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Cryptochromes Regulate IGF-1 Production and Signaling Through Control of JAK2-Dependent STAT5b Phosphorylation

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ROLE OF CRYPTOCHROME IN IGF-1 PRODUCTION AND CALORIE RESTRICTION MECHANISM

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DEDICATION

I dedicate my dissertation work to my family and friends. A special feeling of gratitude to my loving parents, my sister ‘PRITI’ and brother ‘MANESH’ who have never left my side and supported me throughout the process. I will always appreciate all they have done.

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Knock knock!! “May I come in Sir?” “Come in, but just say me ROMAN only” on which I was stumped for a sec as I wasn’t expecting such a friendly welcome. It took me few days but I was used to it soon. That was my first and coolest experience to meet an adviser on which I would like to express my deep gratitude to my Advisor, Dr. Roman Kondratov, who accepted me to his laboratory five years ago and provided me a scientific atmosphere of independence and creativity. His sincerity and ethics to science taught me what a real scientist should be. His optimism, encouragement and understanding helped me to look at achievements and failures objectively. I hope I lived upto his expectations as graduate student. I appreciate all his contributions of time, ideas, and funding to make my PhD experience productive and stimulating. Besides my advisor, I would like to thank the rest of my thesis committee: Dr. George Stark, Dr. Crystal Weyman, Dr. Aaron Severson, Dr. Olga Stenina for their insightful comments and encouragement, but also for posing hard question which incited me to widen my research from various perspectives. I also want to thank my committee members who were more than generous with their expertise and precious time. I thank my fellow labmates for the stimulating discussions and helping me in the lab, and for all the fun we have had in the last four years. Especially, Rohini and Sonal, who were my seniors, they both are wonderful person and I thank them for teaching me everything in the lab and for mentoring me so well. Last but not the least; I would like to thank the entire Biological, Geological, and Environmental Science department of Cleveland State University.

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ABSTRACT

Insulin-like growth factor-1 (IGF-1) is an essential paracrine and autocrine factor for tissue homeostasis, growth, and development. Decreased IGF signaling plays an important role in mechanisms of calorie restriction (CR) - a lifespan-extending dietary paradigm. Recently we demonstrated the interaction between circadian clock and CR. The circadian clock is an internal time keeping system which is regulated by feeding. We also demonstrated that feeding affects the expression of circadian clock genes. Here we report that transcriptional regulators and key components of the circadian clock, Cryptochromes (CRY's) are essential for the CR-mediated downregulation of IGF-1 production and signaling. Plasma levels of IGF-1 and liver and skeletal muscles production of IGF-1 are significantly reduced in CRY-deficient mice. In agreement with reduced IGF-1 signaling, CRY-deficient mice have reduced body size and changes in gene expression profile similar to other dwarf mice. Downregulation of IGF-1 upon CRY 1,2 deficiency correlates with reduced IGF-1 mRNA expression and reduced phosphorylation of the STAT5B transcriptional factor. We also found that CR downregulates CRY 1 expression on the protein level in the liver and skeletal muscles of wild-type mice. CR also results in reduced phosphorylation of the transcriptional factor STAT5B through unknown mechanisms; in turn, reduced STAT5B activity causes downregulation of IGF-1

expression. At the same time, phosphorylation of the STAT5B upstream kinase JAK2 was not reduced, which places CRY activity downstream from JAK2. Finally, CR did not downregulate liver IGF-1 expression and plasma IGF-1 levels in CRY -deficient mice. We found that CRY-deficient mice demonstrate increased expression of CISH, a negative regulator of the JAK2-STAT5B pathway. We propose that downregulation of CRY's contributes to impaired STAT5B phosphorylation and IGF-1 expression during CR and CR regulate CISH expression through the CRY-dependent mechanism. Thus, CRY's link the circadian clock and calorie restriction mechanisms regulating STAT5B-dependent IGF-1 expression. The calorie restriction reduces the cancer incident by downregulating IGF-1 level but the exact mechanism is not known. Our study will provide significant inputs on the mechanism by which calorie restriction downregulate the IGF-1, and thus, suggest that it might be possible to achieve at least some beneficial effects of CR by targeting the Cryptochrome.

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LIST OF ABBREVIATIONS

AL	Ad-libitum
ALS	Acid Labile Subunit
AMPK	Adenosine Monophosphate Activated Protein Kinase
ARNTL	Aryl Hydrocarbon Receptor Nuclear Translocator-like Protein
BMAL1	Brain and Muscle ARNTL 1
bHLH	Basic Helix Loop Helix
CCG	Clock Controlled Gene
cDNA	Complementary DNA
CKI	Casein Kinase 1
CLOCK	Circadian Locomotor Output Cycles Kaput
CLD	Cytoplasmic Localization Domain
CISH	Cytokine-inducible SH2-containing protein
CR	Calorie Restriction
cAMP	cyclic adenosine monophosphate
CREB	cAMP response element binding protein
CRY	Cryptochrome
Cyp4a12b	cytochrome P450, family 4, subfamily a, polypeptide 12B
DNA	Deoxyribonucleic Acid
dNTP	Deoxy Nucleotide Triphosphate
DTT	Dithiothreitol

E-Box	Enhancer Box
ELISA	Enzyme-linked immunosorbent assay
FBXL3	F-box/LRR-Repeat Protein 3
FOXO	Forkhead box O3
FMO3	Flavin monooxygenase 3
FAD	Flavin adenine dinucleotide
GH	Growth Hormone
GHR	Growth Hormone Receptor
GSK3β	Glycogen Synthase Kinase 3β
GPCR	G-protein coupled receptor
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
JAK	Janus kinase
mRNA	Messenger RNA
Murf1	Muscle RING-finger protein-1
MTHF	Methenyltetrahydrofolate
MAPK	Mitogen-activated protein kinases
MUP 4	Major urinary protein 4
NES	Nuclear Export Signal

NLS	Nuclear Localization Signal
NPAS2	Neuronal PAS Domain- Containing Protein 2
PAS	Period Arnt Sim
PCR	Polymerase Chain Reaction
PDK	Phosphoinositide-dependent kinase-1
PER	Period
PI3K	Phosphoinositide 3-kinase
RGS	Retinal ganglion cells
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
rRNA	Ribosomal RNA
RTqPCR	Reverse Transcriptase Quantitative PCR
STAT	Signal transducer and activator of transcription
SCN	Suprachiasmatic Nucleus
SOCS	Suppressor of cytokine signaling
SD	Standard Deviation

SIRT1	Sirtuin
WT	Wild-type

CHAPTER I

INTRODUCTION

1.1 The Circadian Clock

We have developed and synchronized our life in a clockwork universe. In our daily routine life, we always come across this question, 'What time is it now?' Clock rules our lives. The layout of our solar systems such as the movements of planets and gravity control the clock. Clock defined our daily routine such as when to sleep, wake, eat, play, work and pray. All living organism have tuned their internal clockwork to 24 hours day and night cycle to adapt Earths 24 hour's rotation. These adaptations allow them to synchronize with behavior, physiology, and metabolism.

In most organisms, circadian rhythms are the 24 hours endogenous rhythms which maintain all feature of the physiology such as blood pressure, body temperature, hormonal levels, behavior and metabolism (Gachon et al. 2004; Lowrey et al. 2004). In mammals, light is the major zeitgeber to reset circadian rhythms (Dijk et al. 1995). The clock is also entrained by another clue such as feeding.

Research work also revealed that time of feeding is also another important zeitgeber that entrains the peripheral circadian clocks located in different organs of our body. It

was concluded that central and peripheral clock regulates the physiological and behavioral outputs. Disruption of the circadian clock is associated with the development of several pathophysiological conditions; aging-related disorders; moreover, it is also implicated in reducing longevity (Froy & Miskin. 2010; Fu et al. 2002; Kondratov et al. 2006; Dubrovsky et al. 2010).

1.2 Historical Background

It has been reported that plants and animals exhibit the circadian rhythms. In 1729, the French astronomer performed the first experiment on biological rhythms (De Marian. 1773) which showed that mimosa plant leaves are open during the day and are folded at night. The 18th-century Swedish naturalist architect a living clock in which garden with flowers opened at various times around the clock (Linnaeus. 1758). Charles Darwin also expressed in his book "The power of movement in plants" (Darwin and Darwin. 1880) that folded-leaf state of plants at night as "sleep" and figure out that it was a way for plants to reduce exposure and preserve the energy. Studies from the Karmer and Frisch lab, are the first proof of the existence of a biological clock in which they showed that birds and bees can maintain a given direction throughout the day using a time-compensated sun compass in navigation (Kramer G et al. 1952; Von Frisch K. 1950, 1974). Researchers also proposed that circadian systems are conserved throughout evolution. Research work from the Pittendrigh lab proved that circadian clocks are temperature independent (Pittendrigh. 1954). His work concluded that temperature changes did not affect the clock. Aschoff work constructed the "Circadian Rule", which reveal "that in light-active animals: the

spontaneous frequency (Aschoff.1958), the ratio of activity time to rest time (Aschoff. 1960), and the total activity (Aschoff. 1963) all increase with increasing intensity of continuous illumination." Finally, it was shown that circadian rhythms are present in all major groups of organisms (Pittendrigh and Minis. 1964; Evans. 1966).

1.3 Master Clock – suprachiasmatic nucleus

Initially, these 24-hour processes were seen as part of a time-measuring capacity of the organism. However, Colin Pittendrigh, a co-founder of modern chronobiology, proposed that there was a distinct light-sensitive oscillator that acted as an internal clock for 18 the organism (Pittendrigh, 1960). From this point on chronobiologists sought a central master clock within the mammal. To investigate these further, researchers ablated several regions of the brain to search for the brain region involved in driving circadian rhythm. Research studies on rats with lesions in the suprachiasmatic nuclei (SCN) showed that the animal's locomotor activity results into arrhythmic, showing that the SCN is the structure driving circadian rhythms in behavior (Moore and Eichler, 1972). The suprachiasmatic nuclei (SCN) are located in the brain at the base of the hypothalamus, directly above the optical chiasma. SCN consist of about 10-20,000 neurons per nucleus. The same effect of lesions in the SCN was observed in other mammals like golden hamsters (Refinetti et al. 1994) and mice (Schwartz and Zimmerman. 1991).

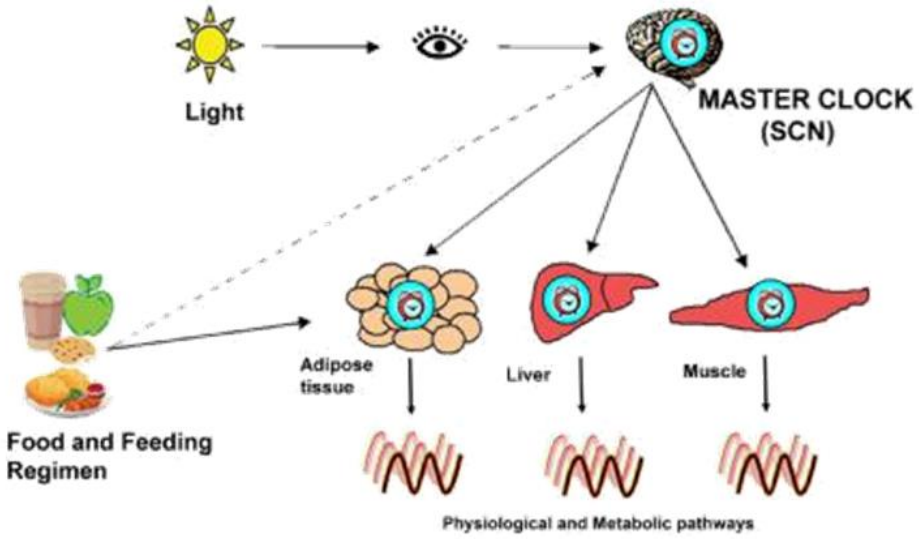

Figure1-1. The mammalian circadian system and the communication between the Master clock and peripheral clock. Light signals the master clock in SCN which in turn synchronize the peripheral clocks via hormonal and neuronal signals, SCN also dictates rhythms in a time of feeding, and behavior. Food and feeding regimen can directly reset the peripheral clocks uncoupling it from the SCN clock.

Later studies clearly defined the SCN as the master clock in mammals came soon after the discovery of the (natural) tau mutant golden hamster, exhibiting circadian behavior with a periodicity of 20 h (for homozygous animals) as compared to 24 h for wild-type animals (Ralph and Menaker. 1988). By transplanting fetal SCN tissue from hamsters with a short period to SCN-lesioned wild type recipient's circadian rhythms were restored with a short period, demonstrating that the SCN is the location of the master clock. The master clock receives the light input from the retina through the retinohypothalamic tract (RHT) that synchronizes internal clock timing to the external

light/dark cycle, which it passes on to the peripheral clock via neuronal and hormonal pathways. (Pando et al. 2002; Yo et al. 2004).

These molecular clocks present in the SCN and clock present the peripheral tissues share the same molecular architecture and generate similar circadian rhythms (Yo et al. 2004). The synchronization between master and the peripheral clock is necessary to maintain rhythms in physiology and metabolism. 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)

1.4 Photoentrainment- Inputs into the SCN

To regulate the daily light and dark cycle, the neurons from the SCN (Suprachiasmatic nucleus) collect photic information as electrochemical signals. These signals are transmitted via direct synaptic connections with the retina, specifically through the retinohypothalamic tract (Ralph and Menaker. 1989; Rusak et al. 1989). Several studies at molecular and genetic level have been performed to describe the light-input pathway for the circadian oscillator in mammals and several candidate photoreceptors have been proposed. It is known that light for vision is absorbed by rhodopsin in rods and for color by opsins in cones, which are located in the outer layer of the retina (Figure 1-2). To investigate whether retinal rods and cones are required for photoentrainment, the effects of light on the regulation of circadian wheel-running behavior were examined in mice lacking these photoreceptors (Freedman et al. 1999). It was concluded that mice without cones or without both rods and cones exhibit

normal phase-shifting responses to light, whereas removal of the eyes abolishes this behavior.

Therefore, it was concluded that neither rods nor cones are required for photoentrainment, and the eye must contain additional photoreceptors that regulate the circadian clock. Studies also have shown that Melanopsin which is an opsin-like protein is an attractive candidate for a photoreceptor. Melanopsin is expressed by a subset of mouse and human retinal ganglion cells (RGCs) (Provencio et al. 1998, Hattar et al. 2002).

The RGCs are situated in the inner nuclear layer of the front part of the retina. Research studies have shown that Melanopsin project the light signals via the retinohypothalamic tract (RHT) to the SCN (Figure 1-2). Studies on Melanopsin-deficient mice concluded that Melanopsin is required for normal circadian responses to the light, as those mice displayed severely attenuated phase resetting in response to brief pulses of light (Panda et al. 2002).

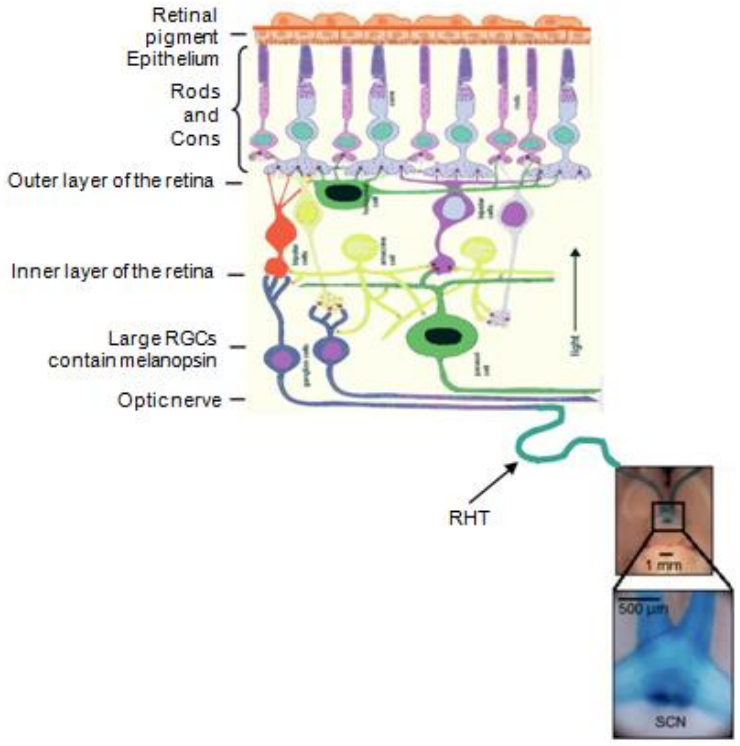

Figure 1-2. Cellular organization of the retina

Visual photoreceptors (rods and cones) located next to the pigment epithelium are stimulated by light that passes various layers of the retina. Retinal ganglion cells (RGCs) are also photosensitive and provide photic input to the circadian system located in the suprachiasmatic nucleus (SCN) via the retinohypothalamic tract (RHT). This diagram is adapted from Dowling (1997) Encyclopedia of Human Biology, vol. 7 pp 571-87.

1.5 The clock in different mammalian tissues-Peripheral clock

It is believed that almost every cell within the mammals contains a molecular clock but the two-known exception known for this rule is the cells within the thymus and testis. In these tissues clock genes expression is not circadian in character and does not oscillate with a 12-hour period (Alvarez et al. 2010). Similarly, mouse embryonic

stem cells also showed a lack of rhythmicity. It has been also shown that peripheral clocks within the peripheral organs with immature or non-differentiated tissues does not function properly (Stratmann & Schibler. 2006).

Molecular oscillators are not only restricted to the master clock, but they are also found in other regions of the brain and in most peripheral tissues (Giebultowicz et al. 2000). It has been found that in peripheral tissue there is a few hours (3-9 h) delay in cycle with respect to the oscillation existing in the SCN (Zylka et al. 1998). The peripheral clocks of Drosophila can be entrained directly by light via non-ocular mechanisms (Plautz et al. 1997) while in mammal's peripheral oscillators do not directly respond to light but are synchronized via neuronal and humoral signals from the SCN (Balsalobre et al. 1998). Studies also showed that immortalized fibroblasts treated with serum or chemicals activate a variety of known signal transduction pathways, exhibit rhythmic expression of several circadian genes persisting for several days (Balsalobre et al. 2000; Yagita and Okamura. 2000). Additionally, peripheral oscillators also respond to other stimuli (e.g. food availability) independently from the SCN (Cermakian and Sassone-Corsi. 2002). Interestingly, it was shown that the molecular composition of the circadian clock in the SCN and periphery is very similar (Yagita et al. 2001). Therefore, it was proposed that the SCN is a master clock that synchronizes the timing of the peripheral oscillators according to light input that SCN receives from the environment and in response to these peripheral oscillators regulate local rhythms in physiology and behavior (Figure1-1) (Reppert and Weaver. 2002; Schibler et al. 2003).

However, studies showed that the function of peripheral clock and specific contribution of each molecular component of the clock is highly tissue-specific. This is clearer when studies demonstrated differential expression patterns of Clock mRNA between tissues. Clock mRNA oscillates within peripheral systems, but it shows constitutive expression within the SCN (Lowry et al. 2004). Similarly, Rev-erb (α, β and γ) present markedly differing expression patterns across tissues (Sato et al. 2003. Akashi et al. 2005). Ror-α display robust oscillation within the SCN while little rhythmic expression is observed in the peripheral tissues. Ror -γ has been shown to contribute within certain peripheral clocks; however, is not expressed or functional within the SCN (Guillamound et al. 2005). In conclusion, it is important to study peripheral clock behavior on a tissue-specific level to fully comprehend their function. This is becoming significantly important as an increasing number of diseases are associated with circadian clock disruption.

1.6 The Molecular Clock

Across the kingdoms the basic structural mechanism of the circadian clock is ubiquitous. Clock gene and their protein products arranged to form an auto-regulatory feedback loop based on transcription and translation. During the past 40 years, dozens of genes have been found regulating circadian rhythms. The interplay of these genes constitutes the molecular pacemaker. In the center of the map are two interlocked transcriptional-translational feedback loops. This is certainly true for the circadian clock in Drosophila, Danio Rerio and rodents (Young & Kay. 2001).

In mammals, the primary loop consists of four components: Two basic helix-loop-helix (HLH), Period-Arntl-Single-minded (PAS) transcription factors, CLOCK, and BMAL1. CLOCK (or its homolog NPAS2) and BMAL1 heterodimerise and interact with E-box cis-regulatory enhancer sequences within the promoters of the genes Period (PER 1, 2 and 3) and Cryptochrome (CRY 1 and 2). PER and CRY proteins forms heterodimer and translocate back into the nucleus, where they act in a negative manner upon the CLOCK: BMAL1 dimers, repressing CLOCK: BMAL1 transcription activity and thus their own transcription (Gekakis et al. 1998. Kune et al. 1999. Shermann et al. 2000. Sato et al. 2006). CLOCK: BMAL1 also act within a secondary loop, promoting the transcription of members of the retinoic acid-related orphan nuclear receptor families, Rev-erb and Ror. REV-ERB (α, β, and γ) and ROR (α and β) subsequently bind to retinoic acid-related orphan receptor response elements (ROREs) within the BMAL1 promoter, repressing and promoting BMAL1 transcription respectively (Guillaumond et al. 2005). These transcriptional-translational feedback falls into approximately ~24 hours rhythms to complete one cycle. The timing of these loops is controlled by post-translational modification, such as ubiquitination and phosphorylation. Both processes governing the degradation of core clock components and the timing of translocation back into the nucleus. These posttranslational modifications are thought to be driven by protein kinases, Casein Kinase epsilon (CK1ε) and Casein Kinase delta (CK1δ) (Lowry et al. 2000. Akashi et al. 2002)

Figure1-3. Molecular mechanism of the mammalian circadian clock.

A current model of the circadian transcriptional-translational loops (TTLs) in mammals. CLOCK/BMAL1 activates E-boxes in promoters of target genes (Pers, CRY s, and CCGs). PER and CRY proteins form a complex which inhibits CLOCK/BMAL1. Additional loops contain Rev-erbα/β and Rorα which regulate CLOCK and BMAL1.Kinase and phosphatase promoting their degradation.

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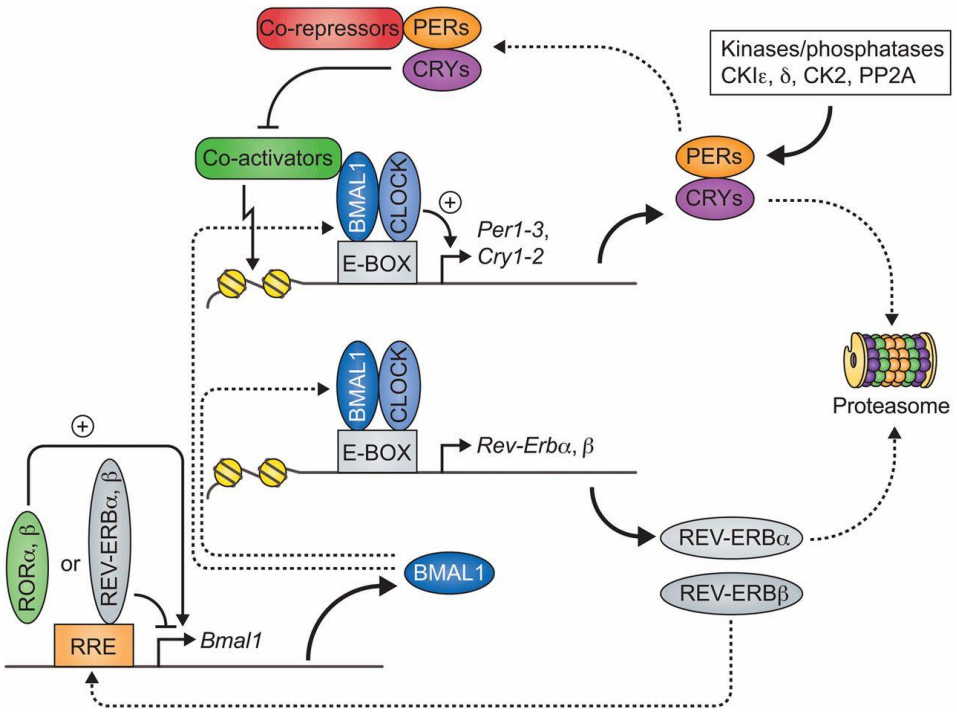


1.7 Circadian Clock and Human diseases

The changes in the body energy state are due to the day-night variation in food consumption, activity, and rest. Pieces of evidence demonstrated that circadian rhythms and human health are tightly interconnected and that disruption in circadian rhythms has profound consequences, many times leading to metabolic disease. The

disruption circadian alignment has been found at many incidents such as changes in the sleep-wake and fasting-feeding cycle. Mostly, these changes or misalignment has been to occur during shift-work, during let lag, and in certain circadian rhythm sleep disorders. Shift-work has been shown to be a risk factor for health problems such as cardiovascular disease, impaired glucose metabolism and obesity (Tenkanen et al., 1997; Morikawa et al., 2005; Suwazono et al., 2008; Suwazono et al., 2009). It is also observed that mice subjected to light exposure during the night and fed exclusively during their rest phase show increased body weight (Arble et al. 2009; Fonken et al. 2010). A Recent study also demonstrated that circadian misalignment has an adverse effect on hormonal factors such as glucose and insulin. Therefore, further research is needed to determine the underlying molecular mechanisms that cause the negative effects induced by the circadian disruption.

1.8 Circadian Clock Proteins

CLOCK and BMAL1 are transcription factors and core components of the circadian clock. Both the proteins consist of one basic helix–loop–helix (bHLH) domain and two PAS (Per-Arnt-Sim) domains. These domains play important role in DNA binding and heterodimerization, respectively. The C-terminal of CLOCK protein also consists of a characteristically active poly-glutamine repeat. The complex formation induces the phosphorylation and the nuclear localization. The NES (nuclear export signal) and NLS (nuclear localization signal) domains are highly conserved in BMAL1 and CLOCK. These domains are located adjacent to the PAS domain. In addition to various interconnected autoregulatory loops, the additional mechanism

regulates and supplement complexity to circadian clock in maintain circadian rhythms some of this mechanism are the post-transcriptional modifications, post-translational modifications, chromatin remodeling and stability of Clock proteins and intracellular localization. (Takahashi et al., 2006). PERIOD proteins are the member of the negative feedback loop; Period proteins are central components of the circadian clock. It has been showed that advances or delays of the molecular oscillator by PER proteins in the feedback loop occur by controlling the early repressive complex assembly. PER proteins consist of two tandemly organized PAS (PER–ARNT–SIM) domains, through which they form homo or heterodimers. 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 and phosphorylates PER1, PER2 and PER3 proteins, regulating each of them in a different manner. The members of the nuclear receptor family Rev-erb α/β and ROR are now accepted as bona fide components of the core clock. Rev-ERB and ROR proteins belong to the retinoic acid-related orphan receptor (ROR) with DNA binding domain recognizing ROR elements (A(A/T)NT(A/G)GGTCA). REV-ERBα and RORα compete for binding to ROR elements in the BMAL1 promoter region. REV-ERBα acts as a negative regulator while RORα acts as a positive regulator of BMAL1 transcription.

The focus of my dissertation is to investigate the physiological role of circadian clock protein, Cryptochrome. Therefore, I will discuss the Cryptochrome and its role in the molecular pathways in more details.

1.9 CRYPTOCHROMES

A. Discovery of mammalian Cryptochromes

Cryptochromes are found in many species such as plants, bacteria, and mammals. Cryptochromes belongs to photolyase/Cryptochrome family. Cryptochrome is structurally similar to flavoproteins and well characterized as blue-light photoreceptors where they play important role in UV-induced DNA damage repair mechanism (Photolyase), regulate growth (Plant Cryptochrome) and importantly in the alignment of circadian clock with daily light/dark cycles (mammalian Cryptochrome). Cryptochrome possesses 50% sequence identity to photolyases (Todo, T. 1999). Cryptochromes are known to regulate some of the blue-light responses including growth and development in plants. Importantly, in mammals, Cryptochromes are important to regulate the circadian rhythm by light-independent mechanisms in animals (Sancar A. 2000).

Based on exhaustive biochemical research data, it has been revealed that humans and other placental mammals do not have photolyase (Li Y. F, Kim S. T, and Sancar A.1993). Cryptochromes have demonstrated both light-dependent photoreceptor activity (bacteria, insects, and plants) like photolyase and light-independent transcriptional repressor activity (mammalian). The result was unexpected and led to a re-evaluation of the previous conclusion. Hsu et al. found that neither this protein nor a second ortholog they subsequently discovered had photolyase activity and suggested that these proteins must, therefore, perform other blue-light-dependent functions in human cells and named them human Cryptochrome 1 and Cryptochrome 2 (hCRY 1 and hCRY 2).

B. Structure and evolution of Cryptochromes

Photolyase/Cryptochrome is monomeric 50-70 kDa protein composed of two non-covalently bound chromophore/cofactor (Sancar.2003). One of the cofactors is always FAD. The second chromophore is methenyltetrahydrofolate (MTHF)1 in the most organism and 8-hydroxy-5-deazariboflavin in a few species that synthesize this cofactor. Photolyase plays important role in repairing UV-induced DNA damage using violet/blue light (350 –500 nm) as the energy source to initiate the reaction.

Cryptochrome consist of the photolyase homology region (PHR) within N-terminal domain and C-terminal domain consists of the canonical flavin-binding site. The N-terminal is conserved in both photolyase and Cryptochrome proteins whereas the C-terminal is somewhat divergent and dispensable for its repressive activity. The C-terminal domain of Cryptochrome possesses the phosphorylation and nuclear localization signal site which can modulate the clock protein functions. It has been showed that Cryptochrome activity can be regulated by phosphorylation. Cryptochromes are phosphorylated by several kinases such as CKI, MAPK, and AMPK. It is concluded that phosphorylation of Cryptochromes by MAPK and CKI reduces the repressor activity of Cryptochromes. It is also proposed that the AMPK mediated phosphorylation target CRY 1 for FBXL3 mediated degradation thus, reducing the CRY 1 stability. The CC helix on the CRY's 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 CRY s when bound to the PERs which restricts the interaction with

the FBXL3 (Hirayama & Sassone-Corsi. 2005).

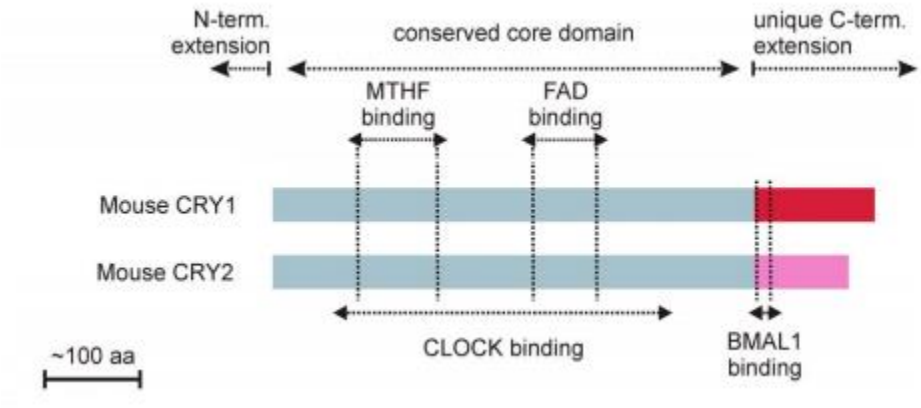


Figure 1-4. Schematic representation of the functional domains present in CRY's. Conserved and unique domain are indicated above, chromophore binding site is indicated by vertical dotted lines. CLOCK and BMAL1 binding regions in mouse CRY.

This figure is adapted from https://www.ncbi.nlm.nih.gov/pubmed/21858120

C. Role of Cryptochrome in circadian clock mechanism

Both hCRY 1 and hCRY 2 has been found to bind with moderate affinity to DNA and with higher affinity to UV light-damaged DNA but have no repair activity (Zhu R S et al. 2003). To understand the biochemical properties of mammalian Cryptochromes, a panel of ten mutant mCRY 1 expression constructs was generated, based on domains identified at the sequence level that is at the carboxy-terminal 100–120 amino acids of mCRY 1, which most distinguishes the protein from mCRY 2 as well as CRY's from other organisms (e.g., Drosophila) and which is lacking in photolyases. Different mutant proteins, which include mutations in the nuclear localization signal (NLS) domains, deletion of the C-tail, deletion of the coiled-coil, and mutation of tryptophan 320 to phenylalanine (in photolyase involved in intramolecular electron transport) was

performed. Using this panel of mutant constructs, it was concluded that the domains involved in protein–protein interactions and subcellular localization of the protein located in the carboxyl terminus of mCRY 1. This domain is involved in association with mPER2 as well as with BMAL1 (Chaves et al. 2006). Another group of researchers performed an experiment with genetically modified mice in which the mCRY 1 and/or mCRY 2 genes were inactivated and revealed that the two mouse Cryptochromes are part of the molecular clockwork generating behavioral and molecular rhythms. Notably, single-knockout mice have opposing circadian phenotypes, as evident from the observation that mCRY 1–/– and mCRY 2–/– mice display short and long behavioral periodicity, respectively, as measured by voluntary wheel-running activity (van der Horst et al. 1999; Vitaterna et al. 1999). Remarkably, behavioral analysis of mCRY 1/2 double- knockout mice indicated a complete loss of the circadian clock in these animals (van der Horst et al. 1999). Thus, mCRY 1 and mCRY 2 proteins not only have an antagonistic clock-adjusting function but are also essential for maintenance of circadian rhythmicity.

The mCRY 1 and mCRY 2 genes are expressed in a circadian manner, whereas simultaneous inactivation of the mCRY genes abolishes cycling of mPER1and mPER2 expression (Okamura et al. 1999). A key observation was the finding that Cryptochromes proteins are much stronger inhibitors of CLOCK/BMAL1-driven transcription of E-box-containing reporter genes than mPER proteins (Kume et al. 1999). This observation placed CRY proteins unequivocally at the core of the circadian oscillator and pointed to them as being the most important factors in maintaining the negative feedback loop of the molecular oscillator.

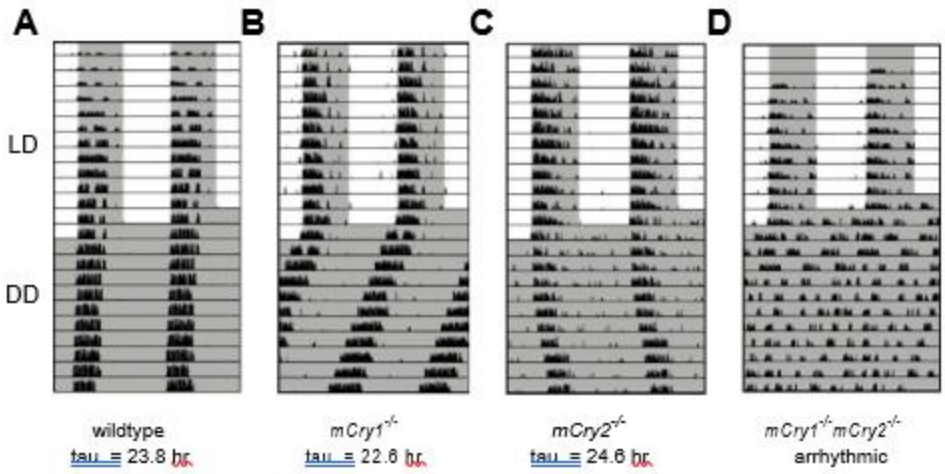


Figure 1-5. Circadian phenotype of CRY mutant mice

Representative double-plotted actogram of A) wild-type animals (n=14), B) mCRY 1 knockout animals (n=9), C) mCRY 2 knockout animals (n=5) and D) mCRY 1 and mCRY 2 double knockout animals (n=8). Mice were under LD (12:12) and DD conditions. Shading indicates darkness and the black vertical lines represent animal activity. This figure is adapted from https://www.ncbi.nlm.nih.gov/pubmed/10217146

Interestingly, the function of mCRY 1 and mCRY 2 in the molecular oscillator is not exclusively confined to the level of transcription repression; the proteins also have a role in the posttranslational control of other core clock proteins. Coimmunoprecipitation studies with transiently expressed clock proteins and yeast two-hybrid experiments have uncovered direct interactions between mCRY proteins and various other core oscillator components (i.e., mPER2, mPER1, CLOCK, and

BMAL1) (Shearman et al. 2000; Yagita et al. 2002; Chaves et al. 2006). In addition, immunohistochemical and biochemical studies revealed synchronous circadian

patterns of abundance, phosphorylation status, and nuclear localization of mCRY and mPER proteins (Lee et al. 2001). Particularly, the observation that mPER2 mRNA levels are increased in mCRY 1/2 double-knockout mice, whereas mPER2 protein could not be detected in the cytoplasm or the nucleus of SCN neurons (Shearman et al. 2000), suggested that mCRY proteins are necessary to stabilize mPER2. Indeed, physical interaction between mPER2 and mCRY proteins was shown to inhibit PER2 ubiquitylation and proteasomal degradation (and possibly changes in subcellular localization) of the mCRY-mPER complex (Yagita et al. 2002). Thus far, known protein–protein interactions of, as well as CLOCK/BMAL1-mediated transcription inhibition by, mCRY proteins have been shown to be independent of light (Griffin et al. 1999).

D. Phenotypes of CRY 1–/– and CRY 2–/– Mice

The analysis of the phenotype of mCRY mutant mice revealed that the mCRY 1 gene drives long-period circadian clockworks, whereas the mCRY 2 gene confers short periodicity to the circadian oscillator (Okamura et al. 1999; van der Horst et al. 1999). It is also proposed that two genes/proteins could be expressed in different circadian time windows, CRY 1 and CRY 2 proteins reach the nuclear compartment in a synchronous manner (Field et al. 2000; Lee et al. 2001). It was concluded that two proteins have opposing circadian effects because of sequence differences translating in different activities in and/or posttranslational regulation of the clock. It was also hypothesized that short and long-period phenotypes of CRY 1–/– and CRY 2–/–

mutant mice could be the result of alterations in the phosphorylation patterns of PER proteins (Vanselow et al. 2006).

E. Biochemical and Physiological properties of Cryptochromes

a. Role of Cryptochrome in metabolism

It is well established that Cryptochromes are essential for circadian timekeeping; the involvement of CRY's in numerous additional signaling pathways is still needed to be established. Cryptochromes are core clock genes and exhibits rhythmic expression in nearly every tissue in the circadian pattern. This time-dependent expression of Cryptochromes is known to impart circadian control on the regulation of downstream pathways throughout the body. Cryptochrome proteins act as a second messenger between the core clock and other cellular processes in which they participate. Cryptochromes regulate many of the signaling pathways involved in the maintenance of cellular, homeostasis such as metabolism, inflammation, and DNA damage, besides regulating CLOCK: BMAL1 complex activity. These pathways help to maintain cellular and genomic integrity by sensing the status of the cell such as metabolic and genotoxic stress. Genetic ablation or knockdown of Cryptochrome revealed many deleterious effects and a large variety of metabolic disturbances. Studies have shown that Cryptochrome double knockout mice exhibit a marked reduction in size and body weight compared with controls (Bur et al. 2009). Disruption of circadian rhythms is correlated with increased incidence of metabolic disturbances (Wang, X. S. 2011). It is also known that Cryptochrome double knockout mice show disturbed sugar metabolism with hyperglycemia and glucose intolerance, indicating a strong link

between the circadian clock protein Cryptochrome and metabolism (Lamia et al. 2011). Although blood triglyceride levels of Cryptochrome deficient mice are reduced, they still develop hepatic steatosis indicating defects in triglyceride metabolism (Cretenet et al. 2010).

Recent studies have shown that Cryptochromes directly regulate glucose homeostasis independent of CLOCK: BMAL1-regulated transcription. CRY 1 and CRY 2 have been shown to interact with the glucocorticoid receptor (GR) to repress glucocorticoid-stimulated changes in transcription (Lamia K. A. 2011). It was confirmed that GR-CRY 1 negatively regulates the rate-limiting gluconeogenic enzyme, phosphoenolpyruvate carboxykinase 1 (Pck1). Consistent with these collective findings, glucose homeostasis is severely disrupted in CRY -deficient mice, highlighting the importance of Cryptochromes in metabolic disease.

b. Cryptochrome in DNA damage response

As mentioned previously Cryptochromes share evolutionary conservation and structural similarity to the DNA damage repair enzyme photolyase. It has been also shown that Cryptochromes do not directly repair DNA lesions like photolyase, but they do interface with pathways that modulate the cellular responses to DNA damage (Kang T. H. 2010; Kang T. H. 2009). In mammals, CRY 1 modulates the ATR-mediated DNA damage checkpoint by interacting with the cell cycle protein TIMELESS (TIM) (Lee, T. H. 2014). To date, mammalian TIM is not considered a central clock component. Instead, TIM mediates DNA damage signaling in the ATR-Chk1 pathway to control cell cycle checkpoints. In mammals, Cryptochromes

maintain their ability to interact with TIM, where it competes with ATR-Chk1 for a binding site on the N-terminus of TIM. Knockdown of TIM attenuates the canonical phase advance of the circadian system upon DNA damage insults. The competitive mechanism of interactions between CRY 1 and TIM and other clock proteins that regulate CRY 1 stability could represent one way in which circadian phase is altered in response to genotoxic stress.

c. Cryptochromes in cancer development

Epidemiological and animal studies show that disruption of circadian rhythms through environmental stimuli (light at night or shift work) or genetic means can lead to an increase incidence of cancer. The mechanisms by which circadian disruption leads to deregulation of cellular homeostasis and cell cycle control are still unknown. It was hypothesized that Cryptochromes play an important role in the circadian timing and regulate vital metabolic processes as well as the UV-induced DNA damage response. Interestingly, Sancar lab's in vivo study showed that CRY 1−/−; CRY 2−/− mice do not show an increase in cancer rate compared to wild-type mice, even after exposure to ionizing radiation (Gauger, M. A. 2005). Further research showed that loss of Cryptochrome reduces cancer risk in p53 mutant mice that are more prone to cancer. Deletion of Cryptochromes extends the median lifespan of p53−/−; CRY 1−/−; CRY 2−/− mice by 1.5-fold compared to p53−/− mice (Ozturk N. J. 2009). Studies in triple knockout fibroblasts demonstrated that they were more susceptible than p53–/– knockout cells to UV-induced apoptosis, implicating Cryptochromes in the transmission of p53- independent apoptotic signals in response to DNA damage.

Cryptochromes are connected to the inflammatory response as downregulation of CRY's modulates the levels of inflammatory cytokines and the NF-κβ-stimulated transcriptional response that sensitizes cells to apoptosis (Lee J. H. 2011).

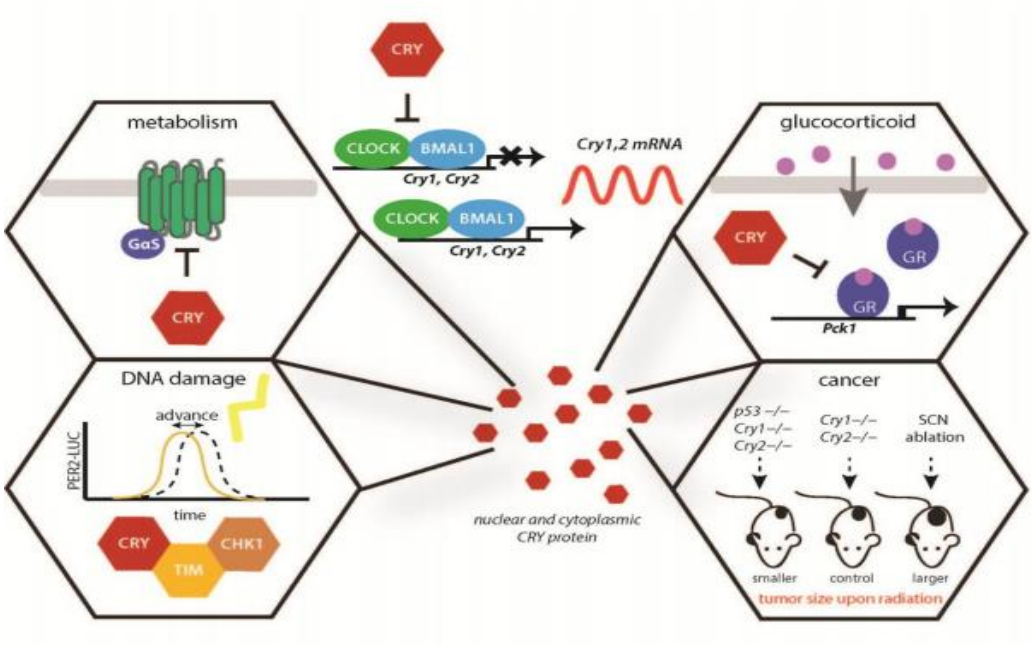


Figure 1-6. Roles of CRY outside of CLOCK: BMAL1 regulation.

In mammals, CRY s negatively regulates CLOCK: BMAL1 activity to generate a ~24hour clock that regulates ~40% of the genome. CRY is also reported to regulate GPCR signaling and downstream metabolism through interaction with the GSα subunit to block glucagon-stimulated increases in intracellular cAMP (top left). CRY negatively regulates the glucocorticoid receptor to maintain glucose homeostasis, partly through regulation of Pck1 expression (top right). Interaction of CRY with components of the ATR-mediated DNA damage checkpoint control phase shifting of the clock in response to DNA damage (bottom left). While ablation of the SCN increases tumor formation in mouse models, deletion of Cryptochromes extends lifespan after ionizing radiation in a p53 null background (bottom right).

This figure is adapted from https://www.ncbi.nlm.nih.gov/pubmed/27891621

1.10 Growth and body size control in mammals

The animal body plan to build through coordinated cell division, cell differentiation, and cell movements. It is also known that hormonal balance and their signaling pathway play an important role during development. Exhaustive research has been done to analyze the endogenous mechanisms that control these basic processes. At its simplest level, the clock can be considered as a three-part system: a pacemaker that generates the circadian rhythm; an input pathway whereby zeitgebers are perceived and adjust the phase of the pacemaker; and finally, an output pathway through which the pacemaker regulates a wide diversity of physiological processes and behavior (Menaker et al. 1978). The discovery of circadian clock mutants has led to the identification of core clock genes in a wide range of model organisms, and we now have detailed models to explain how they interact to generate clock function. Thus, in contrast to earlier beliefs, the clock is not a property restricted to neuronal networks, but ticks in every cell and might, therefore, operate well before the establishment of a functional nervous system. Previous and our finding on Cryptochrome double knockout mice have a reduced body size and body weight brings us to hypothesize that circadian clock gene Cryptochrome may have a different role independent of as a part of the circadian clock. The focus of my work is to address 1. What other molecular pathways are in under control of Cryptochrome 2. What are the regulatory targets of circadian clock protein Cryptochrome? 3. What is the role of Cryptochrome in regulating growth?

One of the most interesting features among various animals is the differences in size. The molecular exact mechanism that controls organ or organ size is not yet known. To date, we know that we are larger than a dog or a mouse, but we do not know why. We also know that our left eye is exactly the same size as our right eye, and our brains or hearts can grow to an exact size big enough to do their job. Many questions on the mechanism of size control remain to be answered. Various factors and signaling pathways have been studied to uncover the truth controlling the body size and growth. For example, GH-IGF1 axis, Mitogens (EGF, PDGF, Survival factors (IL3), Hippo signaling pathways. Our previous investigation on CRY's deficient mice demonstrated that CRY 1,2 -/- mice have reduced and body weight and body size. Therefore, we hypothesized that Cryptochrome might involve in GH-IGF1 signaling. IGF-1 is the major mediator of Growth hormone action in the body. IGF1 is produced in response to GH in liver and around 90% IGF1 produced in Liver. GH-mediated JAK2-STAT5B signaling pathway drives the IGF1 expression. It has been shown that the IGF-1 promotes growth of muscle, bone, and adipose tissues in mammals (Anisimov and Bartke, 2013; Chia, 2014); skeletal muscles are one of the main targets of IGF-1 (Figure 1-10). In my dissertation, I am focusing on the role of Cryptochrome in IGF1 regulation.

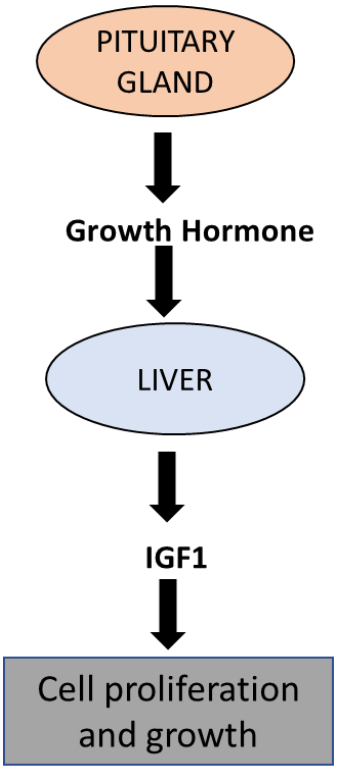


Figure 1-7. GH-IGF1 axis. GH secreted from pituitary induces the IGF1 production in the liver which plays important role in cell proliferation and growth.

1.11 IGF-1 System and Signaling

IGF1 is the mediator of growth hormone action in the body. IGF-I can play an important role as circulating hormone as well as tissue growth factor. The liver is the major source of IGF-1 production. The IGF-1 production from the liver is a complex system and it is known that GH-mediated signaling pathway plays a vital role in upregulating IGF-I gene expression. Initially, it was supposed that virtually all IGF-I originated in the liver and was transported by endocrine mechanisms to sites of action,

but it is now recognized that IGF-I is also synthesized in other organs where it exerts autocrine or paracrine effects. Similarly, to IGF-I, IGF-II is also expressed both in the liver and in extrahepatic tissues but is not tightly regulated by GH (Hackett et al, 1997; Kim et al, 1998). At the surface of target cells, the biological actions of the IGFs are mediated by receptors responsible for the transmission to a highly regulated intracellular signaling network. The IGF system comprises the ligands (IGF-I, insulin, and IGF-II), their receptors (IGF-I receptor, IGF-IR; insulin receptor, IR; six IGF binding proteins (IGFBPs) and their proteases.

Following IGF-I ligand binding, the intracellular tyrosine kinase domain autophosphorylates specific tyrosines on IGF-1R. These proteins then recruit other substrates leading to activation of different signaling cascades, including the phosphatidylinositol 3-kinase (PI3K)–AKT pathway that stimulates growth. Skeletal muscle is the major target of IGF1 signaling. Binding of IGF1 to its receptor leads to activation of its intrinsic tyrosine kinase and autophosphorylation, thus generating docking sites for insulin receptor substrate (IRS); this is also phosphorylated by the IGF1 receptor. Phosphorylated IRS then acts as docking site to recruit and activate phosphatidylinositol-3-kinase (PI3K) which phosphorylates membrane phospholipids, generating phosphoinositide-3,4,5-triphosphate (PIP3) from phosphoinositide-4,5-biphosphate (PIP2). PIP3 acts in turn as a docking site for two kinases, phosphoinositide-dependent kinase 1 (PDK1) and AKT, and in turn, AKT gets phosphorylated leading to AKT activation. AKT, in turn, inhibit FOXO activity. FOXO factors are required for the transcriptional regulation of the ubiquitin ligases atrogin-1, also called muscle atrophy F-box (MAFbx) and muscle ring finger 1

(MuRF1), leading to the ubiquitylation of myosin and other muscle proteins and their degradation via the proteasome.

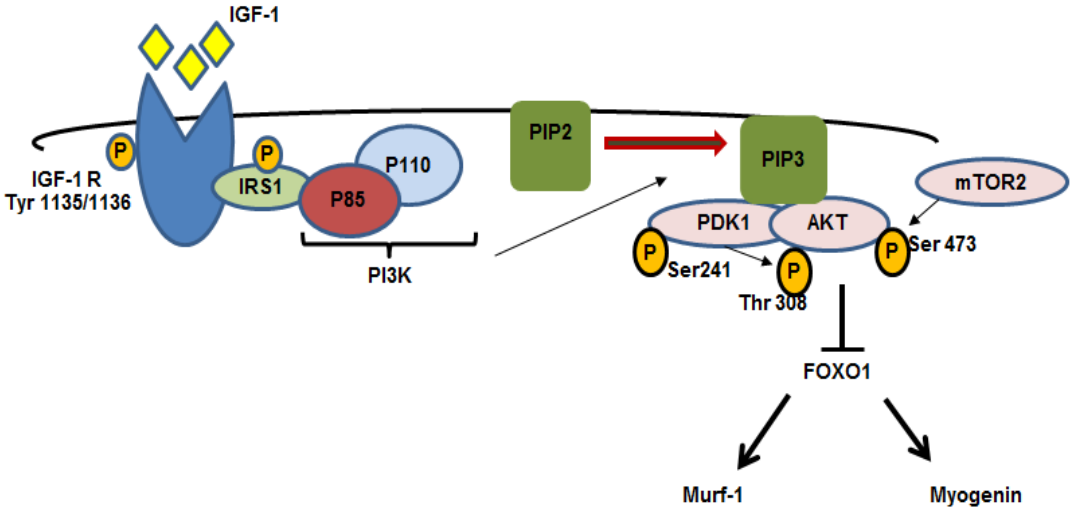


Figure 1-8. IGF1 signaling in skeletal muscle. IGF1 binding causes auto-phosphorylation and activation of IGF1R. Receptor activation stimulates phosphorylation of PDK1(Ser241) and AKT (Ser473) results in the inhibition of FOXO transcription activity.

1.12 Growth hormone-mediated JAK2-STAT pathway

Human growth hormone (GH) is a single chain peptide consisting of 191 amino acids. Growth hormone is the part of the hypophyseal-pituitary axis. Growth hormone is released from somatotroph cells in response to binding of growth-hormone-releasing hormone (GHRH) to its receptor and activating gene transcription by cyclic adenosine monophosphate-dependent mechanisms. Central and peripheral mechanisms are involved in regulating growth hormone's release from somatotroph cells. Growth

hormone has multiple actions which play an important role to promote linear growth, increased muscle mass and reduce fat stores. Direct actions of GH-mediated by binding its receptor, located mostly in the liver and been found in bone, muscle, adipose, brain and immune tissues. The growth hormone binding to its receptor induces intracellular signaling by a phosphorylation cascade involving the JAK/STAT (signal transducing activators of transcription) pathway. Its predominant action is to stimulate hepatic synthesis and secretion of IGF-I, a potent growth, and differentiation factor. The indirect effects are fulfilled through up-regulating hepatic gene expression and production of Insulin-like growth factor-1 (IGF-I). It is also showed that IGF-1 feedbacks to regulate GH production and secretion from the pituitary (Liu J. L. 1999). Clinical study result showed that deficiency in growth hormone or defects in its binding to the receptor is seen as growth retardation or dwarfism. GH-deficient patients or defect in growth hormone action have abnormal skeletal growth (Melmed S. 1995).

Production of IGF-I in the liver and in other tissues is regulated by growth hormone (GH)–dependent control of transcription (Herrington et al. 2000; Herrington and Carter-Su. 2001; Rotwein. 2012; Chia. 2014). GH binds its receptor (GHR) and induces activation of the receptor-associated tyrosine kinase Janus kinase2 (JAK2). JAK2 phosphorylates the intracellular part of GHR, which results in engagement of the JAK-signal transducer and activator of transcription (STAT) signaling pathway (Lanning and Carter-Su. 2006). Transcriptional factors from the STAT family are recruited to the phosphorylated receptor and get phosphorylated on the Tyr residue by JAK2 (Herrington et al. 2000; Rotwein and Chia. 2010; Chia. 2014). After

dissociation from the receptor, phosphorylated STATs form dimers, translocate to the nucleus, and bind to specific sites and regulate transcription. (Figure 1-11)

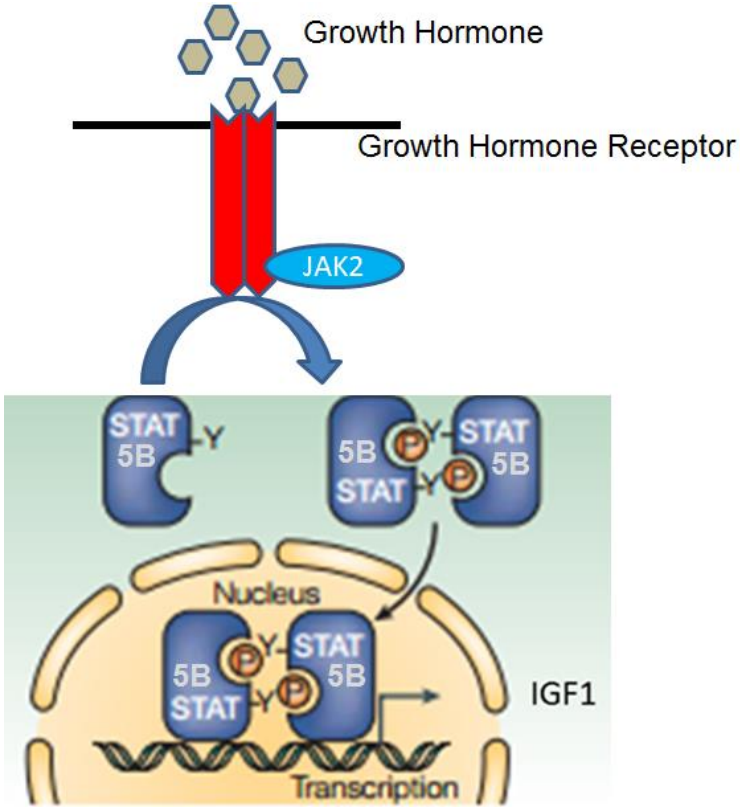


Figure 1-9. GH-mediated JAK2-STAT pathway. The activation of JAKs after growth hormone stimulation results in the phosphorylation of STATs, which then dimerize and translocate to the nucleus to activate IGF1 gene transcription. This figure is adapted from https://www.ncbi.nlm.nih.gov/pubmed/14668806.

Janus kinases (JAK proteins, JAKs) are receptor-associated protein tyrosine kinases. The formation of ligand-receptor complex induces the receptor-associated JAK proteins recruitment at the inside of the cell membrane which in turn induces trans-

phosphorylation of the JAK protein (Leonard and O'Shea. 1998; Yeh and Pellegrini. 1999). Four mammalian JAKs have been identified (JAK1, JAK2, JAK3, and TYK2) (Duhé and Farrar. 1998). Importantly, JAK2 kinase has been found to play important role GH-mediated signal transduction and growth. The JAK family members consist of JH1-JH7 domains.

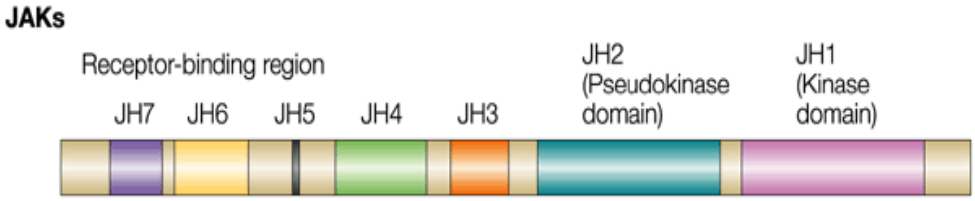


Figure 1-10. Domain structure of Janus kinases (JAKs).

The catalytic activity of the kinase domain JH1 is regulated by interaction with the pseudo-kinase domain JH2. JH3-JH7 mediates protein-protein interactions, e.g. receptor association. This figure is adapted from https://www.ncbi.nlm.nih.gov/pubmed/11905829

It was concluded that GH promotes the association of JAK2 with GH receptor which promotes activation of JAK2 in which JAK2 autophosphorylate itself within tyrosine residue and stimulate the phosphorylation of tyrosines of GH receptor (Argetsinger L. S. et al 1993). Recruitment of these signaling molecules to GH receptor/JAK2 complexes initiates a variety of signaling pathways that lead to the regulation of transcription of specific genes, cellular metabolic enzymes, and the actin cytoskeleton, ultimately leading to GH stimulation of body growth.

Signal transducers and activators of transcription (STAT) proteins are the best-characterized JAK substrates. Seven STAT family members are found in humans, STAT 1-4, STAT5A, STAT5B, and STAT6. Across the diversity of receptors that act via the JAK/STAT pathway, there is no simple relationship between which JAK family members activate which STAT family members.

Structure of STAT

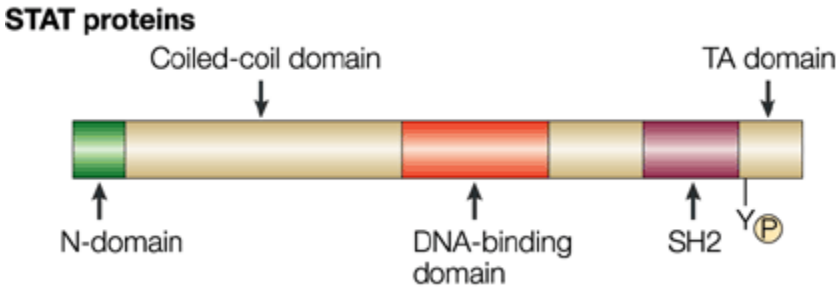


Figure 1-11. Signal transducer and activator of transcription (STAT) proteins consist of six different domains that mediate their interaction with cytokine receptors and upon dimerization with DNA.

This figure is adapted from https://www.ncbi.nlm.nih.gov/pubmed/11905829

STAT5A and STAT5B have been strongly implicated in GH signaling through their participation in transcriptional activation of multiple GH-regulated genes. STAT5 has recently been shown to contribute to regulating several components of the GH-insulin-like growth factor 1 (IGF-I) axis, long recognized as fundamental for growth-promoting actions of GH. Importantly, recent studies showed that the transcription factor STAT5B as a key component in acute GH-stimulated IGF-I gene activation thus extending previous observations pointing to effects of STAT5B on regulating postnatal somatic growth in mice. In summary, The IGF-I gene is a direct target of

STAT5B, and studies also revealed that IGF-I concentrations were reduced in STAT5B-deficient mice.

1.13 Negative regulation of JAK2-STAT pathway

Cytokines induce a variety of biological responses by binding to specific cell surface receptors and activating cytoplasmic signal transduction pathways, such as the JAK/STAT pathway. Although these responses are generally transient, few molecules have been characterized that switch the signal off. Several different steps of the signal transduction pathway appear to be targeted by negative regulators, including the receptor/ligand complex, JAK kinases, and STAT transcription factors. Recent studies identified three major classes of the negative regulator: SOCS (suppressors of cytokine signaling), PIAS (protein inhibitors of activated STAT's) and PTPs (protein tyrosine phosphatases). Many PTPs participate in the regulation of JAKs by ubiquitylation (Ub) has been suggested. The physiological significance of protein ubiquitylation in the regulation of JAKs remains to be determined. Protein inhibitor of activated STAT (PIAS) proteins interacts with STATs in response to cytokine stimulation and they inhibit the transcriptional activity of STATs through distinct mechanisms. (Figure 1-13)

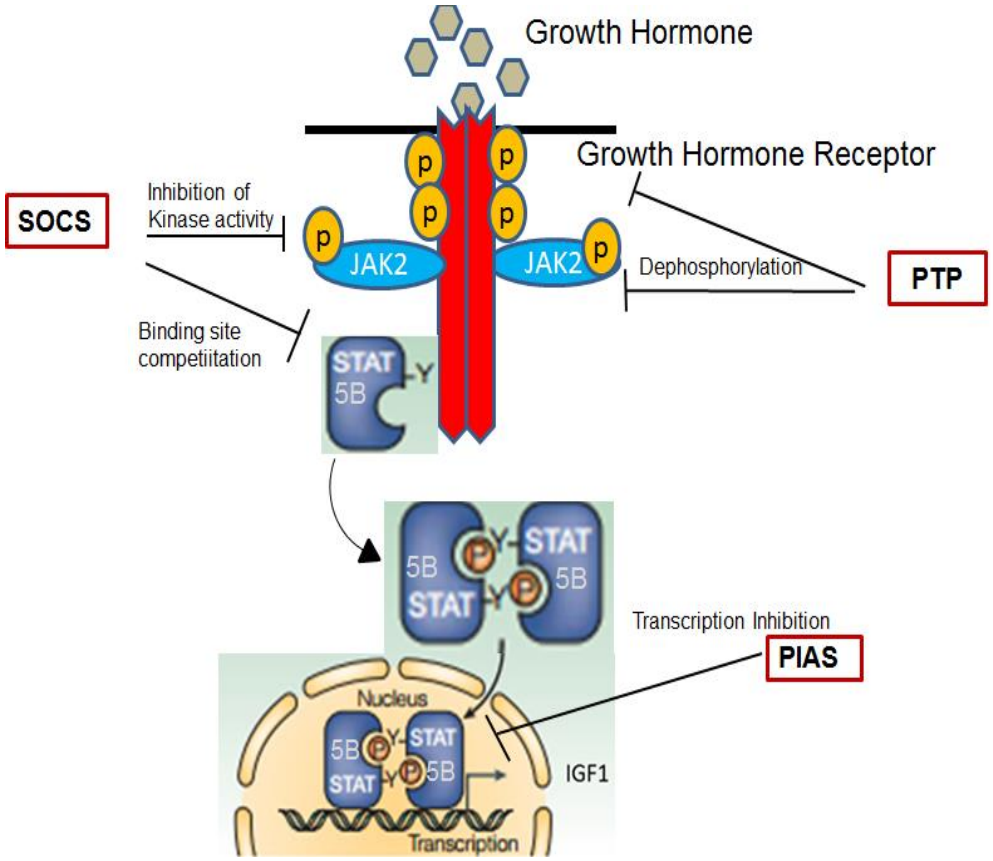


Figure 1-12. Negative regulation of the JAK–STAT pathway. JAKs can be negatively regulated by suppressor of cytokine signaling (SOCS) proteins, protein tyrosine phosphatases (PTPs), such as SRC homology 2 (SH2)-domain-containing PTP1 (SHP1), SHP2, CD45 and T-cell PTP (TCPTP), and ubiquitin-mediated protein degradation. SOCS proteins, which are induced by cytokines, act as a negative-feedback loop to switch off the activity of JAKs. Many PTPs participate in the regulation of JAKs. The regulation of JAK2 by ubiquitylation (Ub) has been suggested. The physiological significance of protein ubiquitylation in the regulation of JAKs remains to be determined. STATs can be negatively regulated by PTPs (such as PTP1B and TCPTP) in the cytoplasm, and by PIAS proteins, as well as PTPs (such as TCPTP and SHP2), in the nucleus. Protein inhibitor of activated STAT (PIAS) proteins interacts with STATs in response to cytokine stimulation

and they inhibit the transcriptional activity of STATs through distinct mechanisms. This figure is adapted from https://www.ncbi.nlm.nih.gov/pubmed/14668806

We hypothesized that SOCS's are the major negative regulators in CRY-deficient mice. Therefore, I will discuss SOCS's proteins in more details.

A. SOCS proteins

The Suppressor of cytokine signaling (SOCS) family consist of 8 SOCS proteins in mammals; SOCS1-7 and the alternatively named Cytokine-inducible SH2-containing protein (CISH). It has been shown that SOCS1-3 and CISH are predominantly associated with the regulation of cytokine receptor signaling. All SOCS proteins can regulate receptor signaling through the recruitment of proteasomal degradation components to their target proteins. SOCS proteins can directly inhibit JAK kinases, binding via their KIR domain to the JAK activation loop to inhibit kinase activity (Naka et al. 1997). CISH, SOCS2, and SOCS3 can also inhibit signaling via their ability to bind to phosphotyrosine residues typically on receptors, thereby blocking access of other SH2-containing signaling molecules.

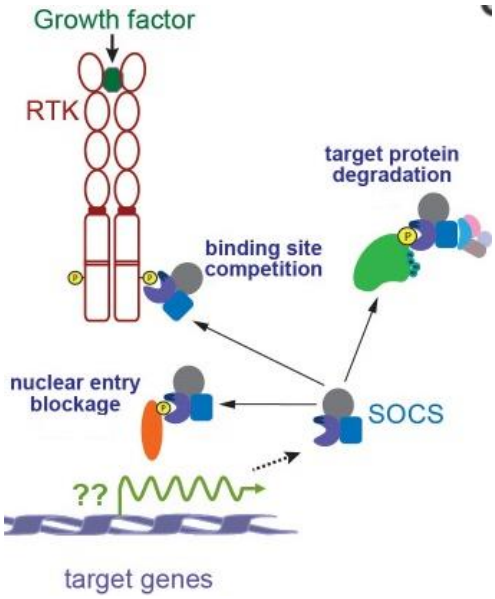


Figure 1-13. Mechanism of action of SOCS. SOCS negatively regulate receptor-mediated signaling. SOCS's can inhibit signaling by several mechanisms: a) Degradation of receptors or associated proteins via the proteasomal pathway. b) Inhibition of JAK tyrosine activity. c) Competition for receptor phosphotyrosine residues thereby blocking other signaling molecules. d) Prevention of nuclear translocation of key signaling molecules.

This figure is adapted from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3714205/.

A. Structure of SOCS's

Each SOCS protein consists of three distinct domains; an N-terminal domain of low conservation, a conserved central Src-homology 2 (SH2) domain, and a more highly conserved C-terminal domain termed the SOCS box. It has been also concluded that the SH2 domains of the SOCS proteins interact specifically with phosphotyrosine residues present on their target proteins, including cell surface receptors, imparting on

SOCS proteins their target specificity (O'Sullivan et al. 2007). Finally, the SOCS box is comprised of two functional sub-domains; a BC box that recruits Elongin B and C and a Cul box that mediates Cullin5 binding required for protein degradation.

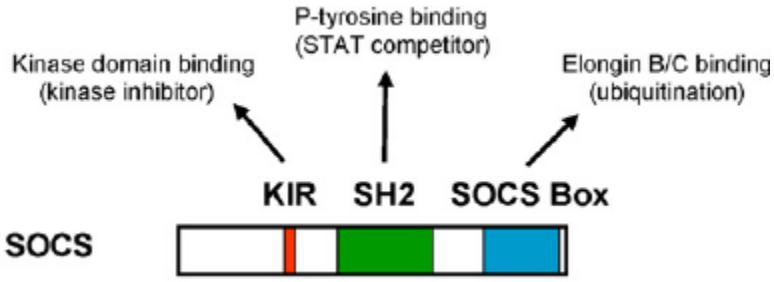


Figure 1-14. All SOCS proteins consist of three conserved domains, the N-terminal, SH2 and SOCS box domains. The N-terminal domain is the kinase inhibitory region (KIR). The SH2 domain is involved in substrate binding via interaction with specific phosphotyrosine residues on the target protein. The SOCS box consists of BC box and Cul box sub-domains mediate proteasomal degradation.

This figure is adapted from ttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC3714205

A. Regulation of SOCS expression

CISH is the founding member of the SOCS family. The study concluded that CISH gene induced by growth hormone (GH) (Adams et al. 1998; Helman et al. 1998; Pezet et al. 1999). It was also shown that STAT5A/B KO mice had no detectable CISH expression (Teglund et al. 1998; Moriggi et al. 1999). CISH has been shown to be a direct STAT5 target gene (Matsumoto et al. 1997; Moriggi et al. 2005). The SOCS1 expression has also been found to induced by growth hormone. (Adams et al. 1998; Hansen et al. 1999). SOCS1 is direct of STAT3. SOCS2 was shown to be highly expressed in the fetal kidney as well as adult kidney, lung, testes, liver, pancreatic

islets (Dey et al. 1998; Metcaf et al. 2000). SOCS2 is most closely related to CISH and like CISH is induced by cytokines that activate STAT5, including GH. SOCS3 has been shown to be expressed in a wide variety of murine and human tissues. SOCS3 has been demonstrated to be induced by the cytokines including growth hormone.

B. Mechanism of regulation GH-mediated JAK2-STA5B pathway

CISH is known to negatively regulate signaling induced by GH. CISH can bind to the same receptor phosphotyrosine sites as STAT5, thereby physically blocking further STAT5 docking, which has been demonstrated for the growth hormone receptor (GHR) (Verdier et al. 1998; Ram et al. 1999; Hansen et al. 1999). CISH can also negatively regulate signaling at the receptor level by facilitating proteasomal degradation of activated receptor complexes via interactions between its SOCS box, Elongin B/C and Cullin5 (Hanada et al. 2003; Piessevaux et al. 2008). SOCS1 is known to regulate signaling via two mechanisms. Firstly, it can bind directly to cytokine receptor-associated JAK1, JAK2, and TYK2 to inhibit their tyrosine kinase activity via its ESS/KIR domains (Yasukawa et al. 1999; Nicholson et al. 1999). However, like other SOCS family members, SOCS1 can also interact with Elongin B/C and Cullin5 via its SOCS box, facilitating ubiquitination and proteasomal degradation of target substrates (Zhang et al. 1999; Vuong et al. 2004). SOCS2 primarily exerts its effects by stimulating ubiquitination of target proteins, including receptors, such as GHR (Vesterlund et al. 2011), Like its closest homologue, SOCS1, the SOCS3 protein can directly inhibit receptor-bound JAKs, although it achieves this via a high-affinity interaction between its SH2 domain and a phosphotyrosine residue

on the receptor rather than the JAK (Sasaki et al, 2000). SOCS3 also regulates signaling via binding site competition.

1.14 Calorie restriction

A. History

Calorie restriction (CR) can be defined as an undernutrition without malnutrition (Black et al. 2001; Dhahbi et al. 2004). In pioneering experiments, McCay et al. provided reduced caloric input to white rats and successfully demonstrated the effect of reducing calorie intake on the total length of life. McCay's initial studies on rats showed that moderate calorie restriction is the only non-genetic method of altering longevity and attenuating biological processes associated with aging. Calorie restriction paradigm has been found to be effective in various species such as yeast, mice, rats, dogs and possibly non-human primates such as squirrel monkeys (Ingram et al. 1990; Lane et al. 1999b; Roth et al. 1999; Weindruch and Walford. 1988).

B. Physiological features

Calorie restriction has been found to affect various metabolic activities. An interplay between the calorie restriction and circadian clock has also been reported. Our lab demonstrated that calorie restriction upregulates BMAL1 expression and our lab also demonstrated that BMAL1 negatively regulate mTORC1 activity (R. V. Khapre et al. 2014); thus, increased BMAL1 activity may contribute to the reduction of mTORC1 activity upon CR.

CR increases SIRT1 activity which is related to a cascade of antithrombotic effects, DNA repair, genomic stability, apoptosis, growth and differentiation, inflammatory inhibition. Additionally, CR was connected to SIRT1 via an alteration in the NAD/NADH ratio; this activity was concluded to have a connection to longevity by amplifying overall SIRT1 activity. Furthermore, CR was shown to attenuate the up-regulation of nuclear factor (NF)-kB, a transcription factor that induces expression of tumor necrosis factor in white adipose tissue. (Lin S. J. et al., 2002). Calorie restriction is also known to reduce body mass and adiposity, body temperature and metabolic rate, blood pressure.

Calorie restriction also reduces glucose and fasting plasma insulin levels while it increases the insulin sensitivity and levels of high-density lipoproteins. Calorie restriction also reduces oxidative stress. (Gredilla and Barja. 2005; Gresl et al. 2001; Heilbronn and Ravussin. 2003)

Extensive research study results revealed that IGF-1 levels decrease upon CR. In contrast, the increased insulin sensitivity and normalized glucose levels in response to a CR result in lowered serum insulin and IGF-1. Surprisingly, GH level is not reduced upon CR (Bruss et al. 2011), and GH sensitivity is also not affected. The exact mechanism of how CR downregulates IGF-1 is not yet known.

1.15 Relationship between Circadian clock and Calorie restriction

The functional circadian clock is necessary for the beneficial effect of calorie restriction. We and others laboratory demonstrated that calorie restriction affects the rhythmic expression and amplitude of BMAL11, PER1and PER2 genes in the liver.

It has been also shown that calorie restriction also affects the expression of several clock genes in the SCN (Mendoza, et al). Thus, calorie restriction regulates circadian clock gene expression in different organisms and in different tissues and might affect both central and peripheral clocks. Studies also investigated if circadian clocks play any role in the calorie restriction effects on metabolism. Disruption of the circadian clock through the knockout of the core clock genes significantly compromised the beneficial effects of calorie restriction including the effect on longevity in both Drosophila and mice. Thus, these studies established that calorie restriction and circadian clock mechanisms are interlinked.

1.16 Role of Cryptochrome in IGF1 production and calorie restriction mechanism

In this dissertation, based on our preliminary results that CRY-deficient mice have reduced the IGF1 level, we are hypothesizing that Cryptochrome might play important role in IGF1 regulation. We are also investigating the role of Cryptochrome in calorie restriction-mediated downregulation of IGF1.

CHAPTER II

MATERIALS AND METHODS

2.1 Experimental Animals

Wild-type and CRY 1, 2−/− mice were previously described (Vitaterna et al., 1999). Mice of all genotypes were of C57B6J background. Mice were maintained on the 12:12 light: dark cycle with lights on at 7:00 am. All groups had unrestricted access to the 18% protein rodent diet (Harlan) and water. All tissue collection experiments were performed on 5-month old mice. For all experiments, at least three animals of each genotype and gender were used, with at least three animals per time point. For all experiments, animals were euthanized using CO2 chamber and all studies were performed with approval from and within the guidelines of the Institutional Animal Care and Use Committee of Cleveland State University.

2.2 RNA isolation and analysis of mRNA expression

For gene expression studies, tissues were collected every 4 h throughout the day and stored at −80°C. Total RNA was extracted from frozen tissue, using Trizol

(Invitrogen) reagent as per manufacturer's instructions and some modifications. Briefly, for every piece of tissue 1 ml of Trizol reagent was used. Tissues were homogenized with the pestle (Sigma-Aldrich) for at least 5 minutes or until the entire tissue was homogenized. This was done keeping tubes on ice. Tubes were spun at 40C at 11500 rpm for 10 min. Supernatants were transferred into new tubes and incubated at room temperature for 5 minutes. After adding 200 µl chloroform (Fischer Scientific), tubes were shaken vigorously and incubated for 2-3 minutes at room temperature. After spinning for 15 minutes at 11500 rpm at 40C, the aqueous phase was transferred to a new tube. The RNA was precipitated by adding 500 µl isopropanol. RNA was washed using 70% ethanol and the pellet was air dried for 5 minutes. The pellet was re-suspended in 30 µL of RNase-free water.

Measurement of RNA concentration

RNA concentration was measured using Nanodrop spectrophotometers. We used RNA's free water to adjust blank reading and dilution. We diluted RNA final concentration to 500ng/uL. RNA integrity was checked on 1% agarose gel run at 90 V for 30 minutes.

Analysis of mRNA expression

Following quantification, a 20µl reverse transcription reaction contained 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 controls, 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. The 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

Gene	Ref No	Product length	Primer	Location
18s	NR_003278.3	69	Forward GCT TAA TTT GAC TCA ACA CGG GA	1235-1258
			Reverse AGC TAT CAA TCT GTC AAT CCT GTC	1281-1305
IGF1	NM_001111274.1	138	Forward GAG ACT GGA GAT GTA CTG TGC	520-541
			Reverse CTC CTT TGC AGC TTC GTT TTC	637-658
Myogenin	NM_031189.2	129	Forward AAC CCA GGG GAT CAT CTG CTC AC	595-618
			Reverse GTT GGG CAT GGT TTC ATC TGG GAA G	699-724
Murf1	NM_001039048.2	66	Forward GTG AAG TTG CCC CCT TAC AA	564-584
			Reverse TGG AGA TGC AAT TGC TCA GT	607-630
CISH	NM_001317354.1	90	Forward CCG ACT GGA CTC CAA TTG CT	307-325
			Reverse CTG CAC AAG GCT GAC CAC AT	361-397
IGFBP3	NM_008343.2	70	Forward GAG TGT GGA AAG CCA GGT TGT C	516-318
			Reverse GCA TGG AGT GGA TGG AAC TTG	565-586
SOCS3	NM_007707.3	119	Forward GGT TCT GCT TTG TCT CTC CTA TG	1987-2010
			Reverse TCC CTC AAC TCT CTG CCT ATT	2085-2106
SOCS2	NM_001168655.1	92	Forward TCC CTC GTC TTA TGC AAC TAA TC	1551-1574
			Reverse GGA TCT CAT GGT GAT GGT TCT T	1621-1643
SOCS1	NM_001271603.1	138	Forward TAA CCC GGT ACT CCG TGA CT	806-826
			Reverse CTC CCA CGT GGT TCC AGA AA	924-944

2.3 Analysis of protein expression and phosphorylation

Western blotting was done on the liver and skeletal muscle samples pooled together from three different mice at each time point for each diet. For tissue lysates preparation, frozen liver and skeletal muscle 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 the sonicator (Fischer Scientific Model 100) for 5-10 seconds on ice. Lysates were centrifuged at 10000 rpm for 12 mins at 4ºC. The 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 the 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 onto 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 a 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).

Antibody table: II

Antibody	Cat No	Company
Phospho-STAT5B (Tyr 699)	9351	Cell signaling
Phospho-jak-2 (Tyr1007)	4406	Cell signaling
phospho IGF-1R β (Tyr 1135/1136)	3024	Cell signaling
Phospho AKT (Ser 473)	4060	Cell signaling
Phospho PDK1 (Ser241)	3061	Cell signaling
Total AKT	9272	Cell signaling
Total STAT5	9363	Cell signaling
Total PDK	3062	Cell signaling
Total IGF-1R	3018	Cell signaling
IGF-1	Ab36532	Abcam
B-Actin	A5441	Sigma
Antibody	Cat No	Company
SOCS-1	3950	Cell signaling
SOCS-2	2779	Cell signaling
SOCS-3	2923	Cell signaling
CISH	8731	Cell signaling
CRY 1	21414	Cell signaling
CRY 2	sc-130731	Santa Cruz Biotechnology

2.4 Measurement of the plasma IGF-I level

Blood/Plasma Collection

We sacrificed the mice in a CO_2 chamber and performed necropsy afterward to collect blood. Plasma separated by centrifuging blood samples at 6000 rpm for 20 min at 4°C.

Measurement of plasma IGF1

Plasma samples were collected every 4 h throughout the day and stored at −80°C. Plasma IGF-I levels were determined using a RayBio Mouse IGF-I 96-well plate enzyme-linked immunosorbent assay (ELISA) kit. Plasma samples were diluted 50-fold with the solution provided by the manufacturer. Sandwich ELISA was done using two different antibodies; the second antibody was conjugated with biotin. For detection, the manufacturer-provided streptavidin-conjugated horseradish peroxidase and TMB substrate were used for the colorimetric reaction, and optical density was detected at 450 nm. Manufacturer-provided mouse IGF-I was used to generate the standard concentration curve; starting concentration was 2 ng/ml, with serial dilutions. All experimental samples and standards were run in duplicate on the same plate; experiments were repeated three times with different plates. The intraassay coefficient of variation was between 4 and 9% for different plates; interassay coefficient of variation was 12%. The detection limit for IGF-I measurement was 4 pg/ml.

2.5 Statistical analysis

For all experiments, we used at least three animals for every time point for each feeding type and each genotype. 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 at each time point for statistically significant difference. IBM SPSS Statistics 20 and GraphPad Prism Version 5.04 software packages were used for statistical analysis. $P < 0.05$ was considered as a statistically significant difference. Data are shown as average +/- standard deviation (SD).

Inter-assay calculation

The same high and low IGF1 controls are run in duplicates on two different plates to monitored plate-to-plate variation. The plate means for high and low are calculated and then used to calculate the overall mean, standard deviation, and % CV. Overall % CV = (SD of plate means ÷ mean of plate means) x 100. The average of the high and low % CV is reported as the inter-assay CV.

Intra-assay calculation

IGF1 concentrations are measured in duplicate. The % CV for each sample is calculated by finding the standard deviation of results 1 and 2, dividing that by the duplicate mean, and multiplying by 100. The average of the individual CVs is reported as the intra-assay CV.

CHAPTER III

CRYPTOCHROMES REGULATE IGF-1 PRODUCTION AND SIGNALING THROUGH CONTROL OF JAK-2 DEPENDENT STAT5B PHOSPHORYLATION. [1]

Amol Chaudhari et al.

3.1Abstract

Insulin-like growth factor (IGF) signaling plays an important role in cell growth and proliferation and is implicated in the regulation of cancer, metabolism, and aging. Here we report that IGF-I level in blood and IGF-I signaling demonstrates circadian rhythms. Circadian control occurs through Cryptochromes (CRY's) transcriptional repressors and components of the circadian clock. IGF-I rhythms are disrupted in CRY-deficient mice, and the IGF-I level is reduced by 80% in these mice, which leads to reduced IGF signaling. In an agreement, CRY-deficient mice have reduced body

weight (~30% reduction) and organ size. Down-regulation of IGF-I upon CRY deficiency correlates with reduced IGF-I mRNA expression in the liver and skeletal muscles. IGF-I transcription is regulated through growth hormone–induced, JAK2 kinase mediated phosphorylation of transcriptional factor STAT5B. The phosphorylation of STAT5B on the JAK2-dependent Y699 site is significantly reduced in the liver and skeletal muscles of CRY -deficient mice. At the same time, phosphorylation of JAK2 kinase was not reduced upon CRY deficiency, which places CRY activity downstream from JAK2. Thus, CRY's link the circadian clock and JAK-STAT signaling through control of STAT5B phosphorylation, which provides the mechanism for circadian rhythms in IGF signaling in vivo.

[1]. As it appears in the Molecular Biology of the Cell Journal, 2017 Mar 15;28(6):834-842. doi: 10.1091/mbc. E16-08-0624

3.2 Introduction

We found that CRY-deficient mice develop dwarfism similar to other models of dwarfism with a reduced level of circulating IGF-1. The main action of Growth hormone is the promotion of growth. IGF1 is the major mediator of growth hormone action. A number of mutations that alter the GH/ IGF-1 axis have been identified in mice and humans and all of them are marked as a dwarf. Mutations in mice have growth hormone deficiency resulted into a reduce IGF1 production and IGF1 signaling. We hypothesized that Cryptochrome might play role in regulation of IGF1 production and signaling.

3.3 Materials and Methods

Details of the Animal experiment, mRNA, Protein analysis and measurement of plasma IGF-1 and statistical analysis have been explained in Chapter II.

3.4 Results

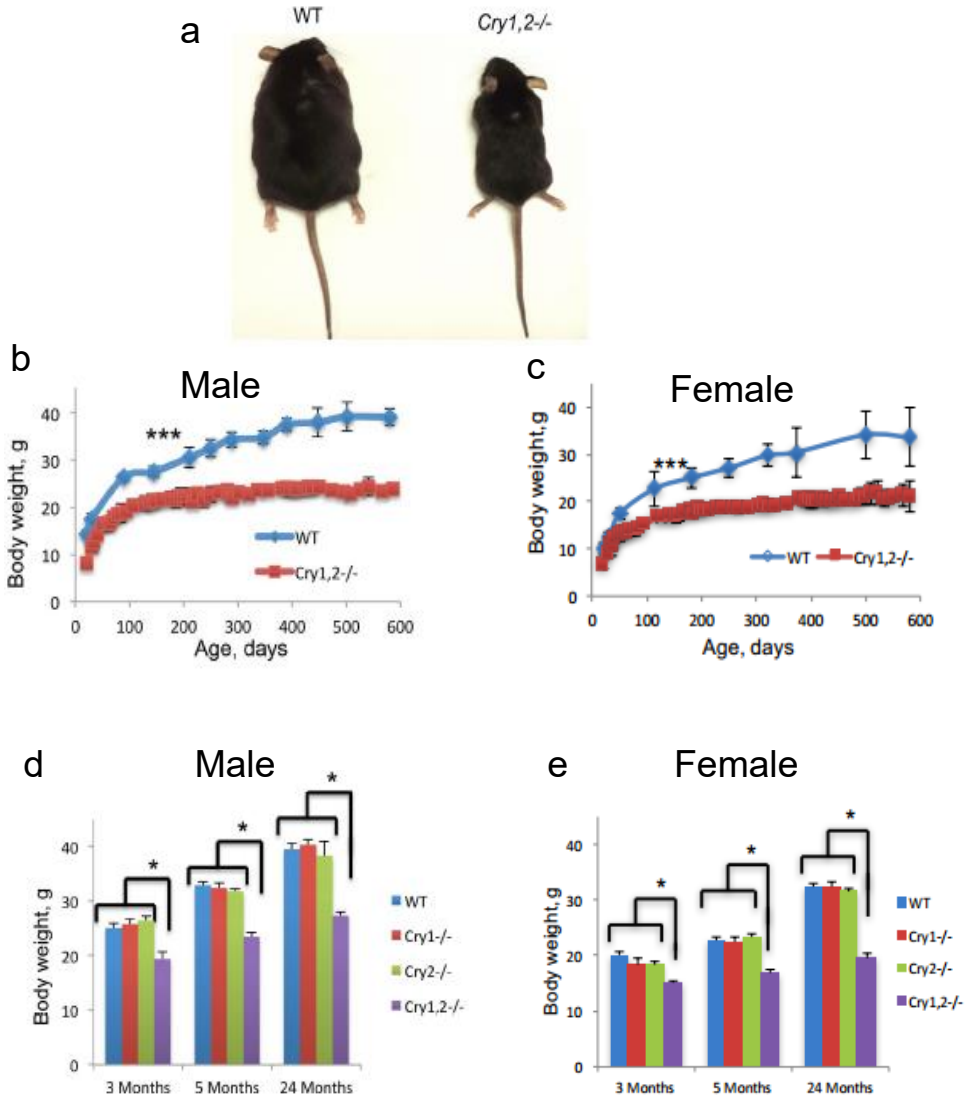
Cryptochromes are the component of the circadian clock and many processes are oscillating across the day, it was important to monitor the effect at several times across the day cycle. We assayed the effect of Cry 1,2-/- on the expression and signaling pathway throughout the day.

CRY 1,2−/− mice develop dwarfism

We noticed that CRY 1,2−/− mice are smaller than wild-type (WT) mice of the same age **(Figure 3-1a)**. The sizes of 5-mo-old WT and CRY 1,2−/− males are compared; a similar size difference was observed in female mice. We decided to investigate the difference in size in more detail. **Figure 3-1b** presents growth curves of male mice of both genotypes and **Figure 3-1c** for females. The difference between genotypes is seen already at weaning (3 wk of age); the observed difference was statistically significant for all ages. The weight of male CRY 1,2−/− mice was ∼70–75% of the weight of WT males; the weight of female CRY 1,2−/− mice was ∼65–70% of the weight of WT females (the difference changed with age). CRY 1 and CRY 2 genes are partially redundant; therefore, we checked whether a deficiency of either of them would be sufficient to induce dwarfism. Body weights of CRY 1−/− and CRY 2−/− mice at three different ages **(Figure 3-1d for males and Figure 3-1e for females)**

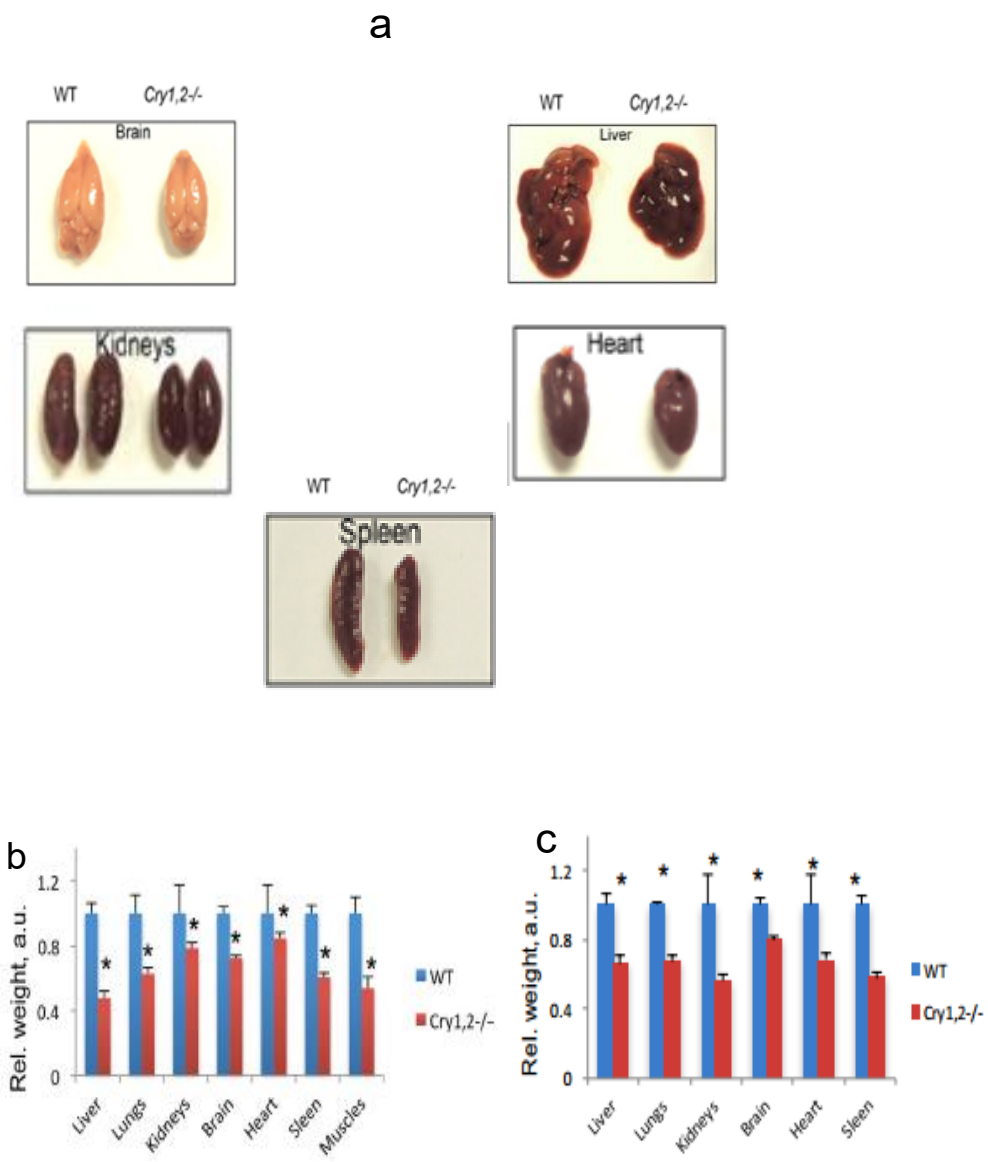
were similar to the body weight of WT mice. Thus, deficiency of both CRY 1 and CRY 2 genes is necessary for body size reduction.

Figure 3-1. CRY 1, 2−/− mice have reduced body weight and size. (a) CRY 1, 2−/− males are smaller than WT males of the same age. (b) CRY 1, 2−/− males (red squares) have reduced body weight compared with WT (blue diamonds) males (N = 16 for each genotype). (c) similar results were observed for females. The difference between genotypes is statistically significant ($p < 0.001$) at all ages, starting at 10 d of age. Weight of WT (blue bars), CRY 1−/− (red bars), CRY 2−/− (green bars), and CRY 1, 2−/− (purple bars) males (d) and females (e) at three different ages (N = 6 for every age group for every genotype). *Statistically significant difference between genotypes ($p < 0.05$).



We also checked whether the size and weight reduction are proportional to the reduction of the weights of some particular organs and tissues. We measured the weights of all major organs in 5-mo-old animals, when both WT and CRY 1,2−/− mice reached their adult size. As shown in **Figure 3-2 a, b for males and 3-2 c for females**, all major organs, including brain, liver, heart, and kidneys, were proportionally reduced in their size and weight; therefore, we conclude that CRY 1,2−/− mice are proportionally smaller than WT mice.

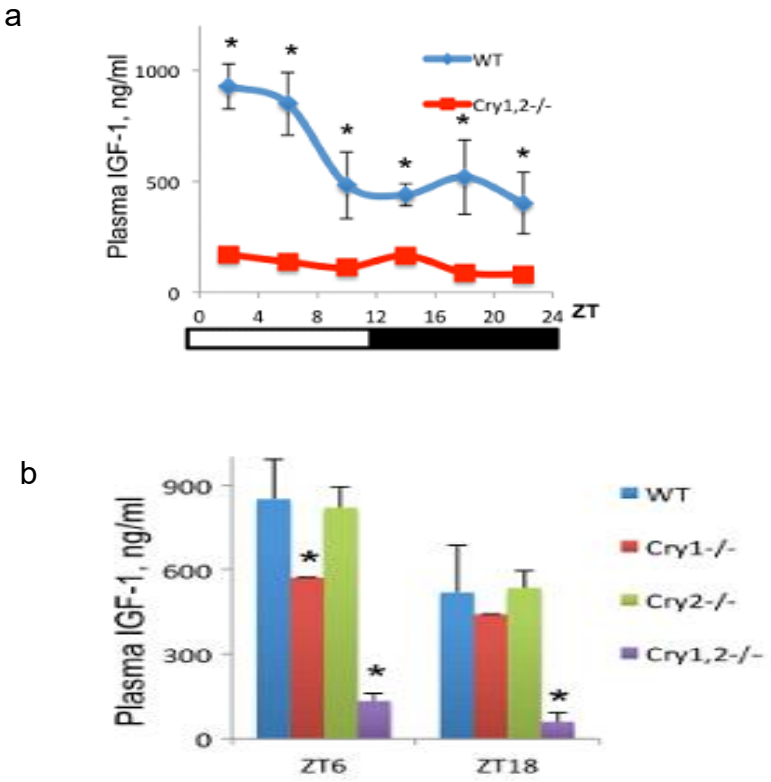
Figure 3-2. The weight of visceral organs is reduced in CRY 1, 2−/− males and females. Organ weights were assayed for WT (blue bars) and CRY 1, 2−/− (red bars) for 5-mo-old males and females (N = 16 for each genotype); the difference between genotypes is statistically significant ($p < 0.001$).



CRY 1,2 -/- mice have a reduced production of IGF-I

Reduced activity of the somatotropic (GH/IGF-I) axis is one of the major causes of dwarfism in rodent and humans (Bartke and Brown-Borg. 2004; Chia. 2014). We assayed the levels of IGF-I in the plasma of WT and CRY 1,2−/− mice. We observed that in WT mice, the levels of the circulating IGF-I demonstrated a statistically significant daily oscillation, with the highest value at zeitgeber time ZT2–ZT6 and the lowest value at ZT14–ZT22 **(Figure 3-3 a)**. We observed a significant reduction in the levels of the circulating IGF-I in CRY 1,2−/− mice at all six-time points tested **(Figure 3-3 a)**. The circulating IGF-I level was reduced by ∼60–80% for CRY 1,2−/− (depending on the time of the day). Thus, similar to other models of dwarfism, a reduced level of circulating IGF-I correlated with the reduced body size of the CRY 1, 2−/− mice (Bartke and Brown-Borg. 2004).

Figure 3-3. CRY's deficiency affects IGF-I level in plasma and tissues. (a) Daily rhythms of plasma IGF-I in WT AL (blue diamonds) and CRY 1, 2−/− AL (red squares) males (N = 3 per time point). (b) Plasma IGF-I levels for WT (blue bars), CRY 1−/− (red bars), CRY 2−/− (green bars), and CRY 1, 2−/− (purple bars) males at ZT6 and ZT18 (N = 6 per time point).

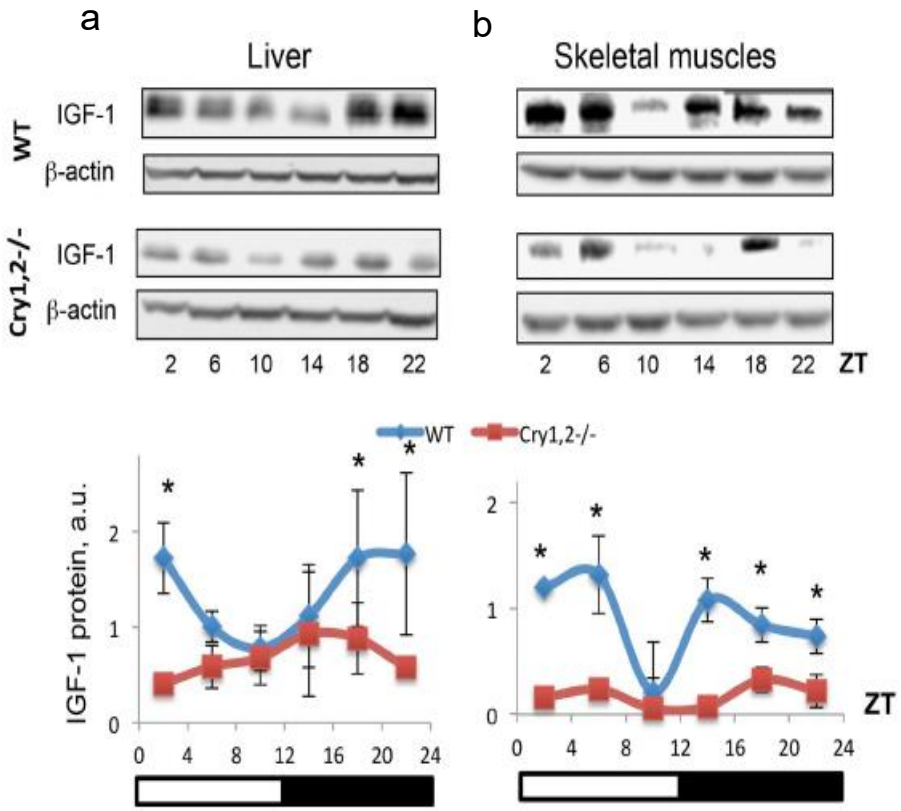


CRY 1−/− and CRY 2−/− mice did not demonstrate any reduction in body size; therefore, we decided to assay whether deficiency of both CRY 1 and CRY 2 is necessary for the effects on the circulating IGF-I. We compared plasma IGF-I levels for WT and single or double CRY's knockouts at two times of the day, ZT6 and ZT18, which corresponded to the high and low levels of plasma IGF-I in WT mice. We found

a 30% reduction of the IGF-I level in CRY 1−/− mice at ZT6 (compare with 80% reduction in CRY 1,2−/− mice; **Figure 3-3 b)**. There was no difference between WT, CRY 1−/−, and CRY 2−/− at ZT18 and no difference between WT and CRY 2−/− at ZT6. Thus, deficiency of both CRY's genes is necessary for down-regulation in the circulating IGF-I level, which correlates well with the effects of CRY's deficiency on the body size. Thus, we observed significant down-regulation of the circulating IGF-I levels in CRY 1,2−/− mice.

The major source of the circulating IGF-I is the liver (Yakar et al. 1999); therefore, we compared the levels of the IGF-I protein in the livers of WT and CRY 1, 2−/− mice **(Figure 3-4 a)**. In WT mice, the liver IGF-I level displayed oscillations (with the highest level of IGF-I expression at ZT18–ZT2), and CRY 1,2 deficiency resulted in the reduction of liver IGF-I levels (the difference between the genotypes was significant at three of six-time points). Many other tissues also produce IGF-I, and, although IGF-I from these sources does not significantly affect the levels of the circulating hormone, it still plays an important role in paracrine signaling (Liu et al. 1998). Therefore, we also determined the IGF-I level in skeletal muscle **(Figure 3-4 b)**. In WT, the level of skeletal muscle IGF-I was significantly lower at ZT10 than at other time points. Similar to the effect in the liver, we observed significant down-regulation of skeletal muscle IGF-I in CRY 1,2−/− mice (the difference between the genotypes was significant at all time points except for ZT10). Thus CRY 1,2 deficiencies resulted in down-regulation of IGF-I production in different tissues.

Figure 3-4. Representative Western blotting (pooled extracts from three mice per time point) of IGF-I expression in the liver figure 3-4 a (left) and skeletal muscle figure 3-4 b (right) of WT and CRY 1, 2−/− male mice. Quantification of IGF-I in the liver (left) and skeletal muscle (right) of WT AL (blue diamonds) and CRY 1, 2−/− (red squares) male mice (N = 3 per time point). The quantification data for the liver and skeletal muscles are presented as relative arbitrary units.

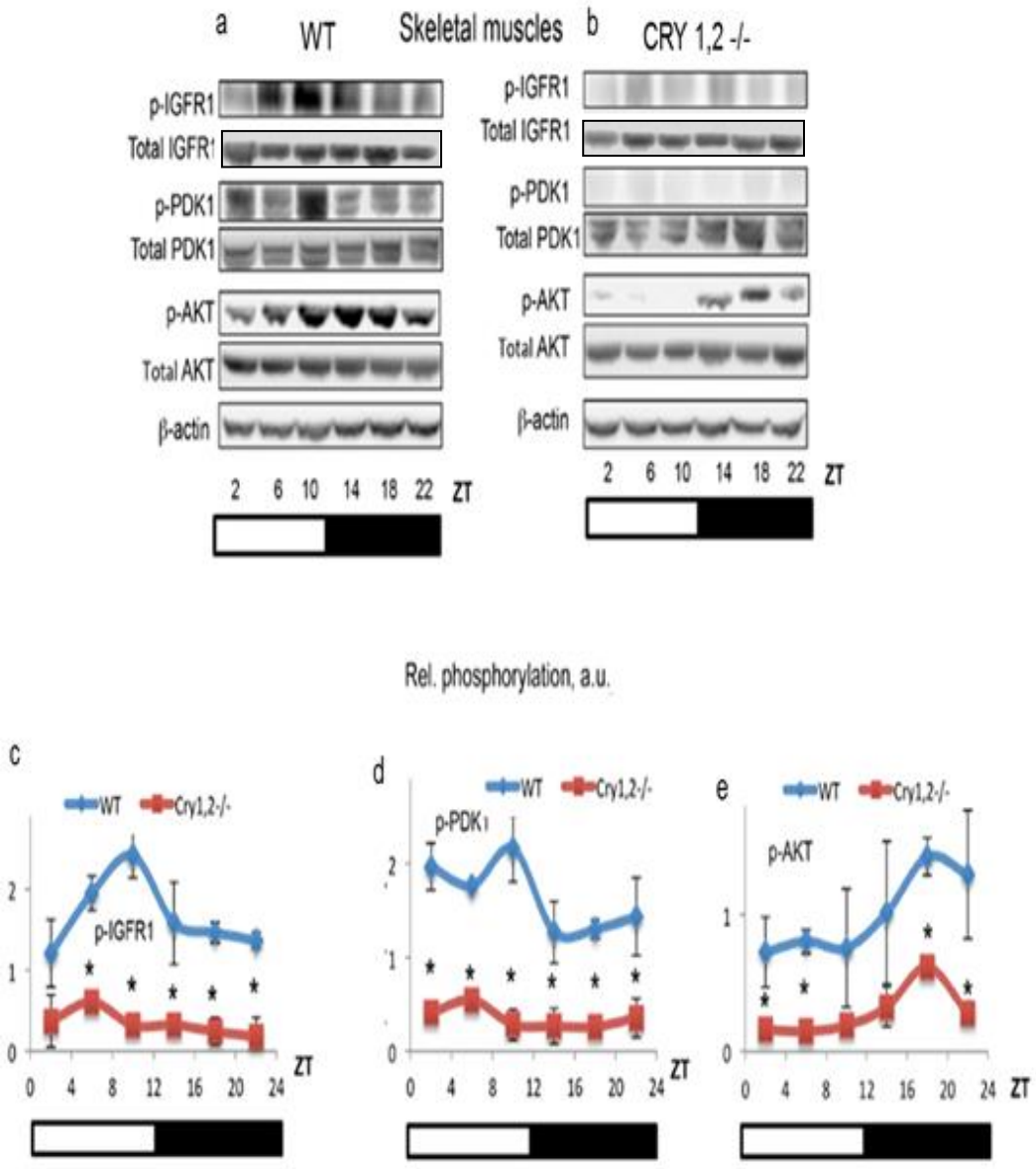


IGF-I signaling is reduced in CRY 1,2−/− mice

IGF-I promotes the growth of muscle, bone, and adipose tissues in mammals (Anisimov and Bartke. 2013; Chia. 2014); skeletal muscles are one of the main targets of IGF-I. We decided to see whether IGF-I signaling is affected in skeletal muscles of

CRY 1,2−/− mice. The receptor for IGF-I is the tyrosine kinase IGF-IR (Kavran et al. 2014). Binding of IGF-I to IGF-IR leads to receptor activation and auto-phosphorylation on Y1137/1138 in mouse (Y1135/1136 in human IGF-IR; Ullrich et al. 1986). We compared IGF-IR phosphorylation skeletal muscles of WT and CRY 1,2−/− mice. In skeletal muscles of WT mice, IGF-IR phosphorylation significantly oscillated during the day, with the highest level of phosphorylation at ZT6–ZT10, in agreement with the highest level of the circulating IGF-I. The level of Y1137/Y1138 phosphorylation was significantly reduced in CRY 1, 2−/− muscles, again in agreement with significantly reduced IGF-I in plasma. PDK1 and AKT are protein kinases that act downstream of IGF-IR (Schiaffino and Mammucari. 2011). They are not direct targets of IGF-IR but are phosphorylated and activated upon IGF-I binding to IGF-IR. We assayed the phosphorylation of PDK1 on S241 and AKT on S473. In WT mice, PDK1 S241 phosphorylation oscillated during the day, with the highest level at ZT10–ZT14, which correlated with IGF-IR Y1137/1138 phosphorylation. There was an additional peak of high PDK1 S241 phosphorylation at ZT2. AKT-S473 phosphorylation was high at the dark phase of the day; thus, AKT phosphorylation was delayed compared with IGF-IR phosphorylation or PDK1 phosphorylation. In skeletal muscles of CRY 1,2−/− mice, phosphorylation of both PDK1 and AKT was significantly reduced (reduction by 30–70% for PDK1 and by 60–80% for AKT, depending on the time of the day). Of interest, AKT phosphorylation was still rhythmic in skeletal muscles of CRY 1, 2−/− mice, with a high level of phosphorylation at night.

Figure 3-5 (a, b, c, d, e). Representative Western blotting (pooled extracts from three mice per time point) of phospho–IGF-IR on Y1137/1138, PDK1 on S241, and AKT on S473 in skeletal muscle of WT (blue diamonds) (a) and CRY 1, 2−/− (red squares) male mice(b). Quantification of phospho–IGF-IR on Y1137/1138 (c), PDK1 on S241 (d), and AKT on S473 (e) in skeletal muscle of WT (blue diamonds) and CRY 1, 2−/− (red squares) male mice (N = 3 per time point).



IGF-I signaling is evolutionarily conserved; in many organisms, reduction of IGF-I signaling leads to activation of the transcriptional factor FOXO. We assayed the expression of FOXO targets Murf1 and Myogenin in skeletal muscles of WT and CRY 1, 2−/− mice **(Figure 3.5 f & g respectively)**. We found that expression was up-regulated at ZT14–ZT22 for Murf1 and at ZT2 and ZT10–ZT22 for Myogenin. These results are in good agreement with reduced IGF-I signaling. Of interest, we also found that expression of the FOXO target genes significantly oscillated across the day in both WT and the circadian mutant, with different patterns, suggesting circadian clock–dependent and –independent effects of time of the day.

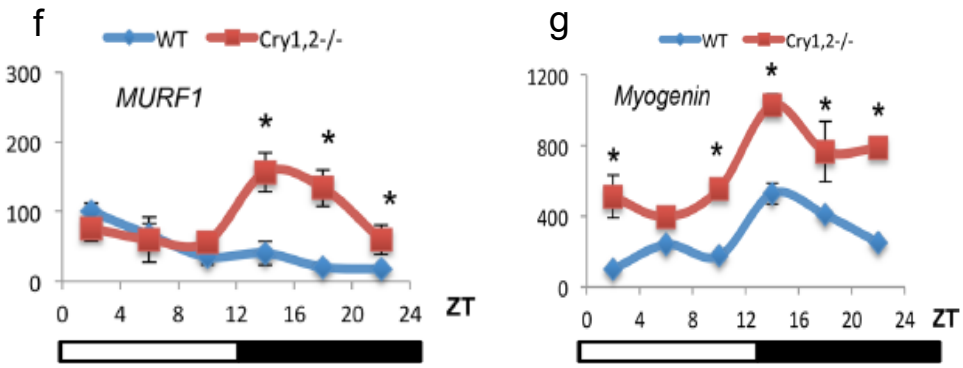


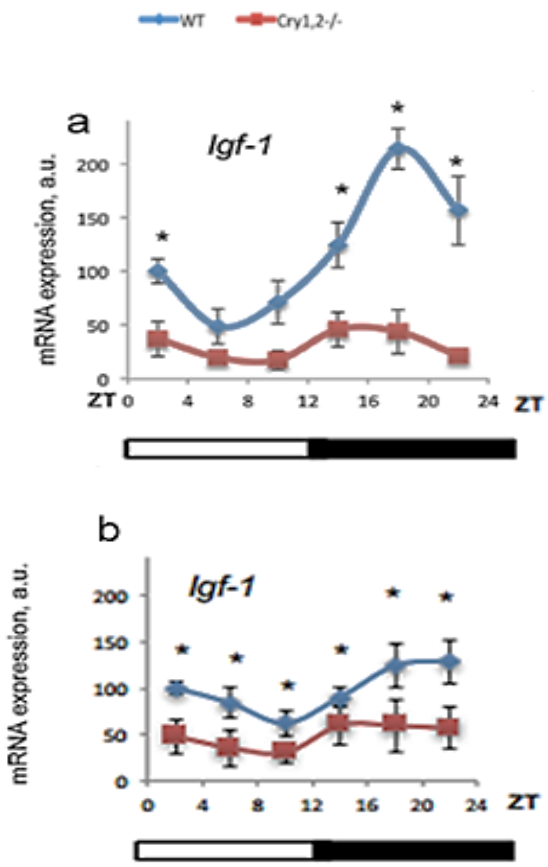
Figure 3-5 (f & g). mRNA expression of FOXO transcriptional targets Murf-1 (Figure f) and Myogenin (Figure g) in skeletal muscle of WT (blue diamonds) and CRY 1, 2−/− (red squares) male mice (N = 3 per time point). *Statistically significant difference between genotypes ($p < 0.05$). The light was on at ZT0 and off at ZT12.

Data in **Figure 3-5** proposes that IGF-I signaling is significantly reduced in skeletal muscles of CRY 1, 2−/− mice. Thus, deficiency of CRY s resulted in reduced plasma IGF-I levels and reduced tissue IGF-I levels and IGF-I signaling in skeletal muscles, which may be a reason for the observed reduced body size of CRY 1, 2−/− mice.

STAT5-dependent regulation of IGF-I expression is disrupted in the liver and skeletal muscles of CRY 1, 2−/− mice

Reduced levels of IGF-I in the plasma and tissues of CRY 1, 2−/− mice indicate that production of IGF-I is compromised in these mice. We assayed the expression of IGF-1 on the mRNA level across the circadian cycle in the liver of CRY 1, 2−/− mice and compared it with the expression in the liver of WT mice. As shown in **Figure 3-6 a**, IGF-1 mRNA expression in the liver of WT mice significantly changed during the day, with the highest expression between ZT14 and ZT22 and the lowest expression at ZT2–ZT6. CRY's deficiency resulted in a significant reduction of IGF-I expression in the liver (the difference was significant at all six-time points). In skeletal muscle of WT mice **(Figure 3-6 b)**, the IGF-I mRNA level did not show any significant rhythms across the day, whereas in skeletal muscle of CRY 1, 2−/− mice, expression was reduced at all six-time points, which correlates with the reduced IGF-1 protein level in skeletal muscles. Thus, reduced IGF-I mRNA expression may contribute to the observed reduced IGF-1 protein level in the tissues of CRY 1, 2−/− mice.

Figure 3-6. IGF-I mRNA expression is down-regulated in the liver (a) and in skeletal muscle (b) of CRY 1, 2−/− mice.



The transcriptional factor STAT5B is the major regulator of IGF-I transcription (Herrington et al. 2000). STAT5B activity is regulated by phosphorylation: phosphorylated STAT5B is transported from the cytoplasm to the nucleus and drives the expression of its target genes (Herrington et al. 2000). We assayed the levels of STAT5B phosphorylation on Y699 (used as a marker of STAT5B activation). We found that in the liver of WT mice **(Figure 3-7)**, STAT5B phosphorylation significantly changed across the day, with the lowest phosphorylation level at ZT2,

which correlates with the lowest level of IGF-I mRNA transcription, and the highest phosphorylation level at ZT10 and ZT18, which correlates with the highest IGF-I mRNA level.

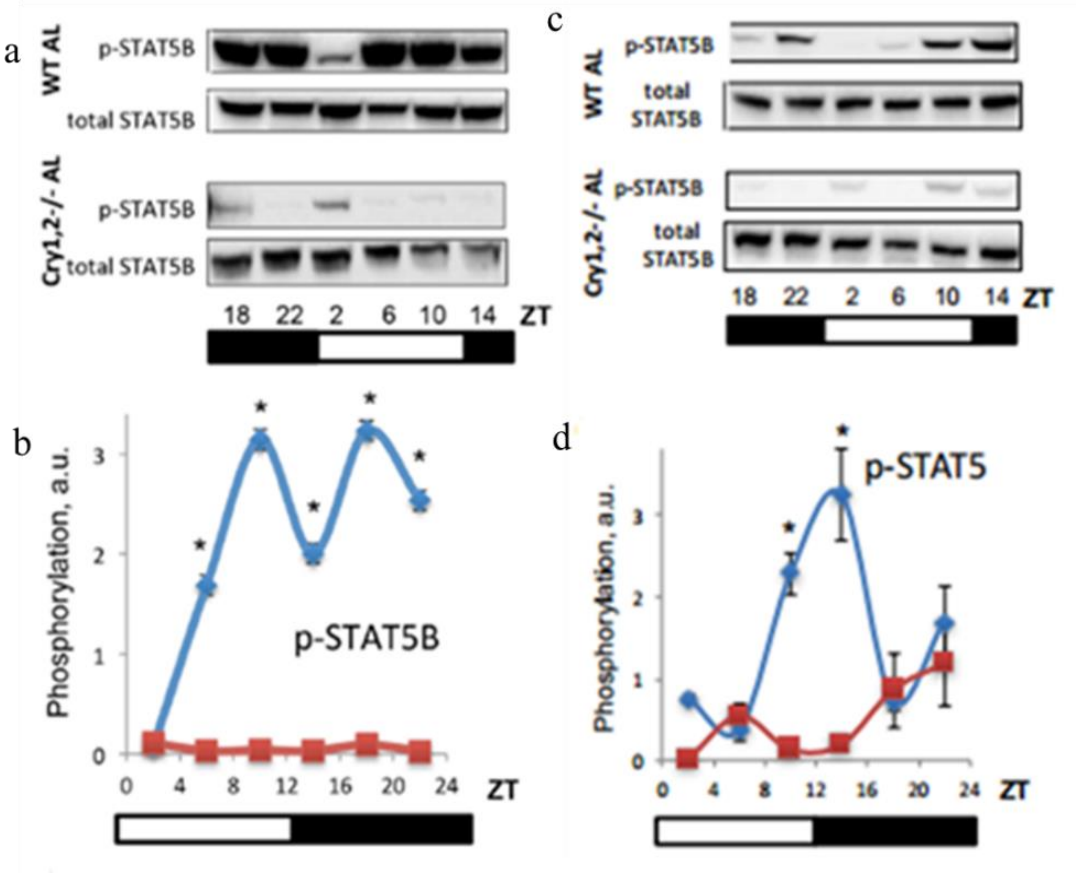
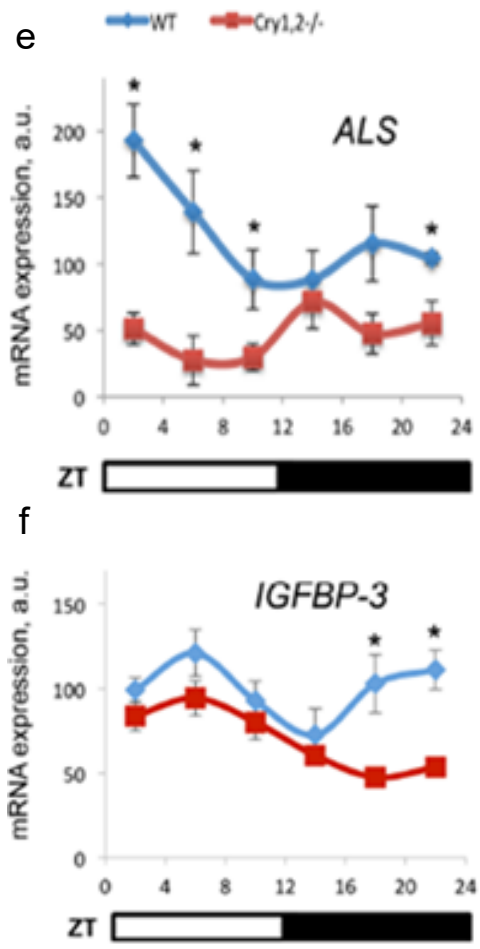


Figure 3-7 (a, b, c, d). Representative Western blotting (pooled extracts from three mice per time point) of phosphorylation of STAT5B on Y699 in the liver (a) and skeletal muscle (c) of WT and CRY 1, 2−/− male mice. (b) Quantification of phosphorylation of STAT5B on Y699 in the liver of WT (blue diamonds) and CRY 1, 2−/− (red squares) male mice. (d) for Skeletal muscle.

Of interest, the level of total STAT5B protein did not change significantly across the day, which suggests that regulation occurs predominantly on the level of phosphorylation. In the liver of CRY 1, 2−/− mice, STAT5B phosphorylation was significantly reduced. We also observed some small reduction in the level of total STAT5B in the liver of CRY 1, 2−/− mice, but this reduction was not significant compared with the effect on STAT5B phosphorylation. The difference in phosphorylation was dramatic even after normalization on total protein level (the quantification of relative STAT5B phosphorylation presented in **(Figure 3-7)**. In skeletal muscles, we observed similar effects of CRY's deficiency on STAT5B phosphorylation **(Figure 3-7).** In WT, STAT5B phosphorylation oscillated across the day, with the highest level at ZT10-ZT14. Phosphorylation was statistically significantly reduced in CRY 1, 2−/− at ZT10 and ZT14.

We also assayed the expression of other known transcriptional targets of STAT5B—acid-labile subunit (ALS) and IGF-binding protein 3 (IGFBP-3; Woelfle and Rotwein. 2004). As shown in **Figure 3-7**, ALS (e) and IGFBP-3 (f) expression were significantly reduced in the liver of CRY 1, 2−/− mice at several times of the day, in agreement with reduced STAT5B transcriptional activity.

Figure 3-7(e & f). Daily rhythms of ALS (e) and IGFBP-3 (f) mRNA expression in the liver of WT (blue diamonds) and CRY 1, 2−/− (red squares) males.

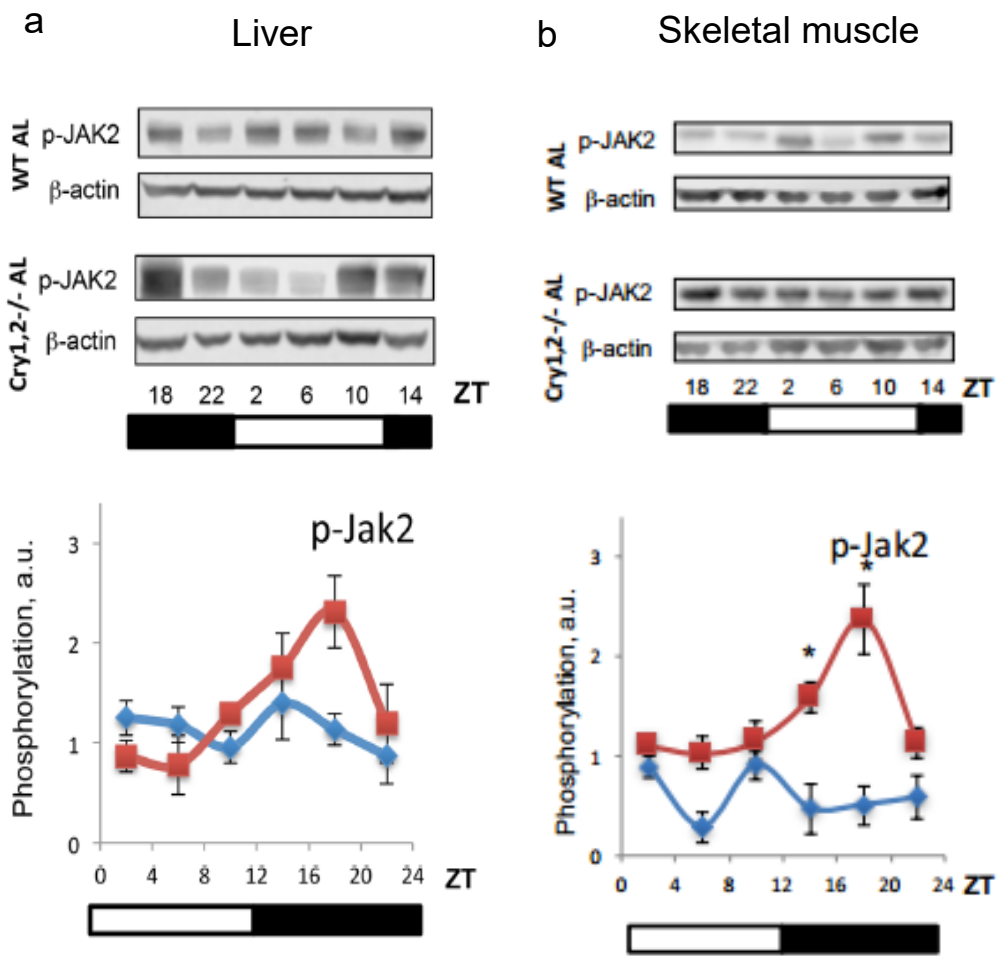


STAT5B is phosphorylated by the protein kinase JAK2. JAK2 kinase is activated by GHR upon binding to GH. Activated JAK2 can be monitored by assaying JAK2 phosphorylation on Y1007. Data on JAK2 phosphorylation on T1007 in the liver are presented in **Figure 3-8 a**. In the liver of WT mice, the level of phosphorylation changed across the day with low amplitude. In the liver of CRY 1, 2−/− mice, we also

observed daily changes in JAK2 phosphorylation. The pattern of JAK2 phosphorylation was different compared with WT, but the statistical analysis did not reveal a significant difference.

In skeletal muscles of WT mice, JAK2 phosphorylation showed some moderate changes **(Figure 3-8 b).** Of importance, we did not detect any reduction in JAK2 phosphorylation in the tissues of CRY 1, 2−/− mice: on the contrary, we observed a significant increase in the phosphorylation at several time points; therefore, the reduced phosphorylation of STAT5B was not a consequence of reduced JAK2 phosphorylation.

Figure 3-8. Representative Western blotting (pooled extracts from three mice per time point) of phosphorylation of JAK2 on Y1007 in the liver (a) and skeletal muscle (b) of WT and CRY 1, 2−/− male mice. quantification of phosphorylation of JAK2 on Y1007 in the liver of WT (blue diamonds) and CRY 1, 2−/− (red squares) male mice (N = 3 per time point). *Statistically significant difference between genotypes ($p < 0.05$). The light was on at ZT0 and off at ZT12.



Expression of CISH is up-regulated in the liver of CRY 1, 2−/− mice

Proteins from the suppressors of the cytokine signaling (SOCS) family are negative regulators of JAK2 signaling. SOCS1, SOCS2, SOCS3, and cytokine-inducible SH2-containing protein (CISH or CIS) have been implicated in the control of the GHR/JAK2/STAT5B pathway. We analyzed the expression of these proteins in the liver of WT and CRY 1, 2−/− mice **(Figure 3-9).** We did not detect any significant changes in SOCS2 expression. Expression of SOCS1 and SOCS3 was reduced at some times of the day (at ZT2 for SOCS1 and ZT10 for SOCS3). Expression of CISH was significantly upregulated at all time points in the CRY -deficient liver, and this change in expression correlated with reduced STAT5B phosphorylation and could be one of the contributing factors.

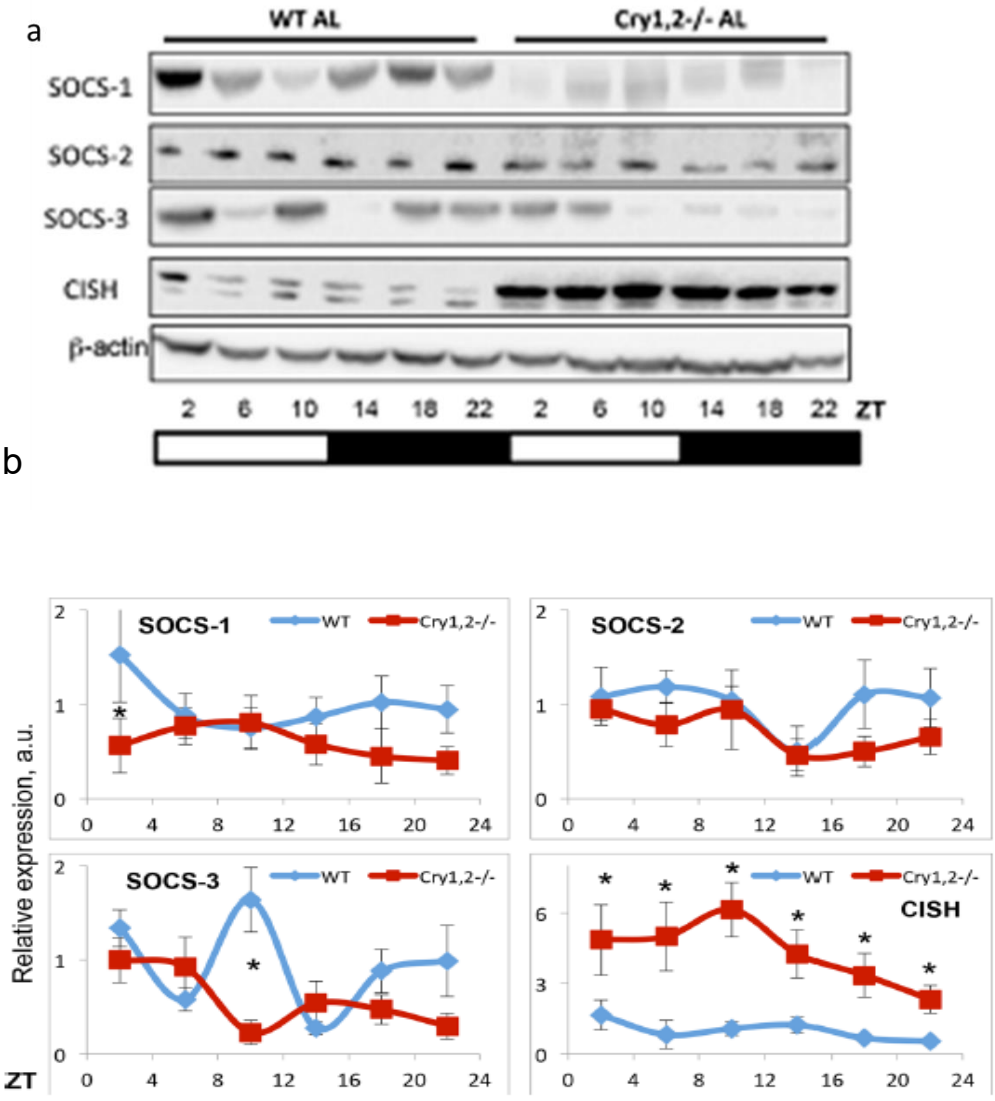
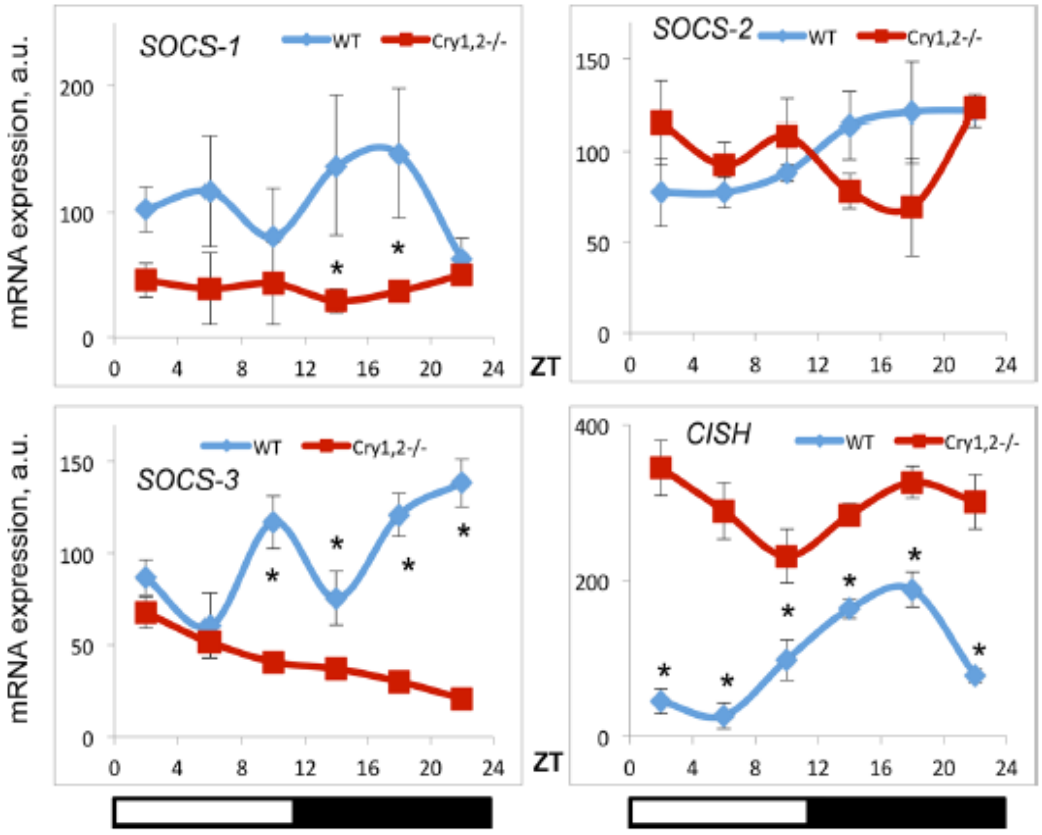


Figure 3-9. The expression of CISH is up-regulated in the liver of CRY 1, 2−/− mice. (A) Representative Western blotting (pooled extracts from three mice per time point) of expression of SOCS1, SOCS2, SOCS3, and CISH in the liver of WT and CRY 1, 2−/− male mice. (B) Quantification of expression of SOCS1, SOCS2, SOCS3, and CISH in the liver of WT (blue diamonds) and CRY 1, 2−/− (red squares) male mice (N = 3 per time point). *Statistically significant difference between genotypes ($p < 0.05$). The light was on at ZT0 and off at ZT12.

To further understand the mechanism of regulation, we also assayed the expression of mRNA for these genes **(Figure 3-10)**. We found that, in agreement with protein data, expression of SOCS2 mRNA was not significantly affected by CRY deficiency. Expression of SOCS1 and SOCS3 genes was reduced at some time points. This down-regulation of expression was not surprising; indeed, the transcription of these genes is positively regulated by STAT5B and therefore their expression is expected to be reduced under conditions of low STAT5B activity. The expression of CISH was significantly increased across the day, which suggests that CRY's known transcriptional repressors might regulate CISH on the level of mRNA expression.

Figure 3-10. Effect of CRY deficiencies on the expression of SOCS and CISH mRNA in the liver. Daily profiles of mRNA expression of indicated SOCS family member genes in the liver of WT (blue diamonds) and CRY 1, 2−/− (red squares) male mice (N = 3 per time point). *Statistically significant difference between genotypes ($p < 0.05$). The light was on at ZT0 and off at ZT12.



3.5 Discussion

CRY deficiency and dwarfism

We observed that CRY 1, 2−/− mice developed dwarfism, manifested by reduced weight and size of all major organs and the body. There are several models of dwarfism in mice, which are associated with reduced IGF-1 secretion and reduced IGF-1 signaling (Bartke and Brown-Borg, 2004; Quarrie and Riabowol, 2004). CRY 1, 2−/− mice represent a novel model of dwarfism. Indeed, Ames and Snell's mice lack cells producing GH in the pituitary; both mice do not produce detectable GH and do not produce prolactin and thyroid-stimulating hormone. Mice with a mutation in GH-releasing hormone receptor, called “Little” mice, have significantly reduced GH levels. Thus, for all of the aforementioned models, reduced IGF-1 production is secondary to reduced GH level. GHR- deficient mice (Laron mice) have increased circulating GH level but are resistant to the action of GH due to the absence of the receptor. These mice were developed as an animal model of human Laron dwarfism syndrome. Thus, in contrast to Ames, Snell, or Little mice, both Laron, and CRY1, 2−/− mice have reduced IGF-1 but normal GH levels. The difference between CRY1, 2−/− mice, and Laron mice, is that Laron mice do not have GHR and downstream signaling; in addition, they lack the GH-binding protein, which is a product of GHR processing.

Cryptochromes regulate CISH expression

We found that CISH expression is significantly increased in the liver of Cry1,2-/- mice, which correlated with reduced STAT5B phosphorylation and reduced body size of these animals. Previously it was shown that mice overexpressing CISH in the liver have STAT5B phosphorylation and transcriptional activity, they have reduced weight and body size, therefore, resembling Cry1,2-/- mice. It is reasonable to hypothesize that reduced size of Cry1,2-/- mice is due to overexpression of CISH.

CISH is a member of SOCS protein family, proteins in this family suppresses the GH/GHR/JAK2 signaling through several different mechanisms. The possible mechanisms by which CISH act are (1) by suppressing JAK2 kinase activity; (2) by targeting JAK2 for proteasomal degradation; (3) by inhibiting binding of STAT5B to phosphorylated GHR. We did not detect any significant reduction in JAK2 total protein level or phosphorylation, we proposed that in the liver of Cry1,2-/- mice CISH regulates STAT5B phosphorylation by inhibiting the interaction of STAT5B with GHR.

How CRY's can regulate CISH expression? Interestingly, CISH, SOCS1, and SOCS3 are known transcriptional targets of STAT5B. The reduced phosphorylation and transcriptional activity of STAT5B in CRY 1,2 -/- mice correlated with the reduced mRNA expression of SOCS1 and SOCS3 genes, which is in an agreement with their regulation by STAT5B. However, the increased mRNA expression of CISH in CRY 1,2 -/- mice do not correlate the reduced STAT5B activity. The exact molecular functions of CRY's are unknown, it was reported that they can suppress the transcriptional activities of several transcriptional factors: circadian clock BMAL1:

CLOCK transcriptional complex (Kume et al. 1999), glucocorticoid receptor (Lamia et al. 2011) and CREB (Zhang et al. 2010). We performed preliminary bioinformatic analysis of CISH promoter region and found two conserved retinoic acid-related orphan receptor response elements, these elements are regulated which suggest that the expression might under control of Circadian Clock.

Do CRY's suppress CISH transcription through direct interaction with some regulatory elements in CISH gene or indirectly through the control of other regulators is a subject for future research.

Circadian disruption, circadian clock proteins, and IGF1 expression

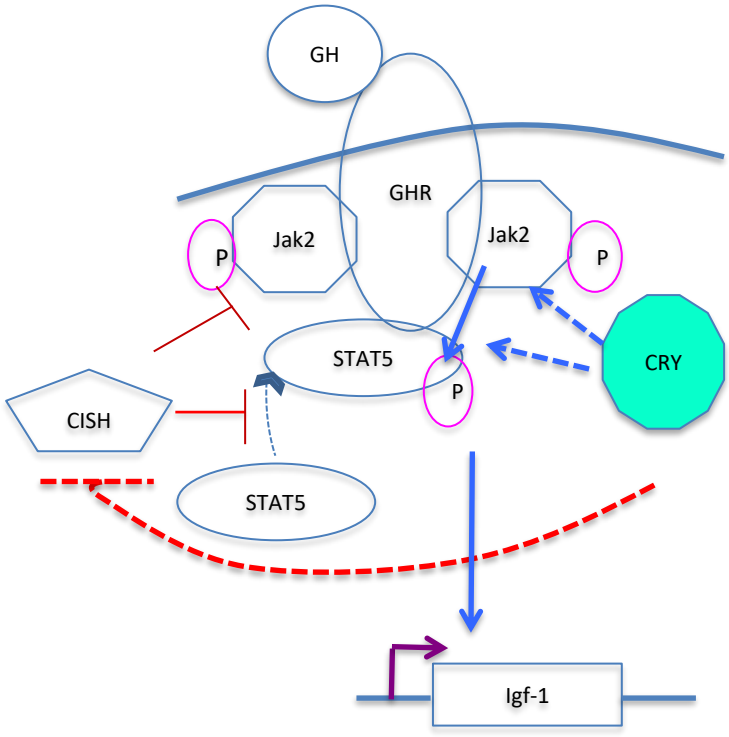
The important question is if the circadian clock disruption will affect IGF-1 production. Recently, we found that CR-mediated effects on IGF-1 plasma levels are impaired (Patel et al. 2015) in the another model of the circadian clock deficiency - BMAL1-/- mice (Bunger et al. 2000). However, the effect of BMAL1 deficiency was rather opposite to the effect of CRY's deficiency: CR did not significantly downregulate circulating IGF-1 levels in BMAL1-/- mice. BMAL1 regulates expression of CRY 1 and CRY 2 (Gekakis et al. 1998; Hogenesch et al. 1998); in turn, CRY's inhibit BMAL1-containing transcriptional complexes (Kume et al. 1999), thus, these proteins play opposite role in the circadian clock mechanism and in the IGF-1 expression control. Impaired regulation of IGF-1 in two circadian clock mutants supports the circadian control of IGF-1 production.

Physiological significance of circadian rhythms in IGF-1 production

We found serum level of IGF-1 oscillated during the day and the expression of Igf-1 mRNA was also rhythmic in the liver and skeletal muscles. Circadian rhythms in IGF-1 level correlated with rhythms in IGF signaling in the skeletal muscles. What can be a physiological importance of these rhythms? In mammals feeding, activity and rest are periodic, therefore, the nutrient supply is periodic and their consumption for physical activity is periodic. At the same time, concentrations of the nutrients in a body fluid, while having some degree of fluctuation, are relatively constant. Tissues must switch from anabolic to catabolic processes to maintain this balance of nutrients. Cell growth and proliferation requires a significant amount of resources. It is extremely important to coordinate nutrient consumption by different tissues during different phases of the day to avoid the energy collapse. Indeed, if all tissues will start to proliferate during the time of physical activity, cells might run out of nutrients. Therefore, in multicellular organisms, cell proliferation in addition to nutrient concentrations, is regulated by multiple extracellular signals such as growth factors and hormones and intracellular checkpoint. IGF-1 is important anabolic hormone, which promotes cell proliferation, continuously high level of IGF-1 can be dangerous, we hypothesized that the circadian clock regulates the IGF-1 production to coordinate cell proliferation in different tissues with organism physiology and metabolism.

Figure 3-11. Model of regulation of IGF-I expression by CRY's in response to GH stimulation. Solid lines represent previously reported activation of STAT5B phosphorylation by JAK2 and suppression of this phosphorylation by CISH members of the SOCS family. CRY's might directly interact with JAK2-containing complexes and

stimulate JAK2 activity toward STAT5B; CRY's might recruit STAT5B to the JAK2/GHR complex, and CRY's might also suppress CISH expression/activity. Thus, CRY's stimulates STAT5B phosphorylation and activation, which in turn drives IGF-I expression in the liver and other tissues.



The previous study also revealed that CISH overexpression in the liver has been to be associated with reduced body weight and suppressed STAT5B activity (Matsumoto, A. et al., 1999).

We also cannot exclude the other possible mechanisms illustrated in **Figure 3-11**. One possibility is that CRY's directly interact with JAK2, STAT5, or GHR and either suppress JAK2 kinase activity or prevent the formation of the GHR/JAK2/STAT5 complex, which is necessary for STAT5 phosphorylation (Herrington et al. 2000). We hypothesized that CRY might involve in the regulation of CISH expression. CRY's

act as transcriptional repressors and suppress the expression of genes controlled by the BMAL1: CLOCK transcriptional complex (Kume et al. 1999). CRY's also modulates the transcriptional activity of glucocorticoid receptor (Lamia et al. 2011) and CREB (Zhang et al. 2010). CRY's, known transcriptional suppressors, might control CISH mRNA expression through direct or indirect interaction. Further study will determine the molecular mechanisms of CRY-dependent regulation.

Future implications

This model system is vital to answering questions about the importance of Cryptochromes in the regulation of IGF-I production. Once this system can be established further modification of this system may help to answer different questions. As a long-term goal of this project would be to find out how CRY's regulate the expression of CISH on the mRNA and protein levels. As we found that increased expression of CISH can be a contributing factor to the observed reduced STAT5B phosphorylation and reduced IGF-1 transcription. The future aim is to determine the importance of Cryptochrome to promote transcription of CISH.

To further confirm that reduced body size and weight in CRY-deficient mice is due to the reduce IGF1 level. We will cross CRY 1,2 -/- mice with mice overexpressing IGF1 level. We expect that it will restore the body weight. OR we will cross CRY 1,2-/- mice with IGF1 negative mice, where we expect that there will be no further reduction in body weight.

Summary

Proposed model for Cryptochrome involved in regulation of IGF-1 production via JAK-2 dependent STAT5B phosphorylation. This study found Cryptochrome play an important role independent of regulating circadian rhythms. This study also suggests Cryptochromes are necessary for growth and development.

The foregoing study found:

I. Cryptochrome deficient mice develop dwarfism and reduced in body weight.

II. Cryptochrome deficient mice have reduced IGF-1 production and IGF-1 signaling in skeletal muscle.

III. Cryptochrome involved in the regulation of IGF-1 production via JAK2-dependent STAT5B phosphorylation.

IV. Cryptochrome might involve in regulation of CISH expression

CHAPTER IV

ROLE OF CRYPTOCHROME IN CALORIE RESTRICTION MEDIATED DOWN-REGULATION OF IGF-1 PRODUCTION AND SIGNALING

AMOL S. CHAUDHARI

4.1 Abstract

Decreased IGF-1 production and signaling plays an important role in mechanisms of calorie restriction (CR) - a lifespan-extending dietary paradigm. The IGF-1 expression is controlled by the transcriptional factor STAT5B, whose activity is regulated through growth hormone receptor-dependent phosphorylation by Janus kinase (JAK2). STAT5B phosphorylation and IGF1 mRNA expression is reduced under CR through unknown mechanisms. Here we report that transcriptional repressor and circadian clock component Cryptochrome (CRY) is essential for the CR-mediated downregulation of IGF-1. CRY controls STAT5B phosphorylation and IGF-1 mRNA

expression by suppressing CISH. CR leads to significantly reduced CRY 1 protein level and the induction of CISH expression on both mRNA and protein levels, which correlates with down-regulation of STAT5B phosphorylation and activity. In agreement with that, CR does not down regulate IGF-1 expression in the liver and plasma IGF-1 levels in CRY-deficient mice. Thus, CRY links the circadian clock and calorie restriction mechanisms by regulating STAT5-dependent IGF-1 expression.

4.2 Introduction

Calorie restriction is a dietary intervention that delays aging in many species (Fontana et al. 2010; Taormina and Mirisola 2014). It was proposed that the anticancer effects of calorie restriction are due to decrease in the activity of the somatotropic axis, and specifically to the reduced production and secretion of the insulin-like growth factor 1 (IGF-I) (Ruggeri et al. 1989; Dunn et al. 1997; Carter et al. 2002). Upon CR, expression of IGF-I mRNA in the liver is reduced (Herrington and Carter-Su 2001; Herrington et al. 2000), which correlates with reduced STAT5b phosphorylation and activity. However, molecular mechanisms connecting CR, STAT5B, and IGF-I expression are still not well determined (Thompson et al. 2014).

We demonstrated that the Cryptochromes (CRY's) are involved in the control of IGF-1 production through the control of GHR/JAK2 mediated phosphorylation of STAT5B. Mice deficient for CRY expression have significantly reduced the level of circulating IGF-1 in the blood and reduced IGF signaling in the skeletal muscles. The phosphorylation of STAT5B is also reduced in the tissues of these mice, while JAK2 phosphorylation was not reduced, which is similar to the effects of CR on

GHR/JAK2/STAT5 signaling. We hypothesize that Cryptochrome might involve in calorie restriction-mediated downregulation of IGF1 level.

4.3 Materials and Method

Experimental animals

CRY 1,2 -/- mice and BMAL1-/- mice were previously described (Vitaterna et al. 1999; Bunger et al. 2000). Mice were on the C57B6J background. Animals were maintained on the 12:12 light: dark cycle with lights on at 7:00 am, and fed the 18% protein rodent diet (Harlan). The ad libitum (AL) group had unrestricted access to food. Calorie restriction (CR) was started at 3 months of age. For the first week, animals have been on 10% restriction, for the second week on 20% restriction and on 30% restriction for the rest of the experiment. The CR group received their food once per day at ZT14 (two hours after light off). After the two months of CR, tissues were collected at six different time points across the day. All groups had unrestricted access to water. All tissue collection experiments were performed on 5 months old mice. For all experiments, three animals of each genotype, gender, feeding regimen and time point were used. All the animal studies were performed with approval from the Institutional Animal Care and Use Committee (IACUC) of Cleveland State University. The care and use of mice were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Cleveland State University.

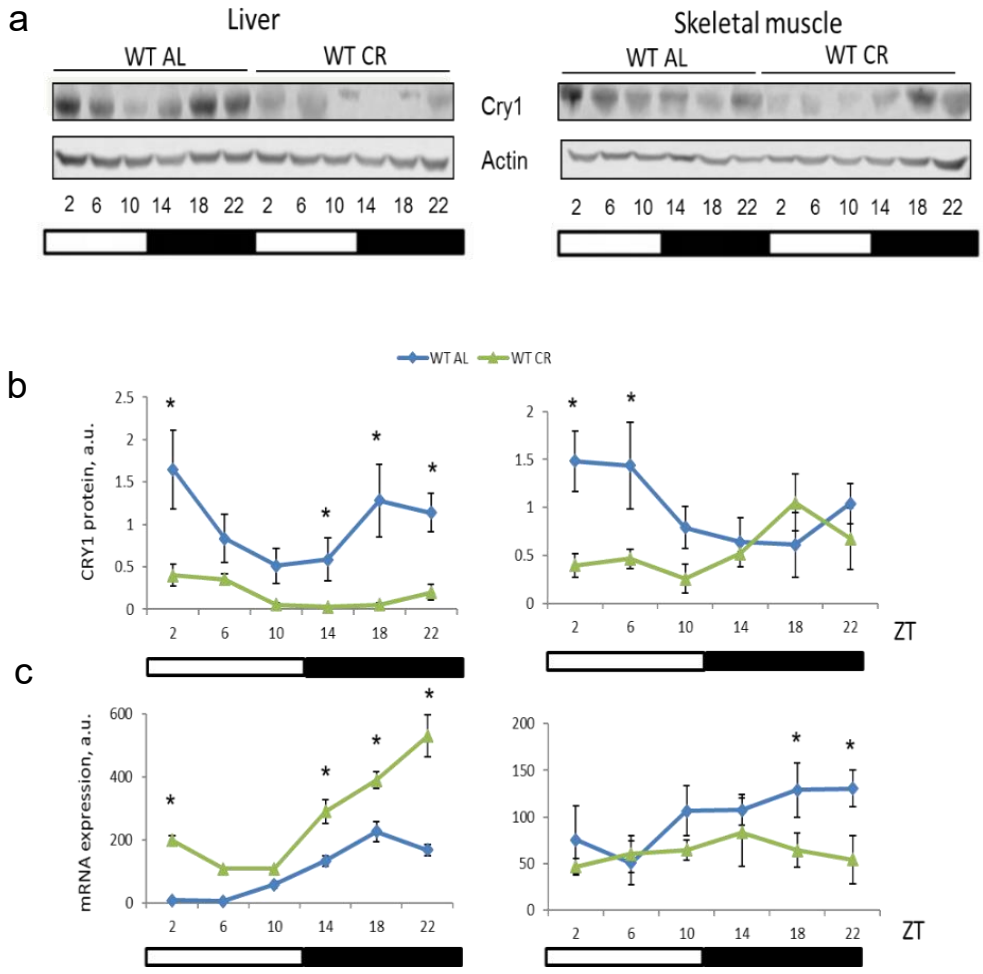
Details of the mRNA, Protein analysis, and statistical analysis have been explained in Chapter II.

4.4 RESULTS: (Unpublished)

CR leads to a reduced level of the CRY 1 protein

CR affects expression and activity of several circadian clock genes in the liver (Patel et al. 2015). We measured the level of the CRY 1 protein in the liver and skeletal muscles of wild-type mice on AL and 30% CR regimes **(Figure 4-1 a)**. In both liver and skeletal muscle, the expression of CRY 1 demonstrated significant changes during the day under both AL and CR feeding conditions. 30% CR resulted in 70 – 90% (depending on time point) down-regulation of CRY 1 protein level in the liver; the difference was significant at all tested time points. In the skeletal muscles, we also detected a significant (30-80% depending on the time point) down-regulation of the CRY 1 protein. Thus, CR resulted in down-regulation of CRY 1 expression in two tested metabolically active tissues. The effect of CR on the CRY 1 protein was different from the effect on the CRY 1 mRNA expression **(Figure 4-1 c)**, in the liver we observed some increase in the expression at two time points, in the skeletal muscles we observed downregulation at ZT22. Thus, regulation CRY 1 expression by CR occur on both mRNA and the posttranscriptional level and mechanisms might be tissue specific.

Figure 4-1. CR leads to down-regulation of tissue CRY 1 on protein but not mRNA level.

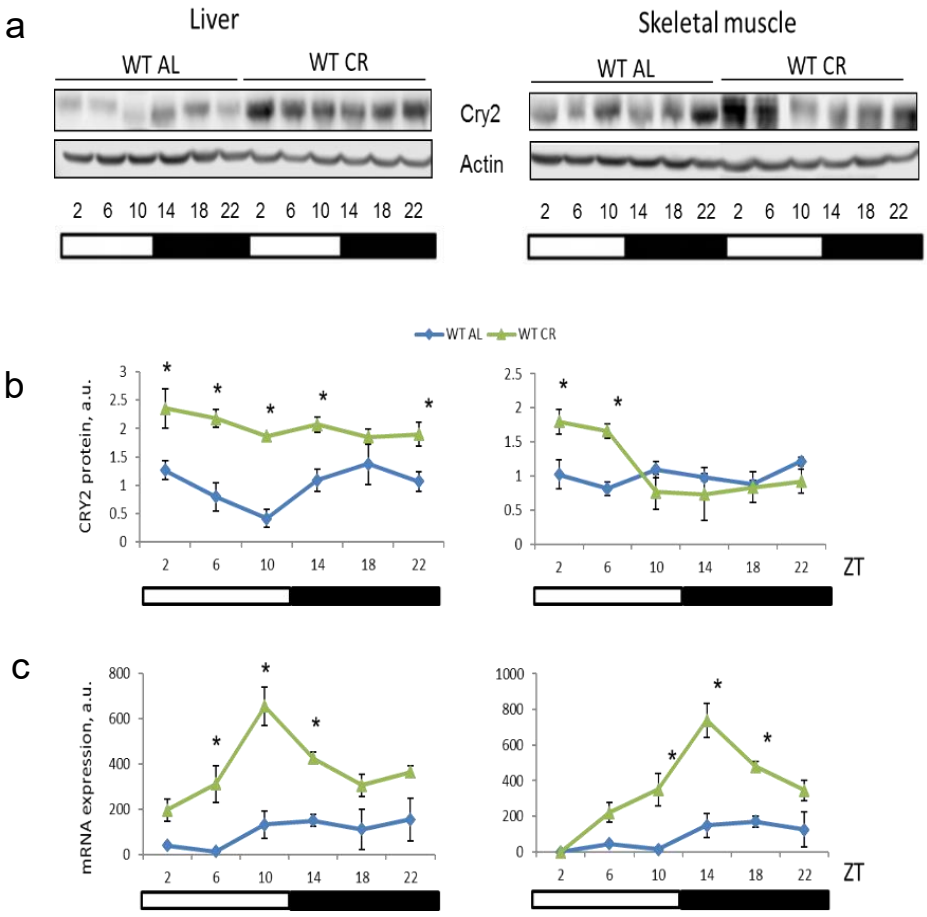


(a) Representative Western blotting and (b) quantification CRY 1 expression in the liver (left panels) and skeletal muscles (right panels) of WT AL (blue diamond) WT 30% CR (green triangle) male mice. Representative WB pooled extracts from 3 mice per time point for treatments were used, for quantification, the extracts from individual animals (N=3 per time point) were analyzed separately and average and errors were calculated. (c) mRNA expression of CRY 1 in the liver (left panel) and in the skeletal muscles (right panel) of WT AL (blue diamond) WT 30% CR (green triangle) male mice (N=3 per time

point). * - The statistically significant different difference between the genotypes ($p<0.05$). The light was on at ZT0 and off at ZT12.

We also investigated the CRY 2 expression in liver and skeletal muscle. In liver, CRY 2 level is significantly changed during the day while there were no changes in skeletal muscle. Interestingly, CRY 2 level significantly increased upon 30% CR at the protein level in the liver. While in skeletal muscle the CRY 2 level increased at only at ZT2 and ZT6 **(Figure 4-2 a)**. Thus, CR resulted in up-regulation of CRY 2 expression in two tested metabolically active tissues. The CR results in increase CRY 2 mRNA expression at all tested points in the liver and skeletal muscle. **(Figure 4-2 c),** Thus, CR regulates CRY 2 expression at mRNA and the posttranscriptional level.

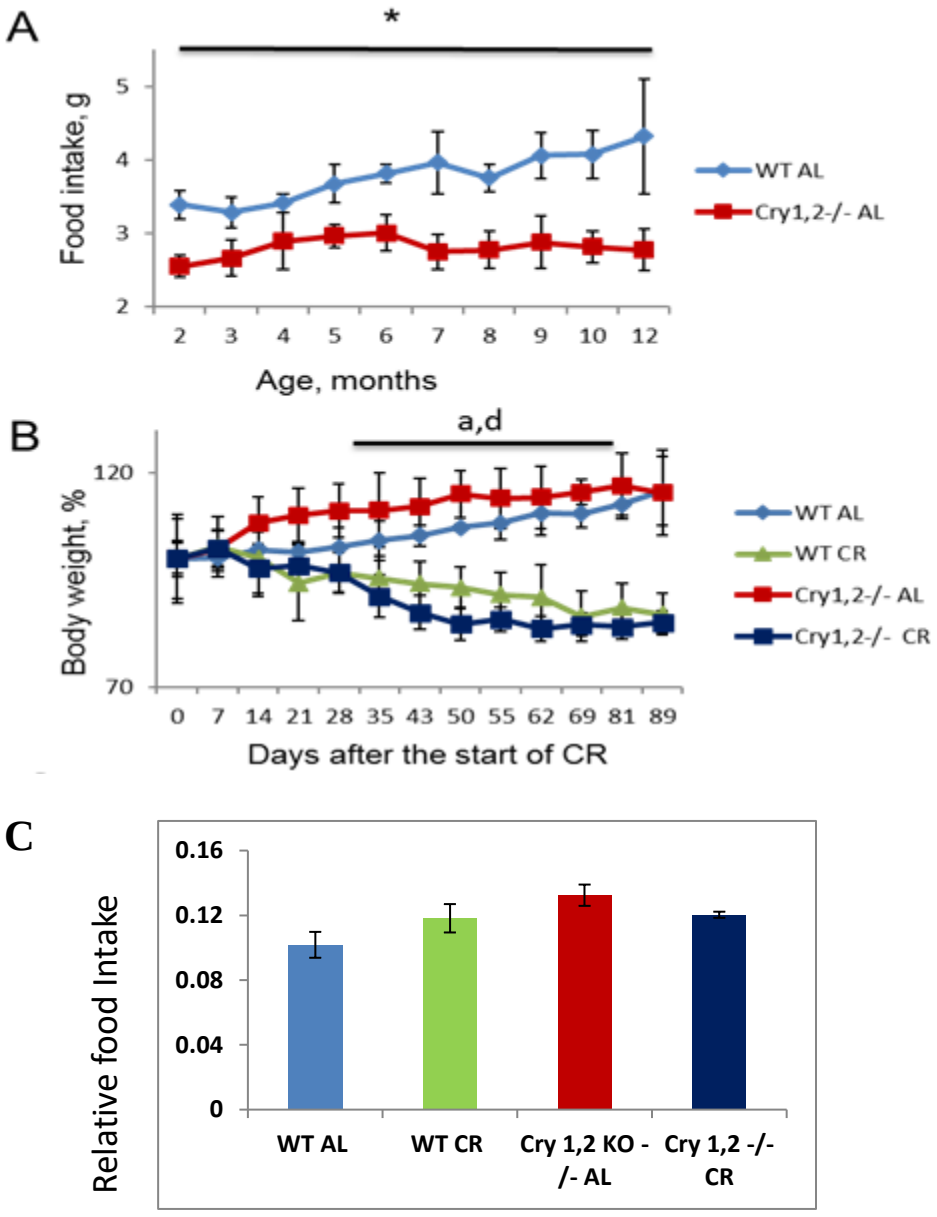
Figure 4-2. CR upregulates CRY 2 at protein and mRNA level. (A) Representative Western blotting and (B) quantification CRY 2 expression in the liver (left panels) and skeletal muscles (right panels) of WT AL (blue square) WT 30% CR (green triangle) male mice. Representative WB pooled extracts from 3 mice per time point for treatments were used, for quantification, the extracts from individual animals (N=3 per time point) were analyzed separately and average and errors were calculated. (C) mRNA expression of CRY 2 in the liver (left panel) and in the skeletal muscles (right panel) of WT AL (blue diamond) WT 30% CR (green triangle) male mice (N=3 per time point). * - The statistically significant different difference between the genotypes ($p<0.05$). The light was on at ZT0 and off at ZT12.



CRY 1,2 -/- mice have a reduced food intake

To investigate if CRY's are a part of CR mechanisms directly we decided to apply 30% CR to CRY 1,2 -/- mice. When we measured food intake of male and female mice we found that ad libitum (AL) fed CRY 1,2 -/- ate about 20-30% less of food compared with AL fed wild-type mice as shown on (**Figure 4-3 a)** for male mice and similar results were obtained for female mice. We reported recently that CRY 1,2 -/- mice are smaller than wild-type mice of the same age (Chaudhari et al. 2016) and this reduced food intake correlates with the reduced weight. Indeed, when we calculated a relative food intake (the food intake divided by the body weight) we found that CRY 1,2 -/- mice consume more food per gram of the body weight. Interestingly that an increased food intake was reported in other circadian clock mutants, thus, reduced food intake was not caused by the disruption of the circadian clock in these mice, but rather unique for CRY deficiency. When CRY 1,2 -/- and wild-type mice were subjected to CR both genotypes demonstrated similar changes in their body weights. After transfer to CR, the body weight of mice of both genotypes decreased for a few weeks and then stabilized. Relative changes in body weight induced by 30% CR are presented in Figure 2B and absolute changes are presented in Supplementary Figure. Interestingly, the stable body weight of WT mice on 30% CR was close to the body weight of CRY 1,2 -/- AL mice of the same age **(Figure 4-3 b)**, which was in a good agreement with 20-30% voluntary reduced food intake for CRY 1,2 -/- mice on AL diet. The relative food intake for wild-type mice on CR was increased in agreement with previously published data, at the same time, the relative food for CRY 1,2 -/- mice on CR was decreased **(Figure 4-3 c).**

Figure 4-3. (a) Food intake for male mice, WT AL male (Blue diamonds) and CRY AL male (Red squares), (b) Body weight of WT AL (Blue diamonds), WT CR (Green triangle), CRY 1,2 -/- AL (Violet square), CRY 1,2 -/- CR (Dark Blue). (c) Relative food intake of WT CR (Green triangle), CRY 1,2 -/- AL (Red square), CRY 1,2 -/- CR (Dark Blue).



CRY-dependent and independent effects of 30% CR on rhythms in gene expression

It is known that CR significantly changes the gene expression landscape (Swindell 2007). And we recently reported the effect of 30% CR on circadian rhythms in gene expression. To study if CRY's are involved in this CR-mediated changes we compared the mRNA expression of several genes, known to be affected by 30% CR, in the liver of WT and CRY 1,2 -/- mice on both AL and CR diet across the circadian cycle. Three of these genes: PER1, PER2, and BMAL1 are the circadian clock genes and four genes: FMO3, MUP 4, CYP4A14A, and CYP4A12B are not the components of the clock. As it was previously reported in the wild-type mice all three tested circadian clock genes showed circadian rhythms in the expression and their expressions were significantly induced by CR at multiple time of the day **(Figure 4-4. A, B, C).** In CRY 1,2 -/- mice under AL conditions the expressions of these genes were not rhythmic in agreement with previously published data; under CR conditions the expression of PER1become rhythmic and the expressions of BMAL1 and PER2 were still arrhythmic. 30% CR induced the PER1expression in both genotypes in a similar way, a significant induction was observed at ZT10 and ZT14. In CRY 1,2 -/- mice CR did not have such strong effect on PER2 or BMAL1 expression as in WT mice; for the PER2 expression significant changes were observed at ZT10, but it was a downregulation, not an up-regulation as in WT mice, for BMAL1 expression some induction was observed only at ZT22. Thus, CR regulated BMAL1 and PER2 expressions in CRY-dependent manner and PER1expression CRY independently. We

proposed that the effect of CR on circadian clock genes expression is Cry dependent and independent mechanism.

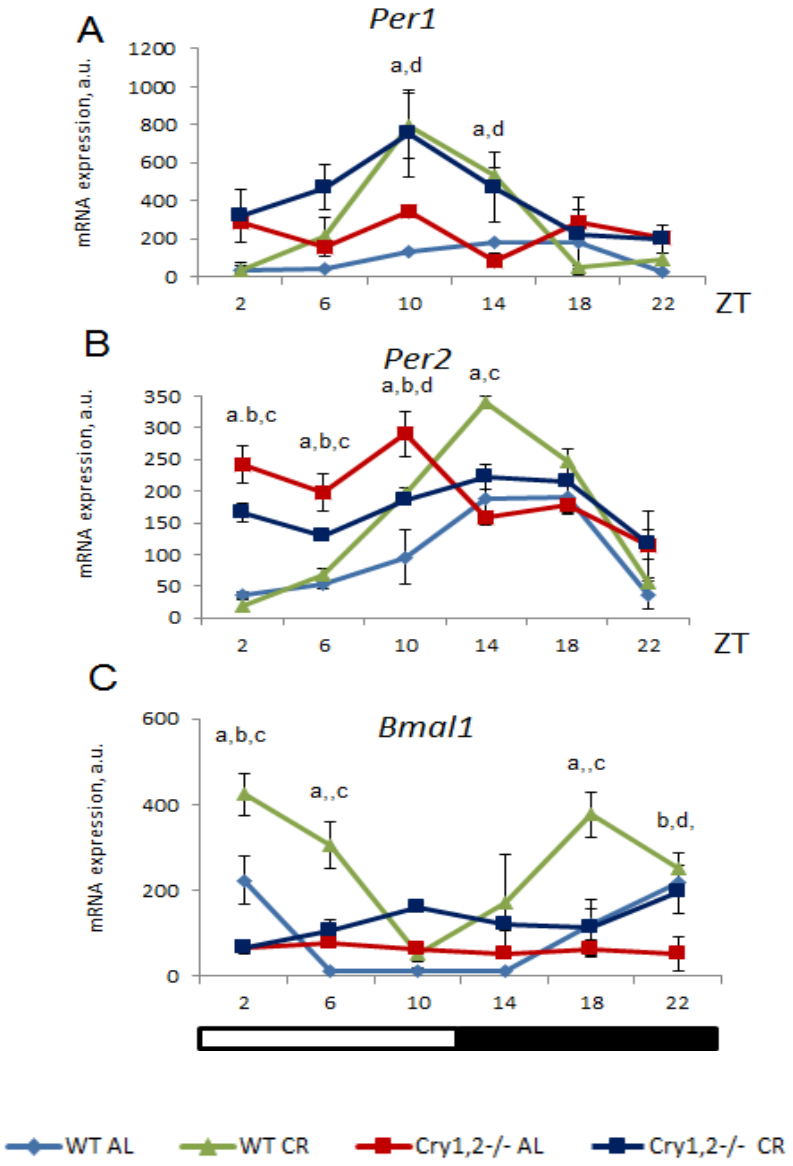


Figure 4-4. Effect of calorie restriction on rhythms of circadian clock genes. mRNA expression of core clock genes (a) PER1, (b) PER2, (c) BMAL1. WT CR (Green triangle), CRY 1,2 -/- AL (Red square), CRY 1,2 -/- CR (Dark Blue). Data represent mean ± SD; statistically significant ($p < 0.05$) effects of the feeding (analyzed by two

ways ANOVA) at a given time point are indicated by (a)- between WT AL and WT CR groups, (b)- WT AL and CRY 1,2 -/- AL, (c)- WT CR and CRY 1,2 -/- CR, (d)- CRY 1,2 -/- AL and CRY 1,2 -/- CR. Light and dark bars at the bottom represent light and dark phase of the day. ZT0 is the time when the light is on and ZT12 is the time when the light is off.

30% Calorie restriction led to a dramatic upregulation (more than 300 folds) of FMO3 gene expression and downregulation (more than 10 folds) of MUP 4 gene expression in the liver of both wild-type and CRY 1,2 -/- mice. The expression of CYP4A14A was also induced by CR in both genotypes, but the induction was higher in CRY 1,2 -/- mice. Thus, CRY's were not necessary for regulation of the FMO3 and MUP4 gene expression by CR and CRY s contributed to the regulation of the CYP4A14A gene expression **(Figure 4-5 a, b, c, d)**. On separate note, we also observed a significant difference in FMO3 gene expression between genotypes under AL conditions: in the liver of CRY 1,2 -/- mice the expression was higher at several time points, however, the magnitude of the induction was not as dramatic as the effect of CR: about 6 folds of difference between WT and CRY 1,2 -/- mice and more than 300 folds of difference between mice on AL and CR diets.

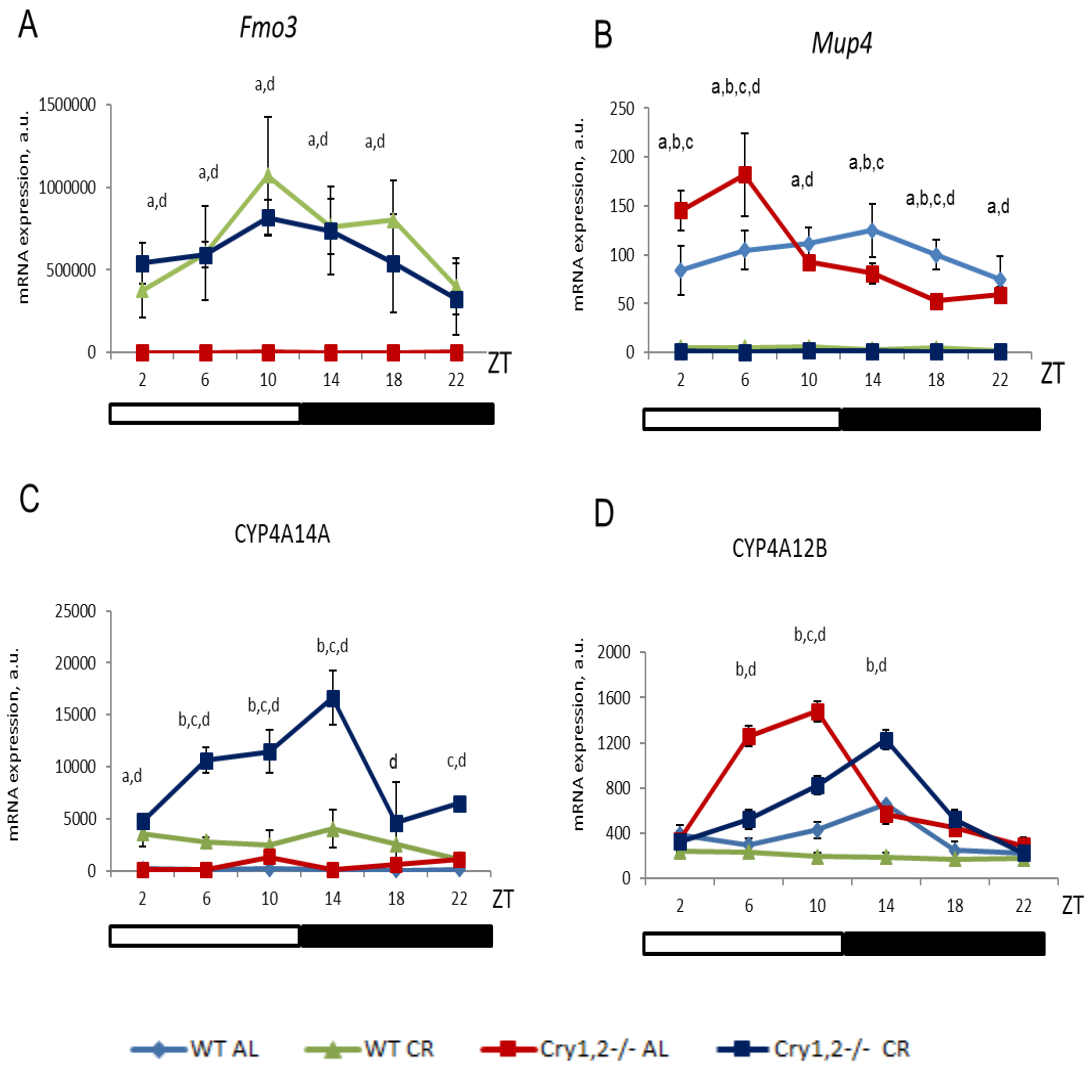


Figure 4-5. Effect of calorie restriction on rhythms of longevity-associated genes. mRNA expression of longevity-associated genes (A) FMO3, (B) MUP4, (C) CYP4A14A, (D) CYP4A12B. WT CR (Green triangle), CRY 1,2 -/- AL (Red square), CRY 1,2 -/- CR (Dark Blue). Data represent mean ± SD; statistically significant ($p < 0.05$) effects of the feeding (analyzed by two ways ANOVA) at a given time point are indicated by (a)- between WT AL and WT CR groups, (b)- WT AL and CRY 1,2 -/- AL, (c)- WT CR and CRY 1,2 -/- CR, (d)- CRY 1,2 -/- AL and CRY 1,2 -/- CR. Light and

dark bars at the bottom represent light and dark phase of the day. ZT0 is the time when the light is on and ZT12 is the time when the light is off.

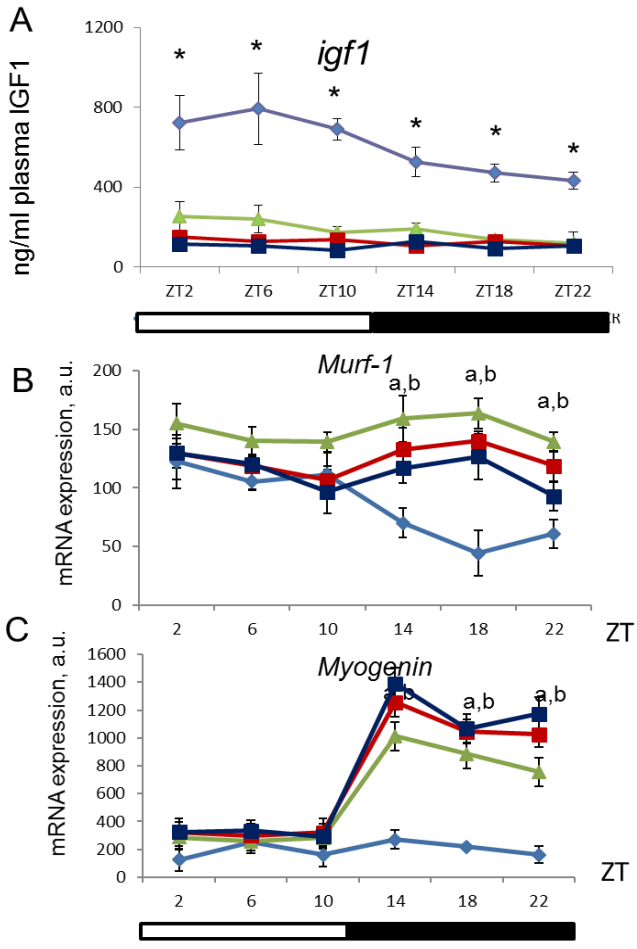
CRY's contribute to CR-induced downregulation of circulating IGF-1 level and IGF signaling

We have reported that CRY's are the important regulator of plasma IGF-1 level and IGF signaling in mammals. Reduced level of CRY 1 protein in response to 30% CR led us to a hypothesis that CRY's are involved in the CR-induced downregulation of IGF-1. We assayed the levels of IGF-1 in the plasma of WT AL, WT CR, CRY 1,2 -/- AL, and CRY 1,2 -/- CR mice. As it was previously reported the levels of the circulating IGF-1 demonstrated statistically significant daily changes **(Figure 4-6 a)**. Also in agreement with previously published data, the levels of the circulating IGF-1 were low in both WT CR and CRY 1,2 -/- AL mice. The circulating IGF-1 levels in WT AL mice were statistically significantly different from IGF-1 levels in WT CR, CRY 1,2 -/- AL or CRY 1,2 -/- CR mice at all 6 times of the day. No significant difference was observed between levels of the circulating IGF-1 between WT CR, CRY 1,2 -/- AL and CRY 1,2 -/- CR mice. Thus, CRY's are necessary for the CR-induced downregulation of circulating IGF-1.

The reduced IGF-1 level in CRY 1,2 -/- mice is associated with the reduced IGF signaling in the skeletal muscles, therefore, we decided to investigate if CRY's are necessary for suppression of IGF signaling upon CR. Reduced IGF-1 signaling results in the increased transcriptional activity of transcriptional factor FOXO. We monitored the expression of two known FOXO targets Murf-1 and Myogenin in the skeletal muscles. As illustrated on (**Figure 4-6 b, c)** the expression of both genes is

significantly lower in WT AL mice than in WT CR or CRY 1,2 -/- AL mice at several times of the days; CR did not have any significant effect on the expression of these genes in the skeletal muscles of CRY 1,2 -/- mice. Thus, CRY's were important for down regulation of IGF-1 level and IGF signaling upon CR.

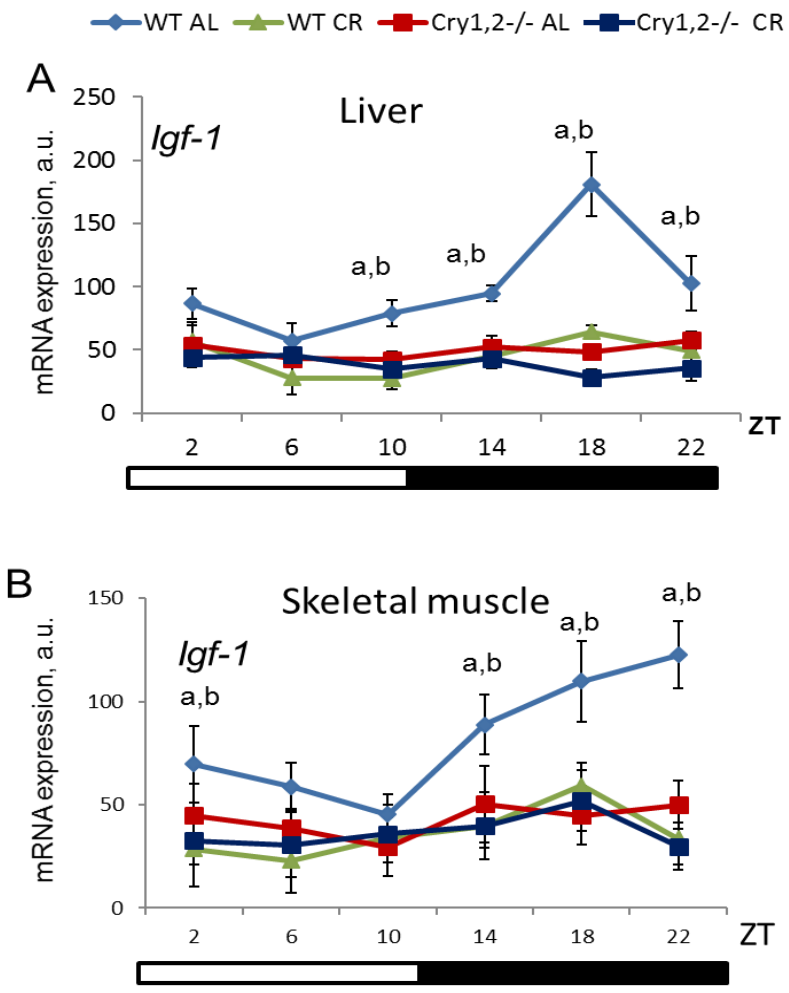
Figure 4-6. Effect of calorie restriction on circulating IGF1 level and IGF1 signaling in skeletal muscle. (a) Plasma IGF1, mRNA expression of (b) Murf1 and (c) Myogenin in skeletal muscle. WT AL (Blue diamond), WT CR (Green triangle), CRY 1,2 -/- AL (Red square), CRY 1,2 -/- CR (Dark Blue). Data represent 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 WT AL and WT CR groups, (b)- WT AL and CRY 1,2 -/- AL, (c)- WT CR and CRY 1,2 -/- CR, (d)- CRY 1,2 -/- AL and CRY 1,2 -/- CR. Light and dark bars at the bottom represent light and dark phase of the day. ZT0 is the time when the light is on and ZT12 is the time when the light is off.



CRY's regulate CR-induced downregulation of IGF-1 mRNA expression

It was proposed that reduction of circulating IGF-1 upon CR is due to reduced IGF-1 mRNA expression in the liver (Herrington et al. 2000). We previously reported that CRY's regulate the expression of IGF-1 gene on mRNA level (Chaudhari et al. 2016). We compared the expression of IGF-1 on the mRNA level across the circadian cycle in the liver of WT and CRY 1,2 -/- mice on both AL and CR diets **(Figure 4-7 a)**. In agreement with previous reports, both 30% CR in wild-type mice and CRY's deficiency resulted in the significant reduction of IGF-1 mRNA expression (the difference was significant at several times of the day), no further significant reduction in the expression was observed CRY 1,2 -/- mice on CR diet. The majority of the circulating IGF-1 is produced in the liver (Yakar et al. 1999) but other tissues also produce IGF-1, which is essential for the paracrine signaling (Liu et al. 1998). IGF-1 mRNA expression was reduced upon 30% CR in the skeletal muscles of WT mice **(Figure 4-7 b)**; in CRY 1,2 -/- mice the IGF-1 expression was significantly lower than in WT and it was not affected by CR. Thus, the reduction of IGF-1 mRNA expression in response to 30% CR was dependent on CRY's in both tested tissues.

Figure 4-7. Calorie restriction affects the IGF1 expression in (a) Liver and in (b) Skeletal muscle. Data represent 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 WT AL and WT CR groups, (b)- WT AL and CRY 1,2 -/- AL, (c)- WT CR and CRY 1,2 -/- CR, (d)- CRY 1,2 -/- AL and CRY 1,2 -/- CR. Light and dark bars at the bottom represent light and dark phase of the day. ZT0 is the time when the light is on and ZT12 is the time when the light is off.



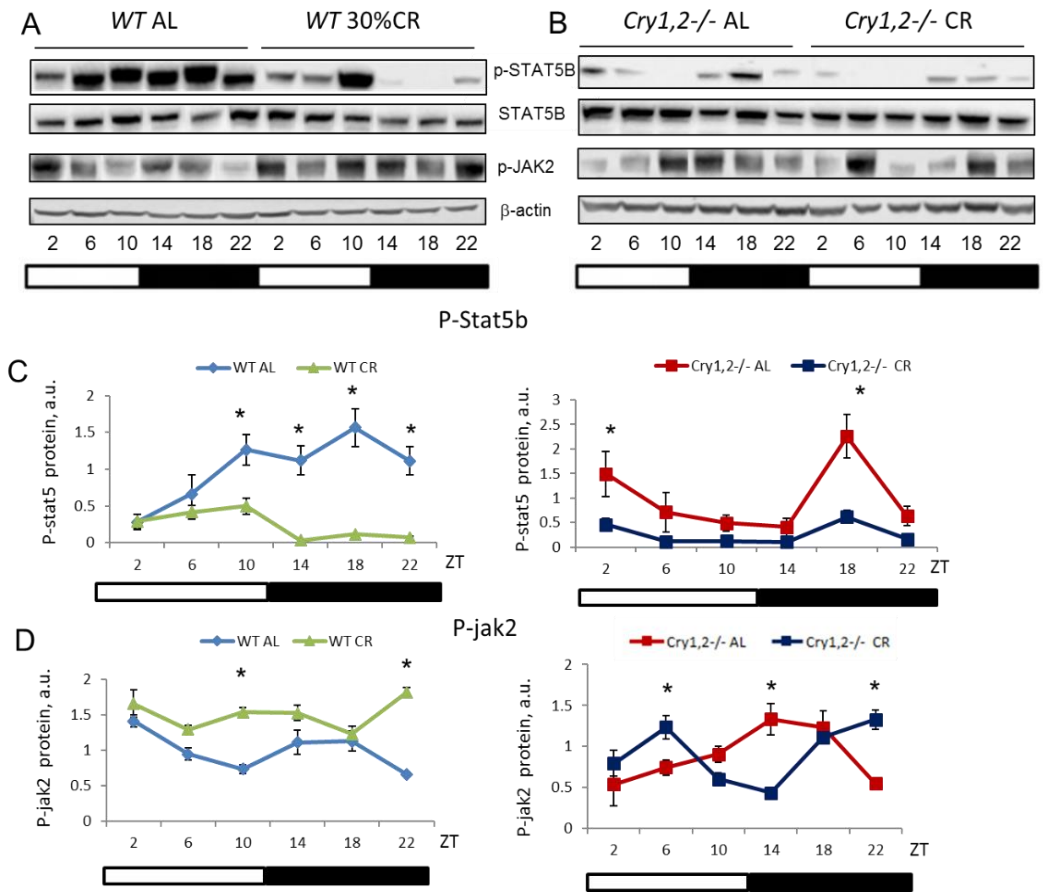
Effect of CR on JAK2-STAT5B signaling

IGF-1 transcription in the liver is regulated by the transcriptional factor STAT5B (Herrington et al. 2000). According to the existing paradigm STAT5B is phosphorylated and active under AL conditions and upon CR both phosphorylation and transcriptional activity of STAT5B is dramatically reduced (by unknown mechanisms), which causes a reduction in IGF-1 transcription (Oberbauer. 2013). We assayed the levels of STAT5B phosphorylation on Y699 (used as a marker of STAT5B activation) in mice of both genotypes on both diets across the clock. As it was expected upon 30% CR, in WT mice the STAT5B phosphorylation was dramatically reduced at several times of the day, which is generally is in an agreement with the previously published data on the effects of CR on STAT5B phosphorylation **(Figure 4-8 a)**. In the liver of CRY 1,2 -/- mice, the STAT5B phosphorylation was significantly affected by 30% CR only at one time ZT18, at all other times of the day, the effect of CR on STAT5B phosphorylation was not significant **(Figure 4-8 b)**. Please note that the level of the STAT5B phosphorylation was significantly lower in the liver of CRY 1,2 -/- mice as we previously reported.

The phosphorylation of STAT5B is regulated by a JAK2 kinase. In the liver of WT mice, CR resulted in a small but significant increase in JAK2 phosphorylation **(Figure 4-8 a)** and in the liver of CRY 1,2 -/- mice no significant difference in JAK2 phosphorylation was observed **(Figure 4-8 b)**. Thus, the reduced phosphorylation of STAT5B upon CR in WT mice was not a consequence of reduced JAK2

phosphorylation, in agreement with previous reports, and CRY s did not regulate JAK2 phosphorylation under CR and, most likely, act downstream of JAK2.

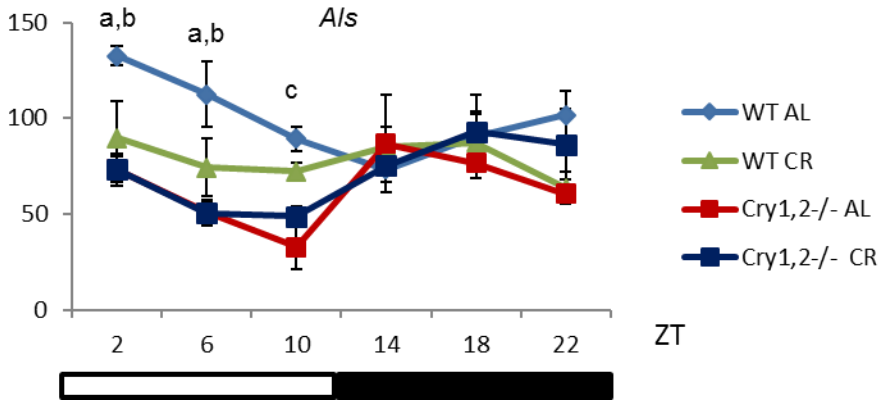
Figure 4-8. Effect of calorie restriction on JAK2-STAT5B activity. (a) Representative Western blot analysis of p-JAK2 and p-STAT5B in the liver of WT AL compared to WT CR (A) and in CRY 1,2 -/- AL compared to CRY 1,2 -/- CR (B). (C) Respective quantification of phosphorylation of STAT5B on Y699 and (D) JAK2 on Y1007. WT AL (Blue diamond), WT CR (Green triangle), CRY 1,2 -/- AL (Red square), CRY 1,2 -/- CR (Dark Blue). Data represent mean ± SD; statistically significant ($p < 0.05$) effects of the feeding (analyzed by two-way ANOVA) at a given time point are indicated by (a)- between WT AL and WT CR groups, (b)- WT AL and CRY 1,2 -/- AL, (c)- WT CR and CRY 1,2 -/- CR, (d)- CRY 1,2 -/- AL and CRY 1,2 -/- CR. Light and dark bars at the bottom represent light and dark phase of the day. ZT0 is the time when the light is on and ZT12 is the time when the light is off.



In an agreement with the effect of CR on the STAT5B phosphorylation, the mRNA expression of STAT5B target gene ALS (Acid labile subunit) was down regulated by CR in the liver of WT but not CRY 1,2 -/- mice. **(Figure 4-9)**.

Figure 4-9. mRNA expression of STAT5B target gene ALS in the liver. WT AL (Blue diamond), WT CR (Green triangle), CRY 1,2 -/- AL (Red square), CRY 1,2 -/- CR (Dark Blue). Data represent 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 WT AL and WT CR groups, (b)- WT AL and CRY 1,2 -/- AL, (c)- WT CR and CRY 1,2 -/- CR, (d)- CRY 1,2 -/- AL and CRY 1,2 -/- CR. Light and dark bars at the bottom represent

light and dark phase of the day. ZT0 is the time when the light is on and ZT12 is the time when the light is off.



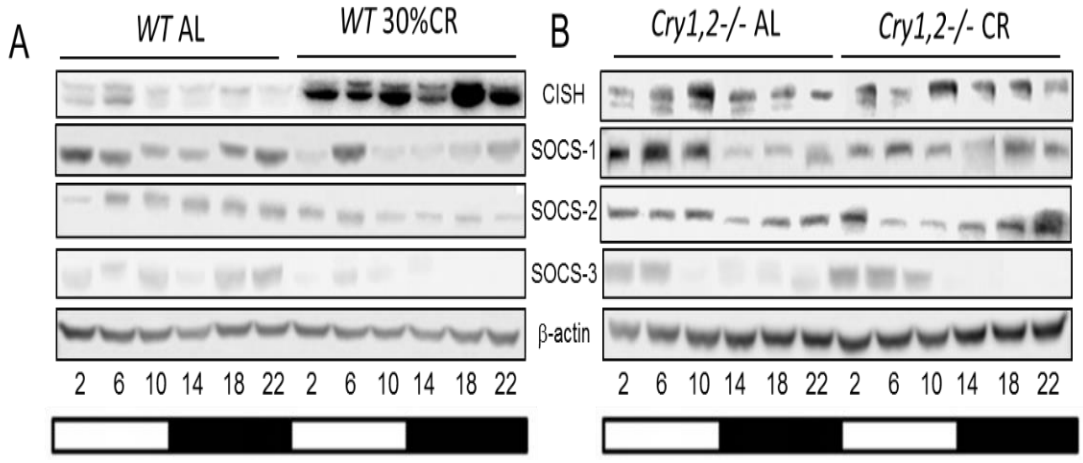
Up-regulation of CISH mRNA and protein expression upon CR is CRY-dependent

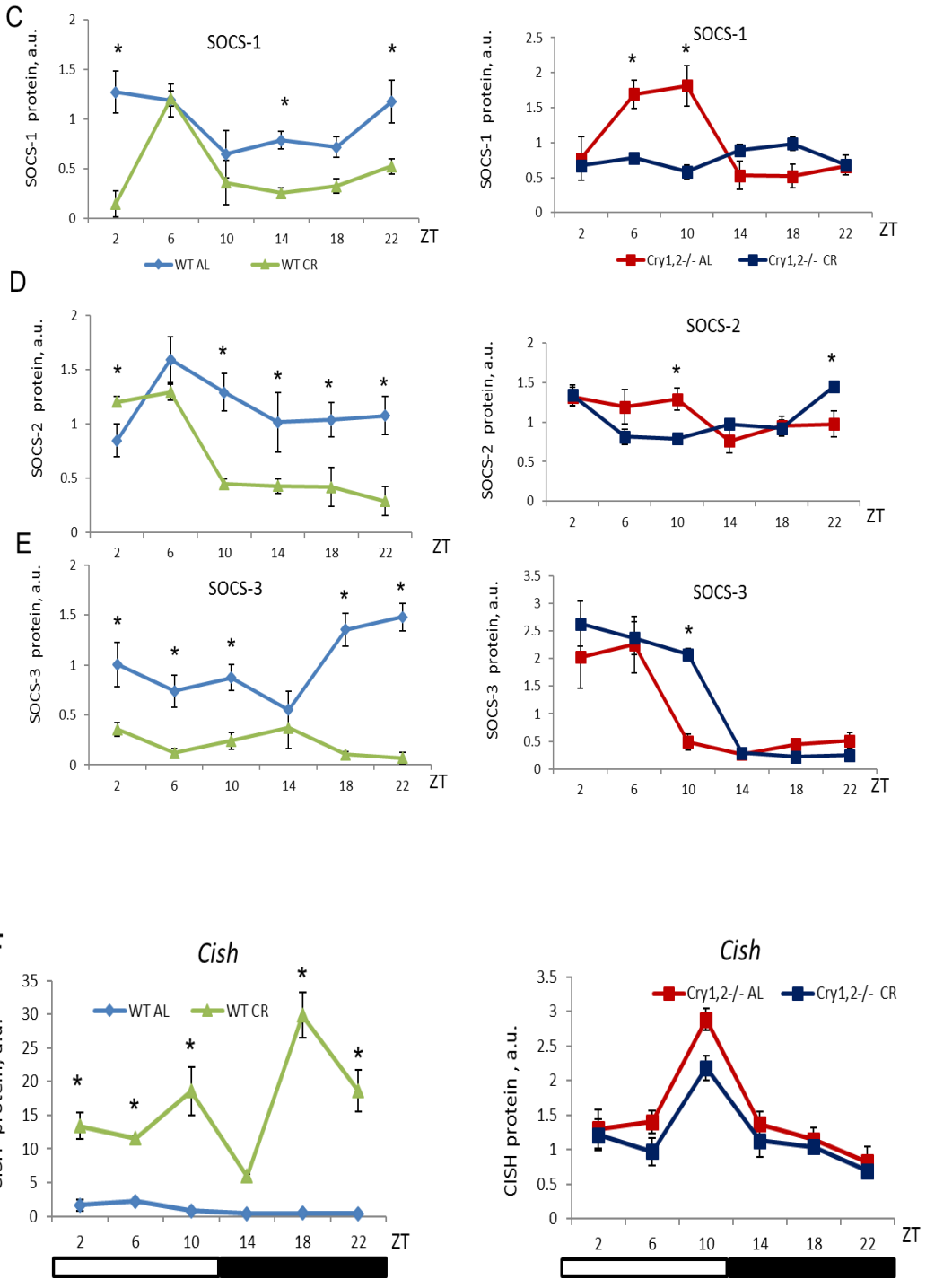
The activity and phosphorylation of STAT5B are regulated by the proteins from SOCS family. We previously reported that the expression of CISH is significantly up-regulated in the liver of CRY 1,2 -/- mice. 30% CR significantly induced the expression of CISH, the induction was from 5 to 30 folds depending on the time of the day. In contrast to that, the expression of CISH was not significantly affected by CR in the liver of CRY 1,2 -/- mice. In agreement with the protein data the mRNA expression for CISH was high in the liver of CRY 1,2 -/- mice and it was not affected by CR **(Figure 4-10 a, right)**. The CISH mRNA expression was significantly induced at multiple time of the day upon CR in WT mice **(Figure 4-11 D)**. Thus, CISH expression was regulated by the CR in the liver in CRY-dependent manner. Upon

30% CR, the expressions of SOCS1 and SOCS3 proteins were significantly reduced in the liver of WT mice at multiple times of the day and the reduction correlated with decreased mRNA expression of both genes **(Figure 4-11 A, C, left)**. In CRY 1,2 -/- mice 30% CR had some significant but mixed effect on the expression of these two proteins, for SOCS1 protein CR resulted in reduced expression at ZT6 and ZT 10 and increased expression at ZT18; for SOCS3 protein CR resulted in increased expression at ZT10. We did not detect any significant effect of CR on the mRNA expression of these two genes in the liver of CRY 1,2 -/- mice **(Figure 4-11)**.

SOCS1 and SOCS3 are known transcriptional targets of STAT5B and the reduced mRNA expression in the liver of CR or CRY 1,2 -/- mice correlated with the reduced STAT5B phosphorylation. The expression of the SOCS2 protein was also affected by CR in both WT and CRY 1,2 -/- mice, but the effect was mixed, we observe up or down regulation of the expression at different times of the day. We did not detect any significant effect of the diet on genotype on the expression of Socs2 mRNA.

Figure 4-10. Effect of CR SOCS's protein. (a) Representative western blotting in WT AL compared to WT CR and (b) CRY 1,2 -/- AL compared to CRY 1,2 -/- CR in the liver. Quantification of expression (c) SOCS1, (d) SOCS2, (e) SOCS3 and (f) CISH. WT AL (Blue diamond), WT CR (Green triangle), CRY 1,2 -/- AL (Red square), CRY 1,2 -/- CR (Dark Blue). Data represent mean ± SD; statistically significant ($p < 0.05$) effects of the feeding (analyzed by two ways ANOVA) at a given time point




C
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SOCS-1 protein, a.u.
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Cry1,2-/- AL
Cry1,2-/- CR
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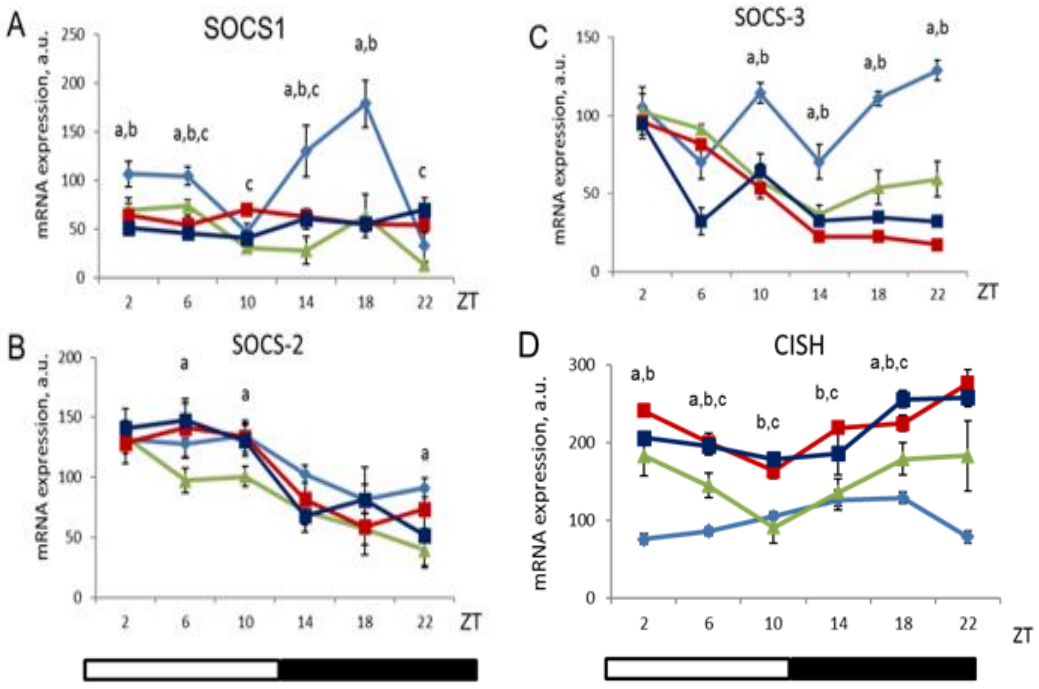
Figure 4-11. Daily profiles of mRNA expression of indicated SOCS family member genes in the liver of WT AL (blue diamonds), WT CR (green triangle), CRY 1,2 -/- AL (Red squares) and CRY 1,2 -/- CR (Dark Blue square) male mice (N = 3 per time point). Data represent mean ± SD; statistically significant ($p < 0.05$) effects of the feeding (analyzed by two ways ANOVA) at a given time point are indicated by (a)- between WT AL and WT CR groups, (b)- WT AL and CRY 1,2 -/- AL, (c)- WT CR and CRY 1,2 -/- CR, (d)- CRY 1,2 -/- AL and CRY 1,2 -/- CR. Light and dark bars at the bottom represent light and dark phase of the day. ZT0 is the time when the light is on and ZT12 is the time when the light is off.



4.5 Discussion

Cryptochromes are necessary for CR-mediated downregulation of IGF-1 expression

CR leads to the downregulation of IGF-1 production and IGF signaling. IGF signaling plays an important conserved role in the longevity. In mammals, one of the main beneficial effects of CR is significantly reduced cancer rate. It was demonstrated that reduced cancer rate is mostly due to the reduced IGF signaling. Therefore, understanding molecular mechanisms of downregulation of IGF-1 production upon CR is extremely important. Upon CR the expression of Igf-1 mRNA is reduced in the liver and other metabolic tissues. The activating phosphorylation of transcriptional factor STAT5B, a major regulator of Igf-1 expression, is also significantly reduced At the same time, GH level is not reduced (Bruss et al. 2011) and GH-dependent phosphorylation of JAK2 is not reduced. Based on these data it was proposed that CR affects the signaling between JAK2 and STAT5B (Thompson et al. 2014), but mechanisms are unknown.

We found that CR did not reduce plasma IGF-1 level and Igf-1 mRNA expression in Cry1,2-/- mice. Importantly, while the levels of plasma IGF-1and Igf-1 mRNA expression is low in Cry 1,2-/- mice on both diets, it is not zero (the daily levels of plasma IGF-1 are between 400 ng/ml and 1400 ng/ml in wild-type mice on AL diet and between 200 ng/ml and 300 ng/ml in Cry1,2-/- mice on both diets and in wild-type mice on CR diet), this level can be further reduced for example by prolonging fasting (as low as 100 ng/ml). In wild-type animals, CR resulted in the

increased expression of CISH on both mRNA and protein levels, while in Cry1,2-/- mice CR did not lead to further upregulation of CISH expression, therefore, CRY's are necessary for the induction of CISH expression by CR. In wild-type mice, the increased CISH expression correlated with the reduced phosphorylation of STAT5B, while Cry1,2-/- mice there is no significant effect of CR on STAT5B phosphorylation. Thus, we hypothesized here that CR regulates downregulation of IGF-1 expression through CRY-dependent control of CISH expression, however, we cannot exclude that there are CRY and CISH- independent mechanisms. To test this hypothesis directly we need to investigate the effect of CR on CISH deficient mice, which is future study.

CR regulates the expression of Cryptochrome proteins

We found that CRY 1 protein level was reduced upon CR in the liver and skeletal muscles of WT mice. This downregulation was due to some posttranscriptional regulation because CRY 1 mRNA expression was not affected. Emerging evidence suggests the importance of CRY's posttranslational regulation for circadian clock function. CRY's are phosphorylated by AMPK, and this phosphorylation leads to CRY's degradation (Lamia et al. 2009). AMPK is implicated as a molecular mediator of CR, but data on its induction by CR are controversial (Cantó and Auwerx 2011). In separate studies, ubiquitin ligases involved in CRY 1 degradation have been reported (Yoo et al. 2013). If these signaling systems are involved in the observed reduction of CRY's level upon CR or not needs to be studied. Interestingly, in a contrast to the effect on CRY 1, the expression of CRY 2 was not

reduced upon CR and even there was some increase in the expression, which suggests different regulation of these two highly homologous proteins.

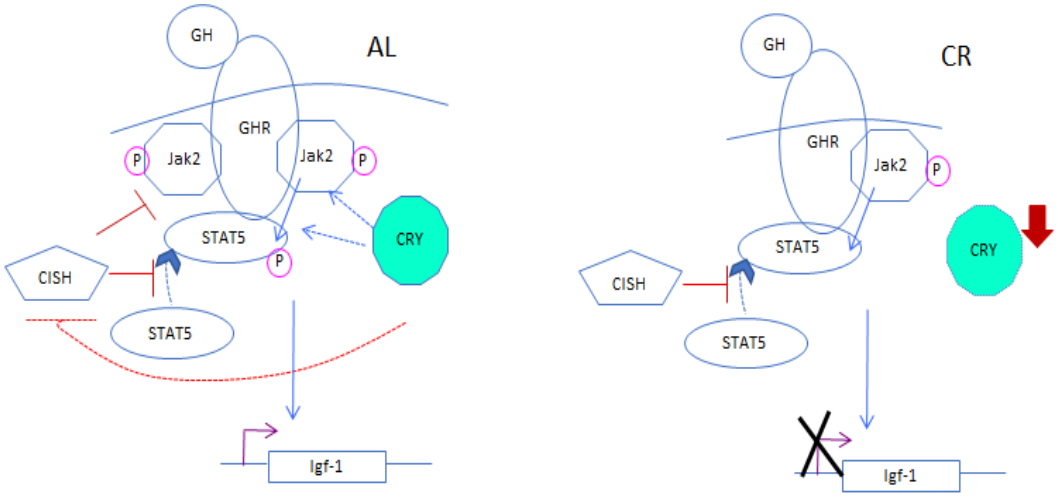
Previously we demonstrated that deficiency of both proteins is necessary for reduced body size downregulation of IGF-1 production. How in this case downregulation only CRY 1 could be sufficient for the downregulation of IGF1 production upon CR in WT mice? Deficiency of CRY 1 results in the modest but statistically significant reduction in circulating IGF-1 level, but the effect is not as strong as deficiency of both proteins. Deficiency of CRY 2 does not have any effect on the level of circulating IGF-1. There is evidence that CRY 1 and CRY 2 have partially redundant functions: for example, their role in the circadian clock mechanisms is not identical - deficiency of individual proteins results in shorter (CRY deficiency) or longer (CRY deficiency) and deficiency of both in rhythms disruption; CRY 2 is implicated as tumor suppressor but CRY 1 is not. CRY 1 and CRY 2 also have a different pattern of tissue expression. It is possible that embryonic deficiency of either protein can be, at least partially, compensated during development, which is often observed for the proteins with redundant functions, when during the development compensatory proteins start to express in the tissue where normally homologous protein is expressed predominantly. Down-regulation of the protein expression in the adult animal cannot be efficiently compensated. The future experiments with CR applied to Cry 1-/- and Cry 2-/- mice will help to understand the role of individual CRY proteins in CR-dependent down regulation of IGF-1 production.

CR regulates IGF-1 production through Cryptochrome/CISH dependent mechanism. Model

We propose the following model **(Figure 4-12)**. Under AL conditions CRY's suppresses the expression of CISH on both mRNA and protein level. When CISH expression is low GH/GHR/JAK2 signaling is active and STAT5B is recruited to the phosphorylated GHR, where it is phosphorylated by JAK2. After that activated STAT5B forms a dimer, translocate to the nucleus where it will drive the expression of targets genes including IGF-1. Under CR conditions CRY 1 protein expression is down-regulated, which releases the suppression of CISH expression. CISH inhibits the interaction between STAT5B and GHR, thus, inhibiting STAT5B phosphorylation and activity. As consequence, the expression IGF-1 mRNA is significantly reduced, which caused the reduced level of serum IGF-1, reduced IGF signaling and, ultimately, will contribute to the reduced cancer rate under CR.

Figure 4-12: Proposed model for the role of Cryptochrome in IGF-1 production and calorie restriction-mediated mechanism.

The solid line represents previously reported activation of STAT5B phosphorylation by JAK2 and suppression of this phosphorylation by CISH members of SOCSs family. Under AL condition level of CRY's is high and CRY's might directly interact with JAK2 containing complexes and stimulate JAK2 activity toward STAT5B, CRY might recruit STAT5B to the JAK2/GHR complex, CRY might also suppress CISH expression/activity. Thus, high level of CRY's stimulates STAT5B phosphorylation and activation, which in turn, drives IGF-1 expression in the liver and other tissues. Under CR conditions level of CRY's in the tissues is significantly reduced, which compromises the above-proposed mechanisms and leads in reduced JAK2 activity toward STAT5, as a result, the IGF-1 expression is reduced.



FUTURE IMPLICATIONS

We found that Cryptochromes are important regulators of CISH expression and IGF-1 production and signaling under AL conditions and these proteins are necessary for CR-induced downregulation of IGF-1 expression. IGF signaling plays an important role in the development of cancer and in control of longevity. Thus, it would be interesting to examine the effect of CR on longevity and cancer development in CRY-deficient mice.

Another direction is regulation of JAK2/STAT signaling by Cryptochromes, we found that GH-dependent phosphorylation of STAT5B is dependent on CRY's, most likely, through the control of CISH expression. CISH is involved in the regulation of phosphorylation of other STAT transcriptional factors such as STAT3 in response to different cytokines such as EPO and IL-2.

Finally, an important question is how CR regulates Cryptochrome expression on translational and posttranslational levels. 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.

CHAPTER V

CONCLUSION

1. Cryptochromes regulated the IGF-1 production and IGF signaling, reduced IGF-1 production in Cry1,2 -/- mice correlated with the reduced body size and body weight of these mice. We proposed that the circadian clock-dependent control of IGF signaling through CRY-dependent mechanism is important for the synchronization of cell growth and proliferation with rhythms in metabolism, daily activity, and feeding.
2. Cryptochromes suppressed the expression of CISH on both mRNA and protein levels. Up-regulation of CISH correlated with the reduced phosphorylation of transcriptional factor STAT5B and might be responsible for the reduced Igf-1 expression. Cryptochromes might be involved in the control of JAK2/STAT signaling through the regulation of CISH expression. Thus, Cryptochromes might

regulate many other physiological systems and processes such as immune response.

3. CR regulated CRY1 expression on the protein level, thus linking the diet with the circadian clock mechanism.
4. Cryptochromes are necessary for calorie restriction induced downregulation of IGF-1 production. IGF signaling plays the central role in cancer development and longevity under CR conditions. Our results suggest that Cryptochromes are essential components of CR molecular mechanism and might be involved in the control of carcinogenesis and longevity in mammals.

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APPENDICES

Table 1. Cosinor Wave Analysis in (A) WT AL and WT CR., (B) CRY 1,2 -/- AL and CRY 1,2-/- CR. 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).

CIRC: CIRCAIDAN, ACRO: ACROPHASE , AMP: AMPLITUDE

A.

GENOTYPE /DIET	WT AL			WT CR		
	CIRC	**Acro**	**Amp**	**CIRC**	**Acro**	**Amp**
BMAL1	YES	22.91635	18.59649	YES	23.56465	8.32047
PER1	YES	14.43865	6.88812	YES	11.06454	20.28089
PER2	YES	15.02812	5.326099	YES	14.27397	17.25972
FMO3	NO	13.83491	3.441873	YES	12.23519	1.784454
MUP 4	YES	11.85188	1.492938	YES	8.08063	3.853585
CYP4A14A	YES	5.474312	15.16344	NO	10.45299	3.627251
CYP4A12B	YES	9.754743	3.545335	YES	12.60116	22.51527
ALS	YES	2.408401	1.806876	NO	16.07329	1.369674
IGFBP-3	YES	2.501775	1.787465	YES	1.582492	1.404963
IGF1	YES	18.37578	3.162794	YES	20.3743	2.345746

B.

GENOTYPE/DIET	CRY AL			CRY CR		
	CIRC	**Acro**	**Amp**	**CIRC**	**Acro**	**Amp**
BMAL1	YES	5.460247	1.533264	NO	16.8326	2.36377
PER1	NO	2.321969	4.027954	YES	9.593249	3.816822
PER2	YES	7.891592	2.515398	YES	14.37423	1.917164
FMO3	NO	21.78263	20.97115	YES	8.734285	2.528742
MUP 4	YES	5.426368	3.461747	NO	12.10274	4.765915
CYP4A14A	NO	15.97276	22.05058	YES	11.33757	3.616333
CYP4A12B	YES	8.870595	55.96446	YES	12.80597	40.7383
ALS	YES	18.98527	2.64	YES	19.75206	1.901505
IGFBP-3	YES	3.170331	1.72	YES	4.144528	1.94486
IGF1	YES	21.34849	1.355818	YES	5.68356	1.627443