumed more food relative to their bodyweight (**Figure 4-5a**). Daily food consumption relative to body weight for WT males and females was about 10% of their body weight, whereas for *Bmal1-/-* males it was about 12% and for Bmal1-/- females about 14% of their body weight. We further monitored the water consumption of both the genotypes. Although, there was no significant difference observed between the genotypes in AL controls i.e. with constant access to food, on 30% CR Bmal1-/- consumed about 30% less water than WT **(Figure 4-5b)**. It would be further interesting to understand the cause of this reduced water consumption upon CR in Bmal1-/- mice. However, this could be one of the reason for the reduced stability in body weight.

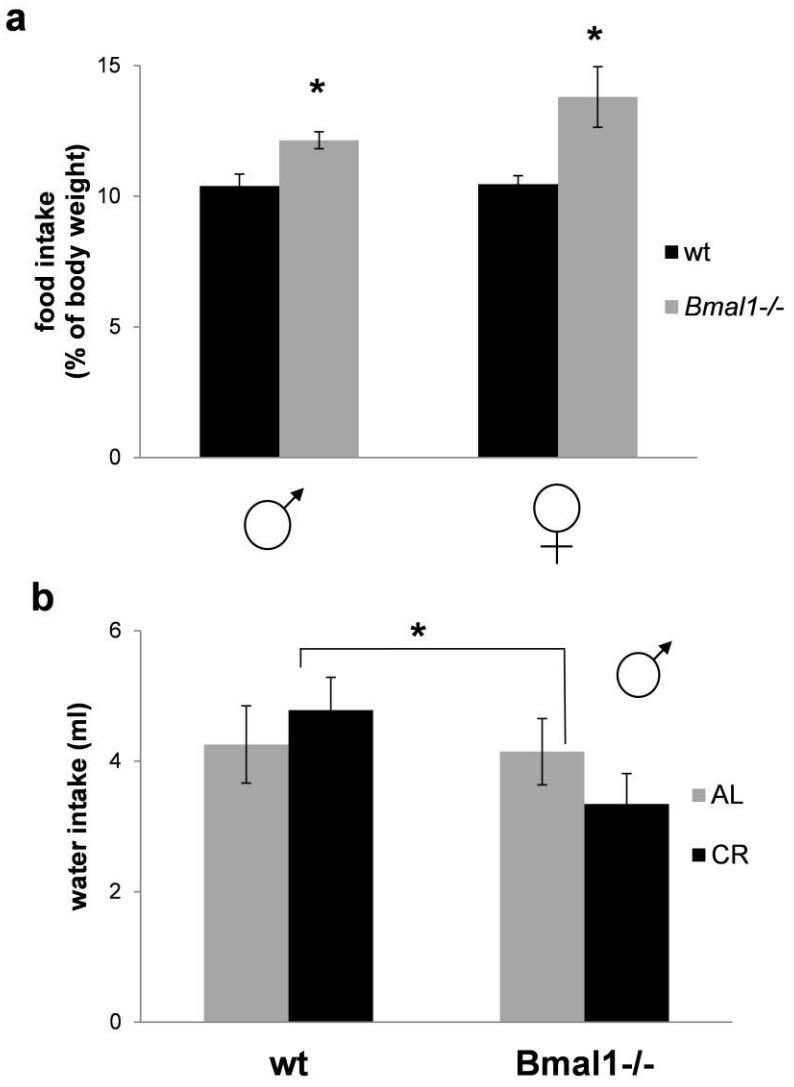


Figure 4-5. Daily food and water consumptions in WT and Bmal1-/- mice on 30% CR. a) Relative daily food intake of WT (black bars) and Bmal1-/- (light gray bars) for male and female mice on 30% CR. Relative food intake was calculated by dividing daily food consumption by mouse body weight. *Statistically significant difference between genotypes ($P < 0.05$). b) Daily water consumption of WT and Bmal1-/- mice on AL (light gray bars) or 30% CR (black bars) feeding. *Difference between genotypes statistically significant ($P < 0.05$).

Effect of 30% CR on lifespan of Bmal1-/- mice.

For longevity experiments, food for CR groups was provided at ZT14 (2 h after light was off, which is a normal physiologic time of feeding for mice). Several studies have demonstrated increased lifespan upon 30% CR in C57B6 strain of mice. Our study further confirmed this effect on lifespan extension in WT mice compared to the AL control group **(Figure 4-6)**. Since Bmal1-/- mice have dramatically shortened lifespan compared to the WT mice, we checked the effect of 30% CR on these mice **(Figure 4-7).** We found that in contrast to the WT mice, 30% CR did not extend lifespan of Bmal1-/- mice, while we actually observed a reduction in the lifespan that was statistically significant. Average lifespan of *Bmal1-/-* mice on the AL diet was 8 months but on 30%CR diet it was only 6 months. We also noticed a significant number of deaths within few weeks of the start of CR, when, most likely, metabolic changes occur to allow the animal to adjust to a novel feeding regimen. Within around 40 days from the start of CR about 40% of *Bmal1-/-* animals (15 of 36) died, while only 1 animal died in the WT group. We further excluded the animals that died during the adaptation period (shown in **Figure 4-7b** as the CR-adjusted group), yet we did not detect any increase in the lifespan on CR. Even then the average lifespan of the CR-adjusted group was about 7.5 month compared to 8 months on AL diet.

In order to study the effect of gender on the outcome of CR, we checked the longevity of *Bmal1-/-* males (13 mice) and females (23 mice) separately **(Figure 4-8)**. Similar to our previous observation of Bmal1-/- lifespan on AL diet, we did not detect any difference in the effect of gender on the survival of *Bmal1-/-* mice on CR. Thus, BMAL1

is not only necessary for CR-mediated life span extension but is also required for the metabolic adaptation to CR. This suggests that BMAL1 is a necessary mediator of the beneficial effects of CR on lifespan.

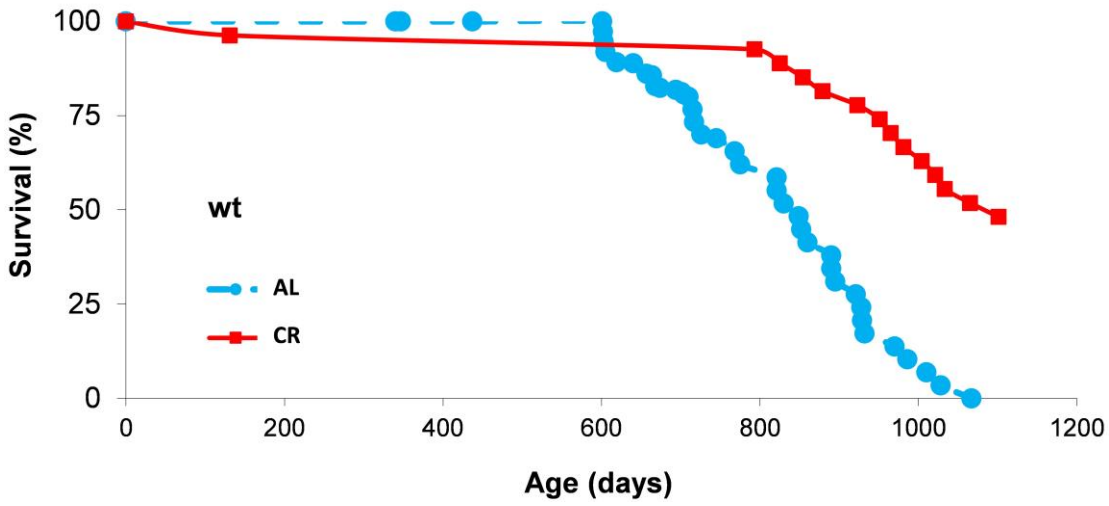


Figure 4-6. Effect of 30% CR on lifespan of WT mice. Kaplan-Meier survival curves of WT mice on AL (n = 73, blue circles and blue dotted line) or CR (n = 31; red squares and solid red line) feeding. Mice of both genders were used. *Difference between survival curves of AL and CR statistically significant according to log rank test.

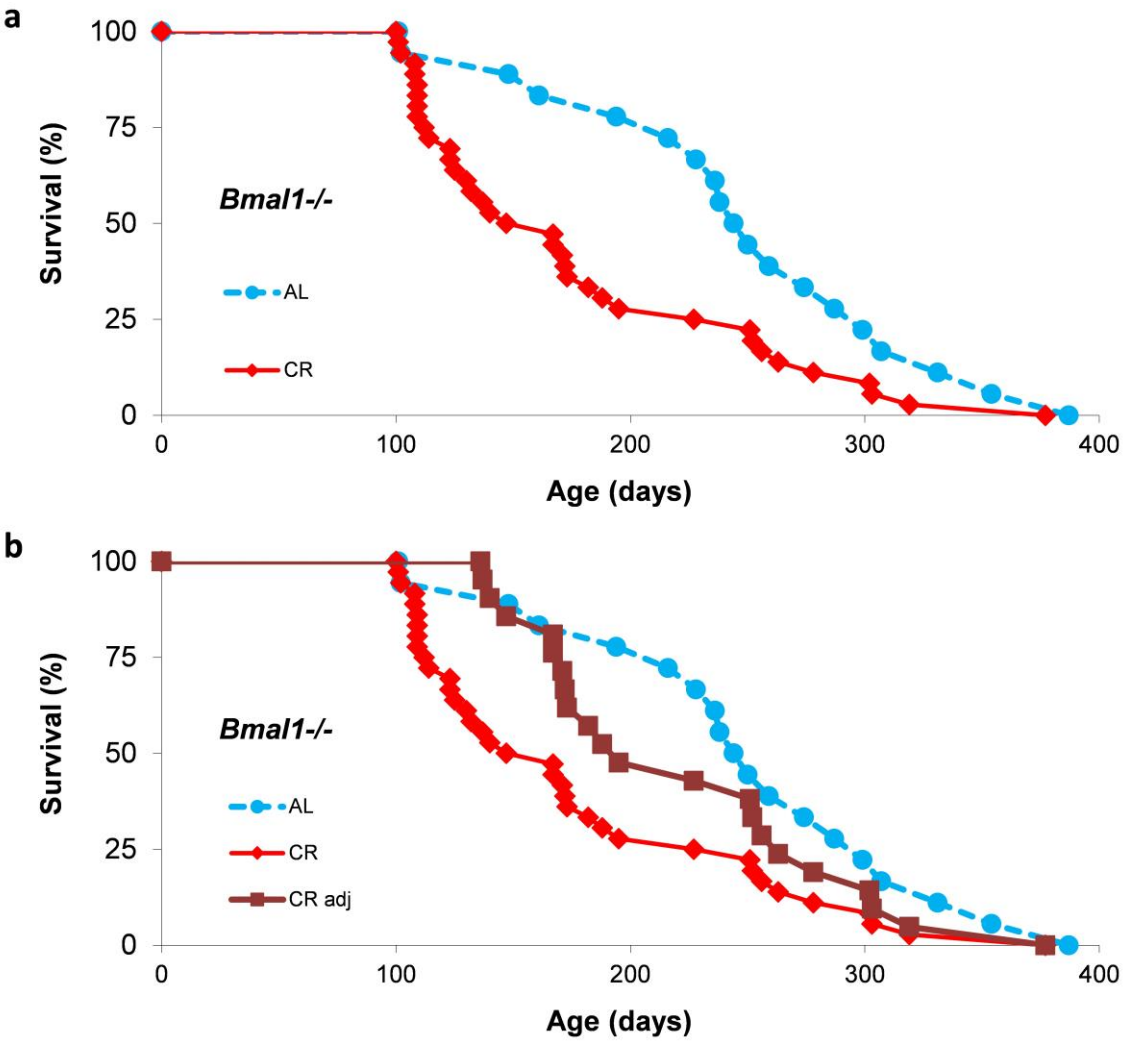


Figure 4-7. Effect of 30% CR on lifespan of Bmal1-/- mice. a) Kaplan-Meier survival curves of Bmal1-/- mice on AL (n = 18, blue circles), 30% CR (n = 36, red diamonds), and b) CR-adjusted (n = 21, dark red squares) feeding. Mice that died during first 3 wk of 30% CR were excluded from analysis. Mice of both genders were used. Difference between survival curves of AL and CR is statistically significant by log rank test; no statistically significant difference between AL and CR-adjusted groups was observed.

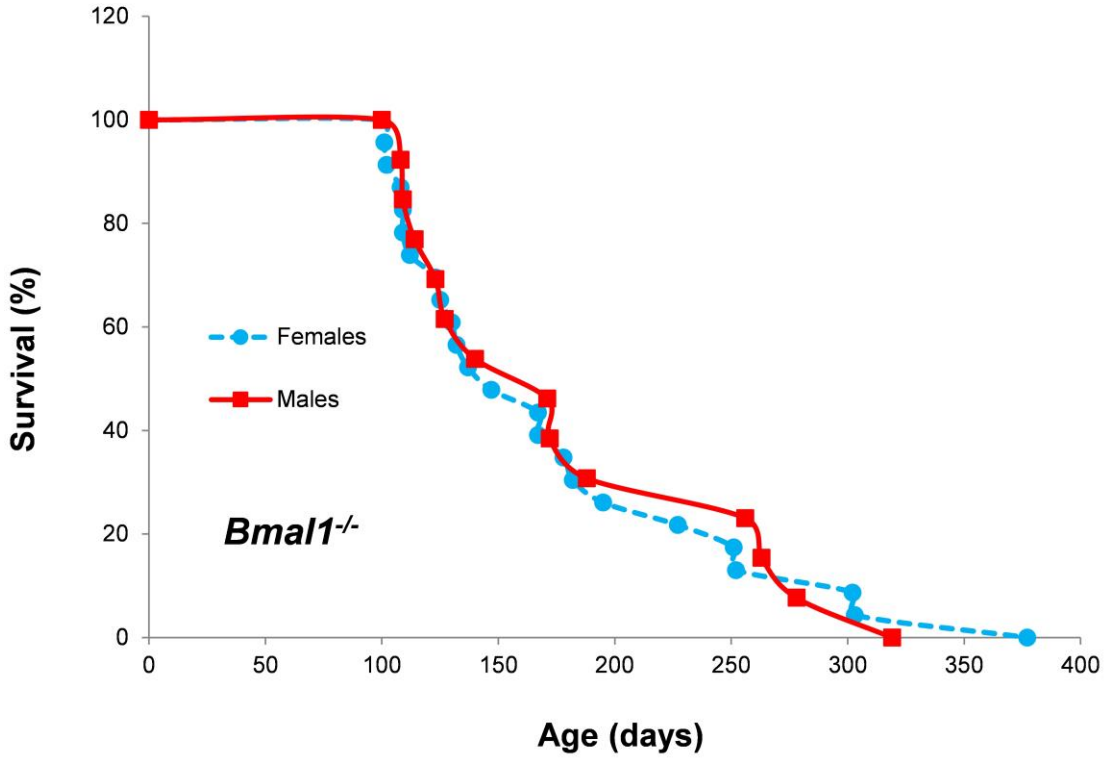


Figure 4-8. Gender based effect of 30% CR on Bmal1-/- mice lifespan. a) Kaplan-Meier survival curves of Bmal1-/- male (n = 13, red squares) and female (n = 23, blue circles) mice on 30% CR. Mice that died during first 3 wk of 30% CR were excluded from analysis. Difference between survival curves of CR is not statistically significant by log rank test; no statistically significant difference between the genders on CR was observed.

Effect of 30% CR on Insulin, IGF-1 and Glucose levels in Bmal1-/- mice.

Reduced Insulin and IGF-1 levels are important characteristics of CR. It has been well demonstrated that CR significantly affects glucose, insulin and IGF-1 levels in mice and thus leads to increased insulin sensitivity (Piper & Bartke 2008; Brown-Borg 2007). Hence, we studied the effect of 30% CR on these parameters in Bmal1-/- mice. As in WT mice, CR significantly lowered the blood glucose levels of Bmal1-/- mice **(Figure 4-9a)**. The reduction in both WT and Bmal1-/- mice was statistically significant when different diets were compared with no difference of the diets between genotypes. Thus, Bmal1-/- mice have normal response for this parameter. While Bmal1-/- mice showed reduced insulin levels on AL diet as expected, in contrast on CR, increased plasma insulin levels were observed **(Figure 4-9b)**. Thus, despite reduced glucose levels, *Bmal1-/-* mice had high insulin levels that might indicate reduced insulin sensitivity on CR regimen.

Next we measured plasma IGF-1 levels in WT and *Bmal1-/-* mice on AL and CR diet. In WT group on AL plasma IGF-1 showed significant changes across the day with highest level at ZT2 to 6 and lowest around ZT14 **(Figure 4-9c)**. However, no such changes were observed in Bmal1-/- mice on AL. Upon CR, plasma IGF-1 levels were significantly reduced in WT mice while no such significant reduction was seen in *Bmal1-/-* mice. Thus, deficiency of BMAL1 resulted resistance to the CR-mediated reduction in the plasma IGF-1 levels. We further analyzed liver mRNA and protein of IGF-1 levels and found that there was time-of-the day dependent effect of diet and genotype on IGF-1 mRNA level with a significant reduction observed at ZT 18 and 22 in WT mice upon CR **(Figure 4-10a)**. Simultaneously the effect of CR on the protein levels of IGF-1 were also comparable

between the genotypes, with the difference between AL and CR groups being statistically significant at ZT18 only for WT mice **(Figure 4-10b)**.

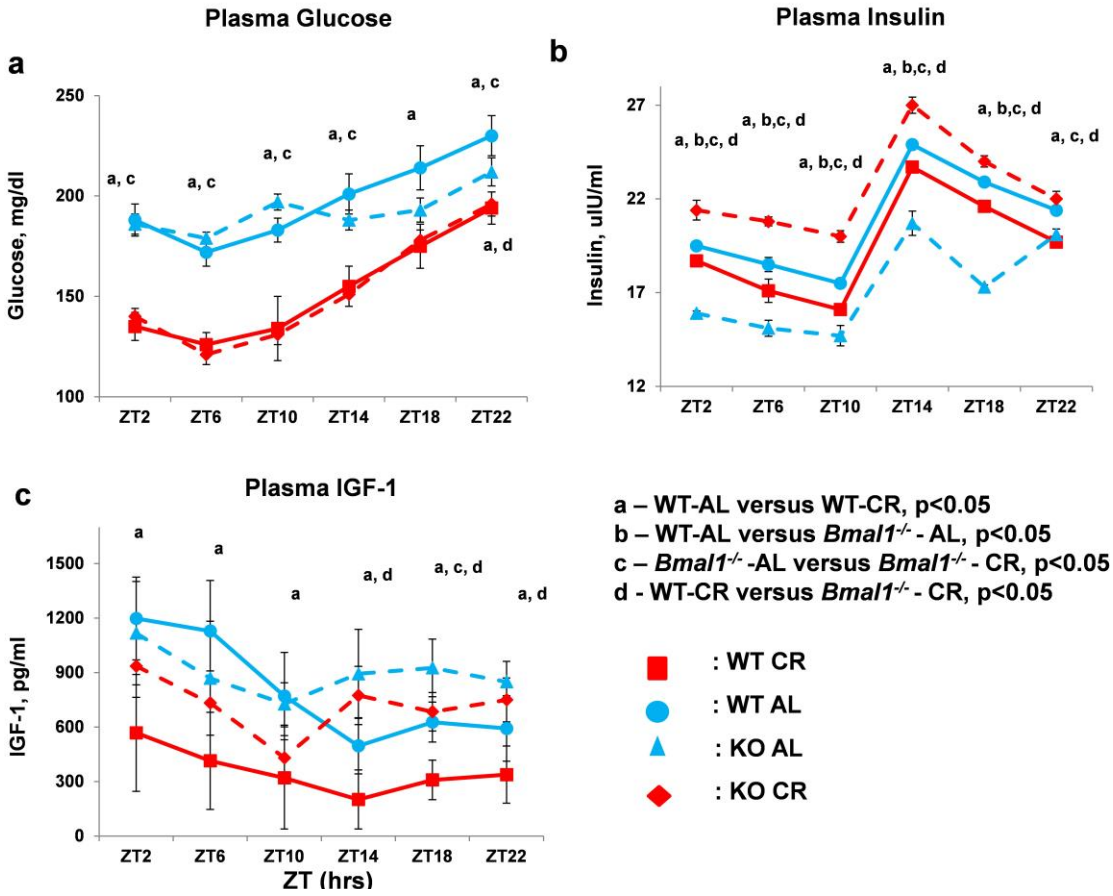


Figure 4-9. Effect of CR on plasma glucose, insulin and IGF-1 levels. Daily profiles of (a) glucose, (b) insulin, and (c) IGF-1 in plasma of WT and Bmal1-/- mice on AL and CR. WT mice on AL feeding (blue circles, solid blue lines); WT mice on CR feeding (red squares, red solid lines); Bmal1-/- mice on AL feeding (blue triangles, blue dashed lines); Bmal1-/- mice on CR feeding (red diamonds, red dashed lines). a) Statistically significant difference ($P < 0.05$) between WT mice on AL and CR feeding. b) Statistically significant difference ($P < 0.05$) between WT and Bmal1-/- mice on AL feeding. c) Statistically significant difference ($P < 0.05$) between Bmal1-/- mice on AL and CR feeding. d) Statistically significant difference ($P < 0.05$) between WT and Bmal1-/- mice on CR feeding.

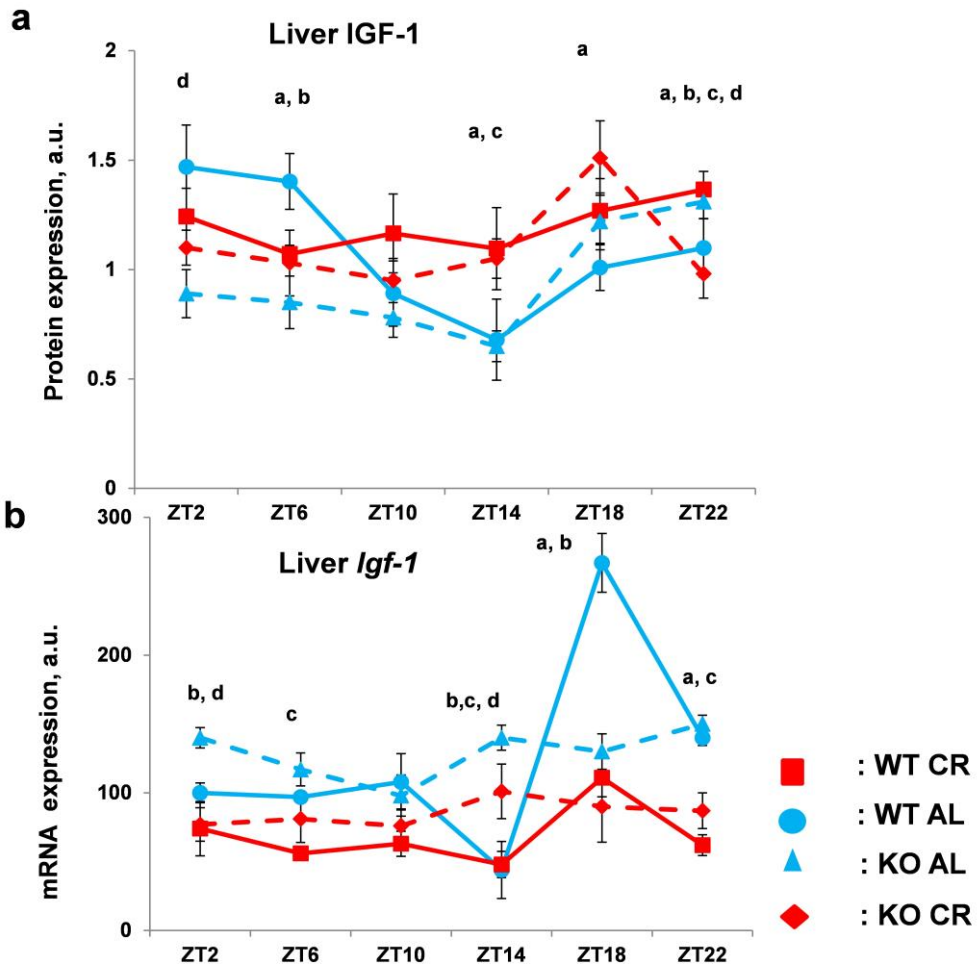


Figure 4-10. Effect of CR on liver IGF-1 mRNA and protein levels. Daily profiles of (a) Igf-1 mRNA, and (b) IGF-1 protein levels of WT and Bmal1-/- mice on AL and CR. WT mice on AL feeding (blue circles, solid blue lines); WT mice on CR feeding (red squares, red solid lines); Bmal1-/- mice on AL feeding (blue triangles, blue dashed lines); Bmal1-/- mice on CR feeding (red diamonds, red dashed lines). a) Statistically significant difference ($P < 0.05$) between WT mice on AL and CR feeding. b) Statistically significant difference ($P < 0.05$) between WT and Bmal1-/- mice on AL feeding. c) Statistically significant difference ($P < 0.05$) between Bmal1-/- mice on AL and CR feeding. d) Statistically significant difference ($P < 0.05$) between WT and Bmal1-/- mice on CR feeding.

4.5 Discussion

Circadian clock as mediator of CR effects

Dozens of reports have shown the CR beneficial effects on longevity in different species, including humans. Out of the many physiological systems proposed as mediators of CR, circadian clock is one such system being involved. Clock proteins, such as BMAL1 and PER2 are involved in control of aging and these proteins are also regulated by SIRT1 that is itself one of the important mediator of CR effects (Nakahata et al. 2008; Asher et al. 2008). In fact both Bmal1 and Sirt1 null mice have significantly reduced lifespan (McBurney et al. 2003; Cheng et al. 2003). Also in our study we have shown CR to affect Bmal1 and its target genes expression in the liver across the day in WT mice, however, in Bmal1-/- mice CR did not have such an effect on all the tested clock genes expression, such as Per1 and Per2. This suggested that BMAL1 could be one of the important factor involved in CR effects. Thus, in this chapter we investigated the effect of CR on behavioral and physiological parameters of *Bmal1-/-* mice.

Normal behavioral and body weight response to CR in the absence of Bmal1.

One of the behavioral response most commonly measured is the locomotor activity in response to the time of food presentation. Previously our lab had demonstrated that Bmal1-/- mice had normal response to food anticipation when on AL diet similar to WT mice. Hence in this chapter we studied the locomotor activity in Bmal1-/- mice in response to CR. Similar to the previous results Bmal1-/- mice had normal food anticipatory activity upon CR as in WT mice. Furthermore, we monitored body weights upon 30% CR and

again observed a significant reduction in body weight upon CR as seen in WT mice. Although at the beginning of CR there was significant reduction in bodyweight for both WT and Bmal1-/- mice, the body weight stabilized after few weeks of CR while in Bmal1-/- weights did not stabilize and was reducing on a constant basis throughout the experiment. Interestingly, the food intake of Bmal1-/- mice was similar to the WT mice, yet Bmal1-/- mice consumed more food when compared to their relative body weights. Upon measuring the water intake upon CR, careful observation showed that Bmal1-/- mice on CR actually consumed less water than their WT counterparts. This phenomenon could be one of the reason for the different body weight response for Bmal1-/- upon CR.

Bmal1 deficiency results in insulin resistance upon CR.

We then tested several physiological parameters such as plasma glucose, insulin and IGF-1 levels upon CR. In WT mice dramatic reduction in plasma levels of glucose, insulin, and IGF-1 was observed that leads to increased insulin sensitivity in WT mice(Piper & Bartke 2008; Brown-Borg 2007). Similarly, in Bmal1-/- mice CR resulted in a comparable reduction in blood glucose level. Though it was reported that BMAL1 is involved in the control of glucose homeostasis (Rudic et al. 2004), our study indicates that it is not necessary for the CR-mediated reduction in plasma glucose. While it is also possible that other factors dictate the effect of CR on glucose homeostasis independent of BMAL1. Simultaneously, we found that CR did not reduce the plasma insulin levels in Bmal1-/- mice as opposed to reduction observed in WT mice. Paradoxically, insulin level in Bmal1-/- mice was increased upon CR compared to the AL group, which, along with the data on reduced glucose levels, indicates reduced insulin sensitivity in Bmal1-/- mice.

It is worth noting that increased insulin sensitivity is implicated in life span extension by CR (Piper & Bartke 2008). Based on the previous report BMAL1 regulates insulin secretion by pancreatic islets (Marcheva et al. 2010) and our results too we observed reduced plasma insulin levels in Bmal1-/- mice on AL diet compared to the WT group. Thus, this difference in the effect of BMAL1 deficiency on insulin demands further studies to understand the role of BMAL1 in pancreatic islet functions. Thus, under CR conditions BMAL1 is required for proper insulin production and most likely for insulin sensitivity, which indeed warrants further study of BMAL1 functions.

IGF-1 being a major regulator of cell growth has been well known to be significantly reduced by CR, however, the molecular mechanisms of this effect are not well studied. Thus, in this chapter we tested the plasma IGF-1 levels and did not observe any significant reduction of circulating IGF-1 in the blood of Bmal1-/- mice (the difference was significant only at one-time point), compared to dramatic reduction seen in WT mice. The mRNA levels of Igf1 in the liver did not change significantly between genotypes of both AL and CR groups, however, it was similarly suppressed under CR condition compared to AL in both WT and Bmal1-/- mice. The effect of CR on the liver IGF-1 protein levels was complicated; IGF-1 protein levels were elevated upon CR in the liver of WT animals at ZT14 and ZT18 while it was reduced at ZT6. CR also affected IGF-1 protein level in the liver of Bmal1-/- mice; the difference being significant at ZT18. These data indicate that CR might be controlling IGF-1 production and secretion since there was no effect or rather opposite effect on IGF-1 protein levels in WT. Additionally, BMAL1 deficiency leads to impaired control of IGF-1 protein secretion. Another possibility is that CR might affect the

plasma IGF-1 rate of turnover. In short, BMAL1 might be involved in the control of IGF-1 secretion and/or turnover.

Bmal1 is required for CR mediated increased lifespan.

Previously our lab has shown that Bmal1-/- mice live a significantly reduced lifespan with premature aging phenotype. Therefore, we decided to study the effect of CR that is well known to extend longevity, on the Bmal1-/- lifespan. We found that 30% CR did not extend lifespan of *Bmal1-/-* mice, when the same amount of restriction actually increased the lifespan of WT mice. Even more so, 30% CR slightly reduced the lifespan of *Bmal1-/-* mice. Significant number of deaths were observed during first week of CR; indeed previous report of CR applied to mice caught in wilderness showed significant deaths of the animals during adaptation to CR, while mice that survived lived longer than their AL-fed counterparts (Cheng et al. 2003). The reason behind this difference in the adaptation is not clearly known; however, from our data it seems that BMAL1 is critical for this metabolic adaptation to CR. It would also be interesting to study if any variation in the rhythms of Bmal1 expression and/or activity could produce differential response to adaptation to CR.

On the other hand, we also found that several behavioral (food anticipatory activity shown above) and physiologic (glucose and IGF-1) changes induced by CR in WT mice were also detected in *Bmal1-/-* animals, indicating that BMAL1 is not involved in all the beneficial effects of CR. Also, in chapter III we showed that Cry1 expression was significantly affected by CR even in Bmal1-/- mice. Thus, we cannot rule out the possibility of the existence of BMAL1 independent mechanisms. These results and looking at the fact

that the Bmal1-/- mice develop premature aging phenotype and have a short lifespan suggest that the role of BMAL1 in the beneficial effects of CR on longevity must be taken with caution. This extreme phenotypical condition of Bmal1-/- might contribute to the failure of CR in this model. Importantly, at the starting point of the CR (3 mo. of age), *Bmal1-/-* mice are very similar with WT mice in terms of gross appearance, food consumption, and body weight. Interestingly, treatment with rapamycin increases the lifespan of these mice(R V Khapre et al. 2014), suggesting that longevity in these mice can be affected by another antiaging intervention.

Future Directions

Studies in future would focus on understanding the role of BMAL1 in the effect of CR on the Insulin/IGF-1 production and secretion. Furthermore, it would be interesting to study the effect of CR on the Insulin/IGF-1 pathway. We have tested the effect of CR on only Bmal1 mutant mice which has gross phenotype however, not all circadian clock mutants demonstrate this gross phenotype and shortened lifespan. Hence, effect of CR on other circadian clock mutants such as Cryptochromes lifespan and physiology also need to be studied.

CHAPTER V

CONCLUSION

In our Chapters III and IV we studied the effect of 30% CR on circadian clock genes expression and on lifespan, behavior and physiology of Bmal1 mutant mice. We observed that 30% CR significantly affected the expression of some of the core circadian clock and clock controlled genes. *Per1* and *Per2* which are direct transcriptional targets of the BMAL1/CLOCK complex were significantly up regulated by CR. However, not all genes were similarly affected by CR which suggests that the effect of CR is not universal for all clock genes.

One of the possible effects of CR is an increase in the BMAL1/CLOCK transcriptional activity which is in agreement with the observed induction of the BMAL1/CLOCK downstream targets. Alternatively, there is increased synchronization of individual cellular oscillators in a tissue due to rhythmic feeding. In which case, we should observe an increase in the amplitude of expression with no significant effect on daily

average expression, which is really not what we observed in our results. However, it is also possible that CR could have different effects on clock gene expression at different times of the day. This may be due to regulation at epigenetic level that have been recently proposed as regulators of the circadian transcriptome (Eckel-Mahan & Sassone-Corsi 2013). Irrespective of the mechanism involved, our data demonstrates that CR significantly increases the expression of Bmal1 gene and its target genes, which indicates that CR may directly or directly affect BMAL1 transcriptional activity, suggesting an important role for BMAL1 for the full beneficial effects of CR.

Our study points out to the importance of studying the gene expression in circadian manner. Discrepancy in gene expression studies affected by CR or other factors, intrigued us to study some of these genes in our CR model applying circadian approach. We thus, selected some of these genes called as longevity candidate genes and tested them in our CR model at different times across the day. Although most of these longevity candidate genes expression was in agreement with the previous microarray results, some of them demonstrated time of the day dependent effect. Since, these longevity candidate genes were affected by CR in circadian way and the clock genes we tested were similarly affected in a gene and time specific manner, we proposed circadian clock genes as longevity candidate genes. Our study is further corroborated by the microarray study showing Per1 and Per2 genes amongst the top genes identified to be affected by CR. Thus, future studies would focus on understanding the mechanism of the effect of CR on some of these clock genes and testing the importance of some of the clock genes for CR effects on health and

longevity. To take one step ahead we started with Bmal1 mutant mice because of the unique phenotype and short lifespan of these animals.

With the expectation that CR would impart its beneficial effects on these Bmal1-/- mice we assayed their behavioral, physiological and longevity response to CR. Bmal1-/- mice did not show any improvement in lifespan nor its premature aging phenotype upon CR while on the contrary it proved to be slightly harsh on these animals. This definitely meant that BMAL1 is at least a part of the mechanism that is necessary for lifespan extension benefits of CR. Yet BMAL1 independent mechanisms could also exist as Bmal1 was not required for all the responses to CR. For example, Bmal1-/- mice demonstrated normal response for glucose levels as in WT mice; also food anticipatory activity was preserved in Bmal1-/- mice as seen in WT upon CR. Thus, other mechanisms of which Bmal1 could be a part of or could be dispensable for CR effects cannot be overlooked.

Other mechanisms which have been proposed to drive benefits of CR include Sirtuins, TOR and Insulin/IGF-1 pathway. CR activates SIRT1, which in turn may regulate CLOCK and BMAL1. Our study also states that BMAL1 contributes to CR-mediated regulation of circulating IGF-1 levels, which is an important mediator of CR effect on longevity (Piper & Bartke 2008; Brown-Borg 2007). Another signaling mechanism considered to be critical for CR driven effects is the mTOR complex 1 (mTORC1) pathway(Blagosklonny 2010). Previously our lab demonstrated that BMAL1 is a negative regulator of mTORC1 (R V Khapre et al. 2014). Thus, it will be important to study if the CR effects on mTORC1 activity are impaired in *Bmal1-/-* mice.

Our study also initializes several important questions that need to be addressed. Future experiments on the effects of CR in other circadian clock mutants will help in addressing the importance of specific clock genes. Another important thing worth noting is that although we clearly observed the role of BMAL1 in CR-mediated longevity, the effect of CR was studied only in the liver, and we do not know if it will be the same in other tissues as well. Overall, our data suggest that BMAL1 is a crucial target of CR, and the BMAL1-dependent pathways ought to be considered for better understanding of CR mechanisms for driving metabolic fitness and prevention of age-associated pathologies and healthy aging.

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APPENDICES

APPENDIX A

Table III. Cosinor Wave Analysis of Clock and Clock-controlled Genes Expression for Figures 3-1; 3-2; 3-3; 3-4 and 3-5. The circadian parameters were assessed by the cosinor analysis that models the circadian rhythms (Circa) as a cosine function with the following attributes: amplitude (amp), and acrophase (acr).

Gene \ Diet	AL			CR			TR			Fasting		
	Circ	Acro	Amp	Circ	Acro	Amp	Circ	Acro	Amp	Circ	Acro	Amp
Per1	NO	-199	0.66	NO	-187	4.4	YES	-195	1.3	NO	-348	0.8
Bmal1	YES	-339	0.98	YES	-325	2.4	YES	-346	0.85	NO	-337	0.62
Per2	YES	-226	0.73	YES	-232	1.6	YES	-250	0.69	YES	-231	0.47
Cry1	YES	-341	0.73	YES	-346	1.56	NO	-3	1.5	YES	-301	2.1
Cry2	NO	-120	0.24	NO	-92	0.39	NO	-161	0.07	NO	-183	0.15
Clock	NO	-353	0.26	YES	-301	0.21	NO	-331	0.125	YES	-303	0.19
Per3	NO	-167	0.68	NO	-155	1.4	NO	-191	1.1	YES	-179	0.38

Rev erbα	**NO**	**-91**	**1.3**	**NO**	**-88**	**1.33**	**NO**	**-115**	**1.3**	**NO**	**-103**	**1.24**
Rev erbβ	YES	-127	0.74	YES	-151	1.3	YES	-150	1.1	NO	-153	0.66
Rorα	YES	-22	0.21	NO	-17	0.15	NO	-333	0.18	NO	-322	0.41
Rorγ	NO	-272	0.57	YES	-304	1.02	YES	-267	0.55	NO	-289	0.56
Dbp	NO	-138	1.3	NO	-137	2.7	NO	-151	1.9	NO	-152	0.64
Dec1	NO	-199	0.19	NO	-211	0.46	YES	-216	0.8	NO	-250	0.4
Dec2	NO	-325	0.8	NO	-357	0.133	NO	-40	0.89	NO	-2	1.2
Hlf	YES	-220	1.04	YES	-244	2.2	YES	-235	1.8	YES	-282	1.09
Tef	YES	-172	0.44	NO	-163	0.59	YES	-78	0.0001	NO	-205	0.63
E4bp4	NO	-342	0.61	NO	-327	0.66	NO	-337	0.57	NO	-317	0.92
Pparα	YES	-162	0.9	NO	-190	1.2	NO	-167	1.2	YES	-107	0.7

Table IV. Cosinor Wave Analysis of Longevity Candidate Genes Expression for Figure 3-9 and 3-10. The circadian parameters were assessed by the cosinor analysis that models the circadian rhythms (Circa) as a cosine function with the following attributes: amplitude (amp), and acrophase (acr).

Diet / Gene	AL			CR			TR			Fasting		
	Circ	Acro	Amp	Circ	Acro	Amp	Circ	Acro	Amp	Circ	Acro	Amp
ALAS2	NO	-320	0.3	NO	-289	0.04	NO	-296	0.11	NO	-275	0.44
CYP4A12B	NO	-356	0.17	NO	-351	0.003	NO	-2	0.679	NO	-228	0.53
FMO3	NO	-357	0.94	NO	-256	975.3	NO	-251	1.1	NO	-342	9.3
HES6	NO	-70	0.15	NO	-255	0.07	NO	-351	0.32	NO	-176	0.01
HSD3β5	YES	-41	0.36	YES	-188	0.8	NO	-44	0.83	NO	-285	0.08
IGFALS	NO	-127	0.05	NO	-314	0.55	YES	-279	0.7	NO	-93	0.06
MUP4	NO	-344	0.17	NO	-81	0.003	NO	-338	0.23	NO	-268	0.28
SERPINA12	YES	-34	0.21	NO	-340	0.05	NO	-345	0.5	NO	-100	0.12
PARP16	NO	-304	0.33	NO	-334	0.35	NO	-355	0.2	NO	-297	0.4
CYP4A14	NO	-55	0.21	NO	-333	124.6	NO	-161	2.2	NO	-63	51.7

Table V. Cosinor Wave Analysis of Clock protein levels for Figures 3-6 and 3-7. The circadian parameters were assessed by the cosinor analysis that models the circadian rhythms (Circa) as a cosine function with the following attributes: amplitude (amp), and acrophase (acr).

Diet / Gene	AL			CR			TR			Fasting		
	Circ	Acro	Amp	Circ	Acro	Amp	Circ	Acro	Amp	Circ	Acro	Amp
Per1	YES	-227	0.51	NO	-10	0.33	NO	-209	0.26	NO	-66	0.3
Bmal1	NO	-355	0.26	NO	-101	0.03	NO	-141	0.64	NO	-74	0.49
Per2	YES	-215	0.5	NO	-167	0.33	NO	-48	0.24	NO	-39	0.22
Cry1	NO	-353	0.56	NO	-20	0.22	NO	-20	0.11	NO	-38	0.49
Clock	NO	-108	0.27	NO	-105	0.245	NO	-160	0.06	NO	-34	0.34

Table VI. Statistical Analysis of Clock and Clock-controlled Genes Expression for Figure 3-1; 3-2; 3-3; 3-4 and 3-5. Two-way ANOVA analysis (time point vs feeding regimen) of rhythmically expressed clock and clock controlled genes in the mouse liver. F and P (Sig) values are presented below for feeding regimen, time and their interaction.

Gene	Feeding regimen		Time		Feeding regimen*Time	
	F	Sig	F	Sig	F	Sig
Clock	49.25	0.0013	9.069	0.0001	1.967	0.0788
Bmal1	37.53	0.0022	17.83	< 0.0001	2.266	0.0444
Per1	34.85	0.0025	9.382	0.0001	5.286	0.0004
Per2	18.87	0.008	25.11	< 0.0001	4.039	0.0021
Per3	44.8	0.0016	54.41	< 0.0001	7.004	< 0.0001
Cry1	1.804	0.286	16.53	< 0.0001	1.333	0.2701
Cry2	342	< 0.0001	3.751	0.0147	1.466	0.2092
Rev erbα	5.061	0.0756	60.11	< 0.0001	2.161	0.0542
Rev erbβ	1.289	0.3926	56.87	< 0.0001	5.216	0.0004
Rorα	8.818	0.0309	5.445	0.0025	1.288	0.2941
Rorγ	7.147	0.0438	47.03	< 0.0001	2.368	0.0366

Gene	Feeding regimen		Time		Feeding regimen*Time	
	F	Sig	F	Sig	F	Sig
Dbp	14.32	0.0132	35.2	< 0.0001	3.643	0.004
Dec1	21.11	0.0065	3.845	0.0133	2.133	0.0572
Dec2	210.6	< 0.0001	14	< 0.0001	6.133	0.0001
Hlf	21.32	0.0064	41.17	< 0.0001	2.611	0.0233
Tef	5.537	0.0659	17.61	< 0.0001	2.216	0.0488
E4bp4	5.991	0.0582	65.04	< 0.0001	3.75	0.0034
Pparα	0.9363	0.5015	25.53	< 0.0001	3.803	0.0031

Table VII. Statistical Analysis of Longevity Candidate Genes Expression for Figure 3-9 and 3-10. Two-way ANOVA analysis (time point vs feeding regimen) of longevity candidate genes expression in the mouse liver. F and P (Sig) values are presented below for feeding regimen, time and their interaction.

Gene	Feeding regimen		Time		Feeding regimen*Time	
	F	Sig	F	Sig	F	Sig
ALAS2	66.2	0.0007	4.043	0.0107	1.87	0.0953
CYP4A12B	63.43	0.0008	2.317	0.0818	4.93	0.0006
FMO3	53.86	0.0011	3.604	0.0174	3.615	0.0042
HES6	41.09	0.0018	1.351	0.284	1.953	0.081
HSD3β5	503.2	< 0.0001	6.746	0.0008	4.563	0.001
IGFALS	33.49	0.0027	5.726	0.0019	2.495	0.0289
MUP4	43.73	0.0016	6.099	0.0014	1.97	0.0784
SERPINA12	8.56	0.0325	2.563	0.06	1.09	0.4206
PARP16	15.25	0.0118	33.42	< 0.0001	5.708	0.0002
CYP4A14	55.65	0.001	4.688	0.0054	3.181	0.0085

Table VIII. Statistical Analysis of Locomotor activity for Figure 4-1 and 4-2. Three-way ANOVA analysis (genotype vs time point vs feeding regimen) of locomotor activity in the mouse liver. F and P (Sig) values are presented below for genotype, feeding regimen, time and their interactions.

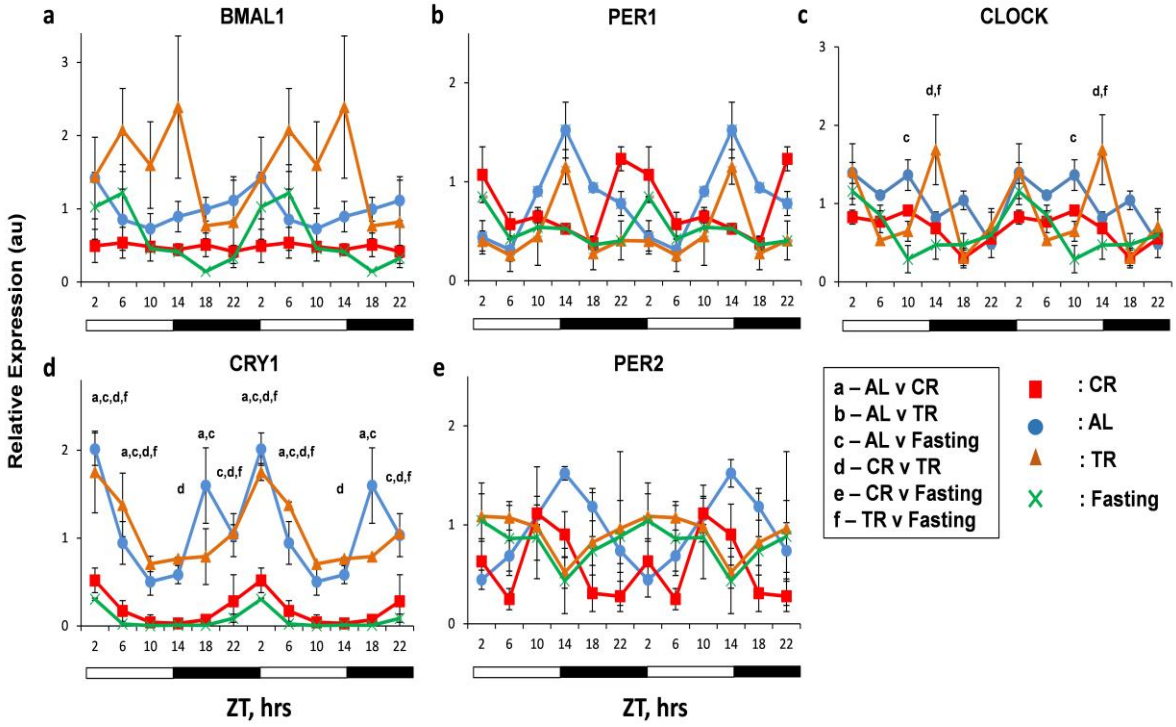
Source	Locomotor activity	
	F	Sig
Genotype	.004	.952
Feeding regimen	7.21	.016
Time	17.506	.001
Genotype*Feeding regimen	.174	.682
Genotype*Time	3.038	.007
Feeding regimen*Time	5.551	.000
Genotype*Feeding regimen*Time	2.688	.015

Table IX. Statistical Analysis of Glucose, Insulin, and IGF1 levels for Figure 4-9 and 4-10. Three-way ANOVA analysis (genotype vs time point vs feeding regimen) of glucose, insulin and IGF1 levels in the mouse liver. F and P (Sig) values are presented below for genotype, feeding regimen, time and their interactions.

Source	Glucose		Insulin		IGF1 plasma		IGF1 mRNA		IGF1 protein	
	F	Sig	F	Sig	F	Sig	F	Sig	F	Sig
Genotype	6.68	.013	12.212	.002	106.729	.000	.526	.476	7.48	.012
Feeding regimen	63.007	.000	79.998	.000	25.194	.000	7.833	.010	1.423	.245
Time	60.409	.000	23.669	.000	3.363	.011	7.67	.000	48.258	.000
Genotype*Feeding regimen	11.712	.001	147.54	.000	19.011	.000	14.006	.001	19.070	.000
Genotype*Time	0.543	.742	2.147	.094	9.709	.000	6.395	.001	1.832	.144
Feeding regimen*Time	5.882	.000	.342	.882	2.162	.074	1.577	.205	7.441	.000
Genotype*Feeding regimen*Time	5.215	.001	.295	.911	3.85	.005	.095	.992	8.660	.000

APPENDIX B

Supplementary Fig. S1. Quantification analysis of protein expression of core clock genes in liver.



Supp. Figure 1. Protein levels of (a) BMAL1, (b) PER1, (c) CLOCK, (d) CRY1 and (e) PER2 proteins in the liver of mice (n=3 per time point) subjected to the following feeding regimens: AL – blue circles, solid line; 30% CR – red squares and solid lines; TR – orange triangles and solid lines, Fasting (F) – green cross and solid lines. Data represents mean ± SD (standard deviation); statistically significant ($p<0.05$) effects of the feeding at a given time point are indicated by: a- between AL and CR groups, b – AL and TR, c- AL and Fasting, d- CR and TR, e – CR and Fasting, f- TR and Fasting. Light and dark bars at the bottom represent light and dark phase of the day. ZT0 is the time when light is on and ZT12 is the time when light is off. For convenience all data are double plotted.