Dissertation

submitted to the

Combined Faculties for the Natural Sciences and for Mathematics

of the Ruperto-Carola University of Heidelberg, Germany

for the degree of

Doctor of Natural Sciences

presented by

Ying Li M.Sc.

Born in Hebei, China

Unravelling multiple clock mechanisms in vertebrates

Referees: Prof. Dr. Nicholas S. Foulkes

Prof. Dr. Uwe Strähle

Acknowledgements

First of all, I wish to express my sincere gratitude to my supervisor Prof. Dr. Nicholas S. Foulkes for giving me the great opportunity to do my Ph.D. in his laboratory and to work on this intriguing project. When I started the zebrafish circadian projects in China, reading his papers gave me a highly efficient way to get familiar with this research field. Subsequently I was so lucky to be his Ph.D. student, and learned more than knowledge and skills during last three years in his lab. He is a respected professor of profound learning and attractive personality. I am very grateful for his helpful discussions and wonderful advices on my experiments, suggestions about seminar preparation and comments on this thesis. Notably, Nick gave me considerable freedom to think about the project myself. I would like to thank Dr. Daniela Vallone for scientific and technical supporting, and say many thanks to the members of my thesis advisory committee, they are Prof. Dr. Burkhard Luy, Dr. Daniela Vallone and Dr. Thomas Dickmeis. Thanks a lot for their advices and feedbacks on my progress.

I would like to thank my collaborators Dr. Benjamin Görling and Philipp-Michael Eisenmann from Prof. Dr. Burkhard Luy group in the Institute of Organic Chemistry, for their great work, many constructive discussions and friendly attitude. A special thanks to my former supervisor Prof. Dr. Jun Yan and my collaborator Guang Li in the Institute of Neuroscience (Chinese Academy of Sciences), they kindly provided servers for programmes running and offered me many supports for the bioinformatic analysis. I am also grateful for all the help of my present and former lab members, Dr. Jose Fernando Lopez-Olmeda, Dr. Rosa Ceinos, Dr. Cristina Pagano, Gennaro Ruggiero, Rima Siauciunaite and Mareike Restatter. I would like to acknowledge Dr. Felix Loosli, Dr. Olivier Kassel, Dr. Ravindra Peravali, Eduard Gursky and all the staff in ITG fish house. Without their support and help, I can’t finish this project properly.

Last but not least, I want to deeply thank my beloved family. For the past few years, I have been living here alone, but I am missing and concerning my family all the time. Thanks for all their strong support and love they have given me throughout this whole time. My dear father, I am so proud to be your daughter and wish I never disappoint you. I want to thank Werner and Heiker, who took care of me like my German parents. I will remember the time we spent together forever. Thanks a lot to my close friends, Guang, Xiao, Wenshan, Cuiping Tian, Yuanhua Liu, Ying Zheng, Ziwei…whose kindness and patience gave me the strength to overcome many difficulties. Finally, thank CSC for the financial support.

Table of Contents

Publications

1. **Ying Li**, Benjamin Görling, Philipp-Michael Eisenmann, Guang Li, Burkhard Luy and Nicholas S. Foulkes. Unravelling multiple clock mechanisms in vertebrates. *In Preparation.*

2. **Ying Li**, Guang Li, Benjamin Görling, Burkhard Luy, Jiulin Du and Jun Yan. Integrative Analysis of Circadian Transcriptome and Metabolic Network Reveals the Role of De Novo Purine Synthesis in Circadian Control of Cell Cycle. *PLoS Comput Biol*. 11(2): e1004086 (2015).

3. **Ying Li**, Guang Li, Haifang Wang, Jiulin Du and Jun Yan, Analysis of a gene regulatory cascade mediating circadian rhythm in zebrafish. *PLoS Comput Biol*. 9(2): e1002940 (2013).

Abbreviations

AA	amino acids
Aanat2	arylalkylamine-N-acetyltransferase 2
ADP	adenosine diphosphate
Ala	alanine
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
Arg	arginine
Asn	asparagine
Asns	asparagine synthetase
Asp	aspartic acid
ATP	adenosine triphosphate
bHLH-PAS	basic helix-loop-helix, Per-Arnt-Sim
Bmal	brain and muscle ARNT-like
bp	base pair
CCGs	clock controlled genes
cDNA	complementary deoxyribonucleic acid
C/EBPβ	CCAAT /enhancer-binding protein β
Cfb	complement factor B
Chap	chaperones
CIRP	cold-inducible RNA-binding proteins
CK1ε	casein kinase-1ε
CLK	CLOCK
CLOCK1 DN	CLOCK1 dominant negative cells
CNS	central nervous system
CREB	cAMP response element binding protein
CREB-p	phospho-cAMP response element binding protein
Cry	cryptochrome
C_T	critical threshold
Cyc	cycle
Cyp2a5	cytochrome P450, family 2, subfamily a, polypeptide 5
Dbp	D-box binding PAR bZIP transcription factor
DBS	RORE DNA binding sequence
DD	constant darkness
Dec1 (Bhlhe40)	basic helix-loop-helix family, member e40
DL	dark-light cycle

DMH	dorsomedial hypothalamic nucleus
D_2O	deuterium oxide
dpf	days post fertilization
E4bp4 (Nfil3)	nuclear factor, interleukin 3, regulated
FAA	food-anticipatory activity
Fabp10a	fatty acid binding protein 10a
FEO	food entrainable oscillator
Fgg	fibrinogen, gamma polypeptide
FOXO1	forkhead box O1
Frq	frequency
GDP	guanosine diphosphate
Gln	glutamine
Glu	glutamate
Glu1a	glutamine synthetase 1a
Glud1b	glutamate dehydrogenase 1b
Gly	glycine
GMP	guanosine monophosphate
Got1, Got2a, Got2b	glutamic-oxaloacetic transaminase 1, 2a and 2b
Gpt2l	glutamic pyruvate transaminase 2, like
GSH	glutathione
GSK3β	glycogen synthase kinase 3β
GTP	guanosine triphosphate
HAT	histone acetyltransferase
HDAC	deacetylation histone deacetylases
His	histidine
Hnf4a	hepatocyte nuclear factor 4, alpha
^{1}H NMR	hydrogen nuclear magnetic resonance spectroscopy
H_2O_2	hydrogen peroxide
HPA	hypothalamic-pituitary-adrenal
HSF1	heat shock factor 1
HSPs	heat shock proteins
Impdh2	IMP dehydrogenase
IDP	inosine diphosphate
IEGs	immediate early genes
Ile	isoleucine
IMP	inosine monophosphate
ipRGCs	intrinsically photosensitive retinal ganglion cells

LC3-I /LC3-II	microtubule-associated protein1 light chain 3 I, II
LD	light-dark cycle
LEO	light entrainable oscillator
Leu	leucine
LL	constant light
LXR	liver X receptor
MD	middle of dark period
Met	methionine
ML	middle of light period
ml	milliliter
mM	millimolar
MNA	n1-methylnicotinamide
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NAD+	oxidized form of nicotinamide adenine dinucleotide
NADH	reduced form of nicotinamide adenine dinucleotide
NAM	nicotinamide
NaMN	nicotinic acid mononucleotide
Nampta	nicotinamide phosphoribosyltransferase a
Namptb	nicotinamide phosphoribosyltransferase b
NES	night eating syndrome
NMN	nicotinamide mononucleotide
Opn4	opsin4
OX	optic chiasm
OXM	oxyntomodulin
PACAP	pituitary adenylate cyclase-activating polypeptide
PARP-1	poly (ADP-ribose) polymerase 1
Per	period
PGC1α	PPARγ coactivator α
Phe	phenylalanine
Pi	phosphate
PPARα	peroxisome proliferator-activated receptor alpha
PRXs	peroxiredoxins
PRX-$SO_{2/3}$	overoxidised/hyperoxidised peroxiredoxin
qRT-PCR	quantitative RT-PCR
REV-ERBα	nuclear receptor subfamily 1, group D, member 1
RGCs	retinal ganglion cells

RHT	retinohypothalamic tract
RORα	RAR-related orphan receptor alpha
RORE	retinoic acid-related orphan receptor response element
Rorγ	RAR-related orphan receptor gamma
ROS	reactive oxygen species
RT	reverse transcription
SCN	suprachiasmatic nucleus
SEM	standard error of mean
Ser	serine
Sirt1	sirtuin 1
Tat	tyrosine aminotransferase
Tdh	threonine dehydrogenase
Tef	thyrotroph embryonic factor
TEO	temperature entrainable oscillator
Thr	threonine
Tim	timeless
TMT	teleost multiple tissue
TTFLs	transcription-translation feedback loops
TTO	transcription-translation-derived oscillator
Tyr	tyrosine
Val	valine
Wcc	white collar complex
Wee1	WEE1 G2 checkpoint kinase
WT	wild type
YMCs	yeast metabolic cycles
ZT	zeitgeber time
µg	microgram
µl	microliter
64Phr	(6-4)-photolyase
2-Py	n1-methyl-2-pridone-5-carboxamide
4-Py	n1-methyl-4-pridone-3-carboxamide

Abstract

The zebrafish (*Danio rerio*) has proven to be a useful genetic model for studying the circadian timing system. The canonical molecular mechanism underlying circadian rhythms is based upon auto-regulatory transcription-translation feedback loops (TTFLs). It is well established that zebrafish has a TTFLs-dependent light entrainable oscillator (LEO) present in most tissues and even cell lines however, like other vertebrates, they also possess a food entrainable oscillator (FEO). Recent studies have addressed how regular food availability can entrain circadian rhythms of increased locomotor activity that precedes the feeding time (so-called food-anticipatory activity, FAA). Unlike the extensively studied LEO, there is very little known about the molecular components and regulation of the FEO.

My PhD programme focuses on the mechanism whereby food regulates the circadian clock in zebrafish. Using behavior recording, we have revealed that FEO exists in adult zebrafish by showing that scheduled, restricted food availability can entrain FAA independently of the timing of the light-dark (LD) cycle. By maintaining fish under a LD cycle and then feeding fish either in the middle of the light period (ML) or in the middle of the dark period (MD), we have performed a circadian metabolome analysis using hydrogen nuclear magnetic resonance (^{1}H NMR) spectroscopy. We have revealed that most circadian metabolites peak in either the FAA or FAA anti-phase periods and thus there is a strong correlation between metabolite phases and mealtime. Furthermore, by comparing the circadian phases of metabolites between ML and MD, we have identified both light and feeding time-regulated rhythmic metabolites. Light-related circadian metabolites include nucleotides and non-essential amino acids while feeding regulated circadian metabolites include essential amino acids. The oxidized form of nicotinamide adenine dinucleotide (NAD+), which operates as a cofactor and occupies a central position connecting most circadian metabolic pathways, is under the control of both LEO and FEO in adult liver. The rhythmic expression of *nampta*, *namptb* and *sirt1* which encode enzymes in NAD+ salvage pathways are also regulated by both LEO and FEO. Notably, the rhythmic transcripts which constitute elements of TTFLs are not influenced by the feeding time in the fish liver.

In order to explore the cellular metabolism under cell autonomous LEO control, we have performed metabolome measurements in cultured zebrafish primary hepatocytes which were exposed to light-dark (LD) and dark-light (DL) regimes. Unexpectedly, the cycling of nearly all the identified circadian metabolites in primary hepatocyte cultures is independent of the lighting conditions. In comparison, the mRNA expression of metabolism-related genes which oscillate strongly in adult livers are arhythmically expressed in hepatocytes. LEO-independent metabolic oscillations with a 24h period were also observed in zebrafish

fibroblast cells (PAC2 and AB9), indicating that a metabolic oscillator could exist widely in zebrafish cells. Our subsequently studies have demonstrated that the phase of the metabolic oscillator is insensitive to serum refresh treatment. Finally, we have revealed that circadian metabolites in cavefish fibroblasts show aberrant rhythmicity with an infradian period of 40h-45h, which matches the extremely long period (~47h) of core circadian clock gene expression.

In conclusion, the zebrafish endogenous timekeeping system can be synchronized by diverse external signals and is under a multi-oscillatory control. The FEO is genetically and functionally independent of the classical core circadian clock TTFL of the LEO in zebrafish liver. Cellular metabolic oscillations could be driven by a non-transcriptional mechanism in both zebrafish and cavefish cell lines. Thus, the study of non-photic oscillators and non-transcriptional dependent circadian periodicity should provide us with a more accurate view of the entire circadian timing system.

Keywords: circadian, zebrafish metabolome, light entrainable oscillator, food entrainable oscillator, cellular metabolism

Zusammenfassung

Zebrafische (*Danio rerio*) haben sich als nützliches genetisches Modell zur Untersuchung des zirkadianen Systems bewiesen. Der dem zirkadianen System zugrundeliegende anerkannte Mechanismus basiert auf selbstregulierenden Transkription-Translation Rückkopplungsschleifen (transcription-translation feedback loops, TTFLs). Wie bereits bekannt ist, besitzen Zebrafische im Gewebe, sowie in Zelllinien, einen TTFLs-abhängigen, durch Licht trainierbaren Oszillator (light entrainable oscillator, LEO). Des Weiteren besitzen sie, wie andere Vertebraten, auch einen durch Nahrung trainierbaren Oszillator (food entrainable oscillator, FEO). Kürzlich wurde in Studien untersucht, wie die Verfügbarkeit von Nahrung zirkadiane Rhythmen in Form von erhöhter Bewegungsaktivität trainiert, welche auf die Fütterungszeit folgt (so genannte food-anticipatory activity, FAA). Im Gegensatz zum ausgiebig untersuchten LEO, ist über die molekularen Komponenten und die Regulierung des FEOs nur wenig bekannt.

Meine Doktorarbeit beschäftigte sich mit dem Mechanismus, bei dem Nahrung die zirkadiane Uhr reguliert. Durch Aufzeichnung der Bewegungsprofile konnten wir zeigen, dass der FEO in ausgewachsenen Zebrafischen existiert. Dabei wurde die FAA durch eine regelmäßige, limitierte Futtermenge trainiert, unabhängig vom zeitlichen Ablauf des Licht-Dunkelheits (LD) Zyklus. Während der Aufzucht der Fische in einem LD Zyklus, wurde die Fische entweder in der Mitte der Lichtperiode (ML) oder in der Mitte der Dunkelheitsperiode (MD) gefüttert und eine zirkadiane Analyse des Metaboloms mittels Protonen NMR (^{1}H-NMR, nuclear magnetic resonance) Spektroskopie durchgeführt. Wir konnten zeigen, dass die Peakzeit der meisten zirkadianen Metabolite entweder in-phase oder anti-phase zur FFA auftrat und daher ein Zusammenhang zwischen der zirkadianen Phase der Metabolite und der Fütterungszeit vorhanden ist. Durch Vergleich der zirkadianen Phasen der Metabolite in den ML und MD Daten konnten Metabolite identifiziert werden, deren Rhythmik sowohl durch Licht, als auch durch Nahrung reguliert wird. Die Licht-regulierten zirkadiane Metabolite beinhalten Nukleotide und nicht-essentielle Aminosäuren, die Futter-regulierten zirkadianen Metaboliten beinhalten essentielle Aminosäuren. Die oxidierte Form von Nicotinamidadenindinukleotid (NAD+), welche als Kofaktor fungiert und eine zentrale Stelle in der Verknüpfung vieler zirkadianer metabolischer Pfade einnimmt, ist in Lebern von ausgewachsenen Zebrafischen unter der Kontrolle sowohl des LEO, als auch des FEO. Die rhythmische Expression von *nampta*, *namptb* und *sirt1*, welche Enzyme in NAD+ Salvage-Pathways kodieren, sind ebenfalls unter der Kontrolle sowohl des LEO, als auch des FEO. Bemerkenswert ist, dass rhythmische Transkripte welche Elemente der TTFLs darstellen, in Fischlebern nicht durch die Fütterungszeit beeinflusst werden.

Um den zellulären Metabolismus unter der Kontrolle des autonomen LEO zu untersuchen, wurden metabolische Studien an gezüchteten, primären Hepatozyten von Zebrafischen durchgeführt, welche einem LD bzw. DL Zyklus ausgesetzt waren. Überraschenderweise war die Rhythmik nahezu aller identifizierten zirkadianen Metabolite in primären Hepatozyten unabhängig von den hell-dunkel Zyklen. Im Gegensatz zur mRNA Expression metabolischer Gene, welche in den ausgewachsenen Zebrafischlebern strak oszilliert haben, waren diese in den Hepatozyten nicht rhythmisch exprimiert. LEO-unabhängige metabolische Oszillation innerhalb einer 24 Stunden Periode konnte ebenfalls in Fibroblast-Zellen (PAC2 und AB9) von Zebrafischen beobachtet werden. Dies weist darauf hin, dass ein metabolischer Oszillator in Zebrafischzellen weit verbreitet ist. Unsere nachfolgenden Studien konnten zeigen, dass die Phase des metabolischen Oszillators unempfindlich gegenüber einem Serumshock ist. Abschließend konnten wir zirkadiane Metabolite in Fibroblasten von Höhlenfischen identifizieren, welche eine abweichende Rhythmik mit einer infradianen Periode von 40-45 Stunden aufweisen. Dies stimmt mit der extrem langen Periode (ca. 47 Stunden) der zentralen clock Genexpression überein.

Abschließend lässt sich sagen, dass das endogene System der Zeitkontrolle in Zebrafischen durch unterschiedliche externe Signale synchronisiert werden kann und unter der Kontrolle verschiedener Oszillatoren steht. Der FEO ist genetisch und funktionell unabhängig von der klassischen zentralen zirkadianen Uhr TTFL des LEO in Zebrafischlebern. Metabolische Oszillatoren in Leberzelllinien, sowohl von Zebrafischen als auch von Höhlenfischen, können durch nicht-transkriptionelle Mechanismen angetrieben werden. Daher sollte die Studien von nicht-lichtinduzierten Oszillatoren und transkriptionell unabhängigen zirkadianen Periodizitäten für ein besseres Verständnis über das gesamte zirkadiane System sorgen.

Stichwörter: zirkadian, Zebrafisch Metabolom, Licht trainierbaren Oszillator, Nahrung trainierbaren Oszillator, zellulärer Metabolismus

Chapter 1

Introduction

1.1 Overview of the circadian timing system

Living organisms have evolved and developed an internal timing system as an adaptation to the daily light-dark cycles generated by the earth's 24h rotation. The biological clock enables organisms to sense environmental time cues and regulates various behavioral, physiological and biochemical processes, including locomotor activity, sleep-wake cycles, body temperature, food intake, hormone secretion and metabolism [1,2]. Disruption of the clock system in organisms may have a serious impact on many physiological functions and lead to a range of pathologies [3]. Although the internal clock of a wide range of organisms exhibits significant differences, it is a highly conserved system and has several common features [1]:

- The period length of the rhythm it generates is "circadian", around 24h. The term "*circadian*" comes from the Latin, meaning "approximately a day."
- Circadian clocks are endogenous and self-sustained mechanisms. Each cell of an organism contains an intrinsic oscillator and circadian rhythms can occur autonomously without any entrainment. Moreover, the circadian rhythm can persist for several cycles under artificial constant conditions (free running) with a period close to 24h.
- The internal clock needs to be synchronized by external environmental timing signals (defined as zeitgebers, such as light, food, temperature). The cell autonomous oscillators are coordinated systematically and temporally by this synchronization. Additionally, because the period of circadian oscillations is not precisely 24h, zeitgebers can prevent the endogenous clock from running out of synchrony with the environmental day-night cycle by adjusting the phase of the circadian system on a daily basis.
- The circadian system is compensated for temperature changes, which allows it to maintain a circadian rhythm with a periodicity of about 24h over a broad range of ambient temperatures.

Generally, the vertebrate circadian system consists of three major components [4]: the input pathways which can sense zeitgebers and convey the signals to the endogenous pacemaker; a core pacemaker (or oscillator) that generates and governs the circadian rhythms; a series of output pathways through which the circadian clock regulates a broad range of physiological functions (Figure 1).

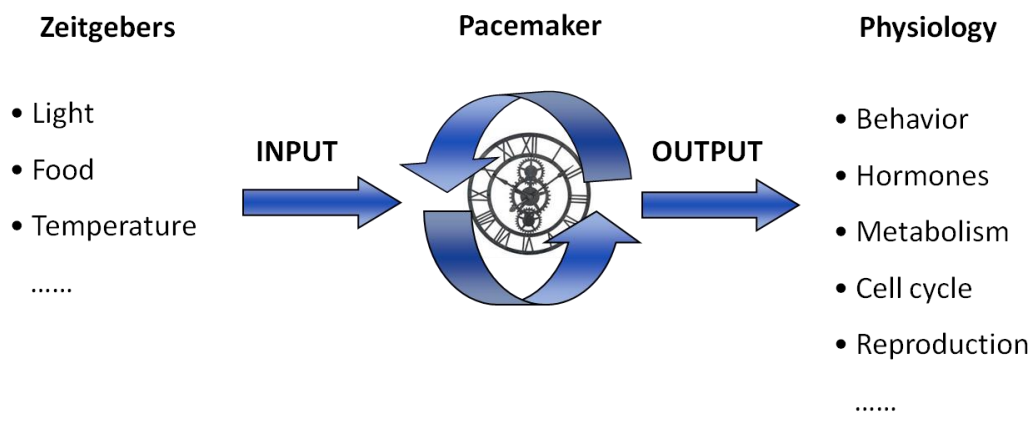

Figure 1: Schematic representation of the circadian timekeeping system

The circadian timing system is composed of three main parts. A pacemaker (oscillator) drives the oscillations with a period of around 24h. Zeitgebers can synchronize the pacemaker via input pathways and physiological outputs are controlled by the pacemaker by output pathways.

1.1.1 Pacemakers

In vertebrates, at the anatomical level the circadian system is comprised of a central pacemaker located in the brain and multiple independent peripheral clocks distributed in almost all subsidiary tissues, organs and cells. Each cell in both central clock and peripheral clocks contains a cell-autonomous and self-sustained circadian oscillator with a similar molecular makeup [1]. However, the status and contribution of the core pacemaker and peripheral clocks in the timing system have big differences. The central pacemaker receives signals from the environment and coordinates the oscillating activity of peripheral clocks via a complex combination of neural or systemic signals [5]. Thus, hierarchy of the circadian system ensures that all body clocks are synchronized with the 24h daily cycle.

1.1.1.1 Central pacemakers

In mammals, the anatomical structure in the brain that generates circadian rhythms is a small area constructed of a network of about 20,000 individual neurons and localized above the optical chiasma in the anterior hypothalamus, named the suprachiasmatic nucleus (SCN) (Figure 2) [5-7]. The isolated rodent SCN shows robust oscillation and this rhythmicity persists for up to several weeks under constant, free running conditions *in vitro* [8]. In addition, single SCN neurons in culture continue to express strong circadian firing activity over a 9 days period [9,10]. These studies have demonstrated that the self-sustained ability of the SCN oscillator is much more powerful than peripheral clocks (see section 1.1.1.2). Animal experiments have shown that lesions restricted to the SCN permanently disrupt behavioral rhythms [11,12], and impair the circadian function of peripheral clocks [13,14].

Moreover, transplantation of an intact fetal SCN into an SCN-ablated hamster restores circadian rhythmicity [6], whereas transplantation of non-SCN cell types does not restore behavioral rhythms to SCN-lesioned rodents [15]. These findings strongly support the notion that the SCN serves as the master circadian oscillator and plays a dominant role in the entire circadian system.

Although the SCN is widely recognized as the main central pacemaker in mammals, in non-mammalian vertebrates central pacemakers are also encountered in the pineal gland and retina (Figure 2) [1]. In mammals, the retina is a photoreceptive organ that transduces the environmental photic signal to the central clock via the retino-hypothalamic tract (RHT) which projects to the SCN [16,17]. The mammalian pineal gland, a tiny endocrine gland in the epithalamus has been shown to have lost direct photosensitivity [18]. Both the mammalian retina [19,20], and pineal gland [21,22] can synthesize and release melatonin which is a principal hormone for promoting sleep onset and regulating circadian rhythms [23] in a light-dependent and time-dependent rhythmic fashion. The role of the retina and pineal organ in mammals is closely linked with the SCN, which is sufficient to drive circadian rhythmicity. In lower vertebrates, such as birds [24] and fish [25], the central pacemakers are dispersed in multiple tissues including the pineal gland, retina and SCN [1,26,27]. The contributions of these three pacemakers to circadian organization vary between species [28]. Unlike mammals, the SCN has a less important, or even a minor role in generating circadian oscillations in non-mammalian species[29]. In lower vertebrates, melatonin production in the retina and pineal gland is controlled by the circadian timing system and suppressed by light *in vivo* and *in vitro* [30-32]. The pineal gland harbors both photoreceptors and circadian oscillators, and thereby retains endogenous oscillatory and pacemaker properties [33]. Paralleling SCN ablation in mammals, lesion of the bird pineal gland eliminates rhythmic behavioral activity, and transplantation of a pineal organ into a pinealectomized animal rapidly reestablishes rhythmicity [34].

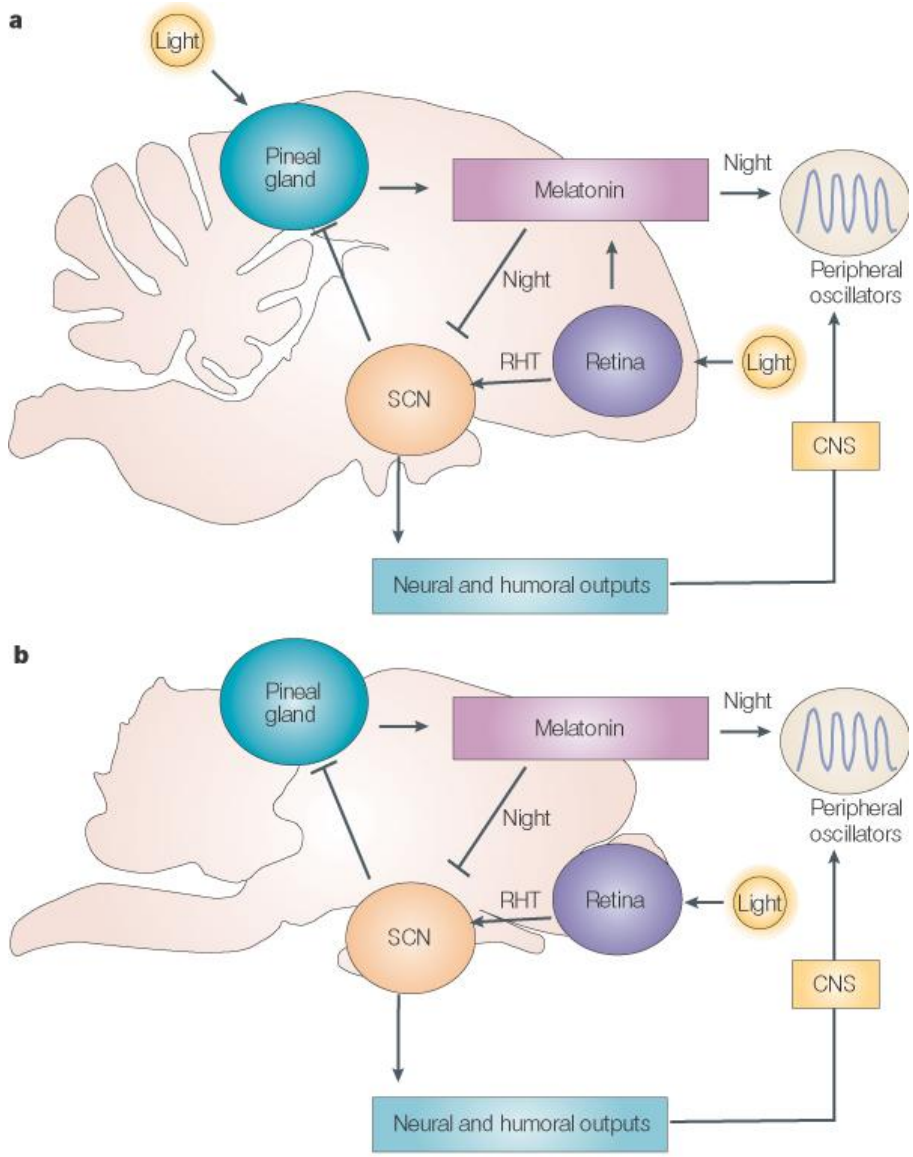

Figure 2: The organization of central pacemakers in non-mammalian vertebrates and mammals

(a). The neuroendocrine-loop model of avian pacemaker organization. SCN, retina and pineal gland are proposed to be central pacemakers of lower vertebrates. In the birds, both retina and pineal gland are photosensitive and capable of melatonin biosynthesis. SCN cannot secrete melatonin directly, but influences pineal secretion of melatonin.

(b). Schematic illustration of the mammalian central pacemaker. The SCN alone serves as a master pacemaker that receives light signals from the retina via the RHT. The pineal organ lacks direct photoreceptive function. The SCN coordinates the peripheral oscillators via neural or humoral outputs.

SCN: suprachiasmatic nucleus, RHT: retinal hypothalamic tract, CNS: central nervous system.

Schematic: Bell-Pedersen D, *Nat Rev Genet*. 2005 [1].

1.1.1.2 Peripheral circadian clocks

In addition to the central pacemaker, numerous circadian clocks exist in extra-SCN brain areas and peripheral organs or cells. In mammals, the basic molecular organization of peripheral oscillators is quite similar to the central pacemaker, but there are some notable differences in regard to the characteristics of oscillators between central and peripheral clocks. First of all, as mentioned above, peripheral oscillators are not as robust as the SCN pacemaker. Yamazaki et al. have revealed that both *in vitro* cultured SCN and liver exhibit bioluminescence rhythms driven by *Per1* promoter, however, the SCN explant rhythm continues to oscillate for several weeks while the liver explant rhythm dampens after several cycles [8]. Secondly, the SCN pacemaker and peripheral clocks are differentially responsive to entrainment by different zeitgebers. In mammals, light represents the predominant external cue for synchronizing the master pacemaker, but it fails to regulate the endogenous clocks in the subsidiary organs directly. In contrast, non-photic signals (food, temperature etc.) can lead to the phase resetting of circadian gene expression in some peripheral tissues independently of SCN rhythms [35,36]. Besides, clocks in *in vitro* cultured peripheral organs or cells can be synchronized by various non-photic external stimuli, such as serum [37], forskolin (active cAMP signal transduction pathway), glucocorticoids [38], dexamethasone (a glucocorticoid receptor agonist) [39], TPA (an activator of protein kinase C) [40], glucose [41], oxygen [42], rhythmic blood-borne signals [43], low amplitude temperature cycles [36] etc. In contrast, the SCN is largely insensitive to these signals. Until now, the mechanism that distinguishes the self-sustaining oscillatory function between central pacemaker and subsidiary clocks has been poorly understood.

In non-mammalian animals, such as zebrafish and *Drosophila*, cellular oscillators in peripheral organs or tissues are sensitive to light exposure and circadian genes can be entrained by the light-dark cycle directly, suggesting that they are less subject to central pacemaker control. This property is described in detail in section 1.4.

1.1.1.3 The interactions between central pacemaker and peripheral clocks

The hierarchical model of the mammalian circadian system has been widely accepted to explain the tight links between the central pacemaker and peripheral clocks. The SCN pacemaker, directly synchronized by external zeitgebers, is able to generate and sustain its own circadian oscillation independently, and in turn acts as a master synchronizer to orchestrate numerous clocks present in peripheral neural and non-neural organs and cells through a variety of nervous and systemic cues. Thereby, the master pacemaker coordinates the overall temporal architecture of the organism [5,44-47]. In mammals, the SCN pacemaker builds phase coherence between subsidiary organs via direct and indirect routes,

mainly relying on autonomic innervations, hormone secretion, temperature cycles and feeding-related cues [5].

(1) Autonomic nervous system

The thousands of neurons in the SCN have extensive connections with diverse types of sympathetic and parasympathetic motor systems by neural projections to the autonomic nervous system [48], which enable the SCN to govern the daily rhythm of peripheral organs, such as the liver directly [49].

(2) Hormone secretion

The SCN synchronizes peripheral circadian oscillators by controlling cyclic hormone production and secretion. A number of hormones have been reported to participate in the phase entrainment of peripheral clocks. Amongst these, glucocorticoids have received the most attention and the hypothalamic-pituitary-adrenal (HPA) axis coupled with the release of multiple hormones has been extensively studied [50-52]. Glucocorticoid secretion as a circadian input can activate its corresponding specific receptors in peripheral organs and in turn, stimulates signaling cascades and ultimately the local circadian machinery is synchronized [53].

(3) Temperature cycles

Daily body temperature oscillation is influenced by the SCN directly and indirectly. While the SCN pacemaker is not sensitive to ambient temperature changes, the phase of the hepatic circadian oscillator can be altered by a smooth thermo cycle *in vivo* [36]. Heat shock factor 1 (HSF1) and cold-inducible RNA-binding proteins (e.g., CIRP) have been reported to contribute to the synchronization. For details see section 1.2.3.

(4) Feeding-related cues

Feeding-fasting cycle is a dominant phase resetting cue for most peripheral organs. Similar to temperature, restricted regular feeding can influence the phase of peripheral oscillators independently of the central clock [35]. Feeding related metabolites and hormones, such as glucose, insulin, are recognized as synchronizers for peripheral clocks. (See section 1.2.2).

Notably, the synchronization of peripheral oscillators requires a combination of multiple systemic cues and regulatory signals, thus, feeding entrainment has tight links with hormone secretion and body temperature (Figure 3). The signaling pathways mediating the phase coherence in local organs can be activated in the absence of input from the SCN. Importantly, most systemic signals can as external zeitgebers for generating circadian oscillations in

cultured peripheral cells (see 1.1.1.2).

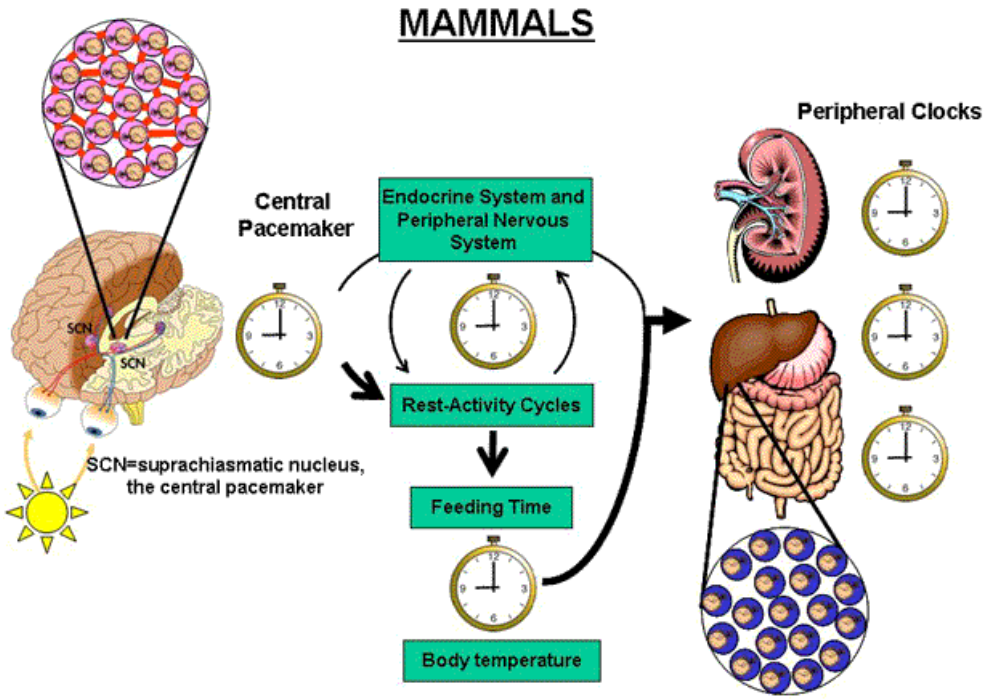

Figure 3: Hierarchical organization of mammalian circadian timing system

SCN function is positioned hierarchically above all other cell-autonomous body clocks. It is entrained by external light signals and then synchronizes the peripheral oscillators through a range of direct and indirect cues, such as autonomic innervation, hormonal signals, feeding-related cues, body temperature etc. Peripheral clocks can response not only to the systemic cues from the SCN, but also non-photic zeitgebers (e.g.feeding time) directly.

Schematic is from: website of Institute of Pharmacology and Toxicology, University of Zurich. (http://www.pharma.uzh.ch/en/research/chronobiology/areas/chronobiology/introduction0.html)

Although the hierarchical organization of the circadian system has been supported by studies in various animal models, the dominant status of the SCN in relation to peripheral clocks is still disputable. From the above description, we know the phase resetting of peripheral oscillators by restricted feeding availability and temperature cycles is uncoupled with the SCN pacemaker in rodents. The contribution of the SCN to the entrainment of local oscillators is still unclear. Moreover, the rhythmic expression of genes in peripheral organs is driven not only by the master pacemaker through systemic signals, but also by local oscillators. Kornmann et al. have reported that 90% of the circadian transcription profiles in the liver are controlled by the hepatic oscillator, with only the remaining 10% governed by

systemic signals, illustrating that in some peripheral tissues the contribution of the SCN as a circadian system organizer is limited [54]. Yoo et al. have showed that organ cultures derived from SCN-ablated animals still exhibit rhythmic clock gene expression, suggesting the self-sustained peripheral oscillators can maintain the rhythmicity independent of the master oscillator *in vitro* [55]. In addition, the routes that transmit the feedback of peripheral clocks to the SCN hierarchical coordination is not clear. All these questions confirm that the interactions between central pacemaker and peripheral clocks are complex.

1.1.1.4 The molecular mechanisms of circadian clocks

The circadian timekeeping requires the activities of a significant number of circadian clock genes. The first identified circadian clock gene was *per* (period) in *Drosophila* [56]. Afterwards, considerable genetic evidence based originally on forward genetic screens led to the identification of many clock mutations that are non-lethal but cause abnormal circadian clock phenotypes. Using this approach, some important circadian genes involved in clock mutants were isolated. The classical molecular mechanism of the circadian clock is based upon auto-regulatory transcription-translation feedback loops (TTFLs), which are composed of several core circadian elements. However, recently an increasing amount of evidence has revealed the existence of novel mechanisms which are independent of canonical TTFLs.

1.1.1.4.1 Transcription-translation feedback loop (TTFL) mechanisms

The clock machinery consists of two coupled transcription-translation feedback loops, a core loop and a stabilizing loop [57-60]. In the mammalian core auto-regulatory TTFL, CLOCK and BMAL (brain muscle ARNT-like) which are transcription factors containing bHLH-PAS (basic helix-loop-helix, Per-Arnt-Sim) constitute a positive feedback loop (Figure 4A) [61]. BMAL and CLOCK forms a heterodimer, then binds to a cis-regulatory element the E-box and activates transcription of the *Per* (period) and *Cry* (cryptochrome) genes. A negative feedback loop is composed of the PER and CRY family of proteins. After dimerization in the cytosol, PER:CRY heterocomplexes translocate back to the nucleus and inhibit the transcriptional activity of the CLOCK:BMAL complex and their own transcription.

The secondary feedback loop is a stabilizing loop, that as the name implies, contributes to stabilize and reinforce the core loop. Specifically, REV-ERBα (nuclear receptor subfamily 1, group D, member 1) [62] and RORα (RAR-related orphan receptor alpha) which belong to the retinoic acid-related orphan receptors family make up the stabilizing loop (Figure 4A) [63]. The transcriptional expression of these two elements is controlled by the CLOCK: BMAL1 heterodimer. In the nucleus, REV-ERBα and RORα as transcription factors compete with each other for the RORE (retinoic acid-related orphan receptor response element)

binding site in the *Bmal1* promoter. RORα activates the transcription of *Bmal1*, whereas REV-ERBα represses it [64]. Thus, the rhythmic expression of *Bmal1* is gated by both positive and negative regulation, respectively.

Furthermore, additional levels of regulatory circuits involving post-translational modification, chromatin modification and aspects of metabolism have been shown to confer precise regulation on this clock mechanism [65,66]. In particular, post-translational modifications of clock proteins mainly including phosphorylation [67-70], SUMOylation [71], and ubiquitination [70], aid to maintain the stability of the core feedback loops [65]. Furthermore, chromatin modification is closely linked with epigenetic mechanisms, which induce dynamic changes in chromatin states via rhythmic histone modifications, DNA methylation and so on [72,73]. Chromatin remodeling regulates transcriptional activation or silencing of clock genes, and thereby coordinates the circadian system [74]. CLOCK itself functions as a chromatin remodeling enzyme, which is able to acetylate histone and nonhistone proteins (such as BMAL) [70,75].

The TTFL mechanism is conserved in a broad range of species, though the core elements of TTFL differ significantly between phylogenetic kingdoms (Figure 4B). In cyanobacteria, KaiA, KaiB and KaiC comprise the feedback circuit elements [76,77]. In *Neurospora*, the central clock mechanism is composed of the positive regulator Wcc (white collar complex), and the negative regulator Frq (frequency) [78]. The *Drosophila* feedback loops are similar to the mammalian TTFLs. CLK (Clock) and CYC (Cycle) bind and activate the transcription of *Per* (Period) and *Tim* (Timeless), and then PER and TIM form a heterodimer and suppress the transcriptional activity of the CLK:CYC complex [79]. As shown in Figure 4B, the organization of the TTFL in different species is quite similar. The PAS domain which serves as a signal sensor is frequently encountered in the proteins constituting the core transcriptional loop [80].

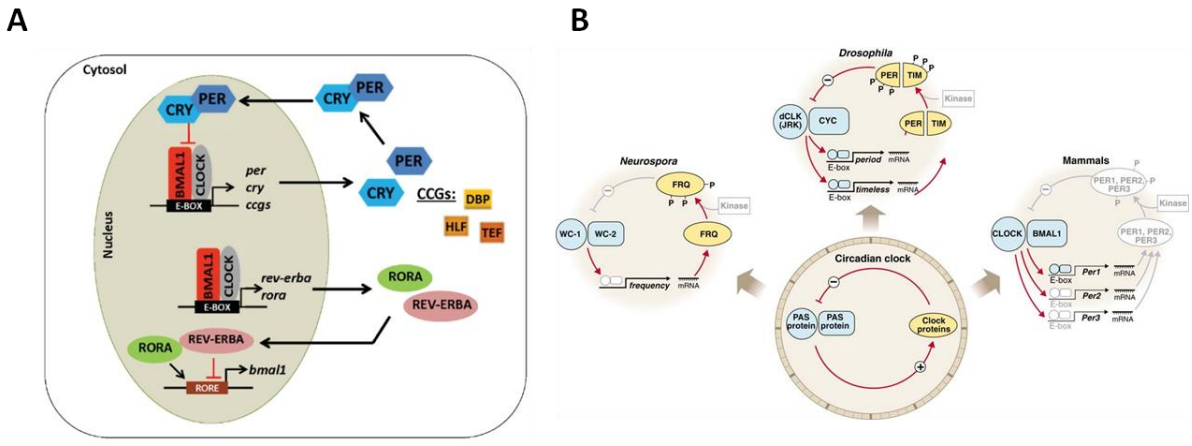

Figure 4: The canonical circadian clock mechanism

(A). The components of transcription-translation feedback loops (TTFLs) in mammals. At a molecular level, the circadian clock is constituted by core loops (BMAL, CLOCK, PER, CRY) and a stabilizing loop (BMAL, CLOCK, REV-ERBα, RORα) which together mediate ~24 h autonomous rhythms. CCGs: clock controlled genes

(B). Conservation of TTFLs in *Neurospora*, *Drosophila* and mammals. Although the components of TTFLs are species-specific, they share the common PAS domain in the clock proteins.

Panel A is from: Udoh US et al. *Biomolecules*. 2015 [81].

Panel B is from: Dunlap J, *Science*. 1998 [80].

1.1.1.4.2 Non-transcriptional mechanisms

Although the function of the canonical TTFL mechanism is sufficient to account for most of the fundamental features of the circadian clock, it is not the only molecular component of circadian timing system. Evidence for timekeeping independently of TTFLs has been reported since the 1960s [82]. Photosynthetic rates showed circadian oscillation in enucleated *Acetabularia*. Subsequently, a series of studies has underlined the existence and important contribution of non-transcriptional mechanisms to cellular timekeeping [83-86]. A most striking example of non-transcriptional feedback in circadian rhythm generation was reported to exist in cyanobacteria (*Synechococcus elongates*) [87]. As mentioned above, cyanobacteria possess an auto-regulatory TTFL (also termed a transcription-translation-derived oscillator (TTO)) which is composed of a protein cluster KaiABC and regulates genome-wide gene expression. In addition, they also have a posttranslational loop comprised of a KaiC autophosphorylation-dephosphorylation cycle with a 24h rhythmic period. In the TTFL, KaiA, KaiB and KaiC display approximately 24 h oscillation at the transcriptional level. KaiA induces KaiC to autophosphorylate, whereas KaiB represses KaiA and then drives KaiC to autodephosphorylate (Figure 5) [88,89]. When cyanobacteria are raised in

constant darkness, the TTFL is almost completely inactive as the rhythmic accumulation of KaiABC genes is absent, but KaiC phosphorylation still shows robust oscillation which is also temperature compensated. Furthermore, the KaiC phosphorylation rhythm persists in the presence of a transcription or translation inhibitor which blocks the TTFL. A molecular clock reconstituted by three Kai proteins and ATP as an energy source exhibits auto-phosphorylation and autodephosphorylation and is temperature compensated *in vitro*. Thus, the KaiC phosphorylation rhythm independently of TTFL harbors the characteristics unique to a circadian clock, such as light entrainability, approximate 24h period length, persistent cycling in free running conditions and temperature compensation. Taken together, all these findings support the notion that *de novo* transcription and translation processes are not necessities for cellular rhythmicity in cyanobacteria.

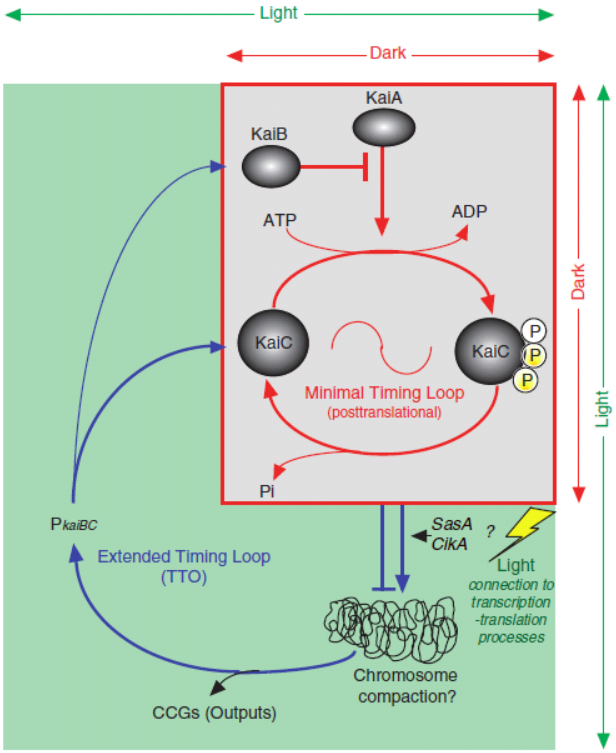

Figure 5: A model of a post transcriptional oscillator coupled with the TTFL in cyanobacteria

The cyanobacteria circadian mechanism consists of a transcription-translation-derived oscillator loop (TTO, like a TTFL) and a minimal timing loop (posttranslational loop, KaiC phosphorylation cycle). The TTO is composed of the KaiABC proteins which are active during the light (green area) and inactive during the dark (grey area). The KaiC phosphorylation cycle can be maintained in the darkness without transcription or translation (grey area). In the dark or under nutrition limited conditions, the posttranslational oscillator may work as a ‘‘time memory’’ process to ensure robust circadian organization in cyanobacteria. Pi: phosphate, CCGs: clock-controlled genes

Schematic: Nakajima M et al. *Science*. 2005 [87].

A non-transcriptional circadian timing mechanism also exists in mammals. A remarkable finding is the presence of circadian rhythmicity in mammalian erythrocytes which lack a nucleus and most other organelles [90,91]. The structure of these cells would be predicted to be incompatible with a mechanism composed of TTFL. Instead, cyclic oxidation of the molecule peroxiredoxins (PRXs) driven by temperature cycles has been implicated as the core of a distinct non-transcriptional clock mechanism (Figure 6) [92]. PRXs are a large and highly conserved family of antioxidant enzymes involved in hydrogen peroxide metabolism in all organisms. The catalytic mechanism of PRXs includes two interlocked oxidation and reduction cycles, where the reduced PRXs catalyze the conversion of H_2O_2 (hydrogen peroxide) to H_2O, and then the oxidized PRXs are reduced again by consuming a reducing equivalent from nicotinamide adenine dinucleotide phosphate (NADPH) [93-95]. In the redox reactions, PRXs are present in different forms according to the different redox status of their active cysteine residue: reduced form (PRX-Cys-SH), oxidized form (PRX-Cys-SOH), over-oxidised (PRX-Cys-SO_2) and hyper-oxidised (PRX-Cys-SO_3) forms [96]. By performing an immunoblotting assay, it was demonstrated that the overoxidised/ hyperoxidised peroxiredoxin (PRX-$SO_{2/3}$) dimer in the light-dark entrained tissues is expressed in a temporal manner with a period of around 24h, and the rhythmicity persists under constant darkness. Further studies showed that the highly and broadly conserved PRX-$SO_{2/3}$ exhibits daily cycling in plants, as well as a range of eukaryotes and prokaryotic models [97]. Additionally, the cyclic expression of PRX-$SO_{2/3}$ has been observed in TTFL mutants of *Drosophila*, *Neurospora crassa*, *Arabidopsis thaliana* and *Ostreococcus tauri*, indicating that the classic molecular basis of the TTFL is dispensable for rhythms in redox metabolism, and non-transcriptional oscillators could be common to all circadian systems. The rhythmicity of PRX-$SO_{2/3}$ governed by the phase of an ambient thermo cycle in human red blood cells reinforces the notion that non-transcriptional mechanisms are sufficient to sustain circadian timekeeping.

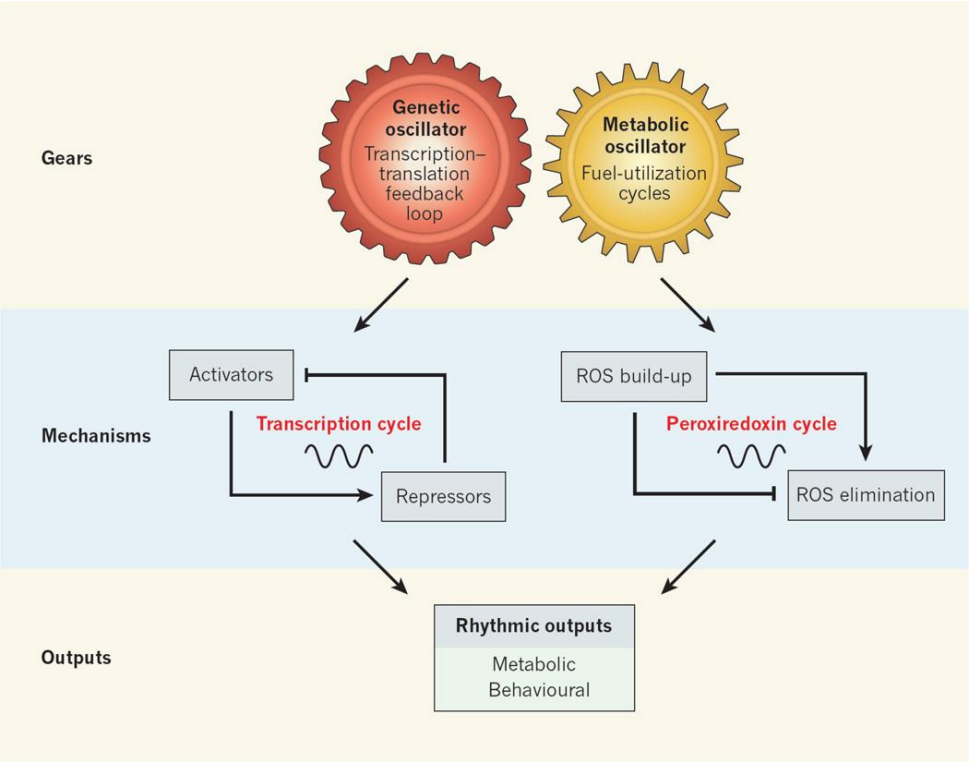

Figure 6: Coupling of genetic and metabolic clocks

The genetic oscillator is based on a TTFL and the metabolic oscillator includes the cycle of peroxiredoxin (PRX) enzymes. PRXs play key roles in maintaining cellular H_2O_2 levels by interacting with ROS (reactive oxygen species) and redox reactions. The two oscillator types are coupled, both driving rhythmic outputs in synchrony with the Earth's rotation.

Schematic: Bass J and Takahashi JS. *Nature.* 2011 [92].

Both the KaiC autophosphorylation-dephosphorylation cycle and the PRX-$SO_{2/3}$ oxidation-reduction process can be explained by protein post-translational modifications, which are an essential mechanism within the circadian clockwork. Notably, non-TTFL regulation can be coupled with TTFL function to different extents. Thus, when cyanobacteria are raised at low temperatures, both the KaiABC TTO cycle and KaiC phosphorylation cycle are required for the circadian timing system [98]. Additionally, the mutation of TTFL elements (PER, CRY) leads to an alteration in period length of the PRX-$SO_{2/3}$ rhythm in blood cells [99]. Taken together, the exploration of non-transcription dependent circadian periodicity will build a more accurate view of the entire circadian timing system. Nonetheless, since the non-TTFL clock mechanisms have only been studied for a relatively short time, ample detailed information about the regulation and the exact relationship between TTFL and non-TTFL oscillators is still not available.

1.1.2 Circadian inputs and zeitgebers

Any external or environmental cues that entrain or synchronize biological rhythms of organisms are termed zeitgebers (German, mean "time giver"). As described in section 1.1.1.3, since the input pathways whereby ambient signals regulate the oscillators are different, the central pacemaker and peripheral clocks are responsive to different zeitgebers. In mammals, the SCN neurons are extremely sensitive to the light-dark cycle (indirectly via input from the retina and the retinohypothalamic tract), but they are relatively insensitive to non-photic stimuli [9]. By contrast, the clocks in peripheral organs or cells cannot be driven by light exposure directly. A multitude of external factors including serum, temperature, hormones (glucocorticoid), signaling molecules (forskolin, oxygen), metabolites (glucose) etc. are able to participate in the phase synchronization of peripheral clocks *in vivo* and/or *in vitro*. Among these synchronizers, food is recognized as the major signal to reset the rhythmicity of peripheral oscillators *in vivo*. In non-mammalian vertebrates such as zebrafish, the light input pathways have been well studied in both the master pacemaker (pineal gland) and peripheral organs, but the circadian inputs of non-photic zeitgebers have been poorly investigated so far. Besides the general external factors, social interactions have also been shown to act as a zeitgeber which is sufficient for circadian synchronization in social species [100-102].

1.1.3 Circadian outputs

Circadian rhythms govern the daily patterns of behavior and most physiological processes, ranging from sleep-wake cycles, body temperature, metabolism, heart rate, blood pressure, hormones secretion, immunity, as well as some basic cellular and molecular processes such as DNA repair, cell proliferation, cell cycles and redox cycles [103-112]. Disruption of circadian rhythms may cause severe pathophysiological effects, including physiologic dysfunctions or disorders, many diseases or even cancers [113-119]. Circadian output pathways are frequently used as molecular targets for drug discovery [120-123]. Metabolism is one of most important outputs of the circadian timing system. As the interaction between circadian clock and metabolic processes is a major topic of my project, a detailed description of the circadian regulation of metabolism is present in section 1.3.

1.2 Multiple clock oscillators in vertebrates

Organisms are subjected to a battery of daily changing environmental stimuli which are able to synchronize the multitude of self-sustained and autonomous cellular oscillators. However, the entraining strength of individual external zeitgebers for the circadian system varies considerably [124,125]. In addition to the oscillators being entrained by exogenous cues, many oscillators are regulated by endogenous signals. In mammals, the master pacemaker

coordinates the phase coherence and interplay of multiple peripheral oscillators. For most life forms, light, food, temperature are widely accepted to be the principal environmental zeitgebers for synchronizing the internal cellular oscillators. Here, I will focus on introducing the properties of the light entrainable oscillators (LEOs), food entrainable oscillators (FEOs), temperature entrainable oscillators (TEOs), as well as metabolic oscillators which direct complex metabolic processes.

1.2.1 Light entrainable oscillators (LEOs)

For most organisms, light represents the dominant external cue for initiating and entraining the entire circadian rhythm. Thus light entrainable oscillators (LEOs) have long been the object of intensive and extensive studies. In mammals, the most important LEO is situated in the SCN central pacemaker.

The photic entrainment of the mammalian SCN occurs through the retina, which has a separate anatomical and functional system mediating photic transmission to the clock. Surprisingly, the classical rod and cone photoreceptors located in the outer retinal layer are not required for photic entrainment [17,126]. A subset of retinal ganglion cells that are intrinsically photoreceptive (ipRGCs) express the photopigment melanopsin (Opn4), which serves as a photoreceptor in circadian photic entrainment. Melanopsin knockout mice ($Opn4^{-/-}$) exhibit severely attenuated phase shifting, a lower magnitude of photic responses, incomplete pupillary light reflex, and loss of direct photosensitivity in ipRGCs [127-129]. In addition, mammalian fibroblasts which are transfected with exogenous melanopsin, display photo responsiveness and cellular rhythmicity, indicating the critical role of melanopsin in circadian photoentrainment [130]. Although rods and cones are not required for photo-entrainment, evidence points to close interactions between these classical visual photoreceptors and the non-visual photoreceptor melanopsin in visual input to the SCN. $Opn4^{-/-}$ mice still show weak circadian entrainment, and rodents lacking rods and cones photoreceptors still maintain partial responses to light. These data reveal that the ipRGCs, rods and cones can all to some degree compensate for loss of circadian photoreception function [127,129]. Moreover, there is strong evidence showing the existence of light input from rods and cones to the SCN [131,132].

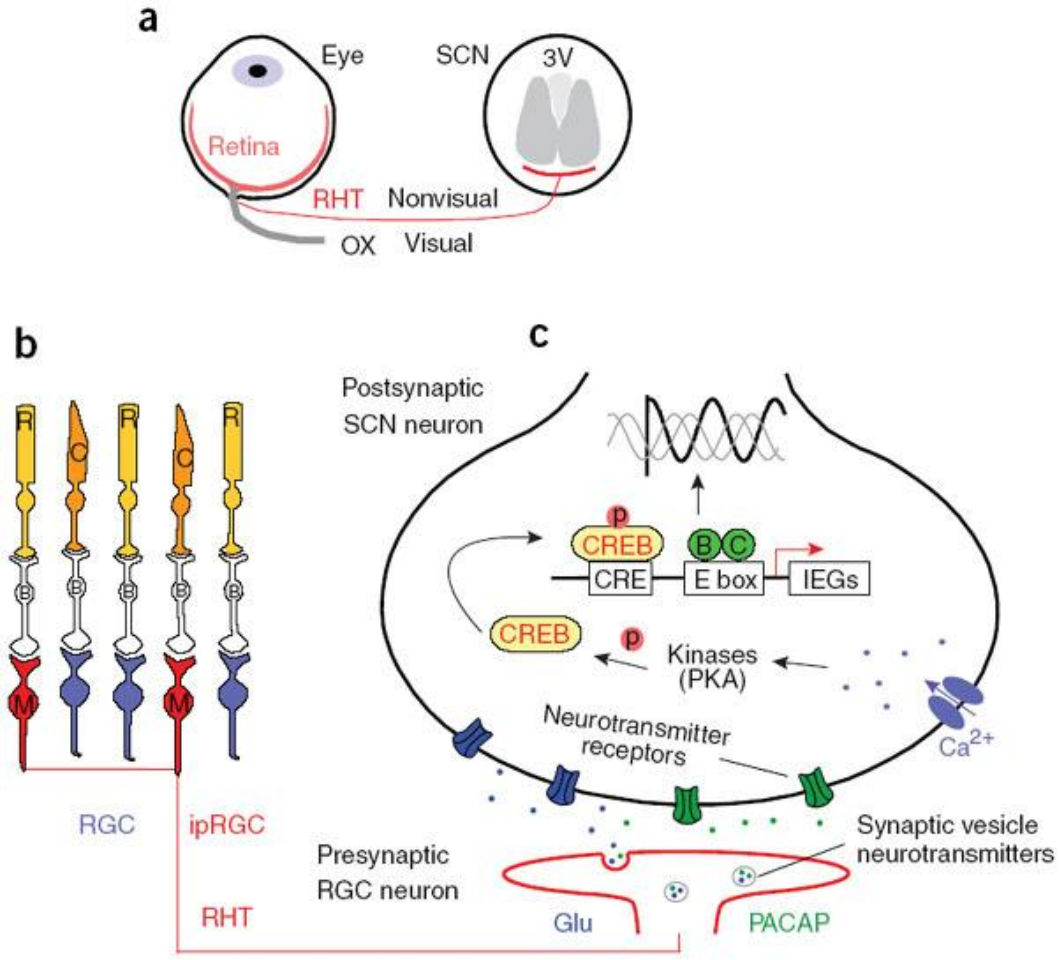

Figure 7: The photic entrainment of mammalian circadian clocks

(a) Photic inputs from the retina to the ventral SCN. The SCN is located in the hypothalamus above the optic chiasm (OX) on either side of the third ventricle (3V). A subset of retinal ganglion cells (RGCs; red) innervate the retinohypothalamic tract (RHT) in the OX, which projects to the neurons in the ventral SCN.
(b) Retinal photoreception. RGCs expressing melanopsin (M) are intrinsically photosensitive, and the ipRGCs also receive light signals from the rods (R) and cones (C) of the retina through bipolar (B) and amacrine cells (not shown). Their axons form the RHT and project to the SCN.
(c) Photic transmission to the SCN and intracellular signal transduction. Glutamate (Glu) and pituitary adenylate cyclase-activating polypeptide (PACAP) are the major neurotransmitters released from the presynaptic RGCs. Glutamate and PACAP bind to the corresponding receptors, and lead to membrane depolarization and an influx of Ca^{2+} in targeted SCN neurons. Within postsynaptic SCN neurons, changes in intracellular Ca^{2+} and cAMP levels stimulate multiple kinases and finally lead to phosphorylation of CREB; then phospho-CREB (CREB-p) activates immediate early genes (IEGs) such as *Per1* via binding to CRE enhancer elements, resulting in establishment of a new circadian phase (depicted in black).
Schematic: Liu AC et al. *Nat Chem Biol*. 2007 [133].

In the retina, lighting cycles stimulate ipRGCs which contain melanopsin and then this light signal is conveyed to the SCN via the retinohypothalamic tract (RHT) [134]. The photic signal can be transmitted via glutamate (Glu) and pituitary adenylate cyclase-activating polypeptide (PACAP), the principal neurotransmitters released from the presynaptic retinal ganglion cells (RGCs) [135,136]. Glu and PACAP are transported into synaptic vesicles and then bind to the respective receptors on the surface of SCN neurons. The synaptic transmission causes the activation of signaling cascades, including a Ca^{2+} influx, increased cAMP levels, activation of multiple kinases, phosphorylation of CREB (cAMP response element binding protein) which finally regulates circadian oscillation in SCN neurons [137]. In turn, the synchronized SCN directly or indirectly adjusts the oscillators in extra-SCN brain regions and peripheral organs (Figure 7).

1.2.2 Food entrainable oscillators (FEOs) and Food anticipatory activity (FAA)

Circadian oscillators exist not only in the central SCN pacemaker, but also in nearly all peripheral tissues. It has been known for decades that regular food availability can entrain the circadian clock effectively in many species, e.g. bees [138], fish [139], birds [140] and mammals [141,142]. Usually lab animals are fed *ad libitum*, however, when feeding is restricted to a certain time every day, animals display circadian rhythms of increased locomotor activity preceding the feeding time, so-called food-anticipatory activity, FAA. FAA may be an important behavioral output of the feeding entrainable oscillator (FEO), an extra-SCN oscillator widely encountered in peripheral tissues (e.g. liver, kidney, heart, pancreas, lung and intestine). In mammals, the FEO is distinct from the central pacemaker anatomically and functionally, as SCN lesioned rodents still exhibit robust FAA [12]. Compared to the LEO located in the SCN, the dorsomedial hypothalamic nucleus (DMH) is hypothesized to receive input and gate the FEO synchronization. However, whether this represents the actual location of the FEO pacemaker is still disputed [143]. Further studies have revealed that daily restricted feeding can reset the phase of some core circadian genes in peripheral tissues, but has little impact on rhythmic clock gene expression in the SCN, which reinforces the notion that the FEO is a SCN-independent local clock [35,144]. Until now, the key circadian genes including *Per1*, *Per2*, *Per3*, *Cry1*, *Dbp*, *Rev-erbα*, *Cyp2a5*, *Bmal1*, *E4BP4*, *Dec1* and *Rorγ* have been reported to be regulated by restricted feeding in the rodent liver. The FEO controlled genes exhibit a reversal of the phase of their cyclic expression when nocturnal rodents are restricted fed during the light period [144-148]. Furthermore, by performing high throughput assays, the rhythmic transcriptome controlled by the FEO has been detected in rodent peripheral tissues. The results reveal that only a small subset of cyclic transcripts is not influenced by the fasting-feeding cycles [149,150]. Although the central pacemaker is insensitive to scheduled feeding entrainment, it still

influences or orchestrates the FEOs in peripheral tissues by systemic signals (rest-activity, or temperature cycles) under natural conditions [151]. The hypothetical relationship between the SCN and feeding behavior is shown in Figure 8.

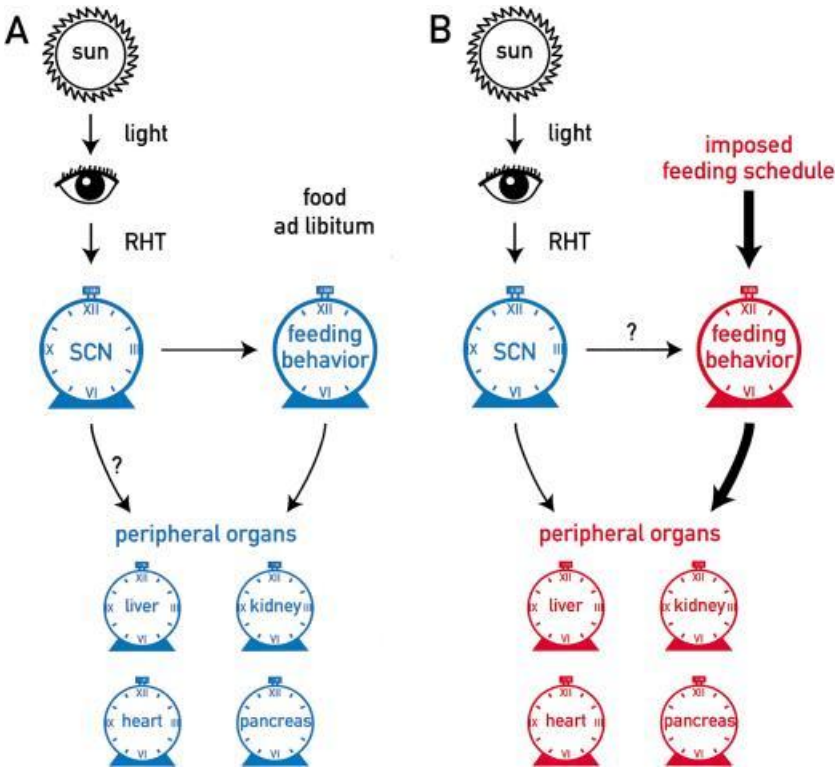

Figure 8: Hypothetical model for the effect of feeding entrainment on peripheral organ clocks

(A) When food availability is under *ad libitum* conditions, the SCN governs the feeding behavior and then synchronizes peripheral clocks via cycles in the secretion of blood-borne factors (e.g., hormones) or temperature rhythms.

(B) When food access is restricted to a certain time (always the period of inactivity), signals triggered by feeding and/or fasting act as dominant zeitgebers on the oscillators of peripheral tissues.

Schematic: Damiola F et al. *Genes Dev*. 2000 [144].

The issue on how periodic feeding entrains peripheral oscillators is still poorly understood. The food entrainable synchronization of peripheral clocks could relate to metabolism or energy homeostasis. Potentially, redox cycles, feeding-dependent hormone signaling or metabolites may contribute to FEO synchronization. NAD+/NADH (oxidized and reduced forms of Nicotinamide Adenine Dinucleotide) are cofactors involved in most redox reactions by transporting electrons and protons. The NAD+ dependent PARP-1 (poly (ADP-ribose) polymerase 1) poly(ADP-ribosyl)ation activity is cycling and regulated by feeding

entrainment in the mouse liver [148]. PARP-1 deficient mice show abnormal peripheral clock and locomotor activity in response to the scheduled feeding cycle. The NAD+-dependent mitochondrial oxidative function and metabolic regulation are in synchrony with the feeding-fasting cycle [152]. In addition to the redox reactions, food intake can activate or inhibit hormone secretion, which probably influences the peripheral FEO entrainment. Glucocorticoids represent the most important hormone for peripheral clock synchronization. Daytime feeding in nocturnal mice not only causes a slow phase resetting of circadian gene expression, but also alters daily glucocorticoid secretion. Glucocorticoid receptor null mice show a rapid adaptation to day time feeding regimens, suggesting signaling through the glucocorticoid receptor blocks the food synchronization in local clocks [153]. Besides glucocorticoids, OXM (oxyntomodulin) has been reported to reset the hepatic transcription rhythms via induction of the core clock genes *Per1* and *Per2* [154]. Insulin levels are rhythmic and adjusted to the inverted restricted feeding cycles. This leads to a phase shift of clock gene rhythms by modulating hepatic *Bmal1* translocation and activity [155]. The synthesis of pineal melatonin exhibits a temporal pattern in SCN lesioned rats, which is responsive to the restricted feeding cycle [156]. Furthermore, metabolites could mediate the phase resetting of circadian genes by the inverted regular feeding. The scheduled food intake may stimulate metabolic reprogramming, which then activates the phase adaptation via multiple signaling pathways. For example, under restricted food access, the accumulated free fatty acids and glucagon activate PPARα (peroxisome proliferator-activated receptor alpha) and CREB (cAMP response element-binding protein) signaling pathways respectively, leading to anti-phase rhythmic expression of *Reverbα*, *Per1* and *Per2* in the liver [145]. In addition, time-restricted feeding regimen stimulate GSK3β (glycogen synthase kinase 3β), DBS (RORE DNA binding sequence), AMPK (AMP-activated protein kinase), mTOR (mammalian target of rapamycin) pathways to mediate FEO related outputs [157]. Notably, the synchronization of local clocks by feeding is nutrient-dependent [158,159]. Finally, the neuropeptide orexin is sensitive to scheduled feeding, suggesting the nervous system might also be involved in the local FEO entrainment [160]. Overall, the molecular mechanisms of FEO input and output are complex since FEO regulation is clearly frequently intertwined with many physiological processes.

FAA, first reported by Curt Paul Richter in 1922 [161] is another important issue relevant for studies of the FEO. Although FAA has been demonstrated to exist in a broad range of species, the underlying mechanism of FAA remains unclear. Lesioning experiments show the brain structures of the SCN [12], hypothalamus (ventromedial, paraventricular and lateral parts) [162], hippocampus (related to memory), amygdale and nucleus accumbens [163], area postrema [164] and olfactory bulb [165] are not required for FAA emergence. Core

genetic elements of the circadian timing system also seem not to be essential for FAA entrainment, as normal FAA is observed in clock mutant mice which lack circadian function in all tissues [166,167]. Nevertheless, FAA closely interacts with circadian control. PER2 mutant mice fail to display anticipation of restricted feeding [168], and ghrelin secretion by the stomach which influences the FAA output is under the control of the circadian system [169]. Consistently, rats entrained by restricted feeding still show robust FAA even when the food is deprived, indicating that FAA persists in free running conditions [170]. The mechanism of FAA generation and regulation is complex, and may be explained by a model that combines food synchronization, circadian clock system and even memory. However, this model remains inadequate to explain many properties of FAA [171].

1.2.3 Temperature entrainable oscillators (TEOs)

One of the key features of circadian rhythms is their temperature compensation, which allows organisms to sustain their circadian periodicity relatively constant over a range of environmental temperatures. However, most physiological processes are susceptible to temperature changes and this includes circadian clocks [172,173]. Indeed the temperature compensated oscillators are entrainable to external or internal (body temperature) low amplitude temperature cycles [174-176]. Temperature compensation and entrainment are conserved characteristics of circadian clocks and occur in a wide range of species, regardless of their homeothermy or heterothermy [87,177-180]. Until now, although several studies have tried to demonstrate the interplay between these two temperature related circadian clock properties [178,181,182], the underlying mechanism remains poorly understood.

In mammals, the temperature entrainable oscillators (TEOs) are mainly present in peripheral organs or cells. The TEOs can be synchronized by the gradual, daily body temperature changes within a physiological range *in vivo*, or induced by square-wave temperature changes (12 hr 37 °C /12 hr 33 °C) *in vitro* [36,183]. In homeothermic rodents, the SCN clock is resistant to ambient temperature changes, but is essential to drive the daily body temperature oscillation and establish the phase coherence of peripheral TEOs utilizing temperature as a systemic signal [184]. TEOs in peripheral organs or cells are quite sensitive to thermo cycles. Similar to FEO entrainment, exposure of animals to temperature cycles results in the phase resetting of hepatic rhythmic circadian gene expression efficiently, without influencing the circadian profiles in the SCN [36]. Subsequent studies revealed that the cyclic body temperature entrains the peripheral TEOs via the HSF1 (Heat Shock Factor 1) related heat shock response pathway [183] (Figure 9). HSF1 is a circadian transcription factor which promotes the expression of heat shock proteins (HSPs, a family of proteins that

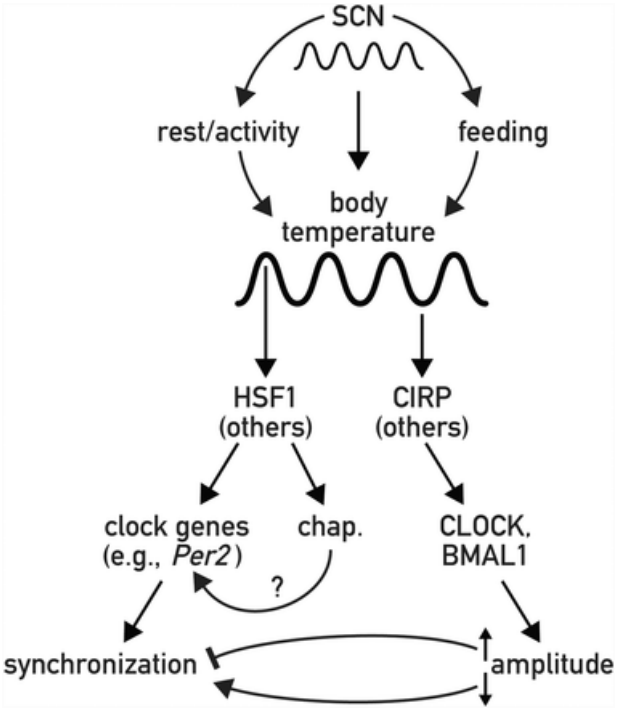

Figure 9: The synchronized pathways of body temperature

Daily body temperature oscillations are controlled by the SCN directly and indirectly. The body temperature rhythm drives the oscillating expression of heat shock factor HSF1 and cold-inducible RNA binding protein CIRP. HSF1 as a transcription factor controls *Per2* expression and then mediates the peripheral clock synchronization. CIRP contributes to the modulation of CLOCK and BMAL1 accumulation and thereby affects the amplitude of circadian gene expression. Chap: chaperones (such as heat shock proteins).

Schematic: Schibler U et al. *Cold Spring Harb Symp Quant Biol*. 2015 [46].

function as chaperones for protein folding) and regulates core circadian gene expression by binding to the BMAL1:CLOCK heterodimer directly. Mutations of the HLF1 binding site in the *Per2* promoter dramatically abolish circadian *Per2* rhythms [185]. HSF1 deficient animals or cultured cells show abnormal temperature regulated clocks, exhibiting a longer circadian period, impaired temperature compensation, slow rate of synchronization, all indicating that HSF1 is necessary for temperature induced entrainment [174,186]. Besides HSF1, CIRP (cold-inducible RNA-binding proteins) also contributes to temperature mediated circadian regulation (Figure 9). The daily expression of *Cirp* mRNA and protein can be induced by temperature cycles, but not serum shock in fibroblasts. CIRP depletion experiments show that it influences the amplitude of circadian oscillation by regulating CLOCK expression [187].

1.2.4 Metabolic oscillators

Metabolic oscillators in living organisms are tightly related to diverse biochemical processes, which represent the basic elements of metabolic networks. Most biochemical reactions are controlled by the circadian timing system. In 1964, Ghosh and Chance reported that autonomous oscillations in the concentrations of glycolytic intermediates were observed in the unicellular organism, yeast [188]. The glycolytic cycles can be synchronized by extrinsic glucose and potassium cyanide stimulation, or acetaldehyde [189,190]. Nevertheless, the oscillations of glycolysis are distinct from canonical circadian rhythms, since glycolytic cycling is not temperature compensated and the frequency of these oscillations is only around a few minutes (Figure 10) [191]. The glycolytic oscillation is not restricted to lower organisms. Transient glycolytic cycles with extremely short periods also exist in mammalian cells [192-195]. Besides the glycolytic cycles, robust yeast metabolic cycles (YMCs) involved in multiple reductive and oxidative processes have been shown to exist in a continuous culture system, with a period from minutes to few hours, depending on the nutrient-limited growth conditions (Figure 10) [196]. Genes associated with YMCs display similar temporal expression patterns, indicating that the metabolic oscillations are always coupled with transcriptional regulation [197]. Similar respiratory cycles also have been revealed in unicellular cyanobacteria, and the ultradian period is influenced by the culture conditions (temperature, pH, oxygen density and glycogen content) (Figure 10) [198]. In addition to ultradian metabolic cycles, infradian metabolic oscillations have been observed in hibernating animals (Figure 10) [199].

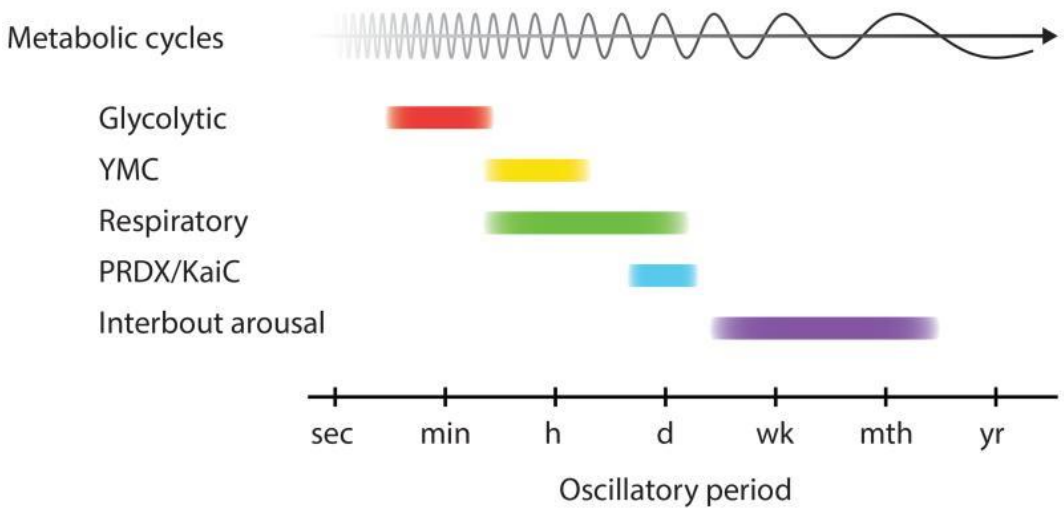

Figure 10: The oscillatory period of metabolic oscillators varies from minutes to months

Metabolic oscillators spanning the oscillatory period range from minutes to months have been described in various species. Glycolytic oscillations in yeast and mammalian cells have a short period of seconds or

minutes. The YMC is a metabolic oscillator with a period ranging from dozens of minutes to a few hours depending on the oxygen consumption. Respiratory cycles in cyanobacteria have a period from 10 h to 24 h. KaiC phosphorylation and peroxiredoxin (PRDX) oxidation cycles are two examples of oscillations based on non-transcriptional mechanism. Interbout arousal indicates periodic increase in body temperature during hibernation, it is an example of metabolic oscillation with an infradian period (from a few days to several weeks).

PRDX: peroxiredoxin, YMC: yeast metabolic cycle.

Schematic: Reddy AB and Rey G. *Annu Rev Biochem*. 2014 [96].

In mammals, metabolic cycles are suggested to be an underlying basis for various biological oscillations. Metabolome measurements of different tissues from various species have been widely performed [200-206]. By metabolomic profiling assays and cluster analysis, the metabolic clusters including glucose homeostasis, lipids, bile acids, and amino acids have been shown to be clock controlled. Most rhythmicity in metabolite levels has a circadian period of around 24h. The circadian timing system always appears to govern biochemical processes or metabolites through transcriptional regulation. A majority of metabolites enriched in the same metabolic pathways have similar circadian phases, which are consistent with their transcripts expression patterns [200]. The metabolic oscillations and circadian clock system are closely linked. On the one hand, the circadian clock governs the metabolome through transcriptional control, the disruption of circadian components of TTFLs causes aberrant expression of metabolic enzymes at the transcriptional or/and translational levels, and thereby leads to metabolism related disorders [207-210]. On the other hand, some metabolites, such as nicotinamide NAD+ [211], heme [212-214] and AMP/ ADP [215] are able to participate in the circadian clock regulation directly or indirectly.

Despite the fact that metabolic oscillations have been extensively studied in mammals, the molecular details of metabolic oscillators remain incompletely understood. Thus, for example the highly conserved peroxiredoxin (PRX) was reported to show cyclic expression in anucleate cells [90,91] (See section 1.1.1.4.2). As PRXs play key roles in maintaining cellular H_2O_2 levels by interacting with ROS reactions and redox cycles, it has been hypothesized that the rhythmic expression of hyper-oxidized PRX (PRX-$SO_{2/3}$) could be used as an output of metabolic oscillator (Figure 10) [99]. Furthermore, daily PRXs expression has been observed in a wide range of species, including metazoans, plants, fungi, cyanobacteria, fly, mouse and human [97], suggesting that this metabolic oscillator system could be a basic element of the biological clock. However, more fundamental information about metabolic oscillators, such as their molecular components, location, regulation, and

precise properties of their oscillation remains unclear.

1.3 Interplay between regulators of the circadian clock and metabolism

The circadian clock temporally orchestrates many aspects of metabolism. The circadian control is achieved by regulating the expression of rate-limiting enzymes, or by integrating nuclear receptors and nutrient sensors (e.g. SIRT1 and AMPK) with the clock machinery, or by gating the abundance of several metabolites [216]. Meanwhile, this mechanism also feeds back to the circadian timekeeping mechanism itself based on cycling cellular energy states. Here we take NAD+ circadian regulation as an example to show the interplay between circadian timing system and metabolism.

The NAD+ biosynthesis and salvage pathway, which is conserved from yeast to mammals has been shown to be controlled by the circadian timing system [211]. In mammals, NAMPT (nicotinamide phosphoribosyl transferase) which acts as a rate-limiting enzyme catalyzing the first step in the biosynthesis of NAD+ from NAM (nicotinamide) exhibits strong circadian rhythmicity. NAD+ levels also display robust diurnal rhythms in synchronized fibroblasts and mice [211,217]. SIRT1 (Sirtuin1) catalyzes the conversion of NAD+ to form NAM and O-acetyl-ADP-ribose. The major function of SIRT1 is a modulator of the circadian machinery through the interaction with and the deacetylation of core circadian clock factors. As shown in Figure 11, SIRT1 regulates the core elements CLOCK:BMAL1-mediated circadian transcription. SIRT1 can bind to BMAL:CLOCK heterodimers directly, then the BMAL:CLOCK:SIRT1 complex associates with the E-box enhancer in the promoters of key circadian genes (including *Nampt*) and drives their rhythmic expression. Although SIRT1 shows arrhythmic expression at both the mRNA and protein levels, it interacts with the CLOCK:BMAL heterodimer in a time dependent manner [218]. Thus, NAD+, NAMPT and SIRT1 are proposed to constitute a novel enzymatic feedback loop, which serves as an accessory loop of the classical TTFL and mediates rhythmic regulation of multiple physiological processes (Figure 11) [219].

SIRT1 also serves as a NAD+-dependent deacetylase that plays vital roles in clock post-translational modulation. The core clock element CLOCK is not only a transcription factor, but also an enzyme with histone acetyltransferase (HAT) activity. CLOCK is able to acetylate histone H3 and also the nonhistone protein BMAL1 on its Lys 537 residue directly [70,75]. SIRT1 mediates deacetylation and this histone deacetylase (HDAC) activity is used to counterbalance the HAT function of CLOCK, thus ensuring the turnover of clock components and maintaining the stability of the circadian system [218]. Notably, both the HAT activity of CLOCK and the HDAC reaction of SIRT1 are regulated in a circadian

manner *in vivo* and *in vitro*. Subsequent studies have shown that SIRT1 can interact with another key clock element PER2 and promote its deacetylation and degradation [220]. In addition to modulating the core clock machinery, SIRT1 also regulates metabolic processes by deacetylating several metabolic proteins including LXR (liver X receptor) for cholesterol and lipid homeostasis [221], PGC1α (PPARγ coactivator α) [222] and FOXO1 (forkhead box O1) [223] for gluconeogenesis (Figure 11). In this way, SIRT1 functions as a modulator to establish close links between metabolic activities and the circadian timing system. Actually, SIRT1 belongs to a NAD-dependent protein deacetylase family, which contains seven sirtuins (SIRT1 through SIRT7) in mammals [224]. Besides SIRT1, SIRT3 [152] and SIRT6 [225] have been reported to mediate the coupling of circadian control and metabolism.

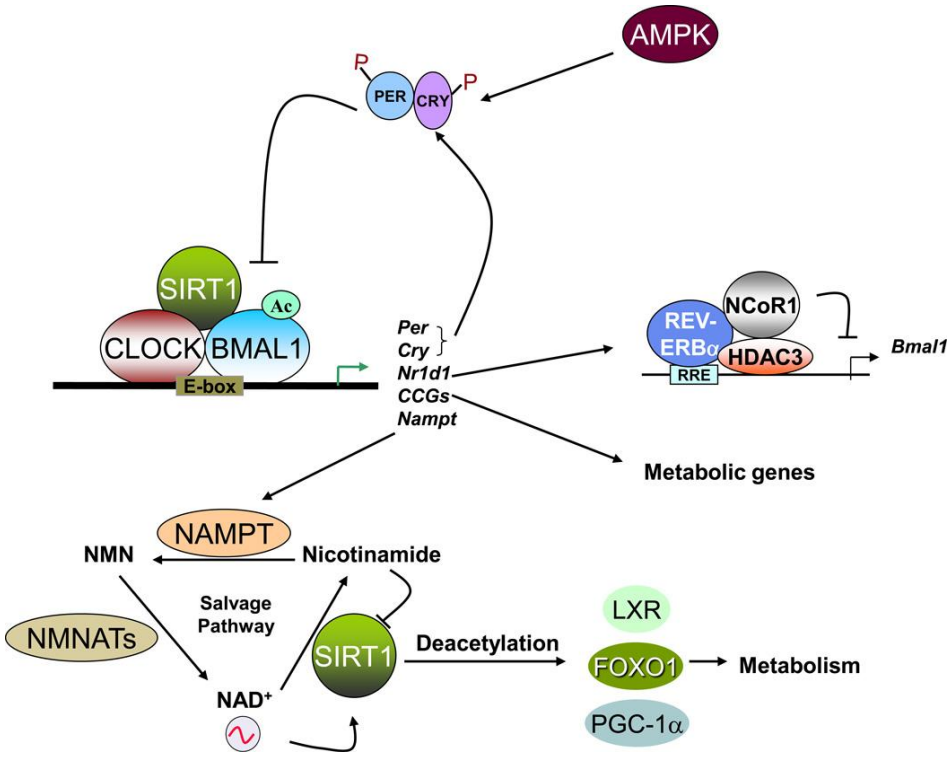

Figure 11. Interplay between regulators of the circadian clock and metabolism

SIRT1, NAMPT and NAD+ are suggested to comprise a novel circadian feedback loop that mediates rhythmic regulation of many physiological events. SIRT1 links the novel enzymatic loop with the classic TTFL. SIRT1 not only contributes to the regulation of circadian genes (*Nampt*, core circadian clock genes, CCGs) by binding to E-box elements of promoters with CLOCK:BMAL1, but also deacetylates the metabolic proteins LXR, FOXO1 and PGC1α and therefore regulates metabolic processes. SIRT1coupled with NAD+, as well as AMPK play as nutrient sensors to modulate the circadian clock. AMPK: AMP-activated protein kinase, CCGs: clock controlled genes

Schematic: Sahar S and Sassone-Corsi P. *Trends Endocrinol Metab*. 2012 [216].

NAD+ is not only involved in many redox pathways as described above, but it also contributes to SIRT1-mediate epigenetic regulation as the HDAC activity of SIRT1 requires NAD+ as a co-enzyme [226]. Another NAD+ dependent enzyme that influences clock control are PARPs (poly-ADP-ribose polymerases), which can rhythmically poly (ADP-ribosyl)ate CLOCK and therefore constitute the interactions between metabolism, the clockwork circuitry and feeding entrainment [148]. Moreover, the NAD(P)+/NAD(P)H redox pairs effect the DNA-binding activity of the CLOCK:BMAL1 and NPAS2:BMAL1 heterodimers, indicating NAD+ could be used as a "sensor" by which the clock system measures the energy status of the cells [227]. Taken together, since NAMPT-mediated NAD+ biosynthesis and salvage pathways are controlled by circadian clock, the rhythmic NAD+ levels and metabolic enzymes represent circadian transcriptional outputs. Meanwhile, NAD+ and some NAD+ dependent enzymes (SIRT1, PARPs) can feedback into the clock machinery, where they function as metabolic cues to modulate the inputs of the circadian timekeeping system.

1.4 The chronobiology of fish

1.4.1 The zebrafish (*Danio rerio*)

1.4.1.1 The zebrafish as a model organism for circadian rhythm studies

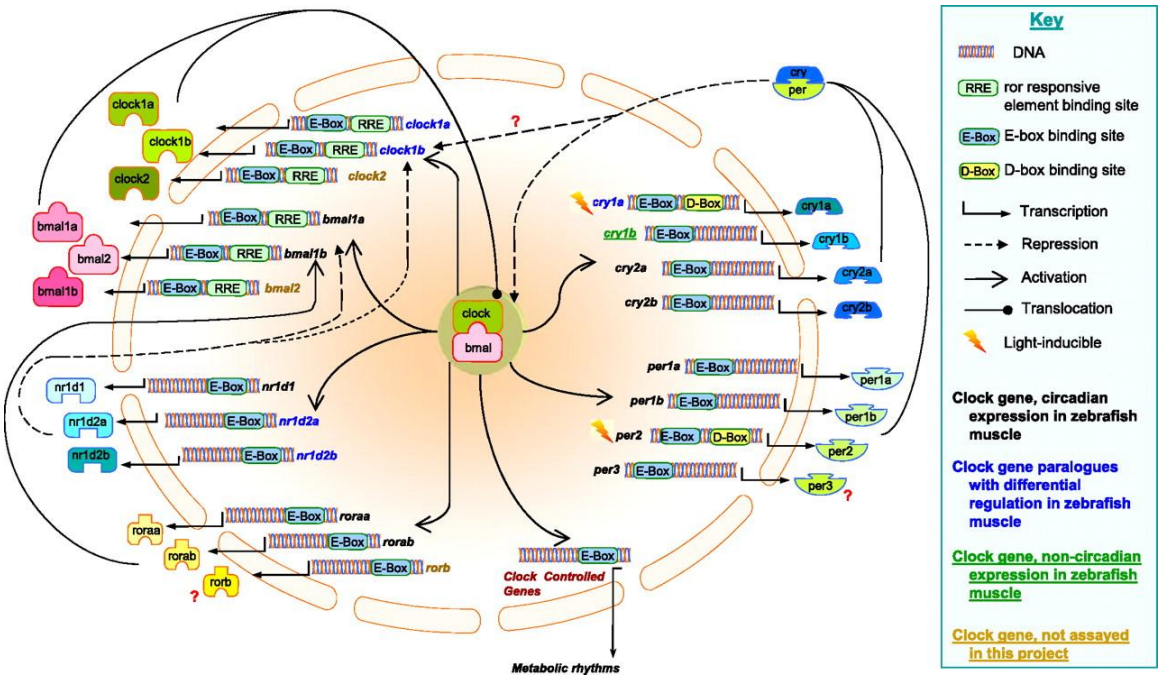

Figure 12. Diagram of the transcription-translation feedback loops in the zebrafish

The organization of TTFLs in zebrafish is quite similar to that of mammals. Due to the genome duplication, zebrafish TTFLs have extra copies of clock components. In zebrafish TTFLs, there are three *bmal* genes, three *clock* genes, four *per* genes, six *cry* genes, three *reverb* (*nr1d*) and three *ror* genes. Significant duplications and expansions of core clock genes make the zebrafish circadian regulatory system much more complex.

Schematic: Amaral IP and Johnston IA. *Am J Physiol Regul Integr Comp Physiol*. 2012 [228].

The zebrafish has proven to be a useful genetic model for studying the circadian timing system. Zebrafish exhibits robust circadian rhythms of locomotor activity both in larvae [229] and in adults [230], with high locomotor activity in the daytime and low activity in the nighttime. The organization of the zebrafish circadian timing system has much in common with the mammalian system. The core mechanism underlying the circadian clock is based on TTFLs. However, because of the genome duplications during early teleost evolution [231], the TTFLs are constituted by more homologues of clock components (Figure 12). Compared to mammals, the zebrafish has many advantages for exploring the regulatory mechanisms of the circadian clock, such as its short generation time, transparent embryos, rapid and external development, sensitivity to external cues, a battery of genetic tools (mutant, transgenic, knock out, knock in fish lines). In addition, applying environmental cues, such as food, light, temperature, or chemicals to zebrafish is technically relatively simple, so the zebrafish is particularly attractive as a model for studying the influence of external signals on the internal clock system.

1.4.1.2 Central pacemakers and peripheral clocks

In non-mammalian vertebrates, the circadian system is composed of multiple pacemakers [232]. Zebrafish has an anatomical counterpart of the mammalian SCN, but whether it contributes to generating circadian rhythmicity is still unclear [233]. The pineal gland, which is a purely secretory organ in mammals, is directly photosensitive and represents the site of a central pacemaker in zebrafish [25]. The retina is also a photoreceptor and can generate self-sustained and robust circadian oscillations [234]. Both the pineal gland and retina rhythmically synthesize and secrete melatonin with high levels during the night and low levels during the day in response to circadian clock and direct light input [235]. Aanat2 (arylalkylamine-N-acetyltransferase 2), a rate limiting enzyme for melatonin synthesis, is expressed exclusively in pineal photoreceptors and controlled by circadian clock at the mRNA level [236]. Melatonin is proposed to constitute a major neuroendocrine signal that has multiple functions and serves to coordinate the circadian outputs in zebrafish (Figure 13)

[237,238]. The cyclic production of melatonin and *aanat2* expression is first observed at around 2 days post fertilization (dpf), much earlier than the appearance of circadian locomotor activity and other clock outputs [239].

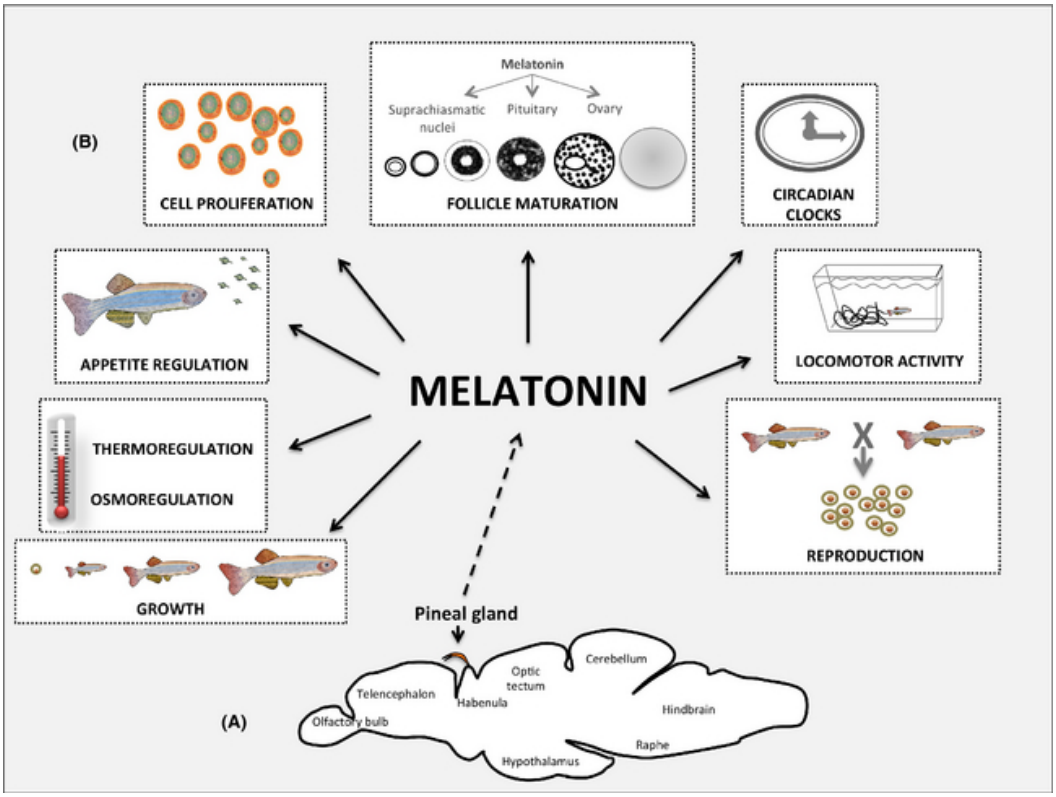

Figure 13: The multiple functions of melatonin in zebrafish

(A) The pineal gland is located on the dorsal surface of the diencephalon in the zebrafish, and it is elongated in shape and composed of ependymal cells, melanocyte-like cells, fibrous astrocytes, and pinealocytes, which represent the morphofunctional unit of the gland.

(B) Melatonin secretion by the pineal gland exhibits a robust circadian rhythm. In zebrafish, the known functions of melatonin are mediated through high-affinity receptors expressed in many tissues and cells. Melatonin participates in the circadian regulation of diverse behavioral and physiological events in the zebrafish.

Schematic: Lima-Cabello E et al. *J Pineal Res*. 2014 [240].

Early studies of the zebrafish circadian clock revealed that it possesses self-sustained clocks in most peripheral tissues and even cell lines that can be entrained by direct exposure to light-dark cycles [241-243]. Core circadian genes *clock*, *cry1a*, *6-4Phr*, *per2*, *wee1*, *per1b*, *per3*, *bmal1* and *bmal2* are rhythmically expressed in cultured tissues or cells *in vitro* under light-dark entrainment conditions [242,244-249]. The direct clock entrainability by light of

peripheral tissues sets fish apart from mammals where light is perceived exclusively through ipRGCs in the retina. The role of the pineal gland as a hierarchical master clock in zebrafish has been under argument, as pinealectomy and ocular enucleation do not impact on the rhythmic behavior of larval fish under LD entrainment [238]. Besides light, whether other zeitgebers such as glucocorticoids and serum, which efficiently entrain mammalian peripheral clocks, could induce circadian rhythmicity in zebrafish cells is unknown.

1.4.1.3 Multiple clock oscillators in zebrafish

Like mammals, the zebrafish endogenous time-keeping system can be synchronized by diverse external cues and is under a multi-oscillatory control. Light is the most powerful exogenous signal for both pineal gland and peripheral clocks entrainment in the fish. Periodic feeding and temperature have also been reported to serve as non-photic zeitgebers resulting in the resetting and phase shifting of internal rhythmicity.

1.4.1.3.1 LEO

Compared to mammals, the zebrafish is much more sensitive to light exposure, perhaps due to its relatively transparent skin. The zebrafish pineal organ contains photoreceptor cells with structural and functional similarities to mammalian retinal photoreceptors [250]. The pinealocytes in the zebrafish pineal organ can detect light via the photoreceptor rhodopsin, transduce the photic information into melatonin synthesis and then regulate circadian rhythmicity through melatonin secretion [251,252]. In the zebrafish retina, the photopigment melanopsin plays roles in light perception and the direct response of the clock to light entrainment [253]. In addition to these central pacemakers, zebrafish peripheral organs and cells are directly photosensitive, but until now the light input pathways to the cellular timing machinery have not been fully understood. Three different peripheral photoreceptors are proposed to mediate the light perception and clock synchronization: 1. non-visual opsins, such as melanopsin, TMT (teleost multiple tissue) opsin, neuropsin, va-opsin [254]; 2. cryptochromes (Crys) and 3. flavin-containing oxidase. The non-visual opsins, especially TMT opsin is expressed widely in zebrafish tissues, even in fibroblast cell lines [255]. In the blind cavefish *Phreatichthys andruzzii*, loss of function mutations in TMT opsin as well as other melanopsin homologs Opn4m1 and Opn4m2 have been linked with the loss of light input pathway function in this species [256]. There are at least six cryptochrome genes in zebrafish, *cry1a*, *cry1b*, *cry2a*, *cry2b*, *cry3*, *cry4*, with different expression patterns and predicted functions. *cry3* and *cry1b* have been proposed to serve as light photoreceptors for entraining the clock by activating the MAPK signaling pathway in zebrafish cells [246]. Flavin-containing oxidase activity in response to blue light exposure leads to an increase in cellular H_2O_2 levels, which in turn may function as a second messenger to activate

expression of core circadian clock genes [257]. Thus, in zebrafish the LEO exists not only in the central pacemaker, but in nearly all cell types (Figure 14). It has been suggested the widespread cellular light sensitivity may be part of a mechanism protecting cells from UV damage. This hypothesis originates from the observation that several genes involved in the repair of UV damaged DNA, also show light-inducible expression [258]. However, many fundamental questions concerning the molecular mechanism of light-responsive entrainment remain unanswered.

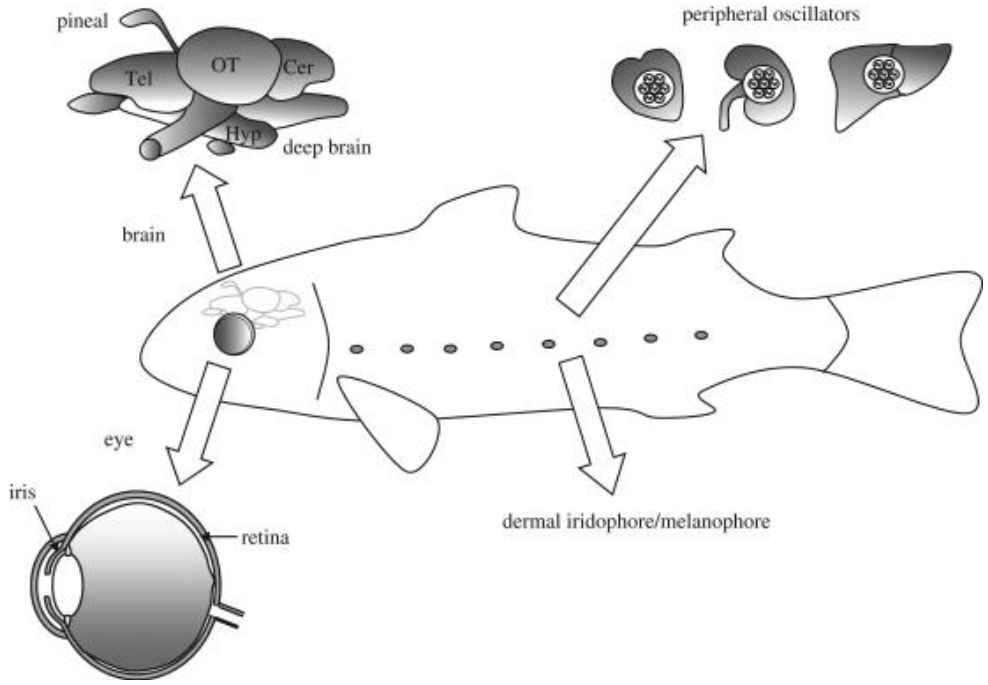

Figure 14: Photoreception in zebrafish

In zebrafish, photoreceptors are located in the retina of the lateral eye, pineal gland and deep brain regions. Additionally, zebrafish peripheral tissues have been shown to be able to entrain their molecular oscillators directly to light.

Schematic: Peirson SN et al. *Philos Trans R Soc Lond B Biol Sci*. 2009 [259].

1.4.1.3.2 FEO

Like mammals, periodic food availability is a potent zeitgeber that entrains circadian rhythmicity in zebrafish [260]. This fish displays FAA (food anticipatory activity) when fed at a fixed time every day, regardless of the lighting conditions. The FEO may be synchronized in the peripheral tissues, but not in the central pacemaker. Rhythmic expression of the core circadian genes (*per1*, *cry1*) in the zebrafish liver has been reported to adjust its circadian phase according to inverted feeding regimes [260], whereas these genes

are insensitive to the feeding schedules in the zebrafish brain [261]. The zebrafish hepatic circadian clock and metabolism appear to be highly susceptible to the feeding time [262]. However, the study of the FEO in zebrafish is still in its infancy and many key issues still need to be addressed: What are the pathways of feeding entrainment? What are the links between metabolism and the FEO? Do the central pacemakers coordinate the peripheral clocks by systemic cues?

1.4.1.3.3 TEO

Zebrafish is a heterothermic animal, where behavior and physiological function are strongly influenced by the ambient temperature. Normally the fish is raised at a constant temperature (28℃). Previous investigations have shown low amplitude thermocycles serve as another non-photic zeitgeber which not only establishes the circadian oscillation in zebrafish cell lines [180] and embryos [263], but also results in the phase shift of several clock genes in developing larval fish [264]. Zebrafish daily behavior is strongly synchronized to the light-dark cycle, regardless of whether the temperature changes are in-phase or anti-phase with the light-dark cycle, indicating the temperature is not such a powerful synchronizer as light in the fish [265,266]. At the transcriptional level, temperature has a strong effect on the amplitude of circadian gene expression induced by light-dark cycles [180], and influences the onset of circadian clock function in larvae [267]. Temperature compensation is also observed in cultured fibroblasts, tissues and organs (retina, spleen, pineal gland, heart, gall bladder, gills) [180,268].

1.4.1.4 The circadian transcriptome and metabolome profiles in zebrafish

Gene expression profiles obtained from high throughput sequence analysis have revealed that approximately 10% of all transcripts in the mouse liver [14,269], mammalian fibroblasts [270], mouse SCN [271] and heart [272] display circadian oscillation. In my previous study, we have demonstrated that more than 10% (2856 circadian genes) of the transcriptome shows robust circadian rhythmicity in larval zebrafish using a microarray assay [273]. Recent work has revealed that in the zebrafish liver, around 10% of the expressed genome is rhythmically expressed [274]. Although a similar number of rhythmic transcripts have been identified in the mouse liver (2530 genes) and adult fish liver (2951 genes), only a small proportion (486) of rhythmic genes overlap between the zebrafish and mouse livers [274].

In zebrafish, metabolism is also an important output of the circadian timing system. Using bioinformatic approaches, we have identified a large cluster of metabolic genes and integrated them with circadian metabolic networks [275]. Until now, hydrogen nuclear magnetic resonance (^{1}H NMR) spectroscopy has been used for fish metabolome studies

[276-279]. However, little research has focused specifically on circadian metabolomic profiling in various adult zebrafish tissues.

1.4.2 The circadian clock in blind cavefish (*Phreatichthys andruzzii*)

Cavefish represent a group of fish eminently well suited for studying the mechanism of non-photic regulated clocks. During evolution in constant darkness over millions of years, these fish have adopted a striking and characteristic set of phenotypes including for example eye loss and the ability to tolerate long periods of starvation [280]. These so-called troglomorphic characters represent one of the classic examples of convergent evolution and there is still considerable debate as to whether they represent adaptations for life in the extreme cave environment [281]. One obvious question is whether isolation from the day-night cycle for millions of years results in the retention of a normal circadian clock that can be entrained by light? Our group has demonstrated that one species, the Somalian cavefish (*P. andruzzii*) which has evolved for 2-3 million years beneath the desert in Somalia and shows an extreme troglomorphic phenotype also displays an aberrant clock [282]. By measuring cycling expression of core clock genes such as *per*, *cry* and *clock*, we have demonstrated that in peripheral tissues this clock mechanism ticks with an extremely long period and has entirely lost the ability to be entrained by light. This clock has been studied in fibroblast cell lines (CF1) derived from adult fin tissue. Mutations in multiple *opsin* genes that appear at least in part to explain the lack of entrainment by light have been identified. In addition, our group has recently identified a mutation that truncates the light-regulated clock gene *per2* and they speculate may explain at least in part, the characteristic long period phenotype (unpublished data).

Tantalizingly, as part of the characterization of the clock in the cavefish *P. andruzzii in vivo*, our group has revealed robust 24 hours rhythms that are entrained in these fish by regular feeding [282]. Specifically, exposure to a regular feeding time of one meal per day, results in the emergence of robust 24 hour rhythms of FAA. In regularly fed animals, high amplitude, 24 hours rhythms of clock gene expression are also observed in various tissues. Cavefish are masters of tolerating starvation, being able to survive weeks in the absence of food without ill effects. During an extended period of starvation following regular feeding, the 24 hours rhythms of FAA persist for many cycles. Interestingly, most recent data have indicated that when food is provided at 48, 72 or 96 hours intervals, 24 hours rhythms of FAA are established. However, feeding with a 36 hours frequency fails to establish rhythmicity in FAA (unpublished data). Thus, the cavefish is a fascinating model for studying the non-photic pacemaker, especially the FEO clock. The cavefish provides a unique opportunity to distinguish the function of the FEO from the LEO, since the LEO is no longer

entrained by light and also it ticks with a characteristic infradian rhythm.

1.5 Aim of the project

The zebrafish circadian system is a multioscillatory system comprised of alternate or parallel oscillators. My research programme attempts to extend the existing comparative studies involving zebrafish and cavefish to tackle some key questions regarding the existence and relationship of multiple clock mechanisms in vertebrates. Specifically, this project focuses on the elements of the LEO and FEO in adult zebrafish liver, as well as the circadian regulation of a metabolic oscillator in zebrafish and cavefish cell lines.

1. What are the elements of the FEO in zebrafish liver?

To tackle this question, we exposed adult fish simultaneously to two different cycling environmental cues, light and food availability (“conflicting zeitgeber” paradigm, see results section 3.1.1). By testing hepatic rhythmic clock gene expression in these animals, we examined the resetting of circadian genes by regular feeding in relation to the timing of the light-dark cycle. Furthermore, using NMR metabolome measurement, we identified differential metabolic targets of the LEO and FEO in the fish liver. A key goal of this study is to construct a database of oscillating genes and metabolites controlled by the FEO. We expected to distinguish the FEO from the LEO functionally and genetically, and explore the molecular basis of the FEO in zebrafish.

2. Besides the LEO, do other circadian oscillators exist in zebrafish or cavefish cell lines?

To assess whether the FEO can be detected in fish cell lines, we treated the cells with fresh serum, which mimicked the feeding entrainment in adult zebrafish. Metabolome assays of different types of treated cells were then used to explore the links between circadian rhythms and cellular metabolic oscillations. The NMR analysis of cavefish fibroblasts represents a strategy to determine whether circadian metabolite oscillations exhibit an infradian period (as previously observed for rhythmic clock gene expression in cavefish peripheral tissues *in vitro*), which could provide unique insight into how metabolic oscillation interacts with the classical clock TTFLs. The overall aim of this work is to investigate the existence and regulation of multiple cellular oscillators in both zebrafish and cavefish cells.

Chapter 2

Materials and Methods

2.1 Animal experiments

2.1.1 Ethics Statement

Zebrafish handling procedures were performed in accordance with the German animal protection standards (Animal Protection Law, BGBl. I, 1934 (2010) and were approved by the Local Government of Baden-Wurttemberg, Karlsruhe, Germany (Az.: 35-9185.81/ G-130/12). General license for fish maintenance and breeding: Az.: 35-9185.64.

2.1.2 Animals

Wild type (WT) Wik strain zebrafish (*Danio rerio*) were raised at 28°C under a 14h:10h light-dark cycle from birth. The light turned on at 8:00, and turned off at 22:00. Normally, the fish were fed twice every day at random time points.

2.1.3 Feeding entrainment and sample collection

6-12 months old WT zebrafish (only male) were used for the feeding entrainment experiments. The fish were maintained in transparent glass aquaria with re-circulating water and normal 14h:10h light-dark conditions. Each aquarium had 15-16 adult fish. Usually, six to nine aquaria were needed for each experiment. According to the feeding time, all the fish were classified into three experimental groups: fish fed once a day at mid-light (ML, 15:00, ZT7), fish fed once a day at mid-dark (MD, 3:00, ZT19), and fish fed once a day randomly (RD). In the RD group, the feeding time was at follows: Mon, 7:00; Tue, 18:00; Wed, 12:00; Thu, 4:00; Fri, 10:00; Sat, 20:00; Sun, 14:00. In such a case, the time delay between one meal and the next one was between 12-36h, and at the end of the week RD fish ate the same amount of food as ML and MD groups.

Fish were fed with commercial flake (Preis aquaristik) by a self-feeding system. The amount of flake given to the fish was fixed at approximately 1% of the fish body weight per day. Before the feeding entrainment, we weighed the fish in a beaker with water and then subtracted the weight of the beaker only with the water. Then, we calibrated the food provided by the automatic feeders (Eheim). For this, we rotated the feeder for several times (8-10) and then calculated the mean amount of food per rotation. The food was given from the middle top of the aquaria.

When all the fish were fully adapted, tissues including livers and brains were collected at 6h intervals starting at ZT0 (zeitgeber time 0, the time of lights on). 2-3 fish were sacrificed per

time point per group. The fish was anesthetized by rapid cooling with ice slush, then tissues were dissected out and frozen immediately in liquid nitrogen and stored at -80°C. The sample collection in darkness was performed under dim red light.

2.1.4 Locomotor activity recording

The locomotor activity of zebrafish was recorded during the feeding entrainment. To quantify the fish behaviour, a photoelectric sensor which illuminated by an infrared source under light and dark conditions (E3Z-D67, Omron) was placed in the front of the aquarium. The top of the sensor was placed close to the water surface of the aquarium. Adult zebrafish moved freely in the tanks. When the fish interrupted the diffused light beam emitted by the sensors, a computer connected with the sensors logged the number of interruptions and exported the data every 10 minutes by specialized software (DIO98USB, University of Murcia, Spain). Fish locomotor activity was analysed and plotted by chronobiology software (El Temps© v.1.179, Dr. Díez-Noguera, Barcelona, Spain).

2.2 Cell culture methods

2.2.1 Zebrafish and cavefish cell culture maintenance

The zebrafish cell lines PAC-2 are derived from 24 dpf embryos while AB9 are derived from adult fins. EPA are cavefish embryonic cell lines and CF1 are derived from the fins of adult cavefish (*P. andruzzii*). These four cell lines are adherent fibroblasts. They were incubated at 26°C in L-15 (Leibovitz-15) medium (Gibco BRL) supplemented with 20% fetal calf serum (Sigma Aldrich F7524) 100 units/ml penicillin, 100 μg/ml streptomycin and 50μg/ml gentamicin (Gibco BRL). PAC2, AB9 and EPA were propagated every 7 to 10 days at a ratio of 1:4 by trypsinization with 0.25% trypsin-EDTA (Gibco BRL). CF1 cells were propagated every 25 days at a ratio of 1:2. All the cells were propagated in 175cm^2 flasks (Greiner).

2.2.2 Primary hepatocyte cultures

Primary cultures of hepatocytes were obtained from adult male zebrafish (WT Wik fish, 9-12 months old). 16-17 fish were anesthetized by rapid cooling and livers were dissected under a stereo microscope. Groups of 3-4 fish livers were pooled and kept in 10ml 1×PBS with 100 units/ml penicillin and 100 μg/ml streptomycin (1% P/S) in a 15 ml Falcon tube. The tissues per tube were washed 5 times with 5ml 1×PBS plus1% P/S, and then digested in 400μl 0.25% trypsin without EDTA (Gibco BRL) at room temperature for 5 min with gentle pipetting to aid tissue breakdown. The reaction was terminated by the addition of 6ml L-15 culture medium (Leibovitz-15 medium, 20% fetal calf serum, 1% P/S and 50μg/ml gentamicin). The cell suspensions were centrifuged at 800g for 20s at room temperature to collect liver pieces

and fibers at the bottom of the Falcon tubes. The hepatocytes were seeded equally in 15× 6cm petri dishes. The dishes were maintained at 26 °C in an atmospheric CO_2, non-humidified cell culture incubator. After 16 h of seeding, the primary cells were attached to the plate and the medium was replaced by fresh L-15 culture medium, then the hepatocytes were cultivated and entrained for the further experiment.

2.3 Molecular experiments

2.3.1 RNA extraction

Total RNA of zebrafish tissues was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. 1ml Trizol reagent was added to individual frozen aliquots of tissue. The samples were then homogenized by passing 10 times through a 1 ml syringe with a 25G needle. After addition of 200 µl of Chloroform, the samples were mixed violently and then centrifuged at 12000 rpm for 15 minutes at 4 °C. The supernatant was transferred to a new 1.5 ml eppendorf tube and 500 µl of Isopropanol was added to precipitate the RNA. The RNA pellet was washed once with 75% ethanol, and then dissolved in RNase-free water (Promega) and stored in -80°C freezer. The concentrations of RNA samples were assessed with a NanoDrop ND-1000 spectrometer (PeqLab). The quality of the RNA was determined after electrophoresis on an agarose gel to visualize the integrity of the ribosomal 28S and 18S RNA bands.

Total RNA was extracted from cells using Trizol reagent without homogenization. In general, cell lines (PAC2, AB9, EPA and CF1) in 25cm^2 flasks or hepatocytes in petri dishes were harvested with 1ml Trizol. Then Chloroform was added to extract the total RNA, and Isopropanol was used to precipitate the RNA.

2.3.2 RT and Real-time PCR

The first strand cDNA synthesis of total RNA was performed according to the manufacturer's protocol (Promega). 600ng total RNA was diluted in 7µl of RNase-free water, and to this was added 1 µl of 10x RQ1 DNase buffer (Promega), 1 µl of DNase (Promega, 1u/1µl) and 1 µl of RNase inhibitor (Promega ,40 U/µl). The mixture was incubated at 37 °C for 30 minutes. After addition of 1 µl of EDTA (Promega) which stops the DNase reaction, the solution was incubated at 65 °C for 10 minutes. Then 1 µl of random hexamers (Sigma Aldrich, 200 ng/µl) were added and the mixture was incubated at 70 °C for 5 minutes. RT was performed by adding cDNA synthesis mix composed of 3 µl of RNase- free water, 4 µl of 5x M-MLV RT buffer (Thermo Scientific), 2 µl of dNTPs (Sigma Aldrich, 10 mM) and 1 µl of M-MLV reverse transcriptase (Thermo Scientific, 200 U/µl), and then incubating at 25 °C for 10 min and 42 °C for 60 min. The reaction was terminated by heating the sample

to 70 °C for 15 min. the cDNAs were diluted 1:10 with distilled sterile water and stored at -20 °C until use.

Real-time PCR was performed in a volume of 25 µl including 10 µl 2×SYBR Green I Master Mix (Promega), 9 µl of PCR-grade water, 4 µl of RT product and 2 µl of forward and reverse primer mix (10µM) on an ABI StepOne Plus machine. The amplification cycle consisted of 1 cycle of 15min at 95 °C, 40 cycles of 15s at 95 °C and 30s at 60 °C, followed by the generation of a melting curve. The PCR primers were designed using Primer 3 and *β-actin* was used as a reference. The primer sequences of genes tested are listed in Table 1. The specificity of PCR reactions was checked by melting curve analysis. The critical threshold (C_T) value is the PCR cycle number where the PCR growth curve crosses a defined threshold in the linear range of the reaction. Relative log2 expression values are calculated from C_{max}-C_T , where C_{max} is the maximum value of C_T in tested samples.

Table 1: RT-qPCR primers sequences

genes	Forward oligo	Reverse oligo
zf *β-actin*	F: GCCTGACGGACAGGTCAT	R: ACCGCAAGATTCCATACCC
zf *per1b*	F: CCGTCAGTTTCGCTTTTCTC	R: ATGTGCAGGCTGTAGATCCC
zf *bmal1*	F: TAGAGCGCTGTTTGCTGATG	R: GACCCGTGGACTTCAGTGAC
zf *bmal2*	F: GCATTTGGGTTCTTGCCTTA	R: CCTTAAGCAACAGGACCAGG
zf *bmal3*	F: ATCCTATGCTGTCCTGGCCT	R: CCCTCTAGCTGTGGCTCAAG
zf *per2*	F: CTTCACCACACCATACAGG	R: GTCTGACGGGGACGAGTCT
zf *clock1*	F: CTGGAGGATCAGCTGGGTAG	R: CACACACAGGCACAGACACA
zf *cry1b*	F: TTGGGCTCCTTGCAACTATT	R: TGCAAATGCTGGAAACTCTG
zf *cry2a*	F: TGCCAGTGGTAACAGGAGTG	R: AAATCTACTGTTGTGGCCCG
zf *reverbα*	F: GGACAAGCCAGCAGAATCTC	R: CCTGAAAAACATCAGCAGCA
zf *dbp1*	F: GTCCAGGCCAATTTCTCAAA	R: CACAGCAGTCCTTCCCTCTC
zf *dbp2*	F: AAGATGCTCGTCCCTGAAGA	R: CCAGATGGTGGCTCTCGTAT
zf *tefa*	F: AAAGCTCTGCTTGAGTACCCTTT	R: ACTCCTCCAGATCCATGTACTCC
zf *nampta*	F: GTGTTCAGGAACGGCAAGAT	R: GGACAGAAACCGTGTGTCCT
zf *namptb*	F: AAACTCCGATGGCTTCATTG	R: CCGGATCTCATCCAGAGTGT
zf *sirt1*	F: GCACCGAACCGTTATGTTTT	R: GTCCGTTTCCTTATCCGTCA
zf *got1*	F: TCACACTAAACACCCCGGAA	R: GAGTTCCCAGAGCCTTCAGT
zf *got2a*	F: GGAGGCTTCACAGTGGTTTG	R: AGCCATGCCTTTTACCTCCT
zf *got2b*	F: CCTACCAGGGCTTTGCTAGT	R: GAATCCTCCAACACGCTCAC
zf *asns*	F: AGGAGCACATCGAGTCTGAG	R: CTTGGCCAGGGTAATGCTTC

zf *glud1b*	F: CAACACCCGATGCTGACAAA	R: AGCAGGTGGTAGTTGGAGTC
zf *glu1a*	F: GAAATGCGGGAAGATGGTGG	R: CAGTGAGTCGACGAGCATTG
zf *gpt2l*	F: GGGTCCCGAGTACTCCAAAA	R: GCTTTCACATCCGCATCCAT
zf *tdh*	F: GCTGGCCGATGAACTTTGAT	R: AGCTATGCGGGACTCTGATC
zf *impdh2*	F: TGCCGTCTGCTGTTTGTATC	R: CCGGAGTGAAAATGGTCTGT
zf *tat*	F: CGGCCTGAATCCAGTGAT	R: GAAATGCTCCATCTCGATCC
zf *cfb*	F: GCTTGTGGATGCTGCTTTC	R: TTTGTAACTGCCTCCGTCG
zf *fgg*	F: TGGACGTGGATGGACTGTAC	R: GTCATCGGGTGAGAGGTAAC
zf *hnf4a*	F: GCCGACACTACAGAGCATCA	R: AGGTGTTCCTGGACCAGATG
zf *fabp10a*	F: CACCTCCAAAACTCCTGGAA	R: TTCTGCAGACCAGCTTTCCT
cf *per1b*	F: TGGAAAGCTGTAACGTCCCA	R: ACAACGCACTCTCCTCTACC
cf *glu1a*	F: CATGGCAGAGGGGATGAGAT	R: ACGGAACTGGAGAAGGACTG
cf *gpt2l*	F: GGCAGTGGTTTTGGTCAGAG	R: AGGGAGGTGATTGGTTGAGG
cf *sirt1*	F: TGTTCTTCTCTCTGCTGGGG	R: AACCCATCACAGAAGCCTCA
zf/cf *β-actin*	F: GATGAGGAAATCGCTGCCCT	R: GTCCTTCTGTCCCATGCCAA

zf: zebrafish, cf: cavefish

zf/cf: the primers can be used for both zebrafish and cavefish

2.3.3 Deep sequencing of cavefish mRNA libraries

To detect the genome of cavefish tissues and cells, RNA sequencing was performed by the Partner Institute for Computational Biology Omics Core (Chinese Academy of Sciences). The real-time PCR primers were designed based on the cavefish mRNA sequencing libraries.

2.3.4 Luciferase reporter constructs

Zf *namptb*-Luc reporter plasmid containing 367 bp *namptb* promoter fragment was constructed as previously described [248]. First, a promoter fragment from position -134 to -500 of *namptb* was amplified from PAC-2 cell genomic DNA by PCR using the following primers: FW: ATCAATTCTTACATCAGCTCACACC, RV: TCACCTCGGTATCTTGTAT ATGTGA. The promoter fragment was cloned into the vector pGEM-T easy (Promega) initially, and then subcloned into the vector pGL3 basic (Promega) using the following primers: FW: GTAAGGTACCGACTCACTATAGGG, RV: ATAGAATACTCGAGCTAT GCATCCA. Zf *per1b*-Luc including 3.1kb of the zebrafish *per1b* promoter was amplified by using Genome Walker PCR (Clontech) and subcloned into pGL3 basic (Promega) [248].

2.3.5 Real time bioluminescence assay and data analysis

The real time bioluminescence studies were carried out with PAC-2 or AB9 cells transiently

transfected with the constructed luciferase-reporter vectors. Transfections were performed using Fugene HD (Roche Diagnostics) following the manufacturer's instructions. Briefly, the cells were seeded at a density of $3x10^4$ cells per well in a 96-well fluo-assay plate (Perkin Elmer, 6005680) and maintained at 26°C. The next day, a mixture composed of 80 ng of constructed vectors, 0.32µl of Fugene HD and 3.68 µl L-15 medium per well were prepared and incubated for 30 min at room temperature. The cell medium per well was refreshed with 150 µl L-15 medium with 20% fetal calf serum and the Fugene mixture. The tranfection proceeded for 24 h at 26°C. Then the medium was replaced with 200µl luciferin medium (L-15 with 20% serum, 1% P/S, 50µg/ml gentamicin and 0.5mM luciferin (Promega)). For the lighting entrainment of the cells, the 96-well plate was sealed with an adhesive "Top Seal" sealing sheet (Perkin Elmer, 6050185) and transferred into an EnVision multilabel counter (Perkin Elmer) or a Topcount NXT automatic scintillation counter (2-detector model, Perkin Elmer) for bioluminescence assay. The cells were exposed to 12h: 12h light-dark cycles under constant temperature. For the serum shock experiment, after the transfection the cell medium was replaced with 200µl luciferin medium with a range of serum concentrations (1%, 10%, 20%) and the cells were incubated at 26°C for 2 days in complete darkness. Subsequently, the culture medium was refreshed with 200µl luciferin medium again with different serum concentrations according to the experimental design. The cellular bioluminescence was monitored on an EnVision or a Topcount under constant darkness. Bioluminescence data was exported every 30 min as the frequency of photon emission.

2.4 ^{1}H-NMR assay

2.4.1 Sample preparation for ^{1}H-NMR

According to different experimental designs, time series of adult zebrafish livers were collected and frozen quickly in liquid nitrogen and stored at -80°C. Considering ideal freezing and storage conditions for pH-sensitive samples [283], the frozen tissues were lyophilized in lyophylisator (Christ) overnight. After addition of ceramic beads (91-PCS-CK14, PeqLab) and 1 ml of acetonitrile / H_2O (1:1, HPLC grade, Carl Roth), the samples were homogenized for 25 minutes at 4 °C with a neolab Intellimixer (program: u1, speed: 99 rpm). The homogenates were incubated on ice for 10 min and then centrifuged at 14,000 rpm for 13 minutes at 4°C. 850µl supernatants were transferred to new 1.5 ml eppendorf tubes, frozen immediately in liquid nitrogen and lyophilized overnight.

The extraction protocol for NMR measurement of zebrafish or cavefish cells was established based on the sample preparation for adult fish tissues. When zebrafish and cavefish cell lines propagated in 175cm^2 flasks were confluent, they were split by trypsinization and seeded in 75cm^2 flasks at different ratios as follows: PAC2 and CLOCK1 DN cells were split at a ratio

1:8, AB9 were split at a ratio 1:6 and CF1 were split at a ratio 1:3. When the cells were well entrained under different conditions, they were harvested and extracted for NMR metabolomics analysis. First, the culture medium was discarded and the cells were washed twice with 10 ml 1×PBS. Then 1.8 ml of acetonitrile / H_2O (1:1) were added and the cells were scraped off on ice. The cell suspensions were removed to 2 ml eppendorf tubes by pipetting and centrifuged at 14,000 rpm for 13 minutes at 4°C. The supernatants were transferred to new 2 ml eppendorf tubes, frozen immediately in liquid nitrogen and lyophilized overnight.

For ^{1}H-NMR measurement, the extracts were dissolved in 800 µL of a mixture of D_2O (deuterium oxide) and buffer (1.5 M KH_2PO_4, 2 mM NaN_3, 0.1% (v/v) TSP (3,3-trimethylsilyl-2,2',3, 3'-tetradeuteropro pionic acid) in D_2O, pH 7.1) (9:1 for liver extracts and 99:1 for cell extracts) and mixed for 5 min at room temperature with a neolab Intellimixer (program: u1, speed: 85 rpm). The mixture was centrifuged at 15,000 rpm for 10 minutes and 600 µl of supernatant was transferred into a 5 mm standard NMR tube for spectral acquisition.

2.4.2 ^{1}H-NMR analysis

^{1}H-NMR analysis was performed as described previously [275]. Spectra were recorded on a Bruker Avance II spectrometer using a ^{1}H-BBI double resonance probe (Bruker Biospin GmbH). 1D NOESY spectra were recorded with presaturation for water suppression and 256 scans at 300 K (26.9 °C). A prescan delay of 4 s was used together with a mixing time of 10 ms. Pulse lengths were determined automatically by the Bruker AU program pulsecal. 64k data points corresponding to a sweep width of 12,345.68 Hz were recorded.

2.4.3 Processing of ^{1}H-NMR data

All spectra were treated identically using an exponential apodization function, introducing an additional linewidth of 0.3 Hz and automated phasing, baseline correction and referencing using the Bruker macro apk0.noe. Preliminary peak assignment was done using databases (Chenomx (Chenomx Inc.), BBIOREFCODE (Bruker BioSpin GmbH), The Human Metabolome Database (HMDB)) and confirmed by spiking pure substances in the samples. Integration was done using AMIX (Bruker Biospin GmbH). Spectra were scaled to total intensity and by the probabilistic quotient method [284]. Circadian oscillations of spectra were identified by fitting the intensities across 24 hours to a cosine function with periods between 20-28h and shifting phases $Bj(t)=\cos(2\pi t/T-\varphi j)$, where $T=24$, $\varphi j=2\pi j/144$, and $0\leq j \leq 143$. The circadian phase was calculated from the best fitted $Bj(t)$ as $\varphi j*24/2\pi$.

2.4.4 Analysis of cavefish metabolome data

Analysis of metabolome data of cavefish cell lines is similar to the method we described before [285]. Briefly, cosine functions $A_{ij}(t)=\cos(2\pi t/T_i-\varphi_j)$ where $T_i=40+i$, $\varphi_j=2\pi j/60$, $0 \leq i \leq 5$, and $0 \leq j \leq 59$ were used as the reference time series of circadian oscillation. The time series expression of each spectrum was fitted to each cosine function time series $A_{ij}(t)$ and the cosine function with highest correlation coefficient was chosen. A p value <0.05 in the regression for the best cosine function was used as the criterion for circadian oscillation, and we estimated a false positive rate of about 10% for this cutoff using a random permutation test.

Chapter 3

Results

3.1 The FEO in the adult zebrafish liver

3.1.1 FAA can be entrained by regular feeding in adult fish

In collaboration with the group of Vazquez-Sanchez (University of Murcia, Spain), the Foulkes group has established a "conflicting zeitgeber" paradigm for defining which circadian rhythms of gene expression are linked with the FEO (feeding entrainable oscillator) and which are derived from the LEO (light entrainable oscillator). The first step of my work was to entrain wild type zebrafish (Wik strain) using the same experimental paradigm. As shown in Figure 15A, adult zebrafish were maintained under a 14h: 10h light-dark cycle and then fed once per day (ZT0 is lights-on and ZT14 is lights-off). They were categorized into three different groups according to the feeding time: fish fed once a day at mid-light (ML, ZT7), fish fed once a day at mid-dark (MD, ZT19), and fish fed once a day randomly (RD). In this experiment, the fish were exposed to two different zeitgebers: light is a constant signal and food is a variable one. After one month, the fish in the three different groups were well entrained according to the behavior recording (Figure 15B). Specifically, general locomotor activity in the ML group was predominantly diurnal, in the MD group it was nocturnal and the RD group exhibited irregular activity with characteristic increases in activity after the feeding time. FAA was clearly observed in both ML and MD groups. In our further experiments, we only focused on the measurement on ML and MD fish.

3.1.2 The TTFL is unaffected by feeding entrainment

In order to investigate the influence of feeding entrainment in zebrafish, we first examined the expression of core clock genes which represent elements of the clock TTFLs in the fish liver. When all the fish in ML and MD groups were adapted to the regular feeding cycles as assayed by behavioral analysis, a time series of livers was sampled at 4h intervals (Figure 15A). Then the expression of *bmal1*, *bmal2*, *bmal3*, *per1b*, *per2, clock1*, *cry1b* and *cry2a*, which are components of main feedback loop, *reverbα* that serves in additional feedback loop as well as *dbp1*, *dbp2* and *tefa* which are BMAL:CLOCK-controlled genes were assayed using QPCR. As shown in Figure 16, while they all exhibit robust rhythmic expression, none of them showed a phase shift between the ML and MD groups. This result indicates that rhythmic clock gene expression within the core clock TTFL mechanism is not associated with the feeding regulated clock in zebrafish. It is therefore likely that the FEO has an independent clock mechanism which may be composed of novel circadian elements.

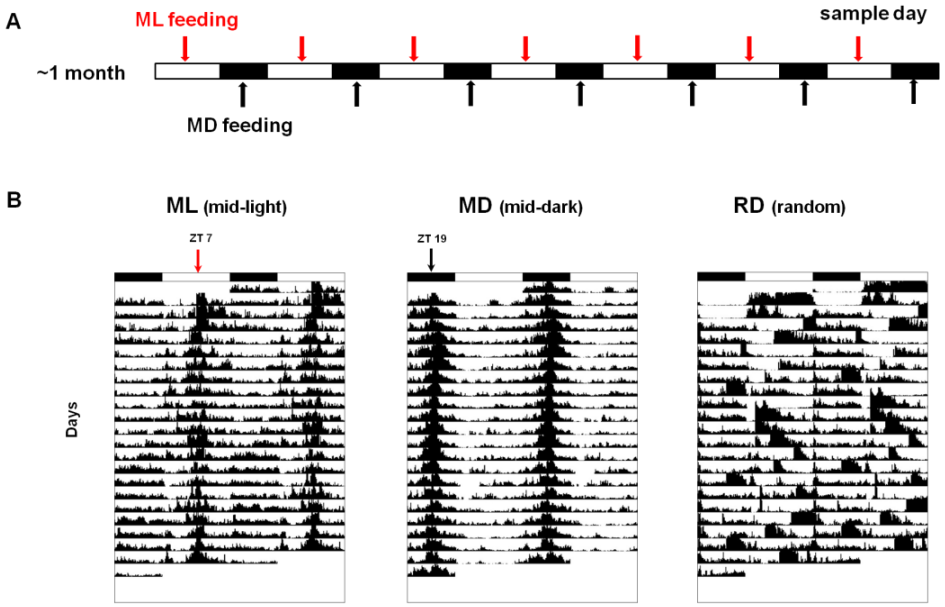

Figure 15: The entrainment of adult fish under light-dark cycles and three feeding regimes (A) The scheme illustrates the "conflicting zeitgeber" paradigm. (B) Locomotor activity in both ML and MD groups synchronizes with periodic feeding phase and regular food availability can entrain FAA. Red and black arrows indicate the mealtime in ML and MD groups, white and black bars represent the light and dark periods, respectively. Behavioral data are double plotted on a 48 h time scale to aid interpretation, the y-axis progresses in single days with each day being plotted twice (day 1 on the right side is repeated on day 2 on the left side). The activity was binned every 10 min, the height of each point represents the number of interruptions of the infrared light beam.

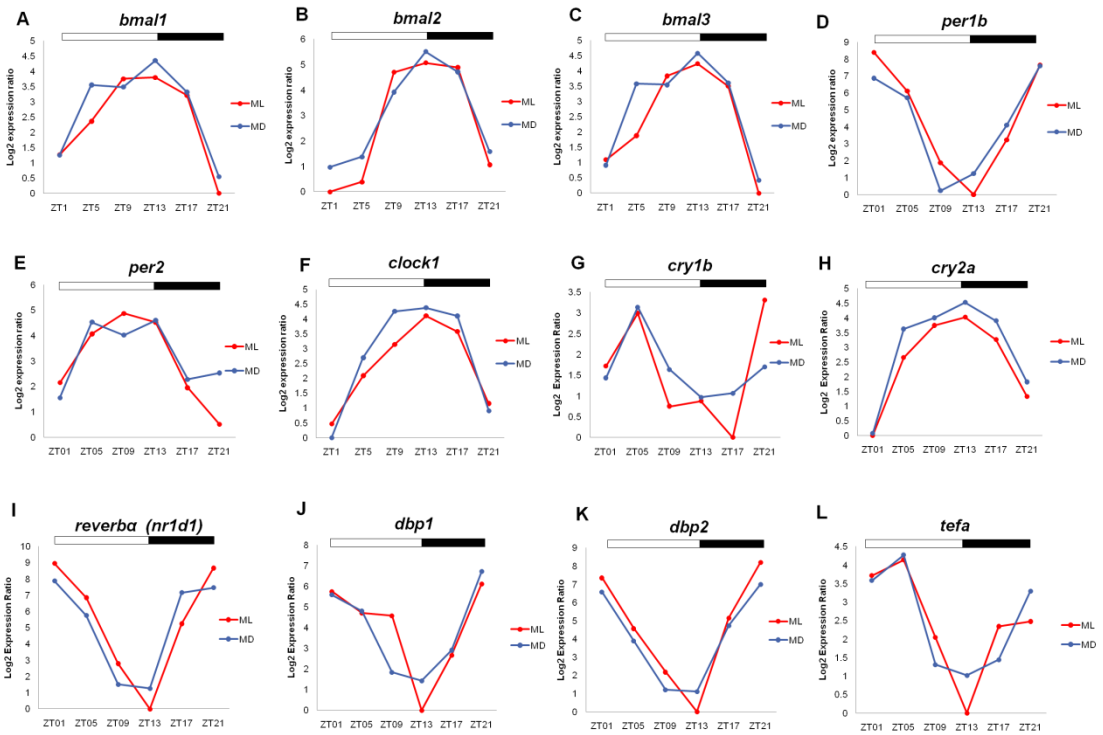

Figure 16. The rhythmic expression of core clock genes of TTFLs under light-dark cycles and different feeding regimes

Each sample is a pool containing RNAs of three livers with equal amounts. The lowest log2-transformed expression level for each gene was normalized to zero. White and black bars represent the light and dark periods, respectively. Cosine fitting, $p<0.05$.

3.1.3 The FEO and LEO control different metabolites in the zebrafish liver

As the circadian metabolic function within the liver is likely to be a key regulatory target of the feeding regulated clock, the fully entrained fish in the ML and MD groups were fasted and livers were collected at 6h intervals spanning 24h for metabolome measurement using hydrogen nuclear magnetic resonance (^{1}H NMR) spectroscopy (Figure 17A). A total of 146 peak signals were quantified based on their spectral intensities. 107 of them could be assigned to known metabolites. As several spectra represent the same metabolite, in total 36 metabolites have been detected. By cosine fitting, we have identified circadian metabolites in both ML and MD livers ($p\leq 0.05$) and then clustered them according to their metabolic pathways. All the detected metabolites belong to seven metabolic pathways: carbohydrate, nucleotide, essential amino acid, non-essential amino acid, co-factor, xenobiotics and energy (Table 2). Essential amino acids and non-essential amino acids in the fish were grouped on the basis of their biosynthetic routes in KEGG databases. Like mammals, the circadian metabolites within the same pathway have similar acrophases in the fish livers.

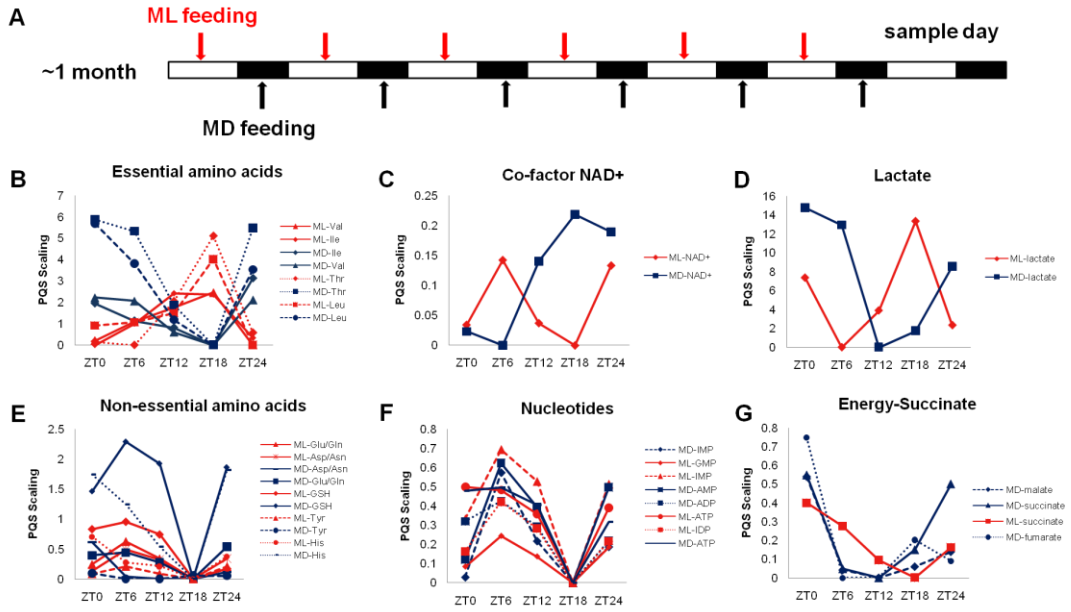

Figure 17. Circadian metabolites in adult fish livers

(A) Schematic illustration of fish entrainment and sample collection for NMR. White and black bars represent the light and dark periods, respectively. The rhythmic levels of FEO regulated metabolites: (B) essential amino acids (C) NAD+ (D) lactate, and LEO regulated metabolites: (E) non-essential amino acids (F) nucleotides (G) succinate in both ML and MD fish are shown. The y-axis is scaled intensity in NMR measurement relative to the sample with the lowest intensity for each metabolite. Every time point per group has at least three biological replicates. For clarity, error bars in each time point per group are not added. Cosine fitting, $p<0.05$.

Table 2: The circadian phases of metabolites in different metabolic pathways

Metabolic Pathway	ML	MD	Phase shift (ML vs MD)
1. Carbohydrate	ZT3, ZT16	ZT3, ZT12	Unclear
	lactate ZT18	**lactate ZT3**	**lactate (15h)**
2. Nucleotide	ZT6,	ZT6,	No
	inosine ZT14	inosine ZT15	
3. Essential AA	**ZT16**	**ZT2-3**	**10-13h**
4. Non-Essential AA	ZT5-6	ZT2-6	No
5. Co-factor	**ZT2**	**ZT17**	**15h**
6. Xenobiotics	**ZT15**	**ZT2**	**13h**
7. Energy	ZT3, ZT7	ZT23	Unclear

AA: amino acids

By comparing the circadian phases, we have identified feeding regulated metabolites including essential amino acids, co-factors (NAD+) and lactate, which show a nearly anti-phase relationship between the ML and MD sets of samples (Figure 17B-17D). In contrast, non-essential amino acids, nucleotides and succinate which display a consistent expression under diurnal and nocturnal feeding entrainment are regulated by the LEO (Figure 17E-17G).

Among all the detected metabolites by NMR, more than 60% of them show rhythmicity and amino acids, carbohydrate and nucleotides account for a large proportion of circadian metabolites (Figure 18A). As shown in Figure 18B, the phases of circadian metabolites display a prominent bimodal distribution with peaks at ZT2-6 and ZT15-17 respectively under both ML and MD conditions. Most circadian metabolites peak during either the FAA period or the FAA anti-phase period. We also illustrate the temporal peaks of metabolites on the "zeitgeber lines" (Figure 18C). For the feeding clock regulated metabolites, the peak time of NAD+ (oxidized form of nicotinamide adenine dinucleotide) is located during the FAA period and others (lactate, essential amino acid, acetoacetate) are enriched at the FAA anti-phase period in both ML and MD zebrafish livers. In these cases, there is a strong correlation between metabolite phases and mealtime.

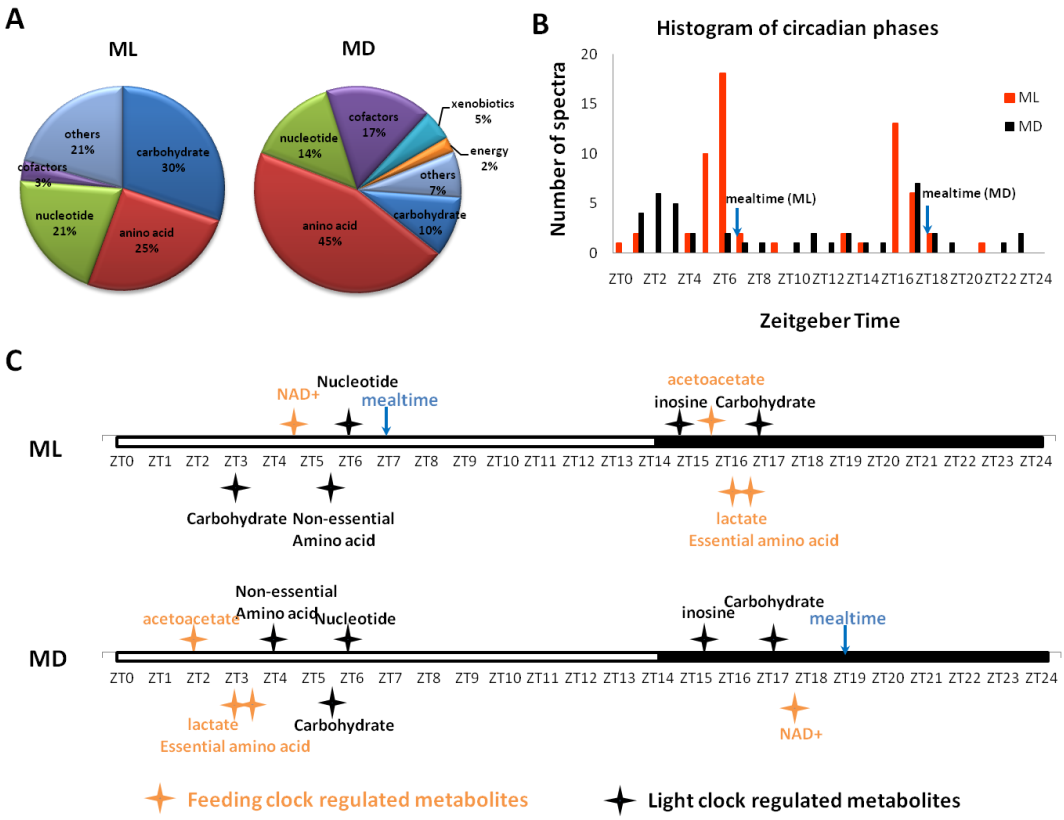

Figure 18. The distribution of circadian metabolites in the adult fish liver

(A) The pie charts illustrate the proportion of circadian metabolites in the fish liver. (B) Bimodal distribution of circadian phases of metabolites and (C) The peak time of metabolic clusters in ML and MD groups are shown. The bar on the x-axis indicates light (white) and dark (black) periods in LD.

3.1.4 FAA persists during food deprivation.

In order to explore the "free running" rhythms of the feeding entrainable clock, we performed short term food deprivation experiments. We ceased food delivery for the well entrained fish in both the ML and MD groups for two days and then returned them to their scheduled feeding regime (Figure 19A). FAA, which is recognized as an important output of the FEO persisted at its usual time during the 2 days of food deprivation (Figure 19B), suggesting that the FEO still functionally persists in the absence of regular food availability. Then we sacrificed the fish during the fasting period and sampled the livers for metabolome measurement using NMR spectroscopy. The temporal expression of essential amino acids regulated by the FEO and inosine controlled by the LEO are shown in Figure 19C. On the first day of food deprivation, rhythmic levels of essential amino acids show an approximate 12h phase shift while nucleotides have no phase shift between the ML and MD samples. On the second fasting day, the phase shift of essential amino acids became narrower (cosine fitting, 10h) and the rhythmic expression of inosine was unchanged and still governed by the light-dark cycle. Although light is the predominant zeitgeber in zebrafish clock entrainment, our data indicate that periodic feeding is also a powerful synchronizer to induce the phase resetting of a subset of metabolites in peripheral tissues.

3.1.5 NAD+ is under the control of both the FEO and LEO in adult fish liver

From our metabolome assay, we found that NAD+ levels appear to be controlled by both the LEO and FEO in the zebrafish liver. Specifically, on the first fasting day, NAD+ levels show inverted rhythmicity between the ML and MD samples, whereas on the second fasting day, the peak time of NAD+ has been delayed by 6h (from ZT6 to ZT12) in ML and advanced 6h (from ZT18 to ZT12) in MD, therefore the phase shift disappeared (Figure 20C). To explore the circadian regulation of NAD+ in depth, we extended the sample time to three days together and examined the expression of genes in NAD+ salvage pathways (Figure 20A). Sample day 1 is the last day of feeding entrainment, from sample day 2, all the fish were food deprived.

The NAD+ salvage pathway, which is conserved from yeast to mammals has been shown to be controlled by the circadian timing system. In mammalian cells, NAMPT is a rate-limiting

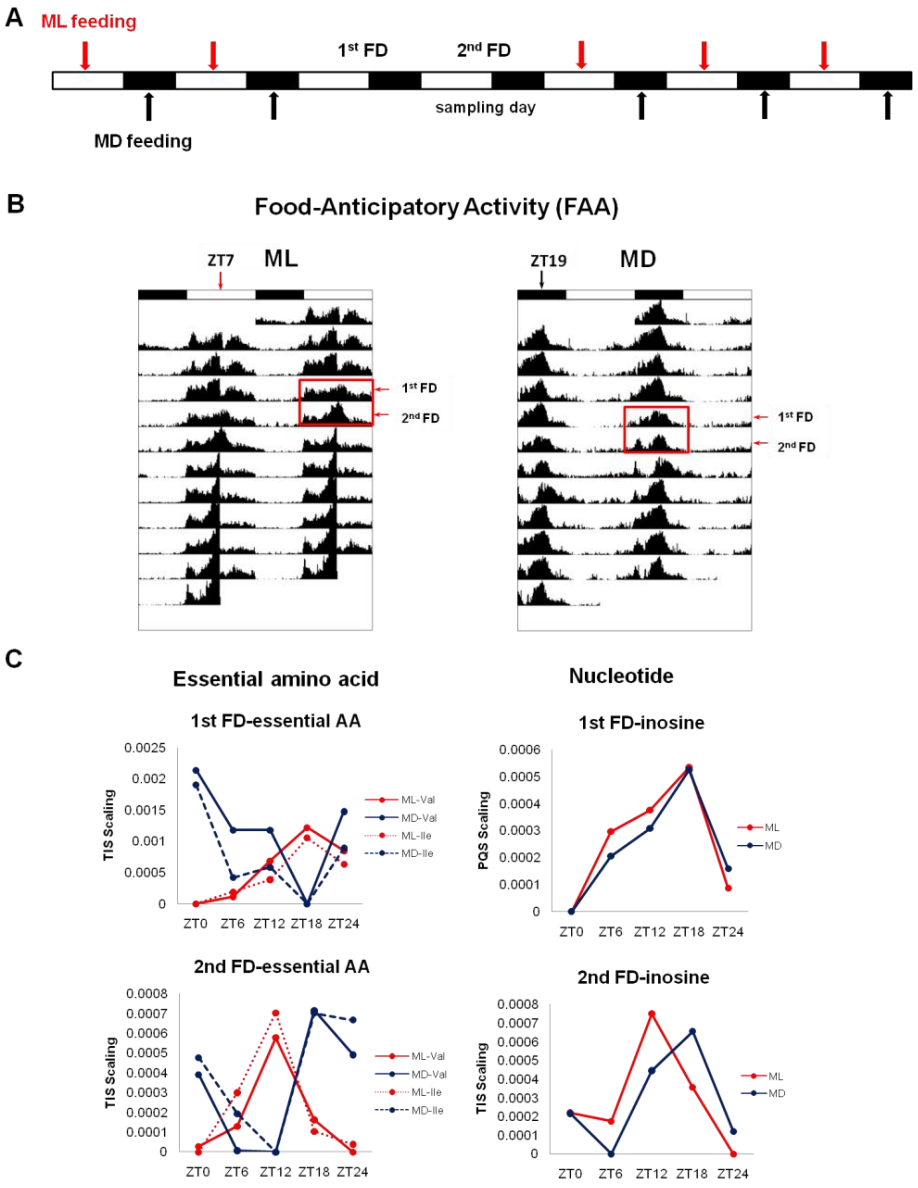

Figure 19: The persistence of FEO under food deprivation

(A) The scheme illustrates the food deprivation and re-feeding protocol for the well entrained adult zebrafish. White and black bars represent the light and dark periods, respectively. The red and black arrows indicate the mealtime of ML and MD group, respectively. FD: food deprivation. (B) The behavior recording shows FAA still appeared at the regular time when the fish were fasted for two days. (C) The temporal expression of FEO controlled essential amino acids and LEO controlled inosine on the first and second fasting day are shown. Each time point has at least three independent biological replicates. For clarity, error bars in each time point per group are not added. Cosine fitting, $p<0.05$.

enzyme for NAD+ biosynthesis and SIRT1 catalyzes the NAD+ degradation to NAM. Due to the gene duplication occuring during early teleost evolution, there are two *nampt* genes in zebrafish (Figure 20B): *nampta* and *namptb*. From the Q-PCR results (Figure 20D), we can see that the rhythmic expression of *nampta*, *namptb* and *sirt1* is specifically reset by the feeding time. The oscillatory patterns of *namptb* and *sirt1* are quite similar. On sample day 1, they present anti-phase cycling between ML and MD, the expression in MD has an apparent phase shift when the fish were starved and it is in-phase with the ML rhythmicity on the last sample day. By contrast, the rhythmic expression pattern of *nampta* in MD is consistent during these three days with peak time around ZT0 (ZT0 is lights-on and ZT14 is lights-off), but the circadian phase of ML is reset by the FEO. Until now, we have not yet determined which *nampt* gene plays a more prominent role for NAD+ biosynthesis in zebrafish, but our results reveal that both the FEO and LEO gate hepatic NAD+ levels through their control of the NAD+ salvage pathway. It seems that NAD+ links the two types of clocks and it could represent a key molecule to study the FEO mechanism.

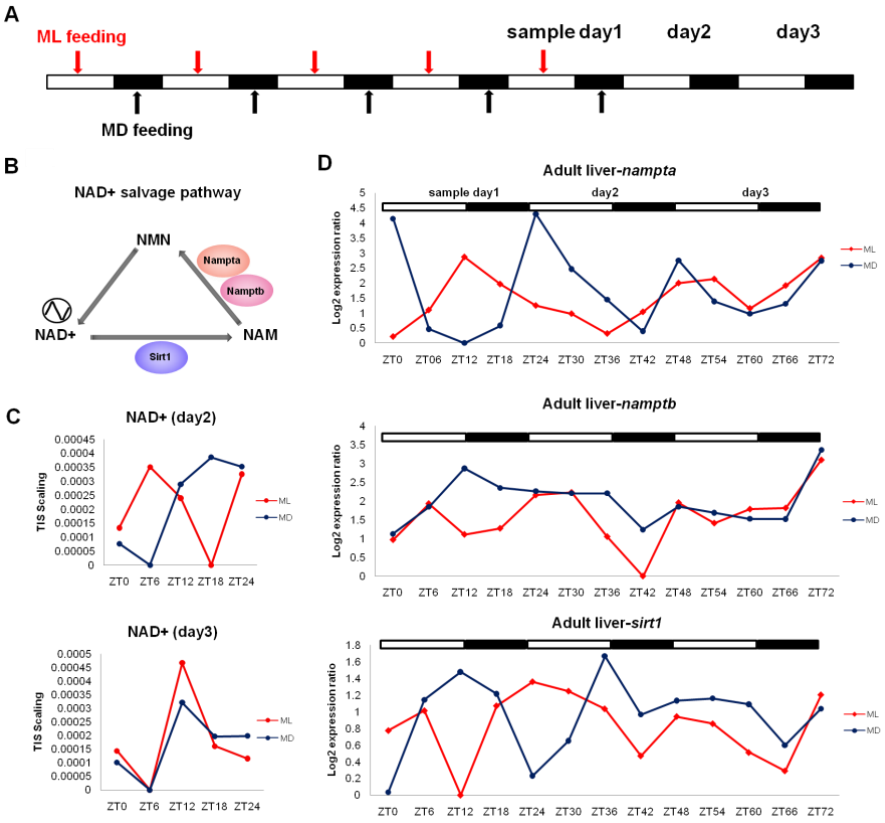

Figure 20. The NAD+ salvage pathway is under the control of both the LEO and FEO in adult zebrafish

(A) Graphic illustration of fish entrainment and three days sample collection. (B) Scheme of the NAD+ salvage pathway. *nampta* and *namptb* encode the rate-limiting enzyme for NAD+ biosynthesis, Sirt1 mediates the NAD+ degradation. (C) The cyclic expression of NAD+ in fasted fish liver was detected by NMR. Cosine fitting, $p<0.05$. (D) The daily expression of *nampta*, *namptb* and *sirt1* in livers from food-entrained and fasted zebrafish was checked by Q-PCR. Each sample is a pool containing RNAs of two independent livers with equal amounts. The lowest log2-transformed expression level for each gene was normalized to zero. White and black bars represent the light and dark periods, respectively.

3.1.6 Metabolic genes show oscillating expression in adult zebrafish liver

We next analyzed the expression of genes participating in the anabolism or catabolism of identified hepatic metabolites. As fish lack the enzymes required for essential amino acids biosynthesis, we examined the genes encoding key or rate-limiting enzymes in non-essential amino acid and nucleotide metabolic pathways. The metabolic genes tested included *got1*, *got2a* and *got2b* (glutamic-oxaloacetic transaminase 1, 2a and 2b) linked to aspartate (Asp) biosynthesis pathway, *asns* (asparagines synthetase) mediating asparagine (Asn) production, *glud1b* (glutamate dehydrogenase 1b) for glutamate (Glu) synthesis, *glu1a* (glutamine synthetase 1a) catalyzing glutamine (Gln) formation, *gpt2l* (glutamic pyruvate transaminase 2, like) involved in alanine (Ala) biosynthetic reaction, *tdh* (threonine dehydrogenase) mediating threonine (Thr) degradation and *impdh2* (IMP dehydrogenase) encoding a rate-limiting enzyme in *de novo* purine synthesis. The liver samples collected on sample day 1 were used for the Q-PCR analysis (Figure 20A). As shown in Figure 21, all the detected metabolic genes are expressed in a rhythmic manner. The cyclic expression of *got1*, *got2a*, *got2b*, *asns*, *glud1b*, *glu1a* and *gpt2l* that encode the enzymes contributing to non-essential amino acid biosynthesis show no phase difference between ML and MD, which correlates with the observed cycling levels of non-essential amino acids in livers measured in our NMR experiments. Similarly, the phase of rhythmic mRNA expression of enzymes involved in nucleotide metabolism, like the actual levels of nucleotides is not influenced by feeding entrainment. Thus, *impdh2* mRNA expression involved in *de novo* synthesis of GTP peaks at the same time point in both ML and MD conditions. Rhythmic levels of threonine (Thr), an essential amino acid exhibits an approximately 12h phase shift between ML and MD, however, *tdh* which converts threonine to L-2-amino-3-oxobutanoate does not exhibit a phase shift between daytime and nighttime feeding. Thus, together these data suggest that transcriptional rhythms are tightly linked with the levels of circadian metabolites in adult livers.

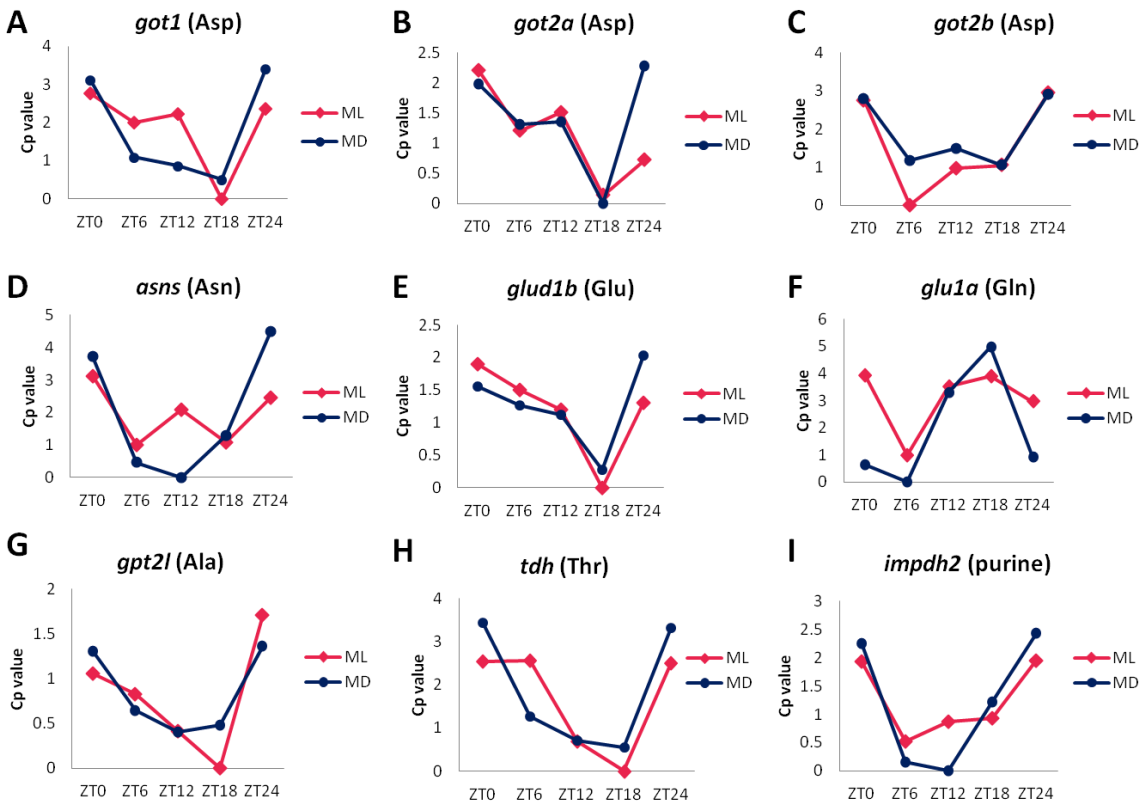

Figure 21. The cyclic expression of metabolic genes in the fish liver

(A) *got1* (B) *got2a* (C) *got2b* (D) *asns* (E) *glud1b* (E) *glu1a* and (E) *gpt2l* encode key enzymes mediating anabolism or catabolism of amino acids. (I) *impdh*2 is a rate-limiting gene in *de novo* purine synthesis pathway. The lowest log2-transformed expression level for each gene was normalized to zero. Cosine fitting, $p<0.1$

In summary, we have revealed that a FEO exists in the adult zebrafish by showing that regular, restricted food intake can entrain FAA independently of the timing of the light-dark cycles. By performing a circadian metabolome analysis using NMR and comparing the circadian phases of metabolites between ML and MD, we have identified LEO controlled circadian metabolites including nucleotides and non-essential amino acids, and FEO gated rhythmic metabolites including essential amino acids. NAD+, which mainly operates as a cofactor and occupies a central position connecting most circadian metabolic pathways, is under the control of both oscillators in the adult liver (Figure 22).

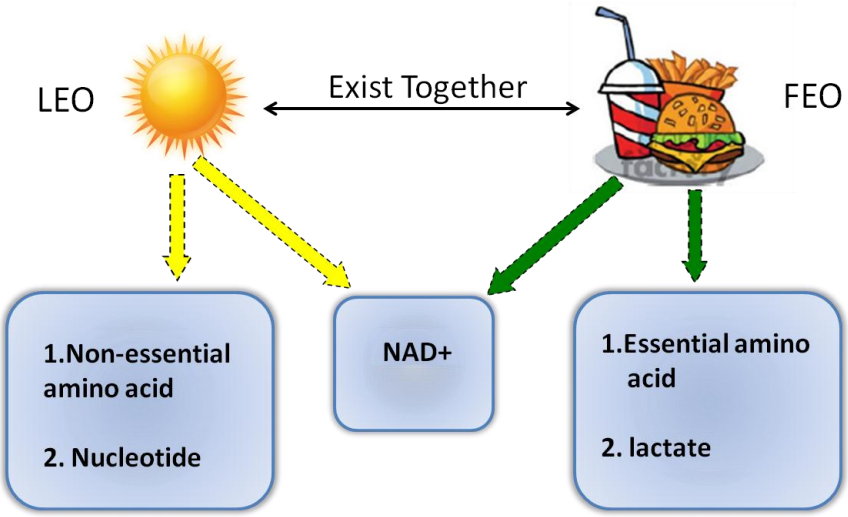

Figure 22: Graphic illustration of the relationships between the LEO and FEO in zebrafish

Both oscillators co-exist in the fish liver and regulate different circadian metabolites. NAD+ could function as a link between the two oscillators.

3.2 Metabolic oscillations in zebrafish and cavefish cells

It is well known that many metabolic processes are regulated by the circadian system in a wide range of species. One of the significant features of the zebrafish circadian system is that its peripheral clocks display direct light responsiveness and rapid entrainment in a cell autonomous manner. The rhythmic expression of some core clock genes of TTFLs can be induced by light-dark cycles in zebrafish peripheral tissues and cell lines directly. Unfortunately, the relationships between cellular metabolism and peripheral clocks have not yet been addressed. In addition, very little research has addressed how the peripheral clocks are regulated by non-photic zeitgebers, such as serum, temperature and signal transduction pathway regulators, in fish cell lines.

3.2.1 The regulation of cellular metabolism in zebrafish hepatocytes is via an oscillator that is regulated independently of light exposure

Does the feeding regulated clock or circadian cycling of metabolites also exist in isolated hepatocytes in the absence of central pacemakers and systemic signals? To answer this question, we prepared primary hepatocytes cultures from adult male zebrafish (Figure 23A). The cultured hepatocytes can be kept *in vitro* for up to 10 days. In order to assess whether hepatic fibrosis or degeneration occurred during *in vitro* culture, firstly we checked the expression of hepatic biomarkers including *cfb* (complement factor B) [286], *fabp10a* (fatty acid binding protein 10a) [287,288], *fgg* (fibrinogen, gamma polypeptide) [289], *hnf4a* (hepatocyte nuclear factor 4, alpha) [290], *tat* (tyrosine aminotransferase) [291], which have

been reported to be expressed specifically in zebrafish livers (Figure 23B). Adult liver and hepatocytes showed similar mRNA levels of these biomarkers, which are much higher than that in PAC2 cells, indicating the primary hepatic cells can be used for metabolic function studies *in vitro*.

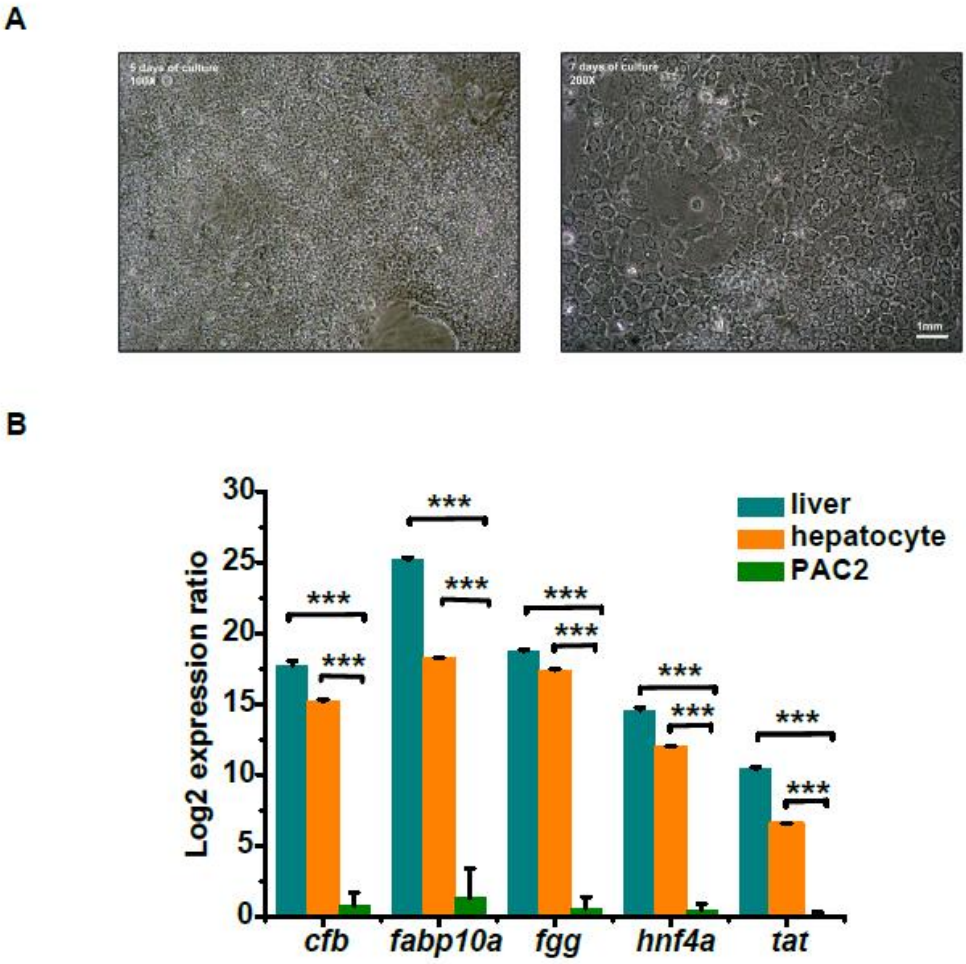

Figure 23. Primary cultures of zebrafish hepatocytes

(A) Morphology of primary cultured hepatocytes from male zebrafish. Images were taken at 5 days and 7 days after seeding. (B) The mRNA expression of hepatic biomarkers in livers, hepatocytes and PAC2. Livers from male fish which were adapted to LD cycle and sampled at ZT0 (8:00), hepatocytes entrained in DD and collected at 8:00 of 5 days post seeding, PAC2 cells incubated in DD and harvested at 8:00 were used for the Q-PCR assay. Error bars represent the standard error of mean (SEM) among independent technical replicates. *** $p<0.001$

Then we entrained the hepatocytes under three different lighting regimes: light-dark cycle (LD, 12h:12h), dark-light cycle (DL, 12h:12h) and constant darkness (DD), harvested a time series of cells and examined the metabolic gene expression by QPCR (Figure 24A). The

light-dark cycles entrained robust rhythmic expression of *per1b* (Figure 24B), and thereby we confirmed that the hepatocytes were fully synchronized by the three lighting regimes. However, the *nampta*, *namptb*, *sirt1* and *asns* which showed oscillating expression in adult fish liver are arrhythmic in primary hepatic cells (Figure 24B). Next we performed a metabolome assay of hepatocytes which were entrained under LD and DL conditions (Figure 24A). A total of 106 peak signals were quantified and assigned to 33 known metabolites. By cosine fitting, we have found about 60 to 70 percent of metabolites show strong circadian periodicity in LD and DL groups (Figure 24C). We then clustered all the circadian metabolites according to their metabolic pathways. Like adult zebrafish liver, the cyclic metabolites within the same pathway display similar expression profiles and surprisingly, circadian metabolites in LD and DL showed no difference in terms of their circadian phases or expression levels, suggesting that the cellular metabolic oscillations are insensitive to light exposure. Unlike most metabolic groups which only peak at a single time point, non-essential amino acids exhibit peaks at two anti-phase time points. Here, combining our NMR data showing circadian cycles of metabolite levels which are phased independently of the lighting conditions with the arrhythmic metabolic gene expression in hepatocytes, we hypothesize that the metabolic oscillations may be driven by a non-transcriptional mechanism.

3.2.2 The canonical TTFLs are not required for metabolic oscillations in zebrafish fibroblasts

To address whether the light-independent metabolic rhythm exist widely in zebrafish cell lines, we next analysed the metabolome of other zebrafish fibroblast cell lines: PAC2 and AB9 by NMR assay.

3.2.2.1 Metabolic oscillations in PAC2 cells

3.2.2.1.1 The daily oscillation of metabolites is independent of light exposure in PAC2 cells

PAC2 is a fibroblast-like cell line derived from zebrafish embryos. It possesses a clock that is very sensitive to light entrainment and exhibits robust oscillation of clock- and light-regulated transcripts in response to exposure to light-dark cycles. In our experiment, the PAC2 cells were initially incubated under constant darkness for three days to ensure the loss of previous cellular clock synchronization, then they were entrained under three different lighting conditions: LD, DL and DD used as a control (Figure 25A). Then the adapted cells were harvested in time series and analyzed using NMR. A total of 33 known

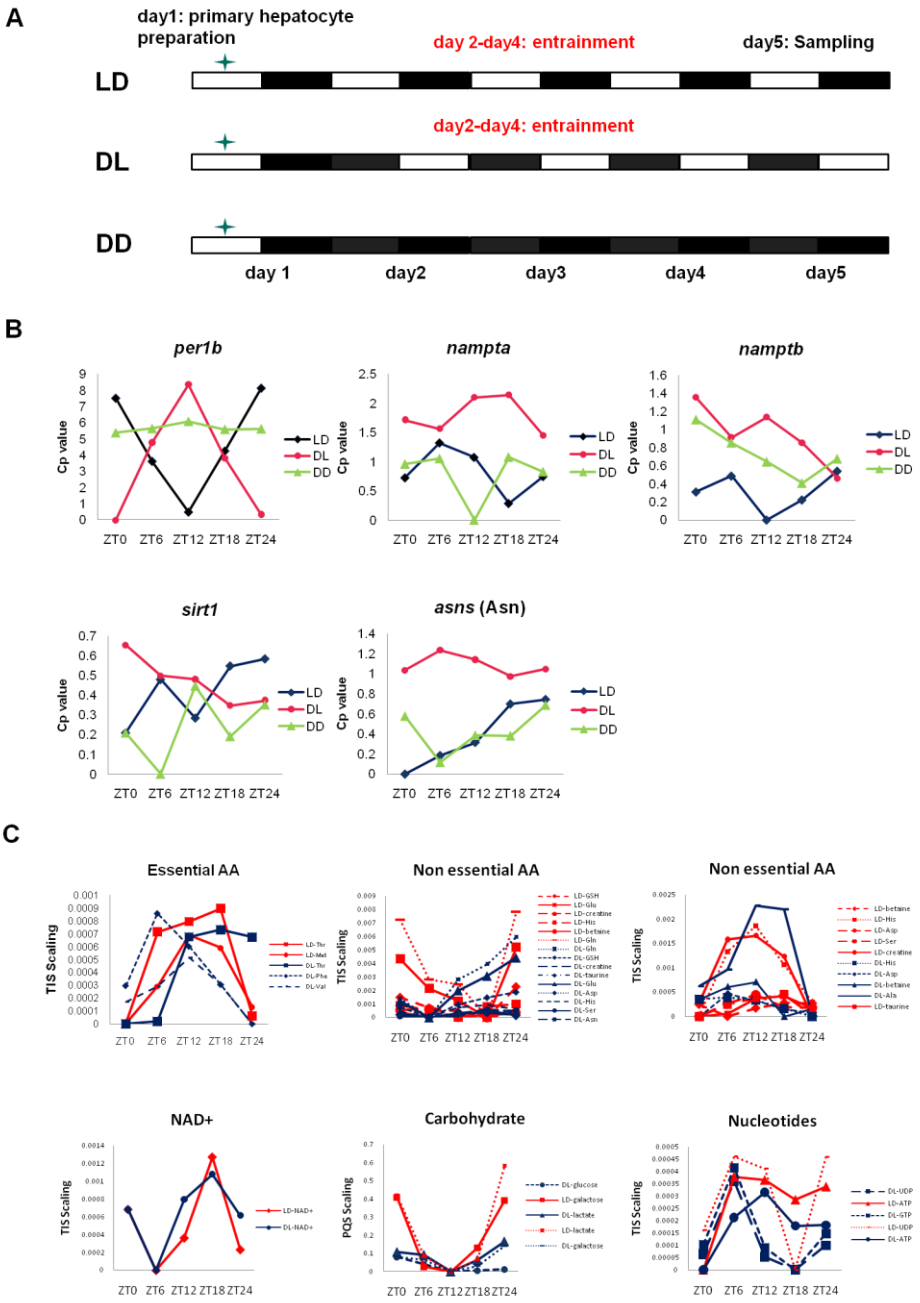

Figure 24. The daily expression patterns of metabolic genes and levels of metabolites in zebrafish primary hepatocytes

(A) Scheme of the three lighting regimes used to entrain the clocks in primary hepatocytes. The bars indicate light (white) and dark (black) in LD or DL, subjective day (grey) and subjective night (black) in DD. (B) The rhythmic expression of metabolism-related genes involved in NAD+ and amino acid biosynthetic pathways is absent in primary hepatocytes. *per1b* is used as a control to indicate all the cells were well adapted to the lighting conditions. (C) The temporal expression of hepatic circadian metabolites under LD and DL. The lights-on in the LD group is defined as ZT0. Each time point has three independent biological replicates. For clarity, error bars in each time point per group are not added. $p<0.05$, cosine fitting.

metabolites have been identified and more than 60 percent of them show rhythmic expression in the LD, DL and even DD groups. Amino acids accounted for a large proportion of all the rhythmic metabolites, (Figure 25B).

We then grouped all the circadian metabolites according to their metabolic pathways. Like hepatocytes, the cyclic metabolites have similar circadian phases between LD, DL and DD. Figure 26 shows the rhythmic expression of clustered metabolites under three lighting conditions in PAC2. Non-essential amino acids also have two anti-phase peaks. The intracellular cycling levels of most metabolites in DD show no significant differences between the metabolites in the LD and DL sets of samples. The phases of circadian metabolites in PAC2 display a prominent bimodal distribution. Essential amino acids, a subset of non-essential amino acids and carbohydrates peak around ZT5-8 (the lights-on in the LD group is defined as ZT0), which coincides with the middle of the subjective day. In contrast, the circadian phases of NAD+, a subset of non-essential amino acids and nucleotides are at ZT17-19, overlapping with the middle of the subjective night (Figure 27).. The results in PAC2 reinforce the notion that cellular metabolic rhythms are independent of the normal light entrainable peripheral clock.

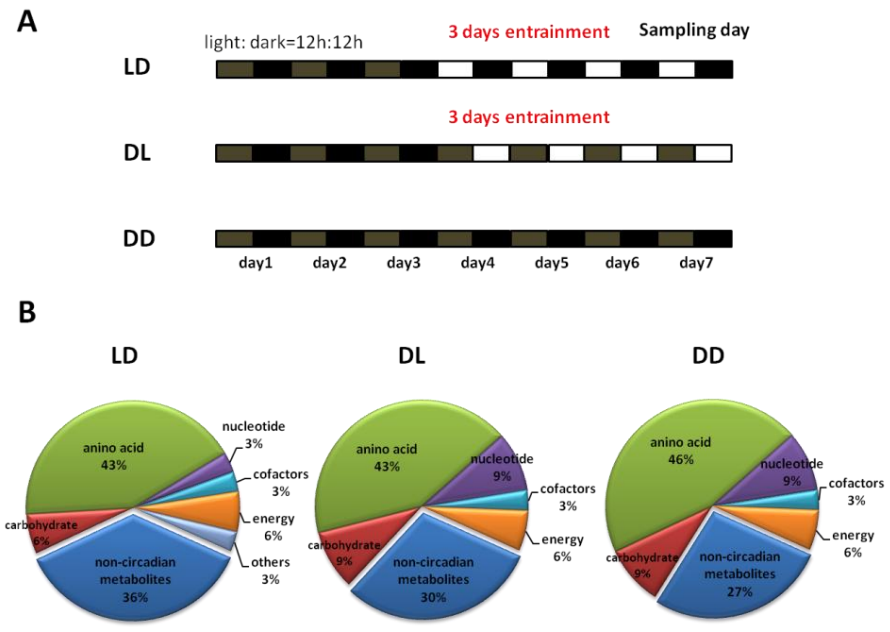

Figure 25. Metabolome measurement in PAC2 cells by NMR spectrometry

(A) Scheme of the three lighting regimes used to entrain the clocks in the PAC2 zebrafish cell line. The bars indicate light (white) and dark (black) in LD or DL, subjective day (grey) and subjective night (black) in DD. (B) The pie charts demonstrate that a large proportion of the identified metabolites in PAC2 cells exhibit circadian oscillation. The bars indicate light (white) and dark (black) periods, respectively.

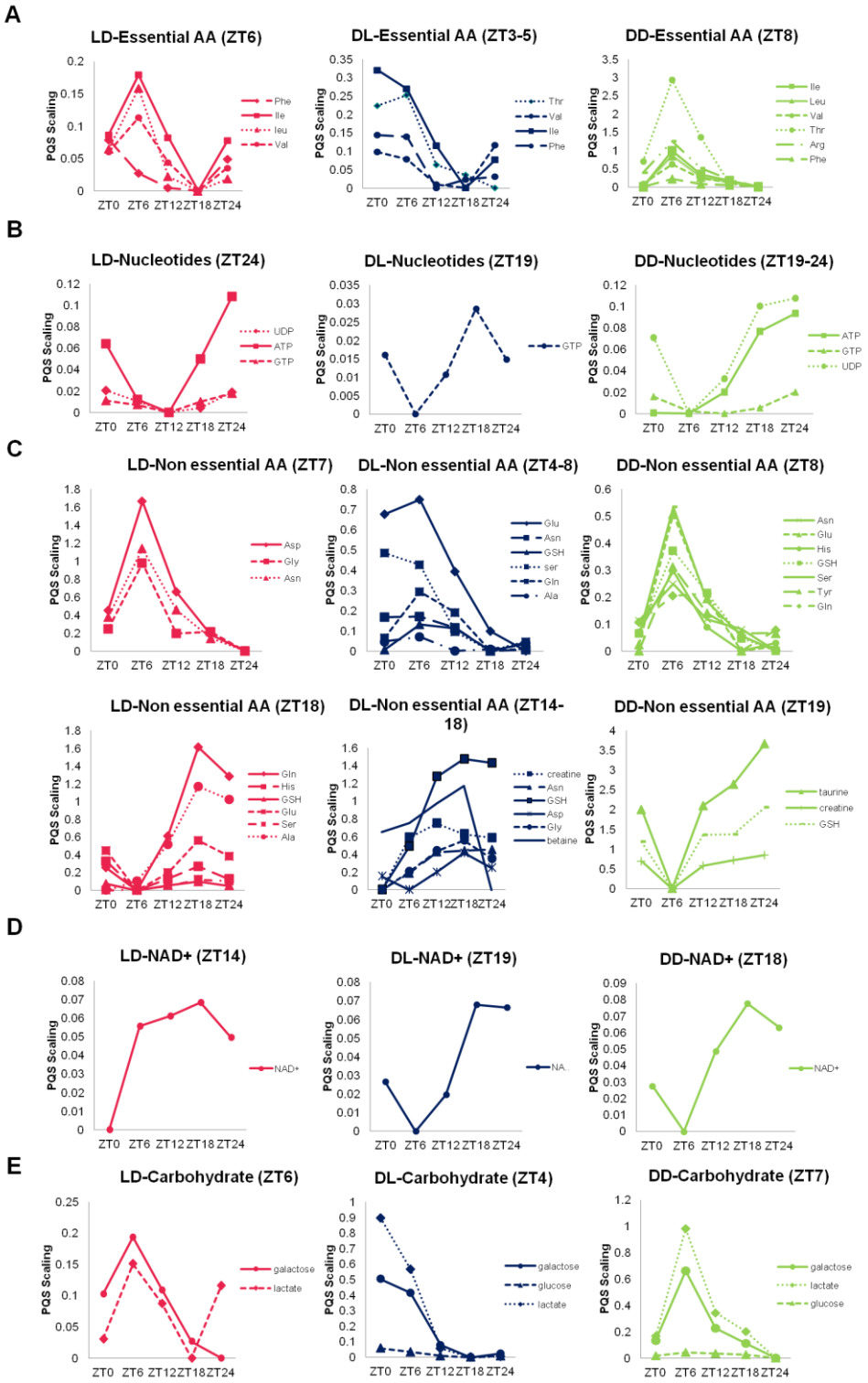

Figure 26. The daily oscillations of circadian metabolites under three lighting regimes in PAC2

The circadian phases of (A) essential amino acids (B) nucleotides (C) non-essential amino acids (D) NAD+ and (E) carbohydrates are similar between LD, DL and DD. The lowest level for each metabolite was normalized to zero. The lights-on in the LD group is defined as ZT0. Each time point has three independent biological replicates. For clarity, error bars in each time point per group are not shown. $p<0.05$, cosine fitting.

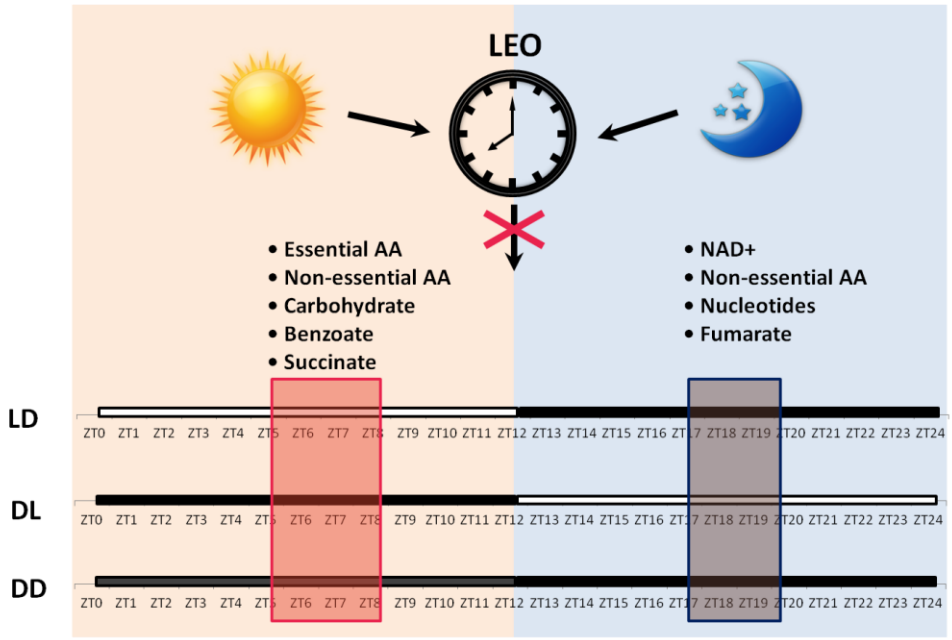

Figure 27. The phase distributions of cellular circadian metabolites in PAC2

The peak time for essential amino acids, some non-essential amino acids, carbohydrates, benzoate and succinate is around the middle of subjective day, and the phase of NAD+, the remaining non-essential amino acids, nucleotides and fumarate coincides with the middle of the subjective night. ZT0 represents lights on in the LD group. The bars represent light (white), dark (black) and subjective day (grey).

3.2.2.1.2 Metabolic genes are arhythmically expressed in PAC2 cells

We harvested a time series of mRNA from PAC2 cells which were fully entrained under LD, DL and DD conditions (Figure 25A). The expression of *nampta*, *namptb*, *sirt1* (NAD+ salvage pathways), *asns*, *got1*, *glu1a*, *tdh* (amino acids metabolic pathways), *impdh* (purine biosynthesis) are present in Figure 28. From the Q-PCR results, high amplitude rhythmic expression of *per1b*, an element of the circadian clock TTFL mechanism was efficiently entrained to the lighting cycles. However, the rhythmicity of all the tested metabolic genes was absent in PAC2 cells. These data again infer that the cellular metabolic oscillations could be driven by a novel mechanism, which is distinct from the classical TTFLs core circadian clock mechanism.

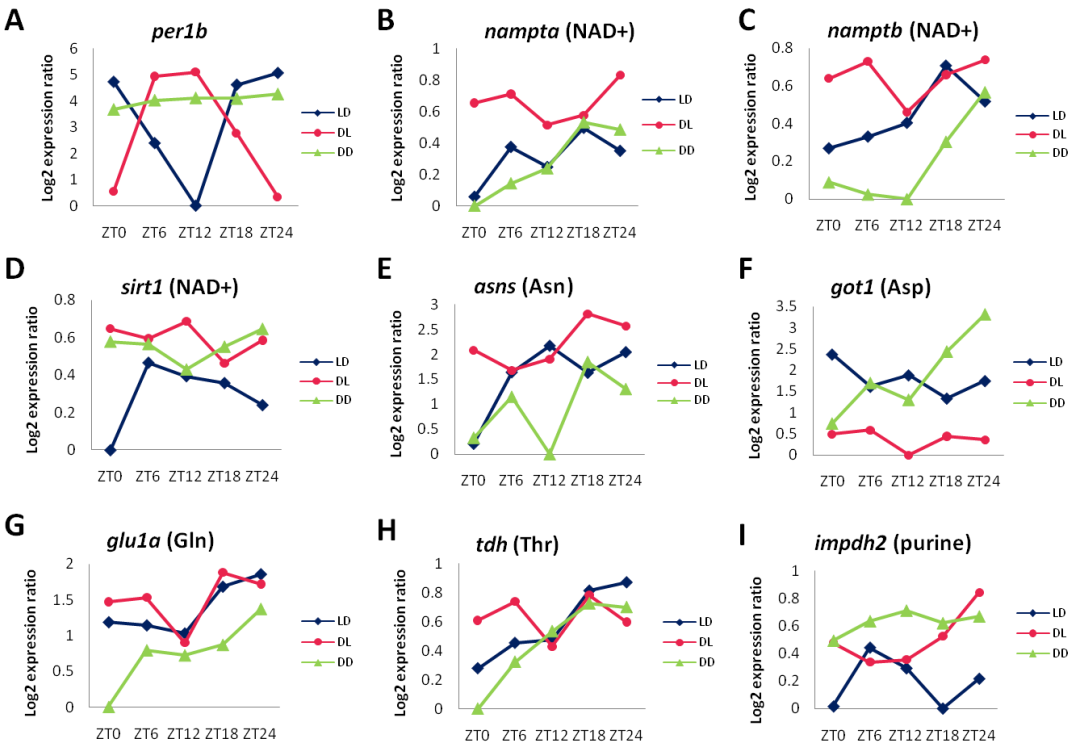

Figure 28. Arhythmic expression of metabolism-related genes in PAC2

(A) *per1b* used as a control reveals that the cells are efficiently entrained to the three lighting conditions. (B) *nampta* (C) *namptb* (D) *sirt1* encode enzymes in the NAD+ salvage pathway. (E) *asns* (F) *got1* (G) *glu1a* (H) *tdh* are involved in amino acid metabolic pathways and (I) *impdh* is an enzyme catalyzing *de novo* purine synthesis. The lowest log2-transformed expression level for each gene was normalized to zero.

3.2.2.1.3 Arhythmic luciferase expression driven by the *namptb* promoter

In the next step, to specifically examine the transcriptional control of metabolic genes, we constructed *zf namptb*-Luc which contains a promoter region extending 367 bp upstream of the *namptb* start codon site. The mammalian *Nampt* promoter has three highly conserved E-boxes which can be activated by associating with a complex composed of CLOCK: BMAL1:SIRT1 and then drives the circadian expression of *Nampt* in cell lines [211]. Our *namptb* luciferase reporter includes the corresponding zebrafish E-boxes sequences, which show some differences compared with the conserved E-boxes in mammals (Figure 29A). Using a promoter transient expression assay in PAC2 and AB9 cells, we observed that expression of the *namptb* promoter reporter construct did not show circadian oscillation under LD cycles. *zf per1b*-Luc including 3.1kb of the zebrafish *per1b* promoter was used as a control (Figure 29B and 29C) and as expected, showed rhythmic expression entrained by the phase of the LD cycle. Together, our findings suggest that the clock regulation of metabolite levels may not be mediated via transcriptional control in zebrafish cells.

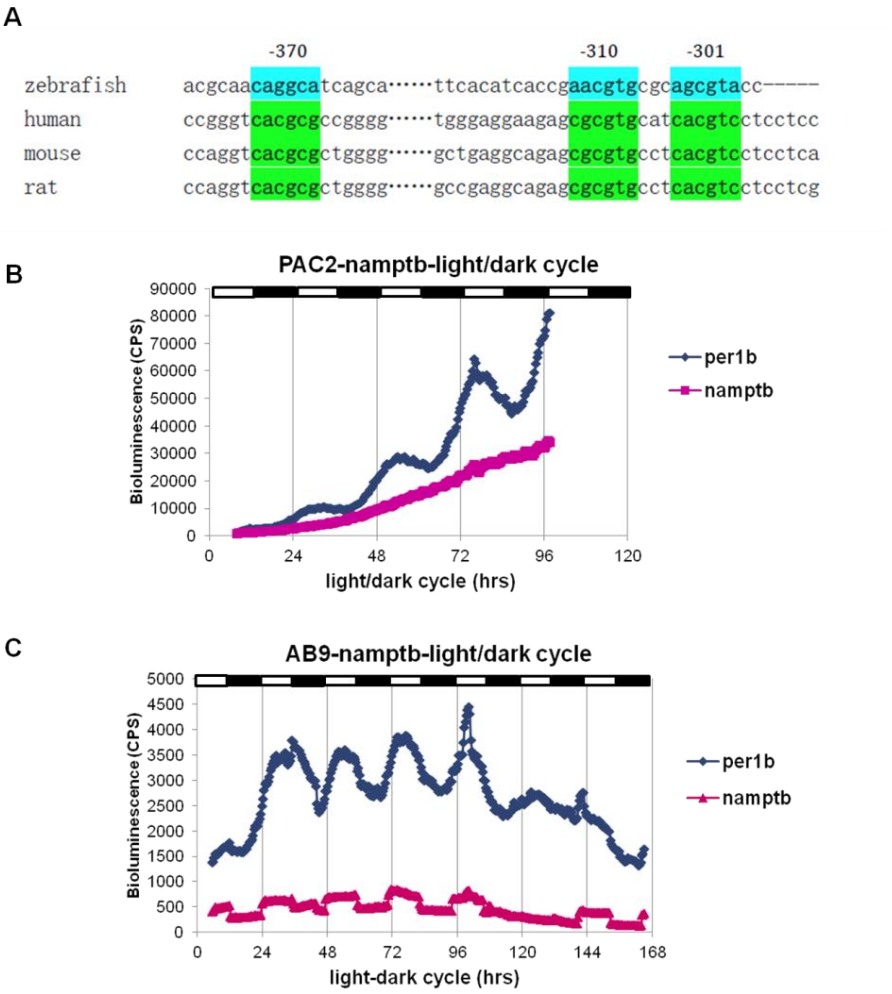

Figure 29. Arhythmic expression of the *namptb* promoter in zebrafish cell lines under LD

(A) Alignment of the mammalian *Nampt* promoter containing three conserved E-boxes with the corresponding sequence of zebrafish *namptb*. Green highlights the E-boxes that are conserved in mammals and blue highlights the corresponding sequence of the zebrafish E-box. (B) PAC2 and (C) AB9 cells transfected with *zf namptb*-Luc (pink trace) and *zf per1b*-Luc (blue trace, control) were exposed to LD cycles. Y-axis represents the relative bioluminescence and the x-axis indicates the zeitgeber time (ZT0 is lights on). Each rhythm is plotted as a mean of n=8 independent wells. The graphic is a representative experiment of three independent biological replicates.

3.2.2.2 Metabolic oscillations in AB9 cells

3.2.2.2.1 The expression patterns of circadian metabolites in AB9 are quite similar to PAC2 cells

Besides PAC2, we also performed metabolomic profiling analysis in AB9, which is a cell line derived from adult zebrafish fins. We exposed AB9 cells to light cycle entrainment and NMR analysis as previously described for PAC2 cells (Figure 30A). A total of 95 spectra representing 28 metabolites have been identified and more than 70 percent exhibit circadian oscillation (Figure 30B). Similar to PAC2, the rhythmic metabolites showed no phase difference between LD, DL and DD (Figure 31), and their phase distribution was quite similar to that of PAC2 cells (Figure 32). The AB9 results imply that the cellular metabolic oscillator may be widely expressed in zebrafish cell lines.

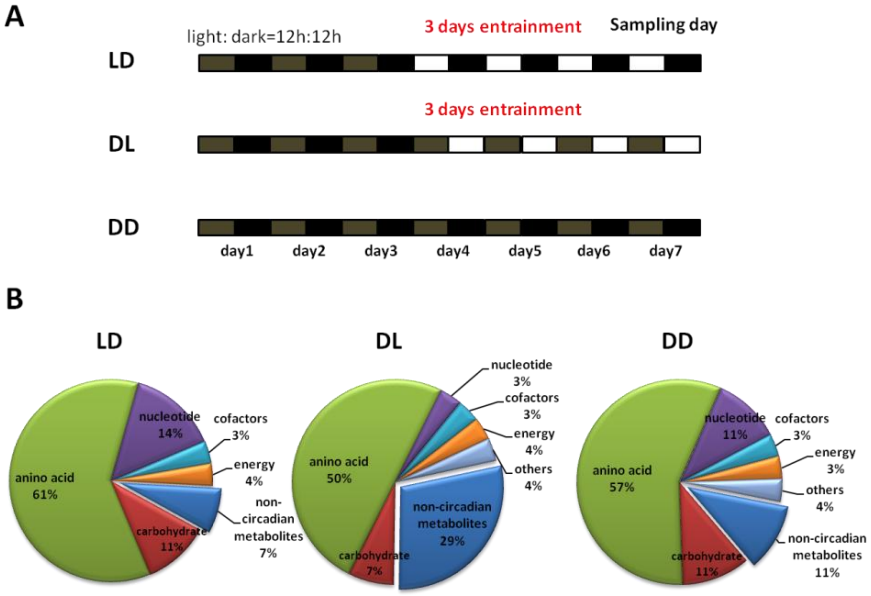

Figure 30. Metabolome measurement in AB9 cells by NMR spectrometry

(A) Scheme of the three lighting regimes used to entrain the clocks in zebrafish AB9. (B) The pie charts reveal that as in PAC2 cells, amino acids still account for a large proportion of circadian metabolites. The bars indicate light (white) and dark (black) periods, and subjective day (grey) and subjective night (black) in DD, respectively.

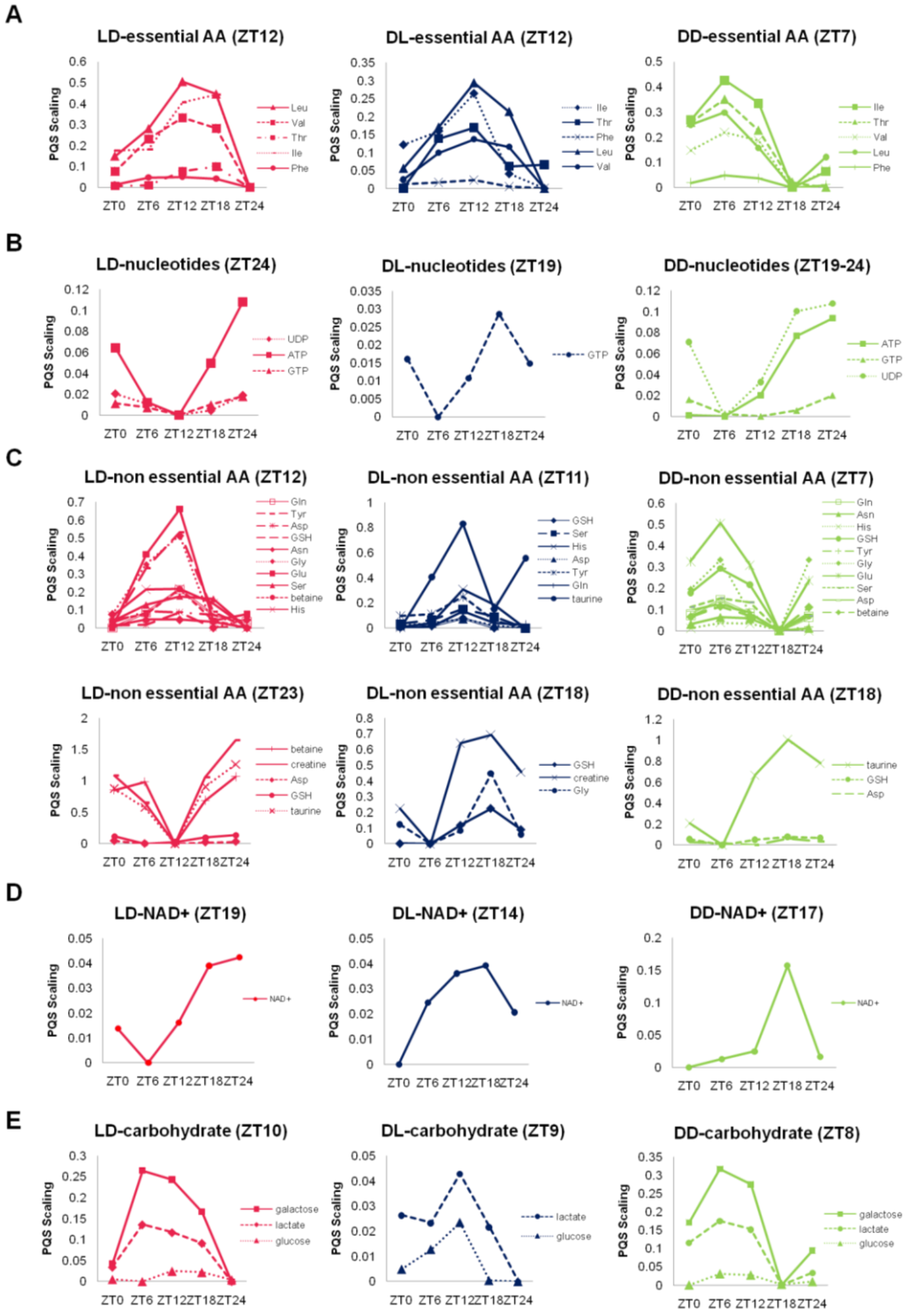

Figure 31. The temporal profiles of circadian metabolites under three lighting regimes in AB9

The circadian phases of (A) essential amino acids (B) nucleotides (C) non essential amino acids (D) NAD+ and (E) carbohydrates are similar between LD, DL and DD. The lowest expression level for each metabolite was normalized to zero. Every time point has at least three independent biological replicates. For clarity, error bars in each time point per group are not added. $p<0.05$, cosine fitting.

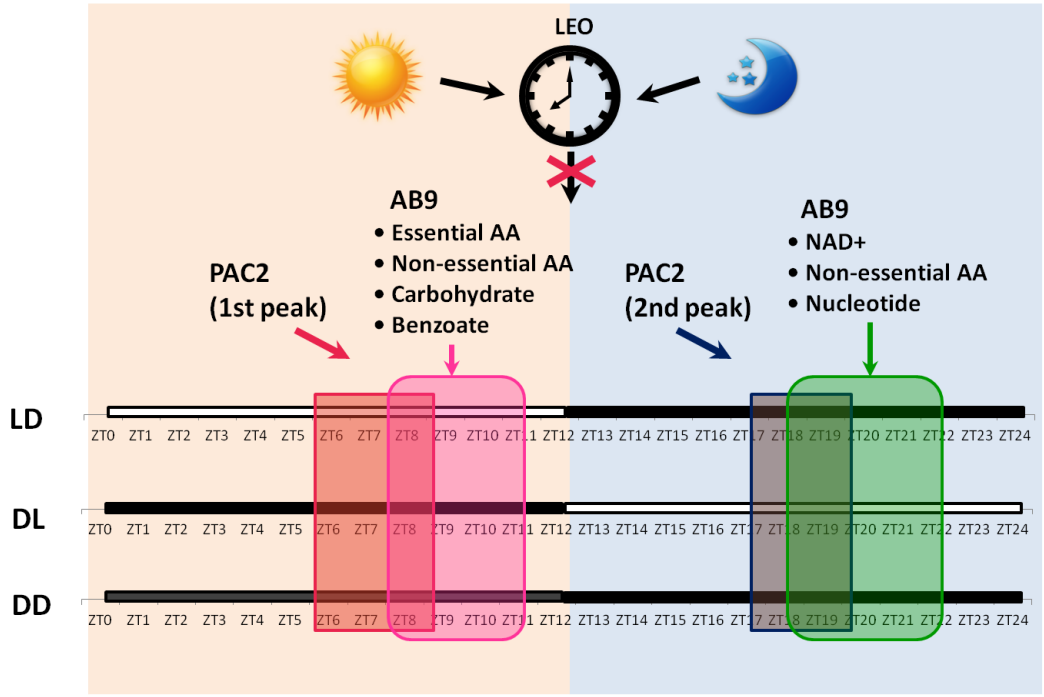

Figure 32. Similar phase distributions of circadian metabolites in PAC2 and AB9 cells

The cellular metabolic oscillation is not influenced by a normal light entrainable circadian clock. ZT0 is defined as lights on in the LD group (8:00).

3.2.2.2.2 Constant light represses the circadian metabolites levels in AB9

It has been widely reported that long term constant light (LL) exposure in animals or plants can disrupt the circadian system and eventually affect circadian physiology. Tamai and colleagues have shown that constant light represses the expression of core clock genes of TTFLs and severely disrupts circadian function in zebrafish cell lines [244,292]. To address whether continuous illumination blocks the circadian metabolome in zebrafish cell lines, we exposed AB9 cells to constant light for three days and then sampled the cells in a time series for NMR detection (Figure 33A). The AB9 cells exposed to DD conditions were used as a control. As shown in Figure 33B, compared to DD, the cyclic metabolites in LL showed no phase differences, whereas their basal levels were much lower. These results imply that continuous light exposure can dampen metabolic oscillators rather than completely disrupt intrinsic metabolic rhythmicity in zebrafish cell lines. Thus, although the metabolic rhythms seem to be regulated independently of the light cycle, there may still be some influence of light.

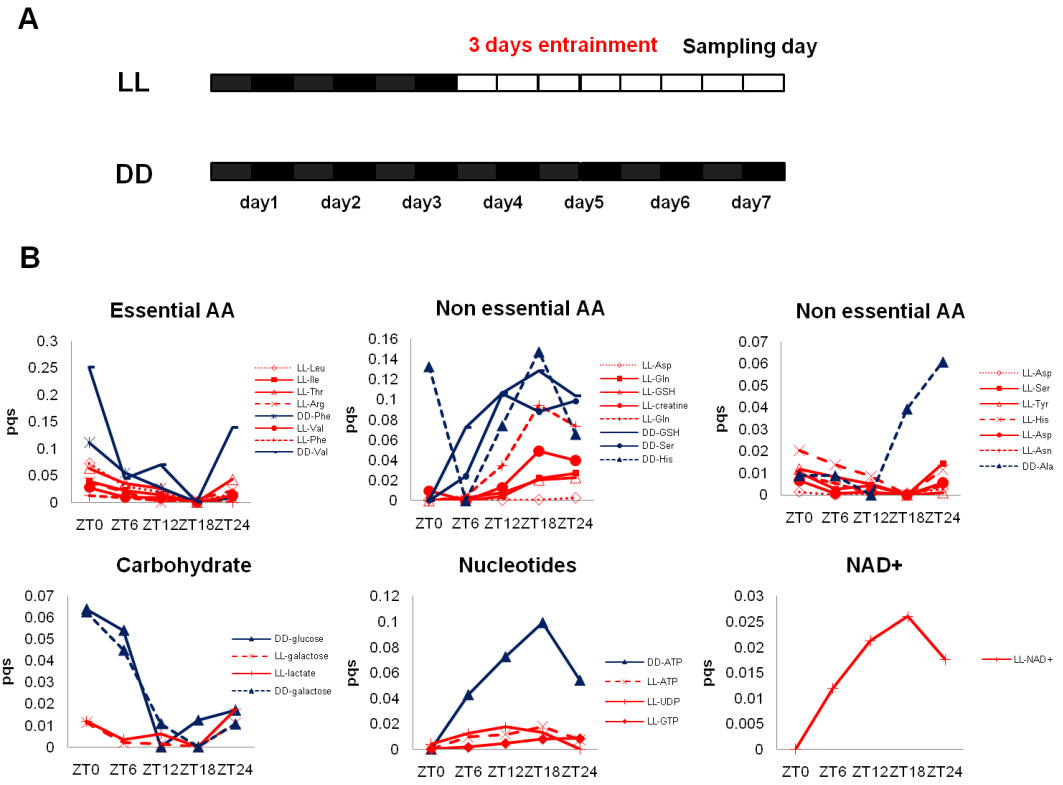

Figure 33. The cycling of circadian metabolites in AB9 cells under LL and DD conditions

(A) Scheme of lighting conditions and sample collection for NMR. ZT0 is defined as lights on in the LL group (8:00). The bars indicate light (white) in LL, and subjective day (gray) and subjective night (black) in DD. (B) The comparison of phases and levels of circadian metabolites between LL and DD. The lowest level for each metabolite was normalized to zero. Every time point has three biological replicates. For clarity, error bars in each time point per group are not added. $p<0.05$, cosine fitting.

3.2.3 Serum stimulation is not sufficient to trigger metabolic oscillations in zebrafish fibroblasts

The key question is which external cues are able to entrain the metabolic rhythms and prevent the endogenous metabolic clock from becoming arhythmic under free running conditions? One candidate environmental cue for cell cultures is serum, which represents a major source of nutrients in cell cultures. The synchronization of mammalian peripheral clocks by serum shock has been well studied and high concentrations of serum are commonly used to synchronize rhythmic clock gene expression in mammalian cell lines. However, since direct exposure to light entrains the zebrafish peripheral clocks so effectively, very little work has been done to explore the regulation of zebrafish clocks by serum and few serum inducible clock genes in zebrafish have been reported. To monitor the influence of serum stimulation on the zebrafish cell lines, we first assayed the luciferase activity from our

zfper1b-Luc reporter construct as a reliable indicator of circadian clock function in PAC2 cells. PAC2 cells were grown in medium containing 20% serum and were transiently transfected with *zfper1b*-Luc (3.1kb). Then the medium was changed with fresh medium of different serum concentrations (1%, 10%, 20%) in complete darkness. After 72h of incubation under DD, the culture medium was replaced by fresh medium as follows: medium with 1% serum was changed to culture medium plus 1% and 20% serum respectively (Figure 34C 1% to 1%, 1% to 20%); medium with 10% and 20% serum were changed to fresh medium with equal amounts of serum (Figure 34C, 10% to 10%, 20% to 20%). Then *in vivo* bioluminescence of the *per1b* reporter was assayed under DD conditions. As shown in Figure 34C, regardless of the different concentrations, none of the serum treatments induced strong oscillation of *zfper1b* promoter, indicating that serum entrainment failed to synchronize the core TTFL circadian mechanism in the individual cell culture cells.

We next performed a serum shock experiment using wild type PAC2 cells, which mimicked our original feeding entrainment studies in adult zebrafish (Figure 34A). Briefly, all the PAC2 cells cultivated in medium with 20% serum were incubated under constant darkness for 5 days to ensure loss of previous clock synchronization. The beginning of the subjective day was defined as ZT0 (8:00). Then the old medium was replaced by fresh medium containing 20% serum either in the middle of the subjective day (ML, ZT6, 14:00) or in the middle of the subjective night (MD, ZT18, 2:00). PAC2 cells maintained in DD without any treatment were used as a control. From ZT0 of the following day, the cells from the ML, MD and DD groups were harvested at 6h intervals for 24h according to the protocols for Q-PCR and NMR assays. The mRNA levels of *per1b* in serum-treated PAC2 were quantified by Q-PCR and are presented in Figure 34B. Circadian cycles of *per1b* mRNA in ML and MD show an anti-phase relationship and the peak time is around 18h after the serum treatment in both ML and MD, suggesting the phase of the oscillations is driven by the timing of the serum treatment. However, unlike the high amplitude oscillation of *per1b* (critical threshold C_T differ~5, Figure 28A) observed under light dark cycles, the mRNA cycling induced by serum treatment is quite shallow (C_T differ~1.5, Figure 34B).

We also performed NMR analysis of serum-treated PAC2 cells. By comparing the metabolomic profiling of the ML, MD and DD samples, we found that serum renewal changes neither the circadian phases nor levels of the cycling metabolome (Figure 35). Thus, we conclude that zebrafish possesses a quite weak serum entrainable clock, which is able to synchronize cell clocks at the gene transcription level but which does not contribute to directing the rhythmic cycles of metabolites.

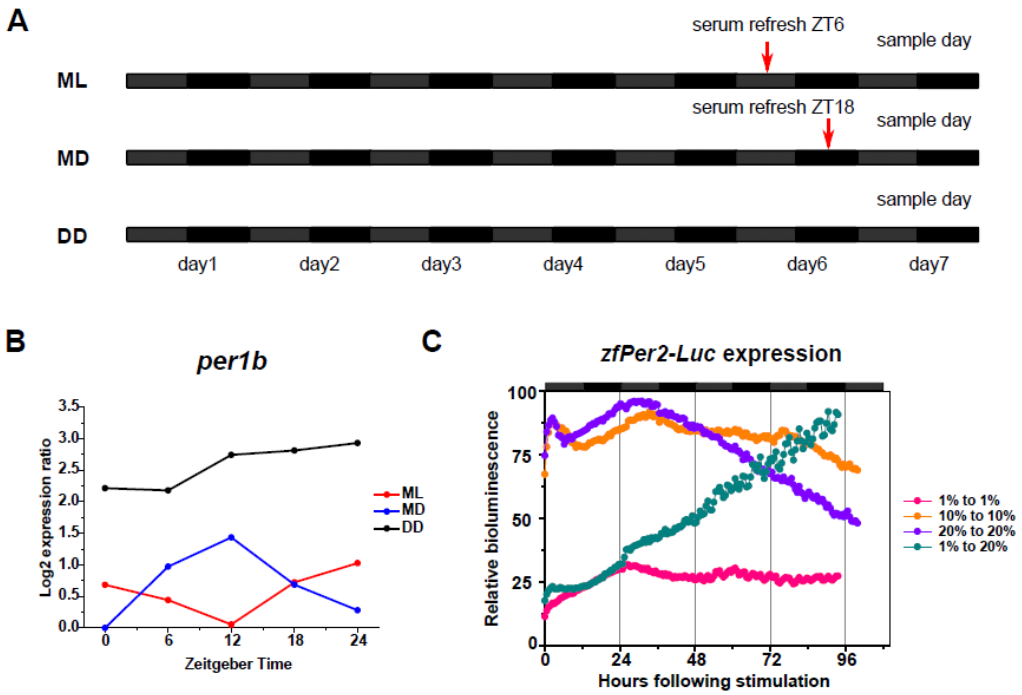

Figure 34. The weak influence of serum treatment on rhythmic *per1b* expression.

(A) Schematic representation of the serum treatment of PAC2 cells and sample collection for Q-PCR and NMR assays. The bars indicate subjective day (gray) and subjective night (black) in DD. (B) *per1b* expression in serum entrained and control PAC2 cells was assayed by Q-PCR. The beginning of the subjective day was defined as ZT0. (C) Real time bioluminescence assays of PAC2 cells transfected with the *per1b*-Luc reporter and treated with serum of different concentrations.

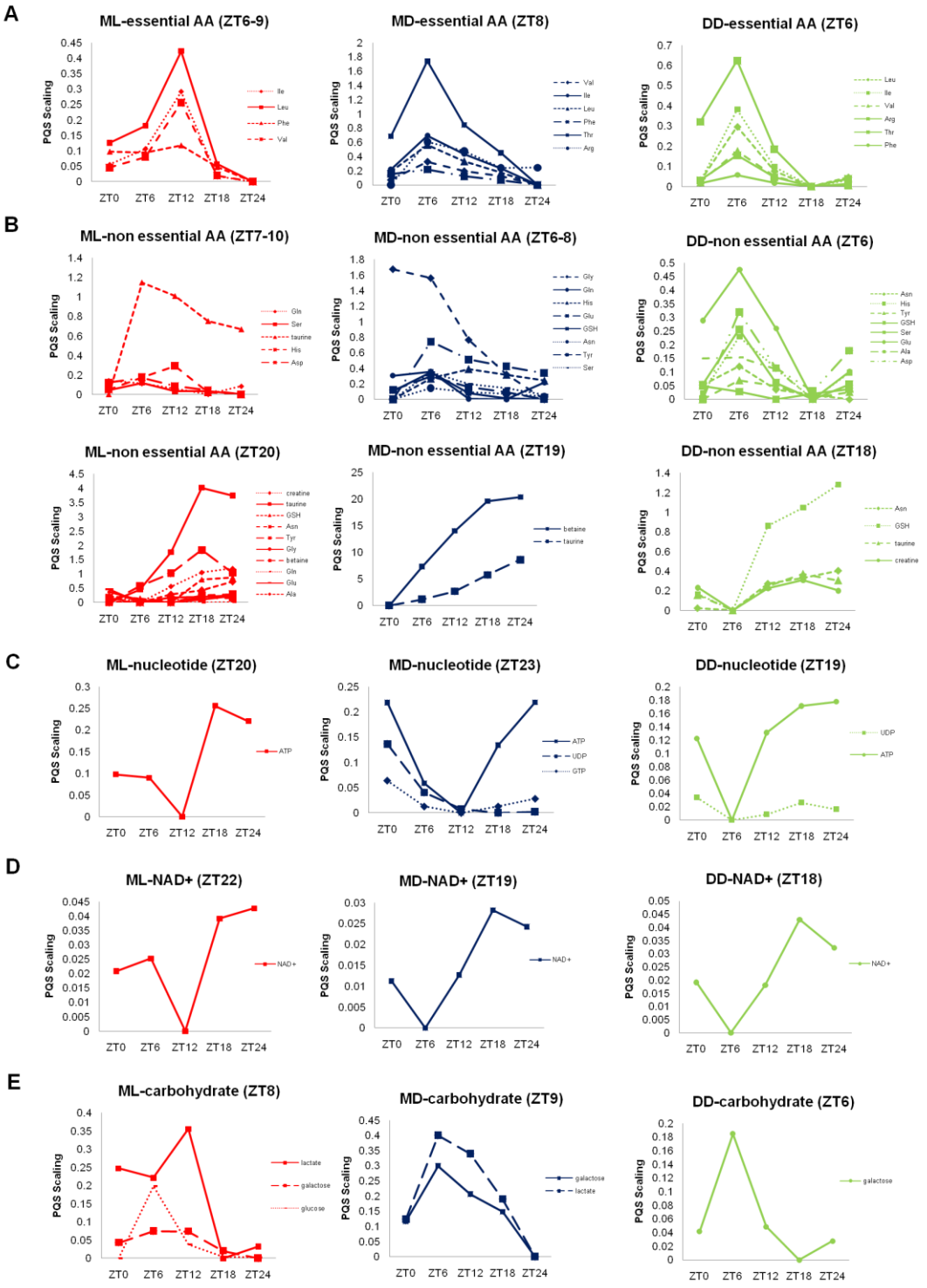

Figure 35: Levels of circadian metabolites in serum treated PAC2 cells

The circadian phases and levels of metabolites show no significant differences between ML, MD and DD. The lowest levels for each metabolite were normalized to zero. $p<0.05$, cosine fitting.

3.2.4 Cavefish fibroblasts possess aberrant metabolic oscillations

The Somalian cavefish (*Phreatichthys andruzzii*) represents a fish eminently well suited for studying feeding-regulated clocks. Furthermore, cavefish-derived cell lines could be a uniquely powerful *in vitro* system to explore the genetic basis of metabolic rhythmicity. Previous studies in our laboratory have revealed the fibroblast-like cell lines (CF1) derived from adult cavefish caudal fins contain clocks which have entirely lost the ability to be entrained by light. However, an aberrant circadian clock has been synchronized in these cells by serum shock or dexamethasone treatment. The CF1 cells transfected with *zfper1b*-Luc and treated with dexamethasone show a strong bioluminescence rhythm with an infradian period of 43h [282]. Metabolome analysis of cavefish cells could be vital for understanding the origin of the metabolic oscillator mechanism, therefore we explored whether cycling levels of metabolites exist in CF1 cells. Specifically, we maintained the CF1 cells in DD for 3 weeks until all the cells were confluent. Then we refreshed the cultures with fresh medium using the same concentration of serum (20%) for the cells in the serum group (the time of serum treatment was defined ZT0), and harvested the cells in serum and control groups (without any treatment) simultaneously at 8h intervals from ZT2 to ZT50 (Figure 36A). The metabolome was analysed by NMR in the same way as for the zebrafish cell lines. Using cosine fitting (see materials and methods), we have identified cycling metabolites with an infradian period of 40-45h in the control group, which matches the period of the *zfper1b*-Luc we have measured in the same cells. Interestingly, this rhythmicity was established 3 weeks before hand and persisted in culture, without any additional entraining signals. The bimodal distribution of rhythmic metabolite levels in CF1 is quite similar to that observed in zebrafish fibroblast cell lines. Essential amino acids, a subset of non-essential amino acids and carbohydrates peak around ZT18, while in contrast, nucleotides, NAD+ and the remaining non-essential amino acids peak around ZT34 (Figure 36B-36G). However, the expression patterns of metabolites in serum group are still not clear, suggesting that the serum renewal influenced the cavefish cellular metabolism significantly. Moreover, we assayed the expression of cavefish *per1b* in the control and serum shock groups. As shown in Figure 37A, expression of *per1b* decreases rapidly within 2h after serum renewal, then it gradually increases and recovers to stable expression levels. However, serum refreshing does not establish rhythmicity of the expression of cavefish *per1b* or the metabolic genes *glu1a*, *gpt2l*, *sirt1* (Figure 37A, 37C-37E). Compared to the serum group, the expression levels of *per1b* and metabolic genes are stable in control group, illustrating that the initiation of metabolic oscillation in control group is not through the transcriptional regulation in cavefish cell lines. EPA is another cavefish cell line which is derived from enzyme-dissociated embryos. Cavefish *per1b* shows a similar expression profile comparing CF1 and EPA (Figure 37A-37B).

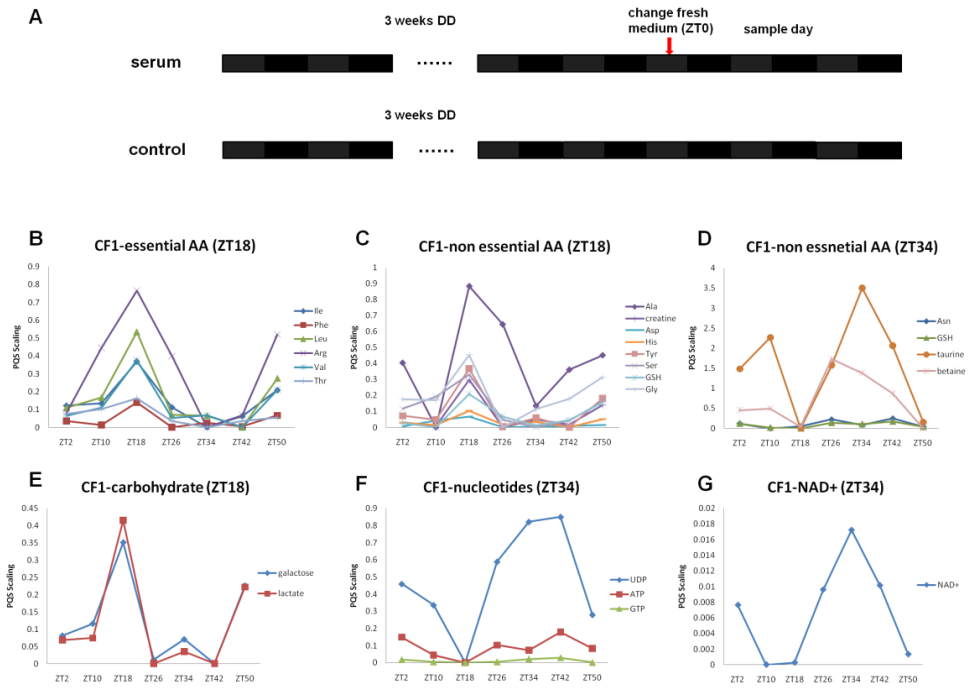

Figure 36: The temporal profile of circadian metabolite levels in serum treated CF1 cells

(A) Schematic representation of the serum treatment regime for CF1 cells and sample collection for Q-PCR and NMR assays. The bars indicate subjective day (gray) and subjective night (black) in DD. The time point of serum renewal was defined as ZT0. (B) The circadian phases of metabolites are around 40-45h in the control group. The lowest expression level for each metabolite was normalized to zero. $p<0.05$, cosine fitting.

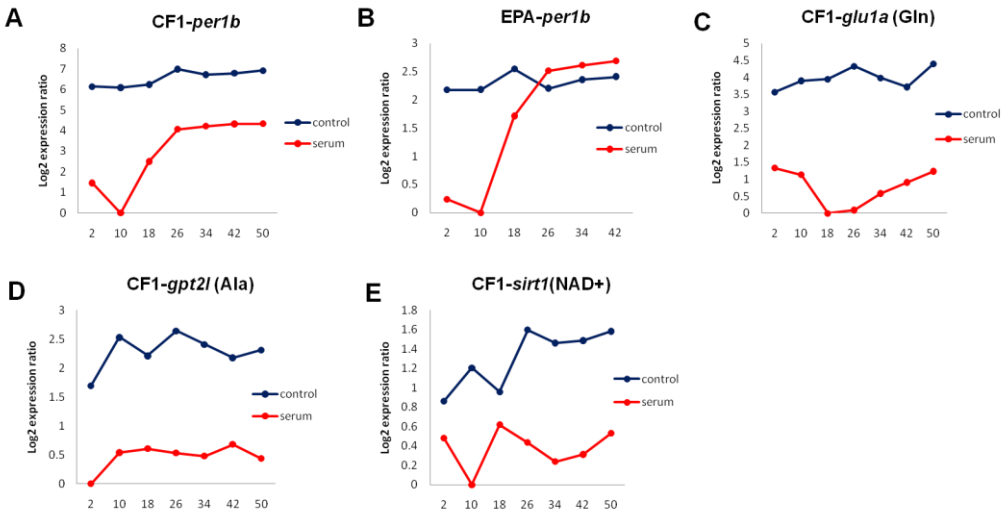

Figure 37: The expression of metabolic genes in serum treated CF1 cells

Cavefish (A) *per1b* and metabolic genes (C) *glu1a* (D) *gpt2l* (E) *sirt1*expression in CF1 cells which were collected over a period spanning 50h, (B) *per1b* expression in EPA cells (Z2-ZT42) were examined by Q-PCR. The lowest expression level for each gene was normalized to zero.

Chapter 4

Discussion

Here I have described the investigation of the LEO and FEO in adult zebrafish liver. By performing Q-PCR assay and metabolome measurement using NMR, we have revealed that temporally restricted food availability serves as a powerful zeitgeber which not only induces robust FAA anticipatory activity, but also sets the phase of rhythmicity in a subset of circadian transcripts and metabolites in zebrafish peripheral tissues while the remainder cycle according to the light cycle regulated LEO. Comparing our *in vivo* and *in vitro* data also suggests that systemic signals are important for the LEO to regulate gene expression and metabolite levels in the liver in response to lighting conditions. Furthermore, our results indicate that the cell autonomous, light regulation hepatocyte clock observed *in vitro* may not be crucial for timing metabolism rhythms according to the light dark cycle *in vivo*. Our cell line data is also of fundamental importance in terms of pinpointing a possible additional mechanism linking cellular clocks with metabolism that is independent of the TTFL mechanism of the circadian clock. Thus, our multiple oscillators study in zebrafish cell lines may provide a broader insight into the molecular basis of circadian timekeeping system.

4.1 FAA onset is recorded at an early stage of feeding entrainment

In order to investigate the onset of FAA, we recorded fish behavior from the onset of our "conflicting zeitgeber" experimental paradigm. As shown in Figure 38, within four days the fish started to exhibit apparent anticipatory activity 2-3h before food availability in both the ML and MD groups. The zebrafish is a diurnal animal, therefore we might predict that nighttime feeding is not a part of its normal (natural) behaviour, however, the kinetics of behavioral adaptation is comparable in the daytime and nighttime feeding groups. In mouse, a nocturnal species, the phase adaptation of peripheral organs is slow when nighttime feeding is changed to daytime feeding, whereas the phase reversal is much faster when the feeding is switched back. The rhythmic secretion of glucocorticoid hormone has been shown to contribute to different kinetics of phase resetting [153]. In zebrafish, the molecular mechanism of feeding entrained synchronization has not been addressed. In our study, to reinforce the influence of scheduled feeding cycle, we entrained the fish for at least a month before the sample collection. Interestingly, the fish entrained for one month with MD feeding were obese compared with ML fish (data are not shown), which is consistent with the reports of night eating syndrome (NES) in human [293,294] and the obesity in nocturnal mice caused by daytime food intake [295]. Together, these findings support the conclusion that disruption of the circadian timing system leads to metabolic disorder and obesity [296,297]. Furthermore, our work implies that the feeding regulated clock could play important roles in

metabolic and energy homeostasis.

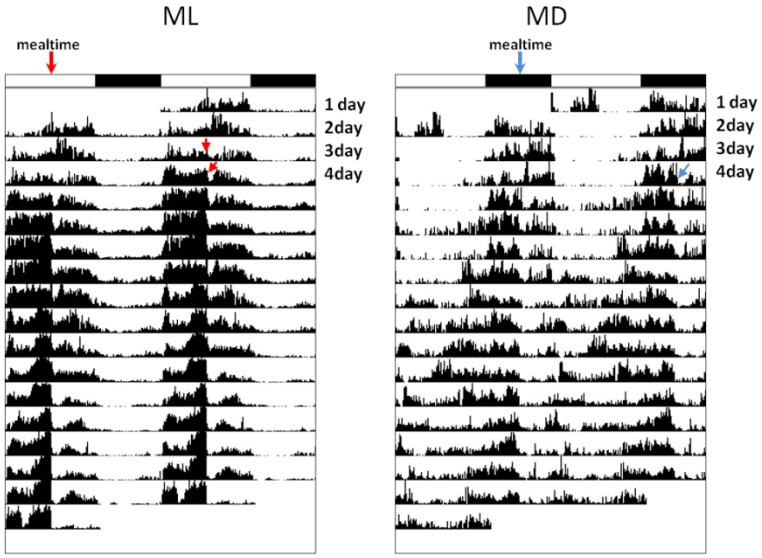

Figure 38: The onset of FAA in adult zebrafish

FAA emerges after around 4 days of feeding entrainment in both ML and MD groups. A weak FAA in ML is seen at day 3 (red arrow), and at day 4 in ML (red arrow) and MD (blue arrow) fish. Behaviour records are double plotted on a 48 h time scale to aid interpretation, the y-axis progresses in single days with each day being plotted twice (day 1 on the right side is repeated on day 2 on the left side). The activity was binned every 10 min, the height of each point represents the number of interruptions of the infrared light beam.

4.2 FEO synchronization is independent of the circadian TTFL in the zebrafish liver

The mouse is a nocturnal animal and restricted daytime feeding has been reported to invert the phase of circadian transcripts such as *Per1*, *Per2*, *Per3*, *Cry1*, *Rev-erbα*, *Bmal1*, and *Rorγ* in the liver independently of the lighting conditions, indicating the hepatic TTFLs are primarily adapted to the timing of scheduled food access [144,145,147,148]. In contrast, we have shown that the rhythmic mRNA levels of most TTFLs components are insensitive to feeding entrainment in zebrafish livers (Figure 16). A possible explanation is that the food-regulated signaling pathways are functionally distinct from the light input pathway and its tight coupling with the TTFL. Indeed, our data point to a genetic and functional separation of the LEO and FEO in the fish liver.

The entraining effect of feeding is clearly central to hepatic function, with key metabolic processes including the NAD+ salvage pathway under FEO control. Indeed, the oscillatory patterns of *nampta*, *namptb* and *sirt1* can be reset by the restricted feeding in the zebrafish liver. However, a key issue is whether this function is restricted to the fish liver or whether it

might also operate in other tissues and organs. To assess whether feeding entrainment has any effect on fish central pacemakers, we also performed Q-PCR and NMR assays using time series samples of the fish brain (including the pineal gland). Unexpectedly, *nampta*, *namptb* and *sirt1* are expressed arhythmically on sample day 1 and the accumulation of NAD+ was too low to be detected by NMR (data are not shown). Moreover, we also examined the expression of *nampta*, *namptb* and *sirt1* in adult fish muscles which were collected on sample day 1, and we observed that they displayed largely arhythmic expression which was consistent between the ML and MD samples. These data suggest that NAD+ regulation by the FEO in the zebrafish may indeed be liver specific. However, a detailed study of more FEO-specific regulatory targets will be required in order to assess the full extent of FEO function.

4.3 NAD+ could be a key link between the LEO and FEO

As described in the introduction, NAD+ acts as a cellular energy status sensor that links circadian clocks to cellular metabolic responses. Via their direct or indirect participation in post-translational modification or chromatin remodeling, NAD+ coupled enzymes enable the clock system to adapt to the cellular nutritional status in multiple tissues. In mammals, NAD+ levels can be affected by the food intake cycle. Thus, the activity of NAD+-dependent PARP-1 poly (ADP-ribosyl)ation is rhythmic and is regulated by feeding entrainment in the mouse liver [148]. Furthermore, NAD+-dependent mitochondrial oxidative function and metabolism are synchronized with the fasting and feeding cycle [152]. However, precisely how the cellular temporal NAD+ levels can be altered by scheduled daytime or nighttime feeding is still uncertain. While the epigenetic functions of Sirt1 and Parp1 have yet to be characterized in zebrafish, our results clearly demonstrate that the highly-conserved NAD+ salvage pathway is regulated by both the LEO and FEO. When fish were fasted, the phase of rhythmic *nampt* and *sirt1* gene expression shifted to match the phase of the light-dark cycle whereas expression of the canonical circadian clock TTFL elements was unaffected, implying that a novel mechanism independent from the clock TTFL governs this process. We hypothesize that NAD+, Nampta, Namptb and Sirt1 constitute an enzymatic feedback loop, which may serve as an accessory loop of the canonical TTFLs but also represents a link between the LEO and FEO in zebrafish. Our hypothesis is based on the physical and functional association between the CLOCK:BMAL heterodimer and SIRT1 in mammals, however, whether Sirt1 has a similar circadian function in zebrafish (histone deacetylase activity, as a transcriptional regulator to drive CLOCK:BMAL-mediated circadian gene expression) needs to be verified in future experiments.

The phase distribution of NAD+ in the fish liver is of particular interest. In both ML and MD samples, the peak time of NAD+ overlaps with the FAA period, whereas other FEO controlled circadian metabolites peak around the FAA anti-phase period (Figure 18C). The abundance of NAD+ is determined by both the activities of the conserved *de novo* biosynthesis pathway and the salvage pathway. As shown in Figure 39, NAD+ can be synthesized *de novo* from tryptophan, which is an essential amino acid and obtained from the diet [298]. Besides tryptophan, nicotinic acid is another major precursor for NAD+ biosynthesis and nicotinamide can be converted to NAD+ by the catalytic activity of NAMPT, the rate-limiting enzyme in the salvage pathway. Like tryptophan, both nicotinic acid and nicotinamide are obtained from the food. Thus, theoretically the cellular NAD+ levels in the liver are diet-dependent and sensitive to feeding time. In zebrafish, the peak time of NAD+ is correlated with mealtime, but is shifted by about 12h with respect to the phase of rhythmic levels of essential amino acids. Thus, the light entrained clock could exert profound effects on NAD+ metabolic pathways during restricted feeding entrainment.

4.4 The supply of essential amino acids during starvation

Another important group of circadian metabolites controlled by FEO in the adult zebrafish liver is essential amino acids. As the fish lack the rate-limiting enzymes for essential amino acids biosynthesis *in vivo*, they obtain the essential amino acids from their diet. However, surprisingly, the rhythmicity and expression levels of essential amino acids persisted even when the fish were fasted for two days. What are the sources of essential amino acids when the fish are food deprived? One possible answer is autophagy, which is an intracellular self-degradation process and promotes the recycling of nutrients and energy under various stresses [299-301]. Autophagy has been reported to be controlled by the circadian system in both mice [302] and zebrafish livers [303]. The numbers of autophagosomes and autolysosomes exhibit daily rhythms and mRNA levels of autophagy genes whose products are involved in autophagic activity are expressed in a time-dependent manner. Transcriptional regulation of most autophagy genes appears to be mediated by a transcription factor C/EBPβ (CCAAT/enhancer-binding protein β), that has cyclic expression regulated by both circadian and nutritional signals [302,304].

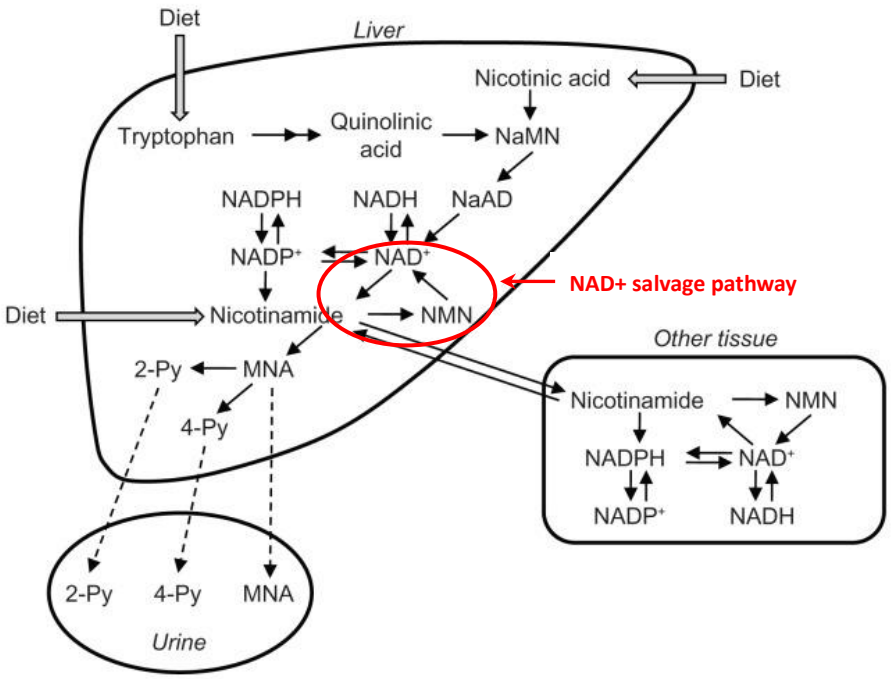

Figure 39: Schematic representation of the NAD+ *de novo* synthetic pathway from tryptophan and the salvage pathway in the mammalian liver

Tryptophan, nicotinic acid and nicotinamide are three main precursors of NAD+ can be obtained from the diet. The loop in the red circle is the NAD+ salvage pathway. Both the *de novo* synthetic pathway and the NAD+ salvage pathway are conserved between fish and mammals.

Abbreviations: NaMN: nicotinic acid mononucleotide; NMN: nicotinamide mononucleotide; MNA: N1-methylnicotinamide; 2-Py: N1-methyl-2-pridone-5-carboxamide; 4-Py: N1-methyl-4-pridone-3-carboxamide.

Schematic: Fukuwatari T and Shibata K. *Int J Tryptophan Res*. 2013 [298].

In our studies, the rhythmic expression of *cebpb* shows no difference between mid-light and mid-dark feeding in zebrafish liver and muscle (unpublished data). Thus, it seems unlikely that *cebpb* would ultimately mediate the effects of feeding time on rhythmic levels of essential amino acids. However, the regulation of autophagy may also rely on post-transcriptional modification. LC3-I/LC3-II (microtubule-associated protein1 light chain 3) is a useful biomarker representing the autophagy activity under different nutritional conditions [305]. The protein levels of LC3-I/LC3-II are rhythmically expressed in multiple mice tissues [304] and larval zebrafish [303]. Whether LC3-I/LC3-II participates in rhythmic essential amino acids supply under nutrient-limited conditions warrants further investigation in the future.

4.5 The comparison of the circadian metabolome between liver and hepatocytes

Hierarchical organization has been widely accepted to be a defining feature of the mammalian circadian system. The SCN clock serves as the central pacemaker and directs the activity of the multiple peripheral tissue clocks via a complex array of signals emanating from the autonomic neural system or systemic cues. In zebrafish, since both central pacemakers and all peripheral cells can be strongly synchronized by direct light exposure, it is argued whether the zebrafish circadian clock is also hierarchically organized, and if so, how central clocks such as the pineal organ coordinate the peripheral tissues or cells? In our results, high amplitude rhythms in the mRNA expression of many clock-controlled, metabolism-regulating genes have been observed in the fish liver, whereas the rhythmic transcription of the same genes is absent in cultured hepatocytes and fibroblasts. Thus, our data predict the existence of systemic signals in the zebrafish which could relay timing information such as light from central clocks to the periphery. In mammals, the systemic cues include hormonal signals (e.g. glucocorticoids, insulin and melatonin), body temperature cycles as well as metabolites (e.g. glucose, NAD+ and heme), which are regulated by the master SCN pacemaker directly or indirectly. In zebrafish, melatonin and glucocorticoids are two important clock controlled hormones that may activate systemic signaling pathways. Cyclic secretion of melatonin from the pineal gland is proposed to be the major neuroendocrine signal that could mediate the phase coherence of circadian outputs. Melatonin has been reported to regulate food intake and lipid metabolism in the zebrafish liver [306]. Glucocorticoids have also been implicated in playing a key role in the coordination of circadian transcription and metabolism in larval zebrafish [277]. However, there is no evidence to date, or illustrate that these hormonal signals are regulated by regular feeding entrainment. Which factors link hepatic clocks and master pacemakers or other peripheral clocks *in vivo* is still unknown.

4.6 Important circadian metabolites in adult fish livers

In our results, the non-essential amino acids and nucleotides are not influenced by feeding entrainment, but regulated by the light-dark cycle in adult fish liver. We have tested the temporal expression of some transcripts encoding enzymes in non-essential amino acid and nucleotide metabolic pathways, which coincides with the oscillatory patterns of corresponding metabolites (Figure 21). We surprisingly observed that *tdh* which converts threonine to L-2-amino-3-oxobutanoate exhibits an in-phase relationship between diurnal and nocturnal feeding entrainment (Figure 21H). Threonine is an essential amino acid which is sensitive to the time of food supply. However, *tdh* which is situated downstream of the threonine metabolic pathway is gated by the light-dark cycle. We have not yet examined the daily expression of metabolites in threonine catabolic pathways, but there is a possibility that

the influence of inverted feeding is diminished or coordinated by the metabolic regulatory networks or the timekeeping system.

In mammals, besides NAD+ and SIRT1, AMP-activated protein kinase (AMPK) serves as another crucial cellular energy sensor to feedback the nutrient signals to the circadian clock [307]. AMPK can modulate circadian rhythms based on the cellular energy state through phosphorylation of core clock components directly, such as clock repressors CRY1, CRY2 [308] and CK1ε (casein kinase-1ε) [309]. In addition, AMPK promotes SIRT1 mediated deacetylation via enhancing cellular NAMPT expression and NAD+ levels, thus activating SIRT1-dependent transcriptional cascades [310]. AMPK has been reported to have a key role in food intake and metabolic entrainment of peripheral clocks, thus it could potentially be an element in the FEO synchronization mechanism [309,311,312]. AMPK is sensitive to the cellular AMP/ATP ratio. Elevation of AMP levels always coupled with ATP depletion can stimulate the enzymatic activity of AMPK [313], therefore a strong rhythm of AMP levels is essential for maintaining the AMPK circadian function. In zebrafish liver, the LEO controlled AMP and ATP have similar expression patterns in both ML and MD feeding, but it is difficult to assess the AMP/ATP ratio by NMR. Additionally, dietary formulations also impact on the circadian oscillation. High-fat diets alter the core transcripts such as *Clock*, *Bmal1* and *Per2* in mice brain and peripheral tissues via AMPK-mediated signaling pathways [314]. In our reports, we mainly focus on the phase shifts and levels of circadian metabolites during our conflicting zeitgeber entrainment paradigm. Diet composition (high-fat, high-sugar) and environmental conditions (temperature, light intensity, light duration) are also important factors which may influence synchronization of the FEO and the circadian timekeeping system.

In mammals, glucose in tissues or plasma is an important metabolite which not only maintains energy homeostasis, but also functions as an SCN controlled systemic cue to mediate peripheral clocks synchronization [315,316]. The SCN is essential for glucose rhythmicity as SCN ablation leads to disorders of glucose homeostasis [317,318]. Zebrafish livers contain high levels of glucose. But unlike other circadian metabolites, it shows two peak times: ZT3 and ZT12-16 in ML and MD sets of samples (Table 2). Based on our current experimental results, it is still not clear whether the daily changes of hepatic glucose are regulated by the FEO, LEO or both. Therefore, the circadian function and regulation of glucose may be more complicated in zebrafish.

4.7 Metabolic oscillation in zebrafish cell lines

In our reports, we explored the metabolome in diverse zebrafish cells including primary

hepatocytes, PAC2, AB9 and CF1 cavefish fibroblasts using NMR. The metabolites detected in adult livers and fish cells are shown in Table 3. The relative concentrations of the principle identified metabolites in the livers (from high to low) are: carbohydrate> essential AA (amino acid)> non-essential AA> nucleotides> NAD+ and in the cell lines: non essential AA> essential AA> carbohydrate> nucleotides> NAD+.

By performing NMR measurements on hepatocytes and fibroblast cells entrained under different lighting regimes, we found that the metabolic rhythms in diverse zebrafish cells appear to be driven by a non-TTFL mechanism and this may co-exist with the normal light regulated TTFL clock mechanism. To directly test whether the clock TTFL is necessary for the cellular circadian metabolome regulation, we plan to examine mutant PAC2 cells where normal function of the clock TTFL is blocked by over-expression of a dominant-negative (DN) mutant *CLOCK1* protein [292]. This mutant clock protein retains the normal PAS and bHLH domains at its N terminus, but carries a deletion of the C-terminal glutamine-rich activation domain. Therefore, while *CLOCK1* DN is able to heterodimerize with endogenous BMAL proteins, it is unable to activate transcription upon binding to E-box elements. Now this experiment is being performed. Firstly, we maintained the *CLOCK1* DN mutant cells under the three lighting regimes (LD, DL and DD) already used to characterize the PAC2 (Figure 25A) and AB9 (Figure 30A) cells and also included wild type PAC2 cells exposed to DD as a control. The cells were sampled at the indicated time points and next they will be used for NMR measurement. If the metabolites in *CLOCK1* DN mutant cells are rhythmically expressed and phased independently of the lighting conditions, these data should be strong evidence to confirm that the cycling of rhythmic metabolites in the cell lines is independent of the normal clock TTFL mechanism.

The mechanism underlying the metabolic synchronization in zebrafish cells remains unclear. Peroxiredoxin (PRXs) is an attractive candidate to be involved in a non-transcriptional clock mechanism (see section 1.2.4). The PRXs rhythm and the observed zebrafish cellular metabolic cycling share common features, such as both of them are tightly related to metabolism, and independent of TTFLs. Although transcriptional control does not seem to be required for both the PRXs rhythm and the cellular metabolic oscillations, the TTFL may still play some regulatory role in these processes. The mutation of TTFL elements (PER, CRY) alters the period length of the PRX-SO2/3 rhythm in blood cells [99]. In zebrafish cell lines, constant light exposure which can repress the core circadian clock gene expression [244] also suppresses the levels of circadian metabolites (Figure 33B). Furthermore, in cavefish cell lines, the period of metabolite cycling matches the infradian rhythmicity which

Table3: Metabolites detected in adult liver and cell lines using NMR

metabolites	liver	hepatocyte	PAC2	AB9	CF1
1. Carbohydrate	lactate, glucose, maltose,	galactose, lactate, glucose	galactose, lactate, glucose	galactose, lactate, glucose	lactate, galactose, glucose
2. Nucleotide	AMP, IMP, ATP, ADP, GMP, inosine, GTP, GDP, IDP	ATP, UDP-glucose, GTP, UDP-N-Acetylglucosamine,	ATP, UDP-glucose, GTP, UDP-N-Acetylglucosamine	ATP, UDP-glucose, GTP, UDP-N-Acetylglucosamine	ATP, UDP-glucose, GTP, UDP-N-Acetylglucosamine
3.Essential AA	Thr, Val, Ile, Leu	Ile, Leu, Val, Thr, Arg, Met, Phe	Val, Leu, Ile, Phe, Thr, Met, Phe,	Val, Leu, Ile, Thr, Phe	Val, Leu, Ile, Thr, Arg, Met, Phe,
4.Non-Essential AA	Ala, Glu, Gln, Asp, Asn, creatine, GSH, His, taurine, betaine, Gly, Tyr	Ala, Glu, Gln, Asp, Asn, creatine, GSH, His, taurine, betaine, Gly, Tyr, Ser	Ala, Glu, Gln, Asp, Asn, creatine, GSH, His, taurine, betaine, Gly, Tyr, Ser	GSH, Asn, His, Tyr, Gly, Glu, Ser, Asp, creatine, taurine, betaine, Ala	Ala, GSH, Asp, Asn, creatine, Tyr, His, Gly, taurine, Ser, betaine, Glu, Gln
5. Co-factor	NAD+	NAD+	NAD+	NAD+	NAD+
6. Others	acetate, acetoacetate, malate, fumarate, o-acetylcarnitine, formiate, succinate	citrate, fumarate, benzoate, formate, succinate	citrate, fumarate, benzoate, formate, succinate	citrate, benzoate, formate	citrate, fumarate, benzoate, formate, succinate
Total number	36	33	33	28	33

Abbrevations: AMP: Adenosine monophosphate; **ADP:** Adenosine diphosphate; **ATP:** Adenosine triphosphate; **GMP:** Guanosine monophosphate; **GDP:** Guanosine diphosphate; **GTP:** Guanosine triphosphate; **IMP:** Inosine monophosphate; **IDP:** Inosine diphosphate;

Ala: Alanine; **Arg:** Arginine; **Asn:** Asparagine; **Asp:** Aspartic acid; **Gln:** Glutamine; **Glu:** Glutamic acid; **Gly:** Glycine; **GSH:** glutathione; **His:** Histidine; **Ile:** Isoleucine; **Leu:** Leucine; **Met:** Methionine; **Phe;** Phenylalanine; **Ser:** Serine; **Thr:** Threonine; **Tyr:** Tyrosine; **Val:** Valine;

NAD+: oxidized form of nicotinamide adenine dinucleotide

is observed for cycling expression of core clock gene mRNA (Figure 36B-36G). These results indicate that the TTFL mechanism and non-TTFL cycling elements may be coupled closely to generate circadian timekeeping. Both the metabolic rhythm in zebrafish cell lines and PRXs cycling exhibit the basic characteristics of circadian oscillation: 24h circadian period, persistent rhythm under free running conditions and synchronization by external environmental timing cues. PRXs cycling has been shown to be temperature compensated [91]. However, temperature compensation of the FEO and metabolic oscillations in zebrafish livers or cell lines has not yet been tested. Now the question is whether PRXs cycling has some functional connection with metabolic oscillations? Unfortunately, despite daily rhythms of PRX-$SO_{2/3}$ expression being present in a wide range of species, this has not been reported in zebrafish so far. In the future it will be important to investigate the possible connections between the FEO, PRXs and metabolic oscillations in zebrafish livers and cell lines.

4.8 The synchronization of zebrafish peripheral clocks by non-photic signals

In mammals, the clocks in cultured cell lines are not entrained by light-dark cycles, but can be synchronized by a variety of exogenous non-photic cues, such as serum, forskolin, glucocorticoid, the glucocorticoid receptor agonist dexamethasone, TPA, glucose, oxygen, rhythmic blood-borne signals, low amplitude temperature cycles etc. In comparison, there is little known about non-photic zeitgebers in zebrafish cells. My group previously has reported that ambient temperature cycles are able to entrain rhythmic circadian clock gene expression in zebrafish fibroblasts [180], and that dexamethasone can initiate rhythmic expression of a zebrafish clock reporter construct *(zfPer1b*-Luc*)* in cavefish CF1 cells [282]. In this project, we treated zebrafish fibroblast cell lines with different concentrations of fresh serum, and only a weak mRNA oscillation of *per1b* was observed in PAC2 cells (Figure 34B). While the mammalian circadian clock resetting by serum shock and the day-night cycle share similar mechanisms [37], the pathways that synchronize the clock in response to serum in zebrafish cell lines are unclear.

In our experiment, since the cells used for NMR assays were cultivated and entrained in thermostatically controlled conditions (26°C), the potential effects of temperature changes on the circadian metabolome can be eliminated. Excluding light, serum and temperature, another possible factor that could synchronize the rhythmic cycles of metabolites and specifically act as a zeitgeber in the cells is spitting time. In the NMR experiments we described above, all the confluent fibroblasts were split and propagated during 14:00-18:00 and then the subcultured cells were entrained under various conditions as previously described. The protocol for the preparation of primary hepatocytes started at 14:00 and the

cells were seeded around 18:00. As the metabolomic profiles observed in different types of zebrafish cells are quite similar, we speculate that the time of cell splitting could be a stimulus to initiate and set the phase of the metabolite rhythms. To validate our hypothesis, we are currently performing the following experiment where we split the AB9 cells in the middle of subjective day (ML) or in the middle of subjective night (MD). The cells were incubated in constant darkness for 6 days and then harvested for NMR detection. By comparing the circadian phases of metabolites, we will understand whether the splitting time plays some role in initiating the metabolic oscillations.

4.9 The slow metabolic rate in cavefish cell lines

Cavefish (*Phreatichthys andruzzii*) harbors an abnormal circadian system, where the period lengths of cycling metabolites and core circadian genes are extremely long (~43h). The slow metabolic rate of metabolites is consistent with our observation that the cultured CF1 cells can be kept *in vitro* for up to 2 months without any medium treatment. Another type of cavefish Mexican tetra (*Astyanax mexicanus*) has two different morphs: a surface-dwelling form which has normal eyes and circadian system, and multiple cave-dwelling forms which lose the visual capacity and pigmentation partially or totally [319]. The cave form Pachón is eyeless and shows reduced circadian clock control *in vivo*. Compared with surface cavefish, Pachón has been shown to have much lower metabolic levels and energy expenditure [320,321]. The slow kinetics in metabolism and the timing system may be a special property of cavefish, which could enable the fish to economize in energy expenditure and thereby adapt to their extreme, perpetual dark habitat.

4.10 Future perspectives

To date in our study of the circadian control of the zebrafish liver metabolome, we have only detected a subset of water-soluble metabolites using NMR spectroscopy, however, lipid-soluble metabolites also represent a large fraction of the metabolome. We are in the process of performing lipidomics measurement of adult fish livers by Nano-mass spectrometry in collaboration with the lipidomics platform at Center for Biochemistry (BZH) in the University of Heidelberg. Combining with the organic metabolites results, we aim to expand the scope of our cellular circadian metabolome and food-entrainable rhythmic metabolites study.

By NMR and Q-PCR, we have identified some hepatic metabolites and metabolic pathways that are differentially regulated by the FEO and LEO. Nevertheless, in order to obtain a global insight into the circadian control of food-regulated clocks, more FEO regulated circadian genes, metabolites or other components need to be identified. In the future we will

perform an analysis of cycling gene expression in ML and MD liver samples using a whole transcriptome deep sequencing assay. By computational methods, we will then aim to construct a database of food-entrainable circadian genes. Using this data set as a guide, we will investigate the regulation and function of core FEO elements in depth. For example, we will compare the promoter regions of these FEO regulated genes to search for common, enriched enhancer elements. Studying these FEO target enhancers will provide the first clues as to the nature of the signaling cues linking the FEO with its regulatory targets, and ultimately may shed light on the molecular nature of the FEO itself.

References

1. Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, et al. (2005) Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat Rev Genet 6: 544-556.
2. Richards J, Gumz ML (2013) Mechanism of the circadian clock in physiology. Am J Physiol Regul Integr Comp Physiol 304: R1053-1064.
3. Potter GD, Skene DJ, Arendt J, Cade JE, Grant PJ, et al. (2016) Circadian Rhythm and Sleep Disruption: Causes, Metabolic Consequences, and Countermeasures. Endocr Rev 37: 584-608.
4. Kreitzman L, Foster R (2011) The rhythms of life: The biological clocks that control the daily lives of every living thing: Profile books.
5. Mohawk JA, Green CB, Takahashi JS (2012) Central and peripheral circadian clocks in mammals. Annu Rev Neurosci 35: 445-462.
6. Ralph MR, Foster RG, Davis FC, Menaker M (1990) Transplanted suprachiasmatic nucleus determines circadian period. Science 247: 975-978.
7. Welsh DK, Logothetis DE, Meister M, Reppert SM (1995) Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. Neuron 14: 697-706.
8. Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, et al. (2000) Resetting central and peripheral circadian oscillators in transgenic rats. Science 288: 682-685.
9. Reppert SM, Weaver DR (2002) Coordination of circadian timing in mammals. Nature 418: 935-941.
10. Liu C, Reppert SM (2000) GABA synchronizes clock cells within the suprachiasmatic circadian clock. Neuron 25: 123-128.
11. Stephan FK, Zucker I (1972) Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. Proc Natl Acad Sci U S A 69: 1583-1586.
12. Stephan FK, Swann JM, Sisk CL (1979) Anticipation of 24-hr feeding schedules in rats with lesions of the suprachiasmatic nucleus. Behav Neural Biol 25: 346-363.
13. Moore RY, Eichler VB (1972) Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. Brain Res 42: 201-206.
14. Akhtar RA, Reddy AB, Maywood ES, Clayton JD, King VM, et al. (2002) Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. Curr Biol 12: 540-550.
15. Earnest DJ, Liang FQ, Ratcliff M, Cassone VM (1999) Immortal time: circadian clock properties of rat suprachiasmatic cell lines. Science 283: 693-695.
16. Provencio I, Rollag MD, Castrucci AM (2002) Photoreceptive net in the mammalian retina. This mesh of cells may explain how some blind mice can still tell day from night. Nature 415: 493.
17. Berson DM, Dunn FA, Takao M (2002) Phototransduction by retinal ganglion cells that set the circadian clock. Science 295: 1070-1073.

18. Macchi MM, Bruce JN (2004) Human pineal physiology and functional significance of melatonin. Front Neuroendocrinol 25: 177-195.
19. Tosini G, Fukuhara C (2003) Photic and circadian regulation of retinal melatonin in mammals. J Neuroendocrinol 15: 364-369.
20. Tosini G (2000) Melatonin circadian rhythm in the retina of mammals. Chronobiol Int 17: 599-612.
21. Foulkes NS, Whitmore D, Sassone-Corsi P (1997) Rhythmic transcription: the molecular basis of circadian melatonin synthesis. Biol Cell 89: 487-494.
22. Ganguly S, Coon SL, Klein DC (2002) Control of melatonin synthesis in the mammalian pineal gland: the critical role of serotonin acetylation. Cell Tissue Res 309: 127-137.
23. Pandi-Perumal SR, Srinivasan V, Maestroni GJ, Cardinali DP, Poeggeler B, et al. (2006) Melatonin: Nature's most versatile biological signal? FEBS J 273: 2813-2838.
24. Bailey MJ, Beremand PD, Hammer R, Bell-Pedersen D, Thomas TL, et al. (2003) Transcriptional profiling of the chick pineal gland, a photoreceptive circadian oscillator and pacemaker. Mol Endocrinol 17: 2084-2095.
25. Idda ML, Bertolucci C, Vallone D, Gothilf Y, Sanchez-Vazquez FJ, et al. (2012) Circadian clocks: lessons from fish. Prog Brain Res 199: 41-57.
26. Yoshimura T, Yasuo S, Suzuki Y, Makino E, Yokota Y, et al. (2001) Identification of the suprachiasmatic nucleus in birds. Am J Physiol Regul Integr Comp Physiol 280: R1185-1189.
27. Underwood H, Steele CT, Zivkovic B (2001) Circadian organization and the role of the pineal in birds. Microsc Res Tech 53: 48-62.
28. Underwood H, Groos G (1982) Vertebrate circadian rhythms: retinal and extraretinal photoreception. Experientia 38: 1013-1021.
29. Noche RR, Lu PN, Goldstein-Kral L, Glasgow E, Liang JO (2011) Circadian rhythms in the pineal organ persist in zebrafish larvae that lack ventral brain. BMC Neurosci 12: 7.
30. Cassone VM (1990) Effects of melatonin on vertebrate circadian systems. Trends Neurosci 13: 457-464.
31. Nowak JZ, E ZU, Zawilska J (1989) Melatonin and its generating system in vertebrate retina: circadian rhythm, effect of environmental lighting and interaction with dopamine. Neurochem Int 14: 397-406.
32. Underwood H, Goldman BD (1987) Vertebrate circadian and photoperiodic systems: role of the pineal gland and melatonin. J Biol Rhythms 2: 279-315.
33. Fukada Y, Okano T (2002) Circadian clock system in the pineal gland. Mol Neurobiol 25: 19-30.
34. Zimmerman NH, Menaker M (1979) The pineal gland: a pacemaker within the circadian system of the house sparrow. Proc Natl Acad Sci U S A 76: 999-1003.
35. Stokkan KA, Yamazaki S, Tei H, Sakaki Y, Menaker M (2001) Entrainment of the circadian clock in the liver by feeding. Science 291: 490-493.
36. Brown SA, Zumbrunn G, Fleury-Olela F, Preitner N, Schibler U (2002) Rhythms of mammalian body temperature can sustain peripheral circadian clocks. Curr Biol 12: 1574-1583.
37. Balsalobre A, Damiola F, Schibler U (1998) A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell 93: 929-937.

38. Reddy AB, Maywood ES, Karp NA, King VM, Inoue Y, et al. (2007) Glucocorticoid signaling synchronizes the liver circadian transcriptome. Hepatology 45: 1478-1488.
39. Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, et al. (2000) Resetting of circadian time in peripheral tissues by glucocorticoid signaling. Science 289: 2344-2347.
40. Akashi M, Nishida E (2000) Involvement of the MAP kinase cascade in resetting of the mammalian circadian clock. Genes Dev 14: 645-649.
41. Hirota T, Okano T, Kokame K, Shirotani-Ikejima H, Miyata T, et al. (2002) Glucose down-regulates Per1 and Per2 mRNA levels and induces circadian gene expression in cultured Rat-1 fibroblasts. J Biol Chem 277: 44244-44251.
42. Adamovich Y, Ladeuix B, Golik M, Koeners MP, Asher G (2017) Rhythmic Oxygen Levels Reset Circadian Clocks through HIF1alpha. Cell Metab 25: 93-101.
43. Gerber A, Esnault C, Aubert G, Treisman R, Pralong F, et al. (2013) Blood-borne circadian signal stimulates daily oscillations in actin dynamics and SRF activity. Cell 152: 492-503.
44. Schibler U, Sassone-Corsi P (2002) A web of circadian pacemakers. Cell 111: 919-922.
45. Gerber A, Saini C, Curie T, Emmenegger Y, Rando G, et al. (2015) The systemic control of circadian gene expression. Diabetes Obes Metab 17 Suppl 1: 23-32.
46. Schibler U, Gotic I, Saini C, Gos P, Curie T, et al. (2015) Clock-Talk: Interactions between Central and Peripheral Circadian Oscillators in Mammals. Cold Spring Harb Symp Quant Biol 80: 223-232.
47. Dibner C, Schibler U, Albrecht U (2010) The mammalian circadian timing system: organization and coordination of central and peripheral clocks. Annu Rev Physiol 72: 517-549.
48. Ueyama T, Krout KE, Nguyen XV, Karpitskiy V, Kollert A, et al. (1999) Suprachiasmatic nucleus: a central autonomic clock. Nat Neurosci 2: 1051-1053.
49. Kalsbeek A, Bruinstroop E, Yi CX, Klieverik LP, La Fleur SE, et al. (2010) Hypothalamic control of energy metabolism via the autonomic nervous system. Ann N Y Acad Sci 1212: 114-129.
50. Nader N, Chrousos GP, Kino T (2010) Interactions of the circadian CLOCK system and the HPA axis. Trends Endocrinol Metab 21: 277-286.
51. Spiga F, Walker JJ, Terry JR, Lightman SL (2014) HPA axis-rhythms. Compr Physiol 4: 1273-1298.
52. Dickmeis T (2009) Glucocorticoids and the circadian clock. J Endocrinol 200: 3-22.
53. Ratman D, Vanden Berghe W, Dejager L, Libert C, Tavernier J, et al. (2013) How glucocorticoid receptors modulate the activity of other transcription factors: a scope beyond tethering. Mol Cell Endocrinol 380: 41-54.
54. Kornmann B, Schaad O, Bujard H, Takahashi JS, Schibler U (2007) System-driven and oscillator-dependent circadian transcription in mice with a conditionally active liver clock. PLoS Biol 5: e34.
55. Yoo SH, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, et al. (2004) PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc Natl Acad Sci U S A 101: 5339-5346.

56. Konopka RJ, Benzer S (1971) Clock mutants of Drosophila melanogaster. Proc Natl Acad Sci U S A 68: 2112-2116.
57. Wager-Smith K, Kay SA (2000) Circadian rhythm genetics: from flies to mice to humans. Nat Genet 26: 23-27.
58. Reppert SM, Weaver DR (2001) Molecular analysis of mammalian circadian rhythms. Annu Rev Physiol 63: 647-676.
59. Allada R, Emery P, Takahashi JS, Rosbash M (2001) Stopping time: the genetics of fly and mouse circadian clocks. Annu Rev Neurosci 24: 1091-1119.
60. Young MW, Kay SA (2001) Time zones: a comparative genetics of circadian clocks. Nat Rev Genet 2: 702-715.
61. Ko CH, Takahashi JS (2006) Molecular components of the mammalian circadian clock. Hum Mol Genet 15 Spec No 2: R271-277.
62. Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, et al. (2002) The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 110: 251-260.
63. Ueda HR, Chen W, Adachi A, Wakamatsu H, Hayashi S, et al. (2002) A transcription factor response element for gene expression during circadian night. Nature 418: 534-539.
64. Guillaumond F, Dardente H, Giguere V, Cermakian N (2005) Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. J Biol Rhythms 20: 391-403.
65. Gallego M, Virshup DM (2007) Post-translational modifications regulate the ticking of the circadian clock. Nat Rev Mol Cell Biol 8: 139-148.
66. Bellet MM, Sassone-Corsi P (2010) Mammalian circadian clock and metabolism - the epigenetic link. J Cell Sci 123: 3837-3848.
67. Toh KL, Jones CR, He Y, Eide EJ, Hinz WA, et al. (2001) An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. Science 291: 1040-1043.
68. Lee C, Etchegaray JP, Cagampang FR, Loudon AS, Reppert SM (2001) Posttranslational mechanisms regulate the mammalian circadian clock. Cell 107: 855-867.
69. Tamaru T, Hirayama J, Isojima Y, Nagai K, Norioka S, et al. (2009) CK2alpha phosphorylates BMAL1 to regulate the mammalian clock. Nat Struct Mol Biol 16: 446-448.
70. Hirayama J, Sahar S, Grimaldi B, Tamaru T, Takamatsu K, et al. (2007) CLOCK-mediated acetylation of BMAL1 controls circadian function. Nature 450: 1086-1090.
71. Cardone L, Hirayama J, Giordano F, Tamaru T, Palvimo JJ, et al. (2005) Circadian clock control by SUMOylation of BMAL1. Science 309: 1390-1394.
72. Ripperger JA, Schibler U (2006) Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions. Nat Genet 38: 369-374.
73. Etchegaray JP, Lee C, Wade PA, Reppert SM (2003) Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. Nature 421: 177-182.
74. Masri S, Sassone-Corsi P (2010) Plasticity and specificity of the circadian epigenome. Nat Neurosci 13: 1324-1329.
75. Doi M, Hirayama J, Sassone-Corsi P (2006) Circadian regulator CLOCK is a histone

acetyltransferase. Cell 125: 497-508.
76. Ishiura M, Kutsuna S, Aoki S, Iwasaki H, Andersson CR, et al. (1998) Expression of a gene cluster kaiABC as a circadian feedback process in cyanobacteria. Science 281: 1519-1523.
77. Teng SW, Mukherji S, Moffitt JR, de Buyl S, O'Shea EK (2013) Robust circadian oscillations in growing cyanobacteria require transcriptional feedback. Science 340: 737-740.
78. Froehlich AC, Pregueiro A, Lee K, Denault D, Colot H, et al. (2003) The molecular workings of the Neurospora biological clock. Novartis Found Symp 253: 184-198; discussion 102-189, 198-202, 281-184.
79. Glossop NR, Lyons LC, Hardin PE (1999) Interlocked feedback loops within the Drosophila circadian oscillator. Science 286: 766-768.
80. Dunlap J (1998) Circadian rhythms. An end in the beginning. Science 280: 1548-1549.
81. Udoh US, Valcin JA, Gamble KL, Bailey SM (2015) The Molecular Circadian Clock and Alcohol-Induced Liver Injury. Biomolecules 5: 2504-2537.
82. Sweeney BM, Haxo FT (1961) Persistence of a Photosynthetic Rhythm in Enucleated Acetabularia. Science 134: 1361-1363.
83. Schweiger E, Wallraff HG, Schweiger HG (1964) Endogenous Circadian Rhythm in Cytoplasm of Acetabularia: Influence of the Nucleus. Science 146: 658-659.
84. Mergenhagen D, Schweiger HG (1975) The effect of different inhibitors of transcription and translation on the expression and control of circadian rhythm in individual cells of Acetabularia. Exp Cell Res 94: 321-326.
85. Cornelius G, Rensing L (1976) Daily rhythmic changes in Mg2+-dependent ATPase activity in human red blood cell membranes in vitro. Biochem Biophys Res Commun 71: 1269-1272.
86. Radha E, Hill TD, Rao GH, White JG (1985) Glutathione levels in human platelets display a circadian rhythm in vitro. Thromb Res 40: 823-831.
87. Nakajima M, Imai K, Ito H, Nishiwaki T, Murayama Y, et al. (2005) Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. Science 308: 414-415.
88. Kim YI, Dong G, Carruthers CW, Jr., Golden SS, LiWang A (2008) The day/night switch in KaiC, a central oscillator component of the circadian clock of cyanobacteria. Proc Natl Acad Sci U S A 105: 12825-12830.
89. Rust MJ, Markson JS, Lane WS, Fisher DS, O'Shea EK (2007) Ordered phosphorylation governs oscillation of a three-protein circadian clock. Science 318: 809-812.
90. O'Neill JS, van Ooijen G, Dixon LE, Troein C, Corellou F, et al. (2011) Circadian rhythms persist without transcription in a eukaryote. Nature 469: 554-558.
91. O'Neill JS, Reddy AB (2011) Circadian clocks in human red blood cells. Nature 469: 498-503.
92. Bass J, Takahashi JS (2011) Circadian rhythms: Redox redux. Nature 469: 476-478.
93. Hoyle NP, O'Neill JS (2015) Oxidation-reduction cycles of peroxiredoxin proteins and nontranscriptional aspects of timekeeping. Biochemistry 54: 184-193.
94. Milev NB, Rey G, Valekunja UK, Edgar RS, O'Neill JS, et al. (2015) Analysis of the redox oscillations in the circadian clockwork. Methods Enzymol 552: 185-210.

95. Hall A, Karplus PA, Poole LB (2009) Typical 2-Cys peroxiredoxins--structures, mechanisms and functions. FEBS J 276: 2469-2477.
96. Reddy AB, Rey G (2014) Metabolic and nontranscriptional circadian clocks: eukaryotes. Annu Rev Biochem 83: 165-189.
97. Edgar RS, Green EW, Zhao Y, van Ooijen G, Olmedo M, et al. (2012) Peroxiredoxins are conserved markers of circadian rhythms. Nature 485: 459-464.
98. Kitayama Y, Nishiwaki T, Terauchi K, Kondo T (2008) Dual KaiC-based oscillations constitute the circadian system of cyanobacteria. Genes Dev 22: 1513-1521.
99. Kumar V (2017) Biological Timekeeping: Clocks, Rhythms and Behaviour: Springer.
100. Viswanathan N, Chandrashekaran MK (1985) Cycles of presence and absence of mother mouse entrain the circadian clock of pups. Nature 317: 530-531.
101. Davis FC, Stice S, Menaker M (1987) Activity and reproductive state in the hamster: independent control by social stimuli and a circadian pacemaker. Physiol Behav 40: 583-590.
102. Mistlberger RE, Skene DJ (2004) Social influences on mammalian circadian rhythms: animal and human studies. Biol Rev Camb Philos Soc 79: 533-556.
103. Ray S, Reddy AB (2016) Cross-talk between circadian clocks, sleep-wake cycles, and metabolic networks: Dispelling the darkness. Bioessays 38: 394-405.
104. Eckel-Mahan K, Sassone-Corsi P (2009) Metabolism control by the circadian clock and vice versa. Nat Struct Mol Biol 16: 462-467.
105. Blagonravov ML, Frolov VA, Azova MM, Goryachev VA (2013) Characteristics of circadian rhythm of blood pressure during long-term hypertension development in SHR rats. Bull Exp Biol Med 155: 612-614.
106. Tsang AH, Barclay JL, Oster H (2014) Interactions between endocrine and circadian systems. J Mol Endocrinol 52: R1-16.
107. Refinetti R, Menaker M (1992) The circadian rhythm of body temperature. Physiol Behav 51: 613-637.
108. Curtis AM, Bellet MM, Sassone-Corsi P, O'Neill LA (2014) Circadian clock proteins and immunity. Immunity 40: 178-186.
109. Hogenesch JB (2009) It's all in a day's work: Regulation of DNA excision repair by the circadian clock. Proc Natl Acad Sci U S A 106: 2481-2482.
110. Khapre RV, Samsa WE, Kondratov RV (2010) Circadian regulation of cell cycle: molecular connections between aging and the circadian clock. Ann Med 42: 404-415.
111. Miller BH, McDearmon EL, Panda S, Hayes KR, Zhang J, et al. (2007) Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation. Proc Natl Acad Sci U S A 104: 3342-3347.
112. Rey G, Reddy AB (2015) Interplay between cellular redox oscillations and circadian clocks. Diabetes Obes Metab 17 Suppl 1: 55-64.
113. Zelinski EL, Deibel SH, McDonald RJ (2014) The trouble with circadian clock dysfunction: multiple deleterious effects on the brain and body. Neurosci Biobehav Rev 40: 80-101.
114. Maury E, Hong HK, Bass J (2014) Circadian disruption in the pathogenesis of metabolic syndrome. Diabetes Metab 40: 338-346.
115. Jones SG, Benca RM (2015) Circadian Disruption in Psychiatric Disorders. Sleep Med Clin 10: 481-493.

116. Kelleher FC, Rao A, Maguire A (2014) Circadian molecular clocks and cancer. Cancer Lett 342: 9-18.
117. Papagiannakopoulos T, Bauer MR, Davidson SM, Heimann M, Subbaraj L, et al. (2016) Circadian Rhythm Disruption Promotes Lung Tumorigenesis. Cell Metab 24: 324-331.
118. Casper RF, Gladanac B (2014) Introduction: circadian rhythm and its disruption: impact on reproductive function. Fertil Steril 102: 319-320.
119. Sahar S, Sassone-Corsi P (2009) Metabolism and cancer: the circadian clock connection. Nat Rev Cancer 9: 886-896.
120. Iurisci I, Filipski E, Sallam H, Harper F, Guettier C, et al. (2009) Liver circadian clock, a pharmacologic target of cyclin-dependent kinase inhibitor seliciclib. Chronobiol Int 26: 1169-1188.
121. Bellivier F, Geoffroy PA, Etain B, Scott J (2015) Sleep- and circadian rhythm-associated pathways as therapeutic targets in bipolar disorder. Expert Opin Ther Targets 19: 747-763.
122. Ohdo S (2010) Chronotherapeutic strategy: Rhythm monitoring, manipulation and disruption. Adv Drug Deliv Rev 62: 859-875.
123. Sukumaran S, Almon RR, DuBois DC, Jusko WJ (2010) Circadian rhythms in gene expression: Relationship to physiology, disease, drug disposition and drug action. Adv Drug Deliv Rev 62: 904-917.
124. Nagvekar MD, Dhume RA, Gogate MG (1987) Study of photic versus non-photic cues as entrainers of circadian running activity in rats. Indian J Physiol Pharmacol 31: 91-98.
125. Refinetti R (2015) Comparison of light, food, and temperature as environmental synchronizers of the circadian rhythm of activity in mice. J Physiol Sci 65: 359-366.
126. Foster RG, Hankins MW (2002) Non-rod, non-cone photoreception in the vertebrates. Prog Retin Eye Res 21: 507-527.
127. Panda S, Sato TK, Castrucci AM, Rollag MD, DeGrip WJ, et al. (2002) Melanopsin (Opn4) requirement for normal light-induced circadian phase shifting. Science 298: 2213-2216.
128. Lucas RJ, Hattar S, Takao M, Berson DM, Foster RG, et al. (2003) Diminished pupillary light reflex at high irradiances in melanopsin-knockout mice. Science 299: 245-247.
129. Panda S, Provencio I, Tu DC, Pires SS, Rollag MD, et al. (2003) Melanopsin is required for non-image-forming photic responses in blind mice. Science 301: 525-527.
130. Ukai H, Kobayashi TJ, Nagano M, Masumoto KH, Sujino M, et al. (2007) Melanopsin-dependent photo-perturbation reveals desynchronization underlying the singularity of mammalian circadian clocks. Nat Cell Biol 9: 1327-1334.
131. David-Gray ZK, Janssen JW, DeGrip WJ, Nevo E, Foster RG (1998) Light detection in a 'blind' mammal. Nat Neurosci 1: 655-656.
132. Guler AD, Ecker JL, Lall GS, Haq S, Altimus CM, et al. (2008) Melanopsin cells are the principal conduits for rod-cone input to non-image-forming vision. Nature 453: 102-105.
133. Liu AC, Lewis WG, Kay SA (2007) Mammalian circadian signaling networks and

therapeutic targets. Nat Chem Biol 3: 630-639.
134. Moore RY, Lenn NJ (1972) A retinohypothalamic projection in the rat. J Comp Neurol 146: 1-14.
135. Hannibal J, Moller M, Ottersen OP, Fahrenkrug J (2000) PACAP and glutamate are co-stored in the retinohypothalamic tract. J Comp Neurol 418: 147-155.
136. Hannibal J (2002) Neurotransmitters of the retino-hypothalamic tract. Cell Tissue Res 309: 73-88.
137. Ginty DD, Kornhauser JM, Thompson MA, Bading H, Mayo KE, et al. (1993) Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. Science 260: 238-241.
138. FRISCH B, ASCHOFF J (1987) Circadian rhythms in honeybees: entrainment by feeding cycles. Physiological entomology 12: 41-49.
139. Davis RE, Bardach JE (1965) Time-co-ordinated prefeeding activity in fish. Animal Behaviour 13: 154-162.
140. Abe H, Sugimoto S (1987) Food-anticipatory response to restricted food access based on the pigeon's biological clock. Animal Learning & Behavior 15: 353-359.
141. Edmonds SC (1977) Food and light as entrainers of circadian running activity in the rat. Physiol Behav 18: 915-919.
142. Jilge B, Stahle H (1993) Restricted food access and light-dark: impact of conflicting zeitgebers on circadian rhythms of the rabbit. Am J Physiol 264: R708-715.
143. Gooley JJ, Schomer A, Saper CB (2006) The dorsomedial hypothalamic nucleus is critical for the expression of food-entrainable circadian rhythms. Nat Neurosci 9: 398-407.
144. Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F, et al. (2000) Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev 14: 2950-2961.
145. Mukherji A, Kobiita A, Chambon P (2015) Shifting the feeding of mice to the rest phase creates metabolic alterations, which, on their own, shift the peripheral circadian clocks by 12 hours. Proc Natl Acad Sci U S A 112: E6683-6690.
146. Hara R, Wan K, Wakamatsu H, Aida R, Moriya T, et al. (2001) Restricted feeding entrains liver clock without participation of the suprachiasmatic nucleus. Genes Cells 6: 269-278.
147. Wu T, Ni Y, Zhuge F, Fu Z (2010) Resetting process of peripheral circadian gene expression after the combined reversal of feeding schedule and light/dark cycle via a 24-h light period transition in rats. Physiol Res 59: 581-590.
148. Asher G, Reinke H, Altmeyer M, Gutierrez-Arcelus M, Hottiger MO, et al. (2010) Poly(ADP-ribose) polymerase 1 participates in the phase entrainment of circadian clocks to feeding. Cell 142: 943-953.
149. Atger F, Gobet C, Marquis J, Martin E, Wang J, et al. (2015) Circadian and feeding rhythms differentially affect rhythmic mRNA transcription and translation in mouse liver. Proc Natl Acad Sci U S A 112: E6579-6588.
150. Vollmers C, Gill S, DiTacchio L, Pulivarthy SR, Le HD, et al. (2009) Time of feeding and the intrinsic circadian clock drive rhythms in hepatic gene expression. Proc Natl Acad Sci U S A 106: 21453-21458.
151. Schibler U, Ripperger J, Brown SA (2003) Peripheral circadian oscillators in mammals:

time and food. J Biol Rhythms 18: 250-260.
152. Peek CB, Affinati AH, Ramsey KM, Kuo HY, Yu W, et al. (2013) Circadian clock NAD+ cycle drives mitochondrial oxidative metabolism in mice. Science 342: 1243417.
153. Le Minh N, Damiola F, Tronche F, Schutz G, Schibler U (2001) Glucocorticoid hormones inhibit food-induced phase-shifting of peripheral circadian oscillators. EMBO J 20: 7128-7136.
154. Landgraf D, Tsang AH, Leliavski A, Koch CE, Barclay JL, et al. (2015) Oxyntomodulin regulates resetting of the liver circadian clock by food. Elife 4: e06253.
155. Dang F, Sun X, Ma X, Wu R, Zhang D, et al. (2016) Insulin post-transcriptionally modulates Bmal1 protein to affect the hepatic circadian clock. Nat Commun 7: 12696.
156. Feillet CA, Mendoza J, Pevet P, Challet E (2008) Restricted feeding restores rhythmicity in the pineal gland of arrhythmic suprachiasmatic-lesioned rats. Eur J Neurosci 28: 2451-2458.
157. Ip MM, Ip C, Tepperman HM, Tepperman J (1977) Effect of adaptation to meal-feeding on insulin, glucagon and the cyclic nucleotide-protein kinase system in rats. J Nutr 107: 746-757.
158. Matsumoto E, Ishihara A, Tamai S, Nemoto A, Iwase K, et al. (2010) Time of day and nutrients in feeding govern daily expression rhythms of the gene for sterol regulatory element-binding protein (SREBP)-1 in the mouse liver. J Biol Chem 285: 33028-33036.
159. Hatori M, Vollmers C, Zarrinpar A, DiTacchio L, Bushong EA, et al. (2012) Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet. Cell Metab 15: 848-860.
160. Kaur S, Thankachan S, Begum S, Blanco-Centurion C, Sakurai T, et al. (2008) Entrainment of temperature and activity rhythms to restricted feeding in orexin knock out mice. Brain Res 1205: 47-54.
161. Richter CP (1922) A behavioristic study of the activity of the rat. Comparative Psychology Monographs.
162. Rusak B, Mistlberger RE, Losier B, Jones CH (1988) Daily hoarding opportunity entrains the pacemaker for hamster activity rhythms. J Comp Physiol A 164: 165-171.
163. Mistlberger RE, Mumby DG (1992) The limbic system and food-anticipatory circadian rhythms in the rat: ablation and dopamine blocking studies. Behav Brain Res 47: 159-168.
164. Davidson AJ, Aragona BJ, Houpt TA, Stephan FK (2001) Persistence of meal-entrained circadian rhythms following area postrema lesions in the rat. Physiol Behav 74: 349-354.
165. Davidson AJ, Aragona BJ, Werner RM, Schroeder E, Smith JC, et al. (2001) Food-anticipatory activity persists after olfactory bulb ablation in the rat. Physiol Behav 72: 231-235.
166. Storch KF, Weitz CJ (2009) Daily rhythms of food-anticipatory behavioral activity do not require the known circadian clock. Proc Natl Acad Sci U S A 106: 6808-6813.

167. Pitts S, Perone E, Silver R (2003) Food-entrained circadian rhythms are sustained in arrhythmic Clk/Clk mutant mice. Am J Physiol Regul Integr Comp Physiol 285: R57-67.
168. Feillet CA, Ripperger JA, Magnone MC, Dulloo A, Albrecht U, et al. (2006) Lack of food anticipation in Per2 mutant mice. Curr Biol 16: 2016-2022.
169. LeSauter J, Hoque N, Weintraub M, Pfaff DW, Silver R (2009) Stomach ghrelin-secreting cells as food-entrainable circadian clocks. Proc Natl Acad Sci U S A 106: 13582-13587.
170. Pendergast JS, Nakamura W, Friday RC, Hatanaka F, Takumi T, et al. (2009) Robust food anticipatory activity in BMAL1-deficient mice. PLoS One 4: e4860.
171. Mistlberger RE (1994) Circadian food-anticipatory activity: formal models and physiological mechanisms. Neurosci Biobehav Rev 18: 171-195.
172. Balzer I, Hardeland R (1988) Influence of temperature on biological rhythms. International journal of biometeorology 32: 231-241.
173. Refinetti R (2010) Entrainment of circadian rhythm by ambient temperature cycles in mice. J Biol Rhythms 25: 247-256.
174. Saini C, Morf J, Stratmann M, Gos P, Schibler U (2012) Simulated body temperature rhythms reveal the phase-shifting behavior and plasticity of mammalian circadian oscillators. Genes Dev 26: 567-580.
175. Tsuchiya Y, Akashi M, Nishida E (2003) Temperature compensation and temperature resetting of circadian rhythms in mammalian cultured fibroblasts. Genes Cells 8: 713-720.
176. Ruby NF, Burns DE, Heller HC (1999) Circadian rhythms in the suprachiasmatic nucleus are temperature-compensated and phase-shifted by heat pulses in vitro. J Neurosci 19: 8630-8636.
177. Liu Y, Merrow M, Loros JJ, Dunlap JC (1998) How temperature changes reset a circadian oscillator. Science 281: 825-829.
178. Barrett RK, Takahashi JS (1995) Temperature compensation and temperature entrainment of the chick pineal cell circadian clock. J Neurosci 15: 5681-5692.
179. Takeuchi T, Hinohara T, Kurosawa G, Uchida K (2007) A temperature-compensated model for circadian rhythms that can be entrained by temperature cycles. J Theor Biol 246: 195-204.
180. Lahiri K, Vallone D, Gondi SB, Santoriello C, Dickmeis T, et al. (2005) Temperature regulates transcription in the zebrafish circadian clock. PLoS Biol 3: e351.
181. Kidd PB, Young MW, Siggia ED (2015) Temperature compensation and temperature sensation in the circadian clock. Proc Natl Acad Sci U S A 112: E6284-6292.
182. Rand DA, Shulgin BV, Salazar JD, Millar AJ (2006) Uncovering the design principles of circadian clocks: mathematical analysis of flexibility and evolutionary goals. J Theor Biol 238: 616-635.
183. Buhr ED, Yoo SH, Takahashi JS (2010) Temperature as a universal resetting cue for mammalian circadian oscillators. Science 330: 379-385.
184. Liu S, Chen XM, Yoda T, Nagashima K, Fukuda Y, et al. (2002) Involvement of the suprachiasmatic nucleus in body temperature modulation by food deprivation in rats. Brain Res 929: 26-36.
185. Tamaru T, Hattori M, Honda K, Benjamin I, Ozawa T, et al. (2011) Synchronization of

circadian Per2 rhythms and HSF1-BMAL1:CLOCK interaction in mouse fibroblasts after short-term heat shock pulse. PLoS One 6: e24521.

186. Reinke H, Saini C, Fleury-Olela F, Dibner C, Benjamin IJ, et al. (2008) Differential display of DNA-binding proteins reveals heat-shock factor 1 as a circadian transcription factor. Genes Dev 22: 331-345.
187. Morf J, Rey G, Schneider K, Stratmann M, Fujita J, et al. (2012) Cold-inducible RNA-binding protein modulates circadian gene expression posttranscriptionally. Science 338: 379-383.
188. Ghosh A, Chance B (1964) Oscillations of glycolytic intermediates in yeast cells. Biochem Biophys Res Commun 16: 174-181.
189. Richard P, Bakker BM, Teusink B, Van Dam K, Westerhoff HV (1996) Acetaldehyde mediates the synchronization of sustained glycolytic oscillations in populations of yeast cells. Eur J Biochem 235: 238-241.
190. Wolf J, Passarge J, Somsen OJ, Snoep JL, Heinrich R, et al. (2000) Transduction of intracellular and intercellular dynamics in yeast glycolytic oscillations. Biophys J 78: 1145-1153.
191. Ruoff P, Christensen MK, Wolf J, Heinrich R (2003) Temperature dependency and temperature compensation in a model of yeast glycolytic oscillations. Biophys Chem 106: 179-192.
192. Frenkel R (1968) Control of reduced diphosphopyridine nucleotide oscillations in beef heart extracts. II. Oscillations of glycolytic intermediates and adenine nucleotides. Arch Biochem Biophys 125: 157-165.
193. Tornheim K, Lowenstein JM (1974) The purine nucleotide cycle. IV. Interactions with oscillations of the glycolytic pathway in muscle extracts. J Biol Chem 249: 3241-3247.
194. O'Rourke B, Ramza BM, Marban E (1994) Oscillations of membrane current and excitability driven by metabolic oscillations in heart cells. Science 265: 962-966.
195. Nilsson T, Schultz V, Berggren PO, Corkey BE, Tornheim K (1996) Temporal patterns of changes in ATP/ADP ratio, glucose 6-phosphate and cytoplasmic free Ca2+ in glucose-stimulated pancreatic beta-cells. Biochem J 314 (Pt 1): 91-94.
196. Tu BP, Kudlicki A, Rowicka M, McKnight SL (2005) Logic of the yeast metabolic cycle: temporal compartmentalization of cellular processes. Science 310: 1152-1158.
197. Tu BP, McKnight SL (2007) The yeast metabolic cycle: insights into the life of a eukaryotic cell. Cold Spring Harb Symp Quant Biol 72: 339-343.
198. Cerveny J, Sinetova MA, Valledor L, Sherman LA, Nedbal L (2013) Ultradian metabolic rhythm in the diazotrophic cyanobacterium Cyanothece sp. ATCC 51142. Proc Natl Acad Sci U S A 110: 13210-13215.
199. Carey HV, Andrews MT, Martin SL (2003) Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. Physiol Rev 83: 1153-1181.
200. Eckel-Mahan KL, Patel VR, Mohney RP, Vignola KS, Baldi P, et al. (2012) Coordination of the transcriptome and metabolome by the circadian clock. Proc Natl Acad Sci U S A 109: 5541-5546.
201. Dallmann R, Viola AU, Tarokh L, Cajochen C, Brown SA (2012) The human circadian

metabolome. Proc Natl Acad Sci U S A 109: 2625-2629.
202. Davies SK, Ang JE, Revell VL, Holmes B, Mann A, et al. (2014) Effect of sleep deprivation on the human metabolome. Proc Natl Acad Sci U S A 111: 10761-10766.
203. Bromage TG, Idaghdour Y, Lacruz RS, Crenshaw TD, Ovsiy O, et al. (2016) The Swine Plasma Metabolome Chronicles "Many Days" Biological Timing and Functions Linked to Growth. PLoS One 11: e0145919.
204. Chua EC, Shui G, Lee IT, Lau P, Tan LC, et al. (2013) Extensive diversity in circadian regulation of plasma lipids and evidence for different circadian metabolic phenotypes in humans. Proc Natl Acad Sci U S A 110: 14468-14473.
205. Gooley JJ, Chua EC (2014) Diurnal regulation of lipid metabolism and applications of circadian lipidomics. J Genet Genomics 41: 231-250.
206. Gooley JJ (2016) Circadian regulation of lipid metabolism. Proc Nutr Soc 75: 440-450.
207. Nikolaeva S, Ansermet C, Centeno G, Pradervand S, Bize V, et al. (2016) Nephron-Specific Deletion of Circadian Clock Gene Bmal1 Alters the Plasma and Renal Metabolome and Impairs Drug Disposition. J Am Soc Nephrol 27: 2997-3004.
208. Castro C, Briggs W, Paschos GK, FitzGerald GA, Griffin JL (2015) A metabolomic study of adipose tissue in mice with a disruption of the circadian system. Mol Biosyst 11: 1897-1906.
209. Fustin JM, Doi M, Yamada H, Komatsu R, Shimba S, et al. (2012) Rhythmic nucleotide synthesis in the liver: temporal segregation of metabolites. Cell Rep 1: 341-349.
210. Cho H, Zhao X, Hatori M, Yu RT, Barish GD, et al. (2012) Regulation of circadian behaviour and metabolism by REV-ERB-alpha and REV-ERB-beta. Nature 485: 123-127.
211. Nakahata Y, Sahar S, Astarita G, Kaluzova M, Sassone-Corsi P (2009) Circadian control of the NAD+ salvage pathway by CLOCK-SIRT1. Science 324: 654-657.
212. Dioum EM, Rutter J, Tuckerman JR, Gonzalez G, Gilles-Gonzalez MA, et al. (2002) NPAS2: a gas-responsive transcription factor. Science 298: 2385-2387.
213. Yin L, Wu N, Curtin JC, Qatanani M, Szwergold NR, et al. (2007) Rev-erbalpha, a heme sensor that coordinates metabolic and circadian pathways. Science 318: 1786-1789.
214. Yang J, Kim KD, Lucas A, Drahos KE, Santos CS, et al. (2008) A novel heme-regulatory motif mediates heme-dependent degradation of the circadian factor period 2. Mol Cell Biol 28: 4697-4711.
215. Canto C, Auwerx J (2011) Calorie restriction: is AMPK a key sensor and effector? Physiology (Bethesda) 26: 214-224.
216. Sahar S, Sassone-Corsi P (2012) Regulation of metabolism: the circadian clock dictates the time. Trends Endocrinol Metab 23: 1-8.
217. Bellet MM, Nakahata Y, Boudjelal M, Watts E, Mossakowska DE, et al. (2013) Pharmacological modulation of circadian rhythms by synthetic activators of the deacetylase SIRT1. Proc Natl Acad Sci U S A 110: 3333-3338.
218. Nakahata Y, Kaluzova M, Grimaldi B, Sahar S, Hirayama J, et al. (2008) The NAD+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control. Cell 134: 329-340.
219. Imai S (2010) "Clocks" in the NAD World: NAD as a metabolic oscillator for the regulation of metabolism and aging. Biochim Biophys Acta 1804: 1584-1590.

220. Asher G, Gatfield D, Stratmann M, Reinke H, Dibner C, et al. (2008) SIRT1 regulates circadian clock gene expression through PER2 deacetylation. Cell 134: 317-328.
221. Li X, Zhang S, Blander G, Tse JG, Krieger M, et al. (2007) SIRT1 deacetylates and positively regulates the nuclear receptor LXR. Mol Cell 28: 91-106.
222. Yoon JC, Puigserver P, Chen G, Donovan J, Wu Z, et al. (2001) Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. Nature 413: 131-138.
223. Frescas D, Valenti L, Accili D (2005) Nuclear trapping of the forkhead transcription factor FoxO1 via Sirt-dependent deacetylation promotes expression of glucogenetic genes. J Biol Chem 280: 20589-20595.
224. Blander G, Guarente L (2004) The Sir2 family of protein deacetylases. Annu Rev Biochem 73: 417-435.
225. Masri S, Rigor P, Cervantes M, Ceglia N, Sebastian C, et al. (2014) Partitioning circadian transcription by SIRT6 leads to segregated control of cellular metabolism. Cell 158: 659-672.
226. Imai S, Armstrong CM, Kaeberlein M, Guarente L (2000) Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature 403: 795-800.
227. Rutter J, Reick M, Wu LC, McKnight SL (2001) Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. Science 293: 510-514.
228. Amaral IP, Johnston IA (2012) Circadian expression of clock and putative clock-controlled genes in skeletal muscle of the zebrafish. Am J Physiol Regul Integr Comp Physiol 302: R193-206.
229. Cahill GM, Hurd MW, Batchelor MM (1998) Circadian rhythmicity in the locomotor activity of larval zebrafish. Neuroreport 9: 3445-3449.
230. Hurd MW, Debruyne J, Straume M, Cahill GM (1998) Circadian rhythms of locomotor activity in zebrafish. Physiol Behav 65: 465-472.
231. Postlethwait JH, Yan YL, Gates MA, Horne S, Amores A, et al. (1998) Vertebrate genome evolution and the zebrafish gene map. Nat Genet 18: 345-349.
232. Menaker M, Moreira LF, Tosini G (1997) Evolution of circadian organization in vertebrates. Braz J Med Biol Res 30: 305-313.
233. Granados-Fuentes D, Herzog ED (2013) The clock shop: coupled circadian oscillators. Exp Neurol 243: 21-27.
234. Li X, Montgomery J, Cheng W, Noh JH, Hyde DR, et al. (2012) Pineal photoreceptor cells are required for maintaining the circadian rhythms of behavioral visual sensitivity in zebrafish. PLoS One 7: e40508.
235. Cahill GM (1996) Circadian regulation of melatonin production in cultured zebrafish pineal and retina. Brain Res 708: 177-181.
236. Gothilf Y, Coon SL, Toyama R, Chitnis A, Namboodiri MA, et al. (1999) Zebrafish serotonin N-acetyltransferase-2: marker for development of pineal photoreceptors and circadian clock function. Endocrinology 140: 4895-4903.
237. Zhdanova IV (2011) Sleep and its regulation in zebrafish. Rev Neurosci 22: 27-36.
238. Cahill GM (2002) Clock mechanisms in zebrafish. Cell Tissue Res 309: 27-34.
239. Hurd MW, Cahill GM (2002) Entraining signals initiate behavioral circadian rhythmicity in larval zebrafish. J Biol Rhythms 17: 307-314.

240. Lima-Cabello E, Diaz-Casado ME, Guerrero JA, Otalora BB, Escames G, et al. (2014) A review of the melatonin functions in zebrafish physiology. J Pineal Res 57: 1-9.
241. Whitmore D, Foulkes NS, Sassone-Corsi P (2000) Light acts directly on organs and cells in culture to set the vertebrate circadian clock. Nature 404: 87-91.
242. Whitmore D, Foulkes NS, Strahle U, Sassone-Corsi P (1998) Zebrafish Clock rhythmic expression reveals independent peripheral circadian oscillators. Nat Neurosci 1: 701-707.
243. Tamai TK, Carr AJ, Whitmore D (2005) Zebrafish circadian clocks: cells that see light. Biochem Soc Trans 33: 962-966.
244. Tamai TK, Young LC, Whitmore D (2007) Light signaling to the zebrafish circadian clock by Cryptochrome 1a. Proc Natl Acad Sci U S A 104: 14712-14717.
245. Hirayama J, Miyamura N, Uchida Y, Asaoka Y, Honda R, et al. (2009) Common light signaling pathways controlling DNA repair and circadian clock entrainment in zebrafish. Cell Cycle 8: 2794-2801.
246. Cermakian N, Pando MP, Thompson CL, Pinchak AB, Selby CP, et al. (2002) Light induction of a vertebrate clock gene involves signaling through blue-light receptors and MAP kinases. Curr Biol 12: 844-848.
247. Hirayama J, Cardone L, Doi M, Sassone-Corsi P (2005) Common pathways in circadian and cell cycle clocks: light-dependent activation of Fos/AP-1 in zebrafish controls CRY-1a and WEE-1. Proc Natl Acad Sci U S A 102: 10194-10199.
248. Vallone D, Gondi SB, Whitmore D, Foulkes NS (2004) E-box function in a period gene repressed by light. Proc Natl Acad Sci U S A 101: 4106-4111.
249. Pando MP, Pinchak AB, Cermakian N, Sassone-Corsi P (2001) A cell-based system that recapitulates the dynamic light-dependent regulation of the vertebrate clock. Proc Natl Acad Sci U S A 98: 10178-10183.
250. Falcon J, Gothilf Y, Coon SL, Boeuf G, Klein DC (2003) Genetic, temporal and developmental differences between melatonin rhythm generating systems in the teleost fish pineal organ and retina. J Neuroendocrinol 15: 378-382.
251. Laura R, Magnoli D, Zichichi R, Guerrera MC, De Carlos F, et al. (2012) The photoreceptive cells of the pineal gland in adult zebrafish (Danio rerio). Microsc Res Tech 75: 359-366.
252. Magnoli D, Zichichi R, Laura R, Guerrera MC, Campo S, et al. (2012) Rhodopsin expression in the zebrafish pineal gland from larval to adult stage. Brain Res 1442: 9-14.
253. Matos-Cruz V, Blasic J, Nickle B, Robinson PR, Hattar S, et al. (2011) Unexpected diversity and photoperiod dependence of the zebrafish melanopsin system. PLoS One 6: e25111.
254. Ramos BC, Moraes MN, Poletini MO, Lima LH, Castrucci AM (2014) From blue light to clock genes in zebrafish ZEM-2S cells. PLoS One 9: e106252.
255. Moutsaki P, Whitmore D, Bellingham J, Sakamoto K, David-Gray ZK, et al. (2003) Teleost multiple tissue (tmt) opsin: a candidate photopigment regulating the peripheral clocks of zebrafish? Brain Res Mol Brain Res 112: 135-145.
256. Cavallari N, Frigato E, Vallone D, Frohlich N, Lopez-Olmeda JF, et al. (2011) A blind circadian clock in cavefish reveals that opsins mediate peripheral clock photoreception. PLoS biology 9: e1001142.

257. Hirayama J, Cho S, Sassone-Corsi P (2007) Circadian control by the reduction/oxidation pathway: catalase represses light-dependent clock gene expression in the zebrafish. Proc Natl Acad Sci U S A 104: 15747-15752.
258. Weger BD, Sahinbas M, Otto GW, Mracek P, Armant O, et al. (2011) The light responsive transcriptome of the zebrafish: function and regulation. PLoS One 6: e17080.
259. Peirson SN, Halford S, Foster RG (2009) The evolution of irradiance detection: melanopsin and the non-visual opsins. Philos Trans R Soc Lond B Biol Sci 364: 2849-2865.
260. Lopez-Olmeda JF, Tartaglione EV, de la Iglesia HO, Sanchez-Vazquez FJ (2010) Feeding entrainment of food-anticipatory activity and per1 expression in the brain and liver of zebrafish under different lighting and feeding conditions. Chronobiol Int 27: 1380-1400.
261. Sanchez JA, Sanchez-Vazquez FJ (2009) Feeding entrainment of daily rhythms of locomotor activity and clock gene expression in zebrafish brain. Chronobiol Int 26: 1120-1135.
262. Paredes JF, Lopez-Olmeda JF, Martinez FJ, Sanchez-Vazquez FJ (2015) Daily rhythms of lipid metabolic gene expression in zebra fish liver: Response to light/dark and feeding cycles. Chronobiol Int 32: 1438-1448.
263. Lahiri K, Froehlich N, Heyd A, Foulkes NS, Vallone D (2014) Developmental stage-specific regulation of the circadian clock by temperature in zebrafish. Biomed Res Int 2014: 930308.
264. Egg M, Tischler A, Schwerte T, Sandbichler A, Folterbauer C, et al. (2012) Endurance exercise modifies the circadian clock in zebrafish (Danio rerio) temperature independently. Acta Physiol (Oxf) 205: 167-176.
265. Lopez-Olmeda JF, Sanchez-Vazquez FJ (2009) Zebrafish temperature selection and synchronization of locomotor activity circadian rhythm to ahemeral cycles of light and temperature. Chronobiol Int 26: 200-218.
266. Lopez-Olmeda JF, Madrid JA, Sanchez-Vazquez FJ (2006) Light and temperature cycles as zeitgebers of zebrafish (Danio rerio) circadian activity rhythms. Chronobiol Int 23: 537-550.
267. Kaneko M, Cahill GM (2005) Light-dependent development of circadian gene expression in transgenic zebrafish. PLoS Biol 3: e34.
268. Kaneko M, Hernandez-Borsetti N, Cahill GM (2006) Diversity of zebrafish peripheral oscillators revealed by luciferase reporting. Proc Natl Acad Sci U S A 103: 14614-14619.
269. Hughes ME, DiTacchio L, Hayes KR, Vollmers C, Pulivarthy S, et al. (2009) Harmonics of circadian gene transcription in mammals. PLoS Genet 5: e1000442.
270. Duffield GE, Best JD, Meurers BH, Bittner A, Loros JJ, et al. (2002) Circadian programs of transcriptional activation, signaling, and protein turnover revealed by microarray analysis of mammalian cells. Curr Biol 12: 551-557.
271. Panda S, Antoch MP, Miller BH, Su AI, Schook AB, et al. (2002) Coordinated transcription of key pathways in the mouse by the circadian clock. Cell 109: 307-320.
272. Storch KF, Lipan O, Leykin I, Viswanathan N, Davis FC, et al. (2002) Extensive and

divergent circadian gene expression in liver and heart. Nature 417: 78-83.
273. Li Y, Li G, Wang H, Du J, Yan J (2013) Analysis of a gene regulatory cascade mediating circadian rhythm in zebrafish. PLoS Comput Biol 9: e1002940.
274. Boyle G, Richter K, Priest HD, Traver D, Mockler TC, et al. (2017) Comparative Analysis of Vertebrate Diurnal/Circadian Transcriptomes. PLoS One 12: e0169923.
275. Li Y, Li G, Gorling B, Luy B, Du J, et al. (2015) Integrative analysis of circadian transcriptome and metabolic network reveals the role of de novo purine synthesis in circadian control of cell cycle. PLoS Comput Biol 11: e1004086.
276. Jang ZH, Chung HC, Ahn YG, Kwon YK, Kim JS, et al. (2012) Metabolic profiling of an alcoholic fatty liver in zebrafish (Danio rerio). Mol Biosyst 8: 2001-2009.
277. Weger BD, Weger M, Gorling B, Schink A, Gobet C, et al. (2016) Extensive Regulation of Diurnal Transcription and Metabolism by Glucocorticoids. PLoS Genet 12: e1006512.
278. Mushtaq MY, Marcal RM, Champagne DL, van der Kooy F, Verpoorte R, et al. (2014) Effect of acute stresses on zebra fish (Danio rerio) metabolome measured by NMR-based metabolomics. Planta Med 80: 1227-1233.
279. Akhtar MT, Mushtaq MY, Verpoorte R, Richardson MK, Choi YH (2016) Zebrafish as a Model for Systems Medicine R&D: Rethinking the Metabolic Effects of Carrier Solvents and Culture Buffers Determined by (1)H NMR Metabolomics. OMICS 20: 42-52.
280. Berti R, Durand JP, Becchi S, Brizzi R, Keller N, et al. (2001) Eye degeneration in the blind cave-dwelling fish Phreatichthys andruzzii. Canadian journal of zoology 79: 1278-1285.
281. Colli L, Paglianti A, Berti R, Gandolfi G, Tagliavini J (2009) Molecular phylogeny of the blind cavefish Phreatichthys andruzzii and Garra barreimiae within the family Cyprinidae. Environmental Biology of Fishes 84: 95-107.
282. Cavallari N, Frigato E, Vallone D, Frohlich N, Lopez-Olmeda JF, et al. (2011) A blind circadian clock in cavefish reveals that opsins mediate peripheral clock photoreception. PLoS Biol 9: e1001142.
283. Rist MJ, Muhle-Goll C, Gorling B, Bub A, Heissler S, et al. (2013) Influence of Freezing and Storage Procedure on Human Urine Samples in NMR-Based Metabolomics. Metabolites 3: 243-258.
284. Dieterle F, Ross A, Schlotterbeck G, Senn H (2006) Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in 1H NMR metabonomics. Analytical chemistry 78: 4281-4290.
285. Yan J, Wang H, Liu Y, Shao C (2008) Analysis of gene regulatory networks in the mammalian circadian rhythm. PLoS Comput Biol 4: e1000193.
286. Ung CY, Lam SH, Hlaing MM, Winata CL, Korzh S, et al. (2010) Mercury-induced hepatotoxicity in zebrafish: in vivo mechanistic insights from transcriptome analysis, phenotype anchoring and targeted gene expression validation. BMC Genomics 11: 212.
287. Zhao X, Monson C, Gao C, Gouon-Evans V, Matsumoto N, et al. (2010) Klf6/copeb is required for hepatic outgrowth in zebrafish and for hepatocyte specification in mouse ES cells. Dev Biol 344: 79-93.
288. Sharma MK, Liu RZ, Thisse C, Thisse B, Denovan-Wright EM, et al. (2006)

Hierarchical subfunctionalization of fabp1a, fabp1b and fabp10 tissue-specific expression may account for retention of these duplicated genes in the zebrafish (Danio rerio) genome. FEBS J 273: 3216-3229.

289. Jima DD, Shah RN, Orcutt TM, Joshi D, Law JM, et al. (2009) Enhanced transcription of complement and coagulation genes in the absence of adaptive immunity. Mol Immunol 46: 1505-1516.
290. Cheng W, Guo L, Zhang Z, Soo HM, Wen C, et al. (2006) HNF factors form a network to regulate liver-enriched genes in zebrafish. Dev Biol 294: 482-496.
291. Lyche JL, Nourizadeh-Lillabadi R, Almaas C, Stavik B, Berg V, et al. (2010) Natural mixtures of persistent organic pollutants (POP) increase weight gain, advance puberty, and induce changes in gene expression associated with steroid hormones and obesity in female zebrafish. J Toxicol Environ Health A 73: 1032-1057.
292. Tamai TK, Young LC, Cox CA, Whitmore D (2012) Light acts on the zebrafish circadian clock to suppress rhythmic mitosis and cell proliferation. J Biol Rhythms 27: 226-236.
293. Goel N, Stunkard AJ, Rogers NL, Van Dongen HP, Allison KC, et al. (2009) Circadian rhythm profiles in women with night eating syndrome. J Biol Rhythms 24: 85-94.
294. O'Reardon JP, Ringel BL, Dinges DF, Allison KC, Rogers NL, et al. (2004) Circadian eating and sleeping patterns in the night eating syndrome. Obes Res 12: 1789-1796.
295. Arble DM, Bass J, Laposky AD, Vitaterna MH, Turek FW (2009) Circadian timing of food intake contributes to weight gain. Obesity (Silver Spring) 17: 2100-2102.
296. Shi SQ, Ansari TS, McGuinness OP, Wasserman DH, Johnson CH (2013) Circadian disruption leads to insulin resistance and obesity. Curr Biol 23: 372-381.
297. Marcheva B, Ramsey KM, Buhr ED, Kobayashi Y, Su H, et al. (2010) Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes. Nature 466: 627-631.
298. Fukuwatari T, Shibata K (2013) Nutritional aspect of tryptophan metabolism. Int J Tryptophan Res 6: 3-8.
299. Mizushima N, Levine B, Cuervo AM, Klionsky DJ (2008) Autophagy fights disease through cellular self-digestion. Nature 451: 1069-1075.
300. Rabinowitz JD, White E (2010) Autophagy and metabolism. Science 330: 1344-1348.
301. Hewitt G, Korolchuk VI (2016) Repair, Reuse, Recycle: The Expanding Role of Autophagy in Genome Maintenance. Trends Cell Biol.
302. Ma D, Panda S, Lin JD (2011) Temporal orchestration of circadian autophagy rhythm by C/EBPbeta. EMBO J 30: 4642-4651.
303. Huang G, Zhang F, Ye Q, Wang H (2016) The circadian clock regulates autophagy directly through the nuclear hormone receptor Nr1d1/Rev-erbalpha and indirectly via Cebpb/(C/ebpbeta) in zebrafish. Autophagy 12: 1292-1309.
304. Ma D, Lin JD (2012) Circadian regulation of autophagy rhythm through transcription factor C/EBPbeta. Autophagy 8: 124-125.
305. He C, Bartholomew CR, Zhou W, Klionsky DJ (2009) Assaying autophagic activity in transgenic GFP-Lc3 and GFP-Gabarap zebrafish embryos. Autophagy 5: 520-526.
306. Piccinetti CC, Migliarini B, Olivotto I, Simoniello MP, Giorgini E, et al. (2013) Melatonin and peripheral circuitries: insights on appetite and metabolism in Danio

rerio. Zebrafish 10: 275-282.
307. Hardie DG, Ross FA, Hawley SA (2012) AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat Rev Mol Cell Biol 13: 251-262.
308. Lamia KA, Sachdeva UM, DiTacchio L, Williams EC, Alvarez JG, et al. (2009) AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation. Science 326: 437-440.
309. Um JH, Yang S, Yamazaki S, Kang H, Viollet B, et al. (2007) Activation of 5'-AMP-activated kinase with diabetes drug metformin induces casein kinase Iepsilon (CKIepsilon)-dependent degradation of clock protein mPer2. J Biol Chem 282: 20794-20798.
310. Canto C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, et al. (2009) AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. Nature 458: 1056-1060.
311. Kim EK, Miller I, Aja S, Landree LE, Pinn M, et al. (2004) C75, a fatty acid synthase inhibitor, reduces food intake via hypothalamic AMP-activated protein kinase. J Biol Chem 279: 19970-19976.
312. Barnea M, Haviv L, Gutman R, Chapnik N, Madar Z, et al. (2012) Metformin affects the circadian clock and metabolic rhythms in a tissue-specific manner. Biochim Biophys Acta 1822: 1796-1806.
313. Corton JM, Gillespie JG, Hardie DG (1994) Role of the AMP-activated protein kinase in the cellular stress response. Curr Biol 4: 315-324.
314. Kohsaka A, Laposky AD, Ramsey KM, Estrada C, Joshu C, et al. (2007) High-fat diet disrupts behavioral and molecular circadian rhythms in mice. Cell Metab 6: 414-421.
315. Cailotto C, La Fleur SE, Van Heijningen C, Wortel J, Kalsbeek A, et al. (2005) The suprachiasmatic nucleus controls the daily variation of plasma glucose via the autonomic output to the liver: are the clock genes involved? Eur J Neurosci 22: 2531-2540.
316. Kalsbeek A, La Fleur S, Van Heijningen C, Buijs RM (2004) Suprachiasmatic GABAergic inputs to the paraventricular nucleus control plasma glucose concentrations in the rat via sympathetic innervation of the liver. J Neurosci 24: 7604-7613.
317. la Fleur SE, Kalsbeek A, Wortel J, Fekkes ML, Buijs RM (2001) A daily rhythm in glucose tolerance: a role for the suprachiasmatic nucleus. Diabetes 50: 1237-1243.
318. Yamamoto H, Nagai K, Nakagawa H (1987) Role of SCN in daily rhythms of plasma glucose, FFA, insulin and glucagon. Chronobiol Int 4: 483-491.
319. Beale A, Guibal C, Tamai TK, Klotz L, Cowen S, et al. (2013) Circadian rhythms in Mexican blind cavefish Astyanax mexicanus in the lab and in the field. Nat Commun 4: 2769.
320. Moran D, Softley R, Warrant EJ (2014) Eyeless Mexican cavefish save energy by eliminating the circadian rhythm in metabolism. PLoS One 9: e107877.
321. Moran D, Softley R, Warrant EJ (2015) The energetic cost of vision and the evolution of eyeless Mexican cavefish. Sci Adv 1: e1500363.