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The Naked Mole Rat Possesses a Differently Phased Core Clock System along with a Different Glucose Handling and Mtor Systems Compared to the Common Lab Mouse

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THE NAKED MOLE RAT POSSESSES A DIFFERENTLY PHASED CORE CLOCK SYSTEM ALONGWITH A DIFFERENT GLUCOSE HANDLING AND mTOR SYSTEMS COMPARED TO THE COMMON LAB MOUSE

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DEDICATION

I dedicate this work to my father ***Sri Dilip Kumar Ghosh*** who taught me to look at the world and examine it and enjoy the wonders that follow. I dedicate this work to my mother ***Srimati Sharmistha Ghosh*** who taught me the words and the figures that form the foundations of this work and my life itself. I dedicate this work to my incredibly talented and loving wife ***Srimati Lakshmi Balaraman Ghosh***- a fellow Scientist-whose motivation and encouragement helped me overcome myriad obstacles. I fondly remember my teachers from school days ***Srimati Shikha Rakshit*** and ***Sri Arun Rakhshit*** who helped build the foundations of the curiosity and discipline that go toward making a scientist. Heartfelt thanks are due to my sister ***Srimati Sanchita Roy***, my brother -in-law ***Dr. Tara Shankar Roy*** and my friend ***Dr. Arun Roy*** for loving help during troubled times. ***Dr. Arun Roy*** is a gem of a friend and a fountainhead of strength. Thanks, and Love go out to my uncle ***Sri Pranab Roy***, for many bits that can not be included here for limitations of space. I thank my ***Father-in-Law Dr.Ramachandran Balaraman*** for his trust in me and his motivation which has boosted my confidence many a time in this long and hard journey.

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This work is a gift to all of you. It is a salute to my ***Father*** without whom I would not have been the person who I am today. It is also a small payback to my ***Mother*** and my ***Wife*** who help and guide me in times of confusion. All the credits go to you. The faults,

wherever they lie are all entirely mine. May your love and affection guide and steer me forever and after. Love you all.

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I need to thank all of my lab mates: Sonal, Kuldeep, Nikkhil, Richa, Amol, Artem – every single one of them helped me in different aspects of my research and gave me many important tips which cannot be found in books. Special thanks to Amol for mentoring me in the beginning. At the same time, I want to wish best of luck to our new lab members: Allan and Xhulia. The friendship which formed within our lab team will continue. I want to thank my committee members: Dr. Mazumder ,Dr. Severson, Dr.Li for the invaluable advices and for investing time to read my thesis. I want to thank Dr. Sam-Yellowe and Dr. Kothapalli for reading my thesis and offer their invaluable input.

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THE NAKED MOLE RAT POSSESSES A DIFFERENTLY PHASED CORE CLOCK SYSTEM ALONGWITH A DIFFERENT GLUCOSE HANDLING AND mTOR SYSTEMS COMPARED TO THE COMMON LAB MOUSE

SOUMYADITYA GHOSH

ABSTRACT

INTRODUCTION

The **Naked Mole Rat** [*Heterocephalus glaber*] is a type of rodent -with the highest average lifespan-30 years-among all the rodent species. It lives underground in sealed burrow systems with little to no exposure to day-night transitions on the surface. It shows polyphasic round the clock activity within its subterranean burrow system. The activity and physiology of most animals is governed by the biological circadian clock. The biological circadian clock is a system that oscillates with a rough periodicity of 24 hours. Environmental zeitgebers regulate the entrainment of the circadian clock to the 24-hour day-night cycle and light is the most important zeitgeber. We checked the circadian clock in the naked mole rat for possible modifications due to its special habitat. The oscillating circadian clock regulates various aspects of physiology and metabolism. So, we also investigated aspects of metabolic and environmental sensing systems in the naked mole rat, to build up a comprehensive picture of possible altered circadian physiology in this remarkable species.

CENTRAL HYPOTHESIS

I hypothesized that the naked mole rat, living in a light-sequestered habitat-may have evolved circadian, metabolic and nutrient sensing systems that are similar to that of the mouse but oscillate with much better rhythmicity and these differences positively influence the long life-span of the naked mole rat.

RESULTS

We found that the circadian clock genes oscillate with a different phase within the liver of the naked mole rat from that in the mouse and the glucose metabolic pathway genes show a synchronized phasing in the naked mole rat. We uncovered sharp differences in activity in the mTOR complexes between the two species. The mTOR complex-1 had a lower activity within the naked mole rat compared to that in the mouse whereas the mTOR complex-2 had higher activity within the naked mole rat. To check possible role of promoter differences in driving gene expression, we analyzed the clock-control element motifs in the putative promoters of the naked mole rat clock-genes and found them similar to those in the mouse. It is possible that novel modes of circadian resonance, glucose metabolism and environmental sensing have evolved in this long-lived species.

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Akt	Activated Protein Kinase
AL	Ad-libitum
AMP	Adenosine Monophosphate
AMPK	Adenosine Monophosphate Activated Protein Kinase
ANOVA	Analysis of Variance
ARNTL	Aryl Hydrocarbon Receptor Nuclear Translocator-like Protein
ATP	Adenosine Triphosphate
BMAL1	Brain and Muscle ARNTL
bHLH	Basic Helix Loop Helix
BSA	Bovine Serum Albumin
CCG	Clock Controlled Gene
cDNA	Complementary DNA
CKI	Casein Kinase 1
CLOCK	Circadian Locomotor Output Cycles Kaput
CR	Calorie Restriction
CRY	Cryptochrome
CVD	Cardiovascular Disease
DBP	D site of albumin promoter Binding Protein
DEC	Differentially Expressed in Chondrocytes
DEPTOR	DEP Domain-Containing mTOR-Interacting Protein
DNA	Deoxyribonucleic Acid
dNTP	Deoxy Nucleotide Triphosphate
DTT	Dithiothreitol
E-Box	Enhancer Box
E4BP4 E4	Promoter-Binding Protein
FOXO	Forkhead box O3
G6Pase	Glucose 6 phosphatase
GH	Growth Hormone

H3	H3 Histone
IACUC	Institutional Animal Care and Use Committee
IGF-1	Insulin-like Growth Factor 1
LD	Light/Dark cycle
mg	milligram
mL	milliliter
mM	millimolar
mRNA	Messenger RNA
mTOR	mammalian Target of Rapamycin
mTORC1	mammalian Target of Rapamycin Complex 1
mTORC2	mammalian Target of Rapamycin Complex 2
NAD	Nicotinamide adenine dinucleotide
NAMPT	Nicotinamide phosphoribosyl transferase
NIH	National Institute of Health
NMN	Nicotineamide mononucleotide
PCR	Polymerase Chain Reaction
PEPCK	Phosphoenolpyruvate carboxykinase
PER	Period
PI3K	Phosphoinositide 3-kinase
PPARα	Peroxisome proliferator-activated receptor alpha
PVDF	Polyvinylidene Difluoride
Rev-Erb	Reverse erythroblastosis virus alpha/beta
RNA	Ribonucleic Acid
RORE	Retinoic Acid Related Orphan Response Element
ROR	Retinoic Acid Related Orphan Receptor
qPCR	quantitative Polymerase Chain Reaction
rRNA	Ribosomal RNA
RT-qPCR	Reverse Transcriptase Quantitative PCR
S6K1	Ribosomal protein S6 Kinase 1
SCN	Suprachiasmatic Nucleus
SD	Standard Deviation

SEM	Standard Error of Mean
SIRT1	Silent mating type Information Regulation Two homolog 1
SREBP-1c	Sterol Regulatory Element Binding Protein
STAT5b	Signal Transducer and Activator of Transcription 5B
UV	Ultra Violet
Ub	Ubiquitin
WB	Western Blot
WT	Wild-type
μg	Microgram
μL	Microliter

CHAPTER I

INTRODUCTION

Organisms have evolved to optimize their response to their environment in a way that maximizes their evolutionary fitness. A key aspect of this evolution is the circadian system. It is the inner biological mechanism that ensures every organism has a functioning 24-hour rhythm that primes it for activity or rest in response to geophysical time. The core clock is made up of a transcriptional-translational feedback loop. In this system a protein heterodimer is formed from the products of two core clock genes- *Bmal1* and *Clock*- within the cell nucleus. It binds to the E-box motif of the target genes and drives rhythmic transcription. The target genes include many Clock Control Genes [CCGs] and two more sets of core clock genes. The first such set is made up of the *Per* and *Cry* genes. The products of these genes form into homo and heterodimers within the cytoplasm and ferry back into the nucleus where they disrupt the BMAL1-CLOCK dimer. The gap between these events serves as a time-keeping mechanism to keep track of geophysical time. The third set of core clock genes is the *Rev/Nr1d* and *Ror* genes. The REV/NR1Ds inhibit the transcription of the *Bmal1* gene and the ROR activates/promotes the transcription of the *Bmal1* gene. The antagonistic action of this set of genes helps add a second layer of

periodicity to the *Bmal1* rhythmicity. Superimposed over these three sets of genes is another loop which comprises the *Dbp* gene -its main function is to contribute a delaying factor to the loop to bring it closer to the approximately 24 h time experienced by animals on the planet (Millius & Ueda, 2017; Takahashi, 2015).

It has long been proposed that the circadian clock is crucial to maintaining health in animals. A functioning circadian clock helps the organism keep healthy while a dysfunctional circadian clock causes various disorders. The dysfunction of the circadian clock has also-crucially-been connected to the onset of the early aging phenotype. The connection between clock and aging is potential of great interest as new knowledge in this domain can directly lead to so-called chronotherapies that can modulate life-span through manipulating the body-clock. Mammals of the class *Rodentia* exhibit an enormous diversity of mean life-span. Because of their relatedness in phylogeny, the *Rodentia* can thus provide us with excellent models of a comparative study to examine aging.

In the field of aging, there is an observed correlation between body size and longevity. The heavier an animal, the longer lived it is. While broadly true across the animal kingdom, this relationship also holds true in the case of the *Rodentia*. If an animal were to possess outstanding life-span compared to its peer species [with respect to body-weight]- it would be considered a "Methuselah species" or a preternaturally long-lived species. Given the above considerations, it is interesting to consider if a pair of two species of *Rodentia* -with widely divergent life-spans – yet comparable body-weights-could serve as a model of comparative aging studies. As a matter of fact, such a pair does exist- namely the common lab mouse *[Mus musculus]* and the naked mole rat. [*Heterocephalus glaber*].

As already mentioned, within nature species with larger or heavier body-weight tend to have longer life-spans. However, a few animals have outstanding life-span compared to their peer species in the same weight category. The naked mole rat is one such animal with a life-span which is disproportionately long for its weight class. The average body-weight of the adult naked mole rat is approximately 35 g compared to about 30 g in the mouse. However, the mouse has a mean lifespan of 3 years while the naked mole rat has a recorded *captive* lifespan of about 30 years. Even in the *wild,* the naked mole rat has been observed to live for up to 17 years which is outstanding for its body-weight. It is thought that the naked mole rat does not experience an increase of age-related frailty which other mammals do. The physiological and molecular reasons behind this are unclear.

Researchers have tried to connect the unusual longevity of the naked mole rat to various parameters. The factors examined thus far include unusual skin -hyaluronan, unusual metabolism, unusual immunity, eusocial behavior and even unusual microbiota. We approached this interesting question from a circadian perspective. We carefully considered the habitat and behavior of the naked mole rat in comparison to that of the mouse. The naked mole rat is a strictly subterranean animal [fossorial species] which lives in sealed burrow systems throughout its life. It has been estimated that sunlight penetration in the naked mole rat burrows is minimal and behavioral studies indicate that the naked mole rats display polyphasic, round the clock activity within their burrow-based society. In other words, the naked mole rat experiences highly *uniform environmental conditions* inside their extensive burrow systems- living and eating underground and almost never coming out on the surface. On the other hand, the mouse in the wild, experiences sharp day and night transitions through a nocturnal foraging habit on the surface and a daytime spent

inside subterranean holes. The mouse has a mean lifespan of 3 years while the naked mole rat has a recorded captive lifespan of about 30 years. Given the sharply divergent ecology and ethology of the two species, we wondered if this might mean that the naked mole rat has developed a molecular circadian clock system which is significantly different from that in the mouse. We speculated that if such a system has indeed evolved within the naked mole rat -then it might contribute to the outstanding longevity observed in the species.

Moreover, the circadian clock is known to be a master regulator of metabolism. We reasoned that an altered circadian clock would at least influence the central energy metabolism pathways. The circadian clock has also been demonstrated as a regulator of the mTOR system which enables organisms to sense their environment at the molecular level. Thus, we reasoned that a modified circadian clock would potentially influence the mTOR system also. To develop a comprehensive picture of comparative circadian physiology, we decided to survey and analyze the glucose metabolism system as well as the mTOR system within the naked mole rat in addition to the circadian clock system and compare them with the corresponding systems in the mouse. There is a standing paradigm in the field of circadian biology that a long-lived species lives in circadian resonance with its environment. It is believed that such a long-lived species can sustain sharp daily oscillations within its molecular and physiological systems -oscillations that serve as key drivers to a long and healthy life. ***We hypothesized that the naked mole rat may have evolved circadian, metabolic and nutrient sensing systems that are similar to that in the mouse but oscillate with much better rhythmicity and these differences positively influence the long life-span of the naked mole rat.*** We have indeed observed some

interesting differences between the peer species and we propose that these differences may contribute to the longevity performance of the species.

We identified the core clock genes to be present in the naked mole rat with highly conserved protein sequences between the naked mole rat and the mouse. Our experimental approach confirmed that the naked mole rat liver expresses the core clock mRNAs across the day. Together the above observations indicated that the core clock is at least functional within the fossorial, congenitally micro-ophthalmic naked mole rat. We observed that many of the core clock mRNAs oscillate with a periodicity of 24 h. This was interesting given how little cue from the external day-night transitions reaches the inside of the sealed naked mole rat burrow systems. We were however surprised by the clear phase differences that emerged when we compared the naked mole rat core clock with the mouse core clock. Several of the naked mole rat clock genes maintain a phase relationship that differs from that in the mouse even allowing for a different food-intake time between the two species. We further noticed that the differences we have observed within the naked mole rat clock genes were not a general shift but a gene-specific feature. In other words, core clock genes could be sorted into small and large shift-groups based on their difference from that in the mouse. The amplitude of oscillation -understood as the difference between the daily maximum and minimum- was high for the mouse in most cases. But a few clock genes showed higher oscillation within the naked mole rat. We found this gene-specific difference in oscillation to be an interesting feature.

We have also observed that the expression of the glucose metabolism genes within the liver of the naked mole rat is circadian. However, the phase of peak expression of the genes in the naked mole rat liver is closer to each other in time than that in the liver of the

mouse. Also, the naked mole rat glucose metabolism genes generally showed *strong* amplitudes of oscillations comparable to or stronger than those in the mouse. This was surprising given that the core clock genes were mostly *gently* oscillating in the naked mole rat liver. This hinted at a preference for food-based time-keeping within the naked mole rat over a light-based one, at least in the liver tissue.

Given the molecular level differences in circadian clock and glucose metabolism, we expected similar differences in how mTOR system may be organized. However, we were surprised by the sharpness of the differences between the species that emerged. The mTOR system is organized into two distinct complexes- mTORC-1 and mTORC-2- with clearly demarcated functions for each. Our results showed that the mTORC-1 has a significantly *low-level activity* within the liver of the naked mole rat compared to that in the mouse even if the naked mole rat possesses all the components of complex-1. Intriguingly the picture was exactly reversed in mTORC-2. We noticed that the naked mole rat has a *strongly activated* mTOR-C2 which is significantly more active than that in the mouse. We analyzed both liver tissue and skeletal muscle tissue and noted the same trend. Thus, we found a complex-specific difference in the activity level of the mTOR system within the metabolic tissues of the mouse and the naked mole rat. We suggest that the observed differences be considered in combination with the differences in the circadian clock and the glucose metabolic system. The naked mole rat has evolved to have a core clock system that is phased differently from that in the mouse and also differs in how a few clock genes show strong amplitude of oscillation across the day. The circadian differences likely manifest in the way glucose metabolism genes are expressed within the naked mole rat. The expression of the glucose metabolic genes may also be under the regulation of an

mTOR system which is significantly different from that in the mouse. To sum up, we believe that each system may be having independent as well as coordinated contributions towards the longevity of the naked mole rat. We believe that the naked mole rat may be achieving circadian resonance by some mechanism which is different from that of strong oscillation at the core clock level. Optimized response to the environment -at least in case of the naked mole rat-probably involves anticipating the environment and sensing the environmental cues in a way that is significantly different from another rodent of a comparable weight. Our findings prepare the ground for further studies -to uncover the molecular and physiological consequences of these effects. The resulting knowledge can potentially be translated to life-prolonging therapies.

1.1 Aging and Public Health

Advanced healthcare in developed economies has meant an upward trend of life-span. While this is a boon for the individual the societal costs of an aging society are profound. A decline in fertility coupled with a 20-year increase in lifespan has generated a population aged 65 or elder in developed countries. Never has such a large cohort of the population experienced such long life-span. The enhanced survivorship of this cohort has generated concerns about proper care for the elderly and infirm. There is straining demand on the public health system and on social services. (Centers for Disease Control and Prevention (CDC), 2003) .There is concern that chronic diseases will afflict the aged population disproportionately and cause increase of disability, diminish quality of life, increase health-care costs (Centers for Disease Control and Prevention (CDC), 2003). There is need for more old-age focused health-care as such care will be life-stage specific and more cost-effective to bear for the patient (Cassel, 2000) . It should include better functional assessment and advances in treating the symptoms of aging (Cassel, 2000).

Some diseases like cancer occur disproportionately high in individuals age 65 and higher (Yancik, 2005). Data from epidemiology studies reveal that 56 % of all newly diagnosed cancer patients and a very high 71% of all cancer deaths happen in this age-cohort (Yancik, 2005). This trend is secular across gender and race and cancer type. The median age for cancer death after all factors are taken into account is about 77 years.This is a big indicator of the direction the US healthcare system needs to take (Yancik, 2005). By 2030, the country will require a strong knowledge-base on the management of disease at the intersection of aging and cancer (Yancik & Ries, 2004).

Aging is also associated with the onset of various other disorders like bipolar disorder (Sajatovic, Forester, Gildengers, & Mulsant, 2013) as well as liver disorders (Boland, Dong, Bettencourt, Barrett-Connor, & Loomba, 2014) and kidney disorders Liver diseases like non-alcoholic fatty liver disease affect aged patients much more. The cause of the onset and progress is just starting to be uncovered (Noureddin et al., 2013). Studies show that kidney disorder is associated with hypertension and diabetes interacting in complex ways in aged individuals (Mallappallil, Friedman, Delano, McFarlane, & Salifu, 2014). A lot of knowledge gap exists in this area.

With age, there is a loss of muscle mass and muscle function. This syndrome - known as sarcopenia - produces major health conditions associated with aging and taxes the public health system. The syndromes cause major disabilities in the older population. (Beaudart, Rizzoli, Bruyère, Reginster, & Biver, 2014). The causes of proteolysis in the aged have been connected with a number of intracellular signaling pathways such as IGF/AKT-myostatin/SMAD, PGC1, cytokine / NF-kappaB as well as the AMPK systems. (Wing, Lecker, & Jagoe, 2011). These interact with protein homeostatic systems in

complex ways to modulate muscle aging in the aged people (Wing, Lecker, & Jagoe, 2011). Lots of ground needs to be covered to develop a knowledge-base in this area and any gain can be translated into better public health systems.

Another important domain of aging disorders is impairment of glucose metabolism. Large sections of the population show insulin resistance with advancing age. Post-eating glucose load has shown high correlation with cardiovascular disorders such as atherosclerosis (Brutsaert et al., 2016). Any improvement of knowledge in this domain will help in improving screening and treatment of glucose metabolism-related disabilities in older adults and aged people.

There is important data that connects dietary intake in mid-life stage with healthy aging (Samieri et al., 2013), as well as data that connects healthy aging with healthful eating in advanced age (Clark et al., 2011). Knowledge gains in this area should help in better public health outcomes.

There are studies that show advancing age increases adiposity and this is related to an increase in the risks of ischemic stroke and coronary arterial diseases in advanced age across gender and ethnicity (Kizer et al., 2011). Studies indicate that cardiovascular disease, hypertension, and arterial aging are inter-related. Early intervention and modulation of factors that affect arterial aging can improve cardiovascular health in aging people (Scuteri & Lakatta, 2013).

Thus, from a standpoint of chronic diseases, metabolic disorders, as well as the impairments of specific organ-systems, the process of aging is an important risk factor. An informed public health strategy for this purpose will have to address two fundamental questions. The first is about the biological mechanisms of aging- what happens to the

young, healthy tissue with the time that it develops frailty. The second question is about the reversibility or at least attenuation of the process of aging. In other words, if there is something that can be done to prolong health-span within a chronologically longer time scale. A better understanding of aging can generate know-how that informs clinical and scientific policies to ensure life-stage and age-specific care for the elderly. The properly informed system can screen biomarkers of aging in a community and recommend behavioral interventions. Similarly, in cases where various organ systems show advanced aging phenotype- there is the possibility of therapy to reverse or arrest further deterioration. Together such measures should ensure that a large cohort stays fit and productive over a longer period. It will also prevent frequent and costly hospital bills in an already strained insurance system. The knowledge generated through aging-related studies will also help in related fields like chronic diseases like diabetes and cancer. It should also help in tackling lifestyle diseases like sleep disorders and binge-eating disorders. It is known that aging and the various mentioned diseases have a mutual connection and benefits of aging studies can be translated to the related disorders on a large scale.

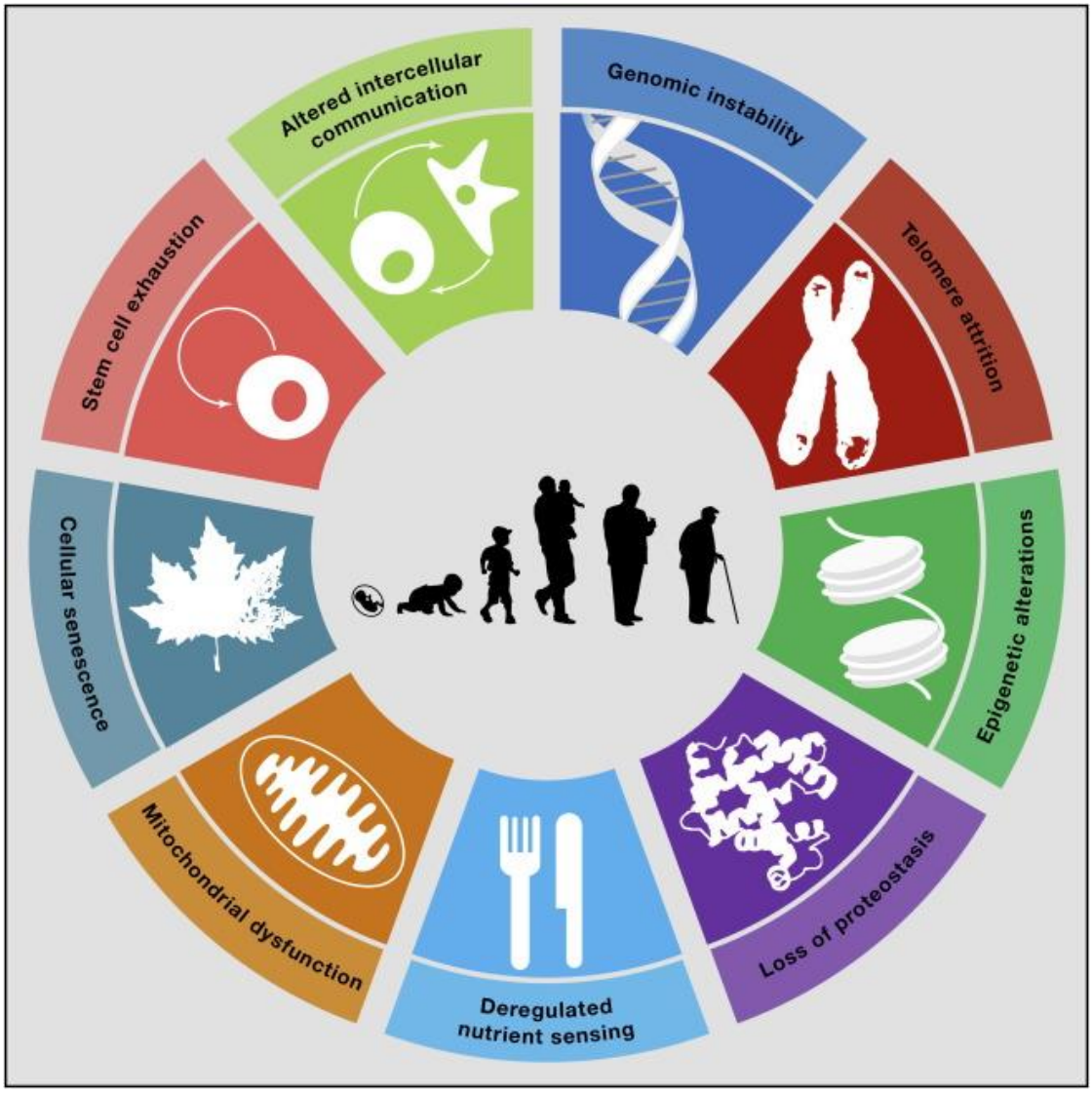


Figure-1-1: The figure above is taken from the review paper "The Hallmarks of Aging" by Lopez Otin et al. [Cell 2013]. It shows nine distinct parameters that are verifiably connected to organismal aging. The general guideline for the selection of the hallmarks is that they should be part of the normal aging process, their aggravation should speed up the aging process and their amelioration should retard the speed of aging.

1.2 Aging and the Body-Clock

Aging is defined as the time-dependent degeneration of an organism's normal bodily functions. According to a comprehensive review from 2013, there are currently up to 9 principal hallmarks of aging. They can be classified into three main categories- primary,

antagonistic and integrative. Of these, the second category is defined as a range of phenomena that evolved as protective mechanisms to support the organism but under deleterious conditions can subvert their original purpose and cause problems for the organism. This category includes-deregulated nutrient sensing, mitochondrial dysfunction, and cellular senescence (López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013).

There is confirmed the connection between clock function and physiological aging in mammals. A mutation in core clock gene Bmal1 causes shorter lifespan in mice. Per mutant animals, there was a shortened lifespan with a free running period. When period mutant flies are exposed to hyper-oxygenic stress, they show higher mortality. Similarly flies lacking period gene showed higher accumulation of damaged proteins and lipids.

It is known that the insulin and insulin-like growth factor signaling can regulate aging and longevity. The IGF signaling has reciprocal links with the circadian system. The suprachiasmatic nucleus(SCN) is the master pacemaker of the circadian clock. Neuronal activity recordings of the SCN have shown age-related damping of electrical amplitude. These changes have been associated with changes to the conductance of the calcium-potassium channels as well as cellular communication (Farajnia, Meijer, & Michel, 2015).There is also evidence of age-related loss of neuropeptides (Krajnak, Kashon, Rosewell, & Wise, 1998). There is also evidence of a decline in the expression of core clock gene Bmal1 with advanced age in the SCN (Hood & Amir, 2017).

With advanced age, the levels of sirtuin1- a protein deacetylase is reduced in the SCN. This sirtuin1 directly regulates circadian rhythms by modulating the activity of circadian proteins-clock and Bmal1. Mice models with loss of clock and or Bmal1 show premature aging (Kondratov, Kondratova, Gorbacheva, Vykhovanets, & Antoch, 2006). A

recent study from our lab has shown that Bmal1 deficiency can increase mTOR complex1 activity. Increased activity of mTOR complex 1 is associated with accelerated aging (Khapre, Kondratova, et al., 2014b). This is a further indication of the inter-connectedness of circadian clock and aging.

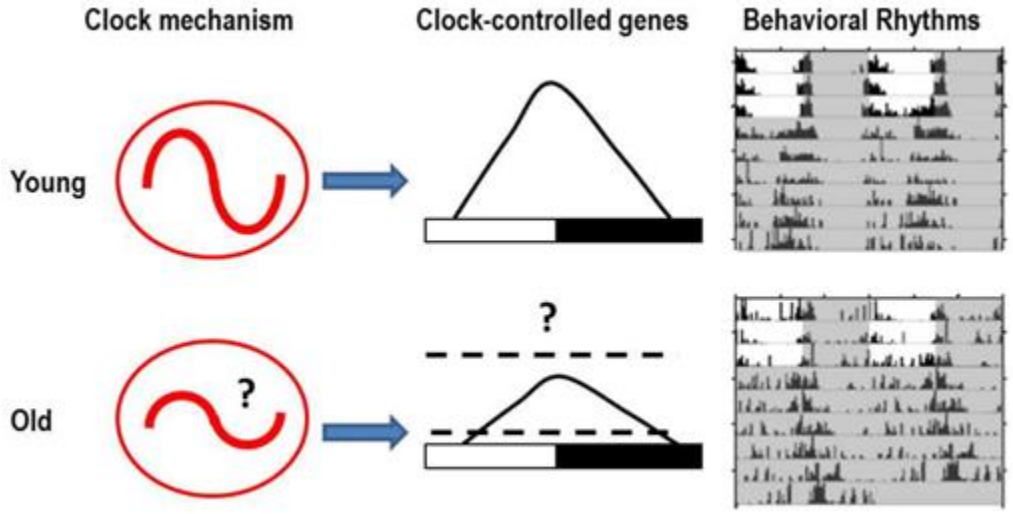


Figure 1-2. The above figure is taken from a study (Jadwiga M. Giebultowicz, Dani M. Long ,2015) that connected changes in the circadian system of *D. melanogaster*. It demonstrates how young flies show stronger clock oscillations than in the old flies. In the case of Clock mutant flies, the oscillations are absent.

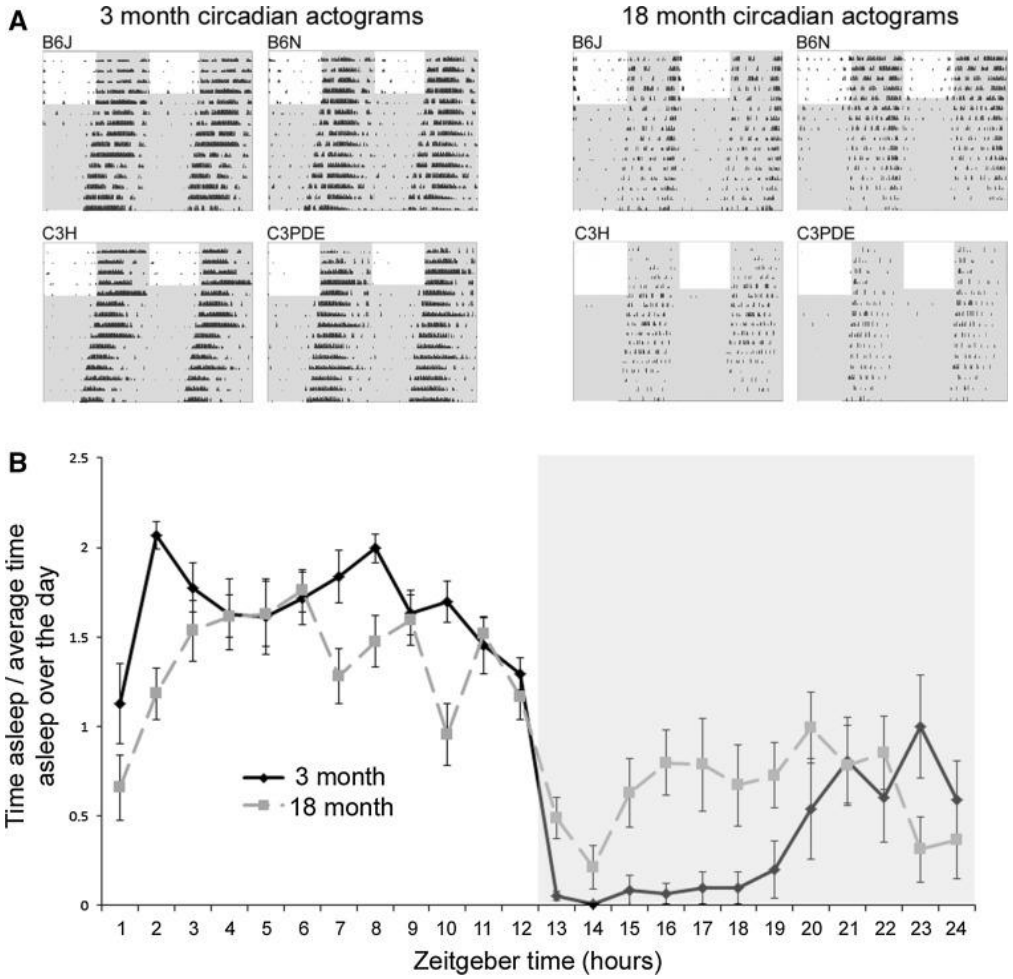


Figure-1-3.The above figure is taken from Banks [2015] It analyzes the circadian profiles of activity of the young [3 months] and old mice [18 months]
A: Older mice show a reduced circadian activity, reduced circadian amplitude and lengthened circadian period compared to younger mice.
B. Older mice show poor sleep rhythms and poor sleep amplitudes

1.3 Molecular basis of the circadian clock

The molecular clock in mammals is based upon the principle of transcription-translation feedback loops (TTFL). The positive arm of this TTFL is made up of 2 activators-*Clock* and *Bmal1*. CLOCK and BMAL1 form a heterodimer with a basic helix loop helix-PAS transcription factor. This dimer initiates the rhythm by activating the

transcription of various clock-controlled output genes. The same positive arm also drives the transcription of the repressors period and cryptochrome.

The period and cryptochrome proteins form various heterodimers in the cytoplasm and translocate back to the nucleus. Within the nucleus, they interact with the activator complex inhibiting further activation of their own transcription. The PER and CRY proteins get destabilized through ubiquitin pathways. This lifts the repression action. This is followed by a resetting of the cycle over a period of 24 hours. A range of kinases like CK1α, CK1δ, CK1 ε and phosphatases such as PP1, PP5 are involved in fine-tuning this cycle. They influence the rate at which the PER- CRY dimers are transported across the nuclear membrane and also the rate at which they are degraded (Partch, Green, & Takahashi, 2014; Takahashi, 2015).

There is a second TTFL that emerges through transcriptional activation of *Bmal1* by the retinoid-related orphan receptors RORs and repression of the same by the REV-ERBs. This *second TTFL* is responsible for generating oscillations in the *Bmal1* transcription rates. It is thought that this introduces a delay in the clock which is critical for 24-hour timing.

It is hypothesized that the inter-connecting TTFL loops provides the core-clock stability against environmental perturbations and help maintain an accurate 24-hour time. The robustness of this system is evidenced by the fact that single knockouts of most integral clock genes do not cause a complete breakdown of behavioral rhythmicity.

There is a *third TTFL* which involves genes like *Dbp, E4bp4* and *Tef*. These genes bind to the D-box element and drive the expression of the other core clock genes. They introduce a delay function to bring the clock to a 24-hour rhythmicity. While it is now

evident that the circadian clock is primarily based upon transcriptional control, there are clues about other modes of rhythmic control. It is known that circadian control of the poly-A tail length can regulate the rate of translation for some genes. Moreover, some protein isoforms that have alternative splicing can display variable expression.

At a genomic level, the CLOCK-BMAL1 complex binds to *several thousands* of sites with maximum frequency at mid-day. This binding happens at consensus E-box motifs. Correspondingly the PER1, PER2, CRY1 and CRY2 bind to many sites on the DNA in the evening. The PER-CRY complex is degraded during the night which lifts the repression on their own transcription - allowing a new transcription cycle to proceed.

The ROR/BMAL/REV ERB feedback loop makes the system robust to disruption. The transcription of RORs and the REV ERBs is done during the subjective day. Both these proteins competitively bind to RRE binding sites in Bmal1 promoter to regulate its transcription. While ROR is an activator of Bmal1, the REV-ERB inhibits Bmal1 transcription. Thus, multiple loops of gene expression constitute the mechanism of the circadian clock.

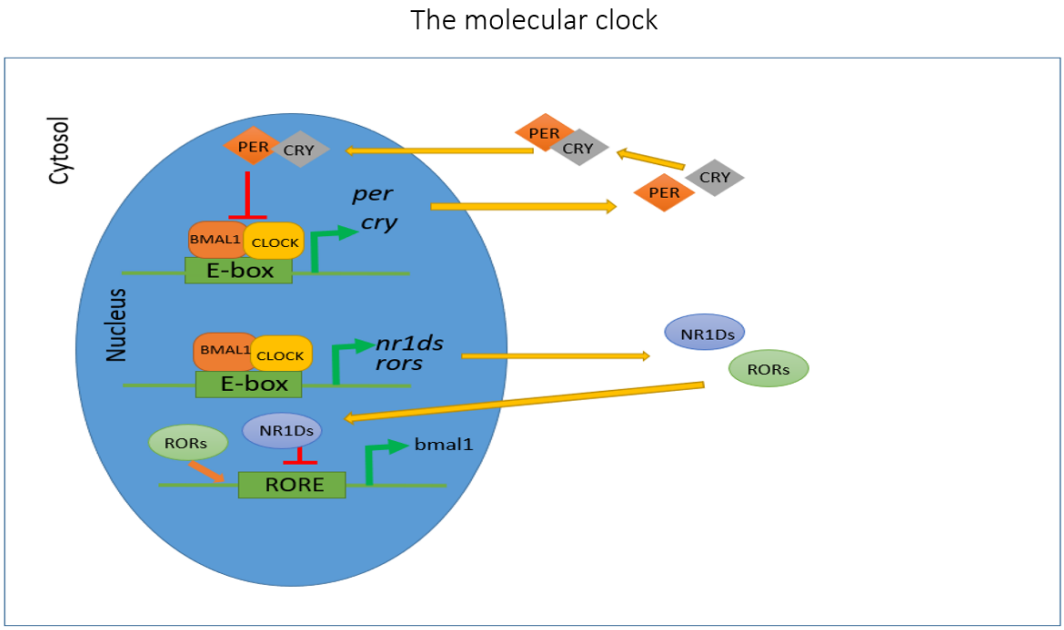


Figure 1-4 Simplified circuit of the mammalian circadian clock

The Transcriptional Factors CLOCK and BMAL1 form a heterodimer .These then bind to the promoters of the clock controlled genes and the *Period* (*Pers*) and *Cryptochrome* (*Crys*) genes PERs and CRYs proteins then form dimers that transport back into the nucleus and inhibit BMAL1-CLOCK transcriptional activity. This thus forms a full transcription-tranlation feedback loop. The products of another group of BMAL1-CLOCK regulated genes, *Rev-Erbs [Nr1ds]* and *Rors*, form a second feedback system whereby the *Nr1ds* repress and the *Rors* activate the transcription of the *Bmal1* gene This figure is adapted from an article by Ko and Takahashi 2006.

1.4 Biological Clock and the Metabolic System

The organismic clock is connected to the biochemical cycles of the animal. A large number of studies show that the circadian clock –the core TTFL system- has reciprocal interactions with the energetics of the body.

The CLOCK protein is one of the participating members of the Bmal1-Clock heterodimer. It was found to directly affect chromatin remodeling though a Histone Acetyltransferase activity (M. Doi, Hirayama, & Sassone-Corsi, 2006).

The NAD^+ -dependent deacetylase SIRT1 was found as to be the key effector that acetylates BMAL1 protein and Histone H3 through the CLOCK protein. Thus, a cellular energetics sensing system helps inform the activation state of BMAL1-CLOCK dimer that is essential to generate oscillating rhythms of circadian system. SIRT1 and CLOCK remain in association through all points in the cell cycle. This is an indicator of the importance of the interplay between the metabolic regulation and the circadian regulation (Nakahata et al., 2008).

Additionally the SIRT1 has been found to promote the destabilization of the Per2 protein which is the component of the primary negative feedback loop in the system (Asher et al., 2008). This plays important role in maintaining the timeliness of the 24 h rhythm.

Various transcriptional activators and co-activators like PPAR-alpha (Purushotham, Schug, & Li, 2009), FOXO3 (Brunet et al., 2004) , PGC1 alpha (Rodgers & Puigserver, 2007), and CRTC2 known to be involved in liver metabolism may also be affected by SIRT1 in a circadian manner.

The nuclear receptor family [PPARs, REV-ERB s, RORs, FXR, SHP, TRs etc.] are involved in various aspects of the anabolic and catabolic metabolism. A large proportion of them [20 out of total 49] displays circadian oscillations (Yang et al., 2006). Of these the REVs and the RORs are involved in the core clock machinery. The REV-ERBs regulate liver gluconeogenesis, liver cell differentiation as well as lipid metabolism. Thus, the nuclear receptors are a further axis of clock –metabolism cross-talk.

It has been shown that the metabolic intermediates in the central energy yielding pathways –like Glucose -6-phosphates and Fructose-1,6-diphosphate etc. can also show rhythmicity (Ghosh & Chance, 1964). It has also been demonstrated that hepatic

gluconeogenesis –responsible for maintaining glucose steady state levels during periods of rest- is under circadian control through the Cryptochrome system.

Another factor connecting the energetic state to the circadian phases is AMPK. It is a highly conserved nutrient sensor that is activated by nutrient stress. When activated it can destabilize the CRY1 – which is a component of the negative feedback loop of the core circadian clock (Lamia et al., 2009).

It is thus clear that there is a metabolism to clock signaling system. However, the clock itself can signal to metabolism. The histone deacetylase enzyme SIRT1 depends on NAD^+ levels to influence core clock activity. The supply of NAD+ is itself governed by the activity of the rate limiting enzyme NAMPT. It has recently been demonstrated that at the transcriptional level the *Nampt* gene is under the direct control of the CLOCK. Thus there is a tight coregulation feedback system of clock and metabolism (Ramsey et al., 2009). At the physiological level glucose metabolism varies in both humans and rodents depending on time of day. At times of fasting non- insulin mediated glucose utilization prevails. Food consumption shifts glucose disposal towards insulin dependence. This behavior dependence of glucose metabolism is anticipated by internal rhythms. Endogenous circadian rhythms exist for whole body glucose metabolism. In such rhythms, circulating glucose levels increase prior to waking up in animals. Blood glucose levels continue to show rhythms in rodents even when they are fasted (Ando, Ushijima, Shimba, & Fujimura, 2016) . SCN ablation experiments lead to the disruption of these systemic physiological rhythms (Yamamoto, Nagai, & Nakagawa, 1987).

Similarly glycogen metabolism enzymes show circadian oscillation (R. Doi, Oishi, & Ishida, 2010). The anabolic glycogen synthase shows peak levels during active phase within rodents.

While the catabolic glycogen phosphorylase shows peak levels during sleep phase. The enzymes of gluconeogenesis like PEPCK show 24-hour rhythms (R. Doi et al., 2010).

Glucose homeostasis shows circadian patterns within other metabolic tissues like muscle (Dyar et al., 2014). Animals use endocrine factors like insulin and glucagon to regulate glucose homeostasis. There is experimental evidence that such endocrine factor release is also under a layer of circadian clock control. Plasma insulin levels, insulin transcripts, insulin sensitivity are all shown to be under circadian clock regulation (Kalsbeek, la Fleur, & Fliers, 2014) . Additionally, the release of glucagon is also under 24-hour clock regulation.

An important axis of circadian glucose homeostasis is cryptochrome regulated gluconeogenesis. The process of gluconeogenesis is a glucose input mechanism where the animal uses endogenous substrate to generate glucose in times of inactivity. During such inactive or fasting phase animal pancreas produces glucagon hormone, This hormone binds to its receptors in hepatocytes. The binding event activates downstream G- Protein coupled receptors. (GPCR). This in turn generates cascades of cAMP -CREB signaling. The phosphorylated CREB activates pck1 and g6pc enzymes of the gluconeogenesis pathway. As such the levels of phosphor-CREB transcriptional activity is of central importance in gluconeogenesis. Zhang. et al experimentally verified that CREB activity is gated by CRY1 and CRY2 both core clock genes (Zhang et al., 2010).

Nuclear receptors like REV-ERB alpha can regulate the expression of gluconeogenic enzymes. Mice lacking REV-ERB alpha display hyperglycemia over circadian time (Delezie et al., 2012). Another nuclear receptor is ROR gamma. The chip-seq analysis confirms ROR gamma is recruited in a circadian matter to various genes of glucose metabolism such as Pepck, Glut2, Pklr, Gck, Gckr, Ppar gamma, Pcx. There was a corresponding decrease in the mRNA expression of these genes in the liver upon loss of ROR- gamma (Yang et al., 2006).

It has also been shown that disruption of clock genes in mouse liver causes loss of post-translational modification like acetylation within mitochondrial proteins. Many of these proteins were members of glycolysis gluconeogenesis pathway, citric acid cycle pathway, amino acid metabolism pathway and fatty acid metabolism pathway. Thus the circadian clock and metabolism have connections even at the level of the mitochondria (Masri et al., 2013).

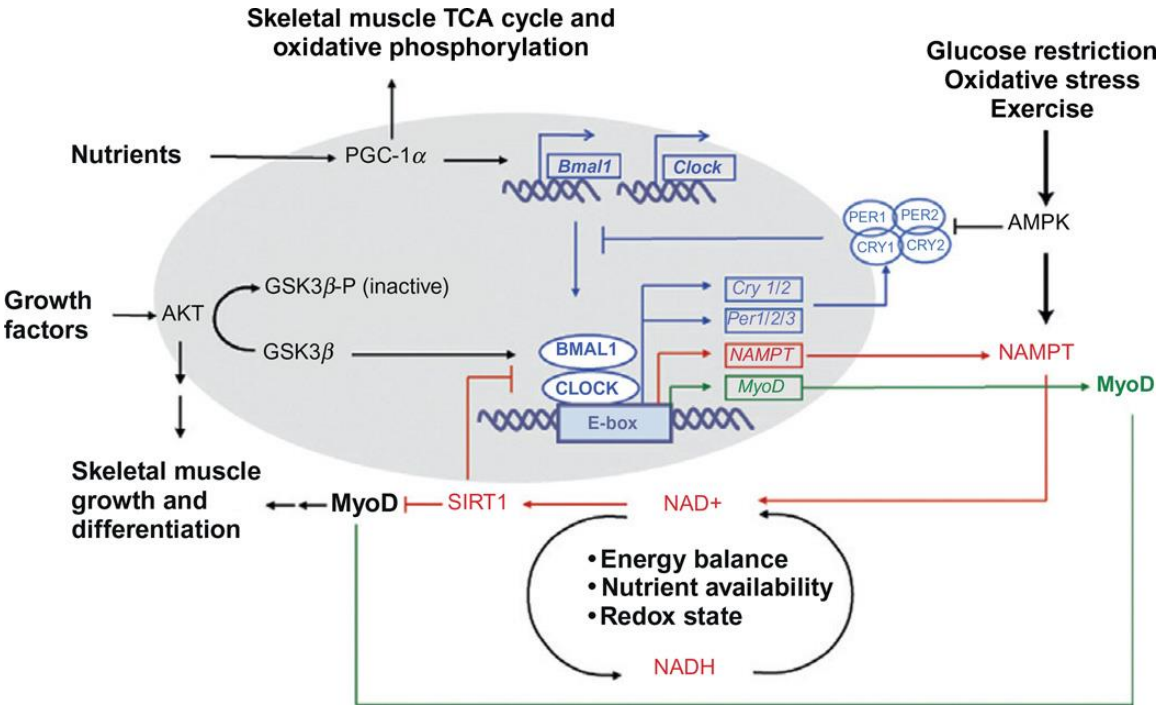


Figure1.5 The above figure (Lefta, Esser,2011), shows interactions between the biological clock and a metabolic tissue-the skeletal muscle. AMPK, Sirtuin1, PGC1alpha are all metabolic sensors that regulate muscle metabolism and the core clock. The core clock, in turn, helps regulate the rhythmic expression of these sensors.

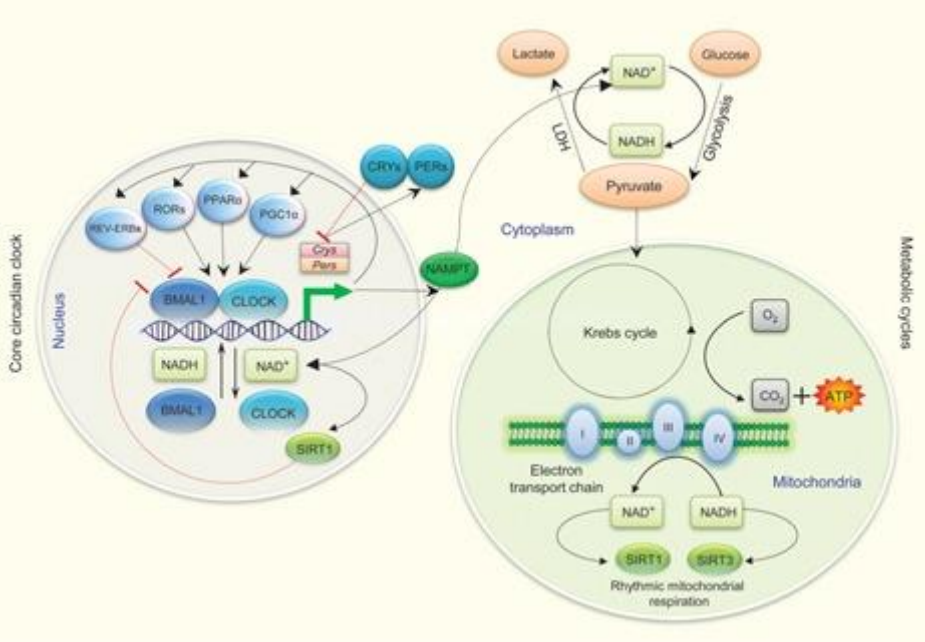


Figure 1.6 The above figure (Ray, Reddy 2016), describes multiple points of interaction between the circadian oscillators and the metabolic oscillators. Core clock proteins such as Bmal1 and Clock regulate the NAD + biosynthesis which is a key metabolite. In turn, the BMAL1-CLOCK binding to DNA is affected by the NAD+/NADH levels. The enzyme LDH can regulate NAD/NADH concentration levels. The NAD/NADH levels can activate Sirtuins1 and 3 which in turn can regulate circadian gene expression. Abbreviations: BMAL1, brain and muscle ARNT-Like 1; CLOCK, circadian locomotor output cycles kaput; Cry, cryptochrome; LDH, lactate dehydrogenase; NAD, nicotinamide adenine dinucleotide; NAMPT, nicotinamide phosphoribosyl-transferase; NPAS2, neuronal PAS domain protein 2; Per, period; PPAR, peroxisome proliferator-activated receptor; PGC-1α, PPAR gamma coactivator-1 alpha; ROR, retinoic acid orphan receptors; SIRT 1, sirtuin 1; SIRT 3, sirtuin 3.

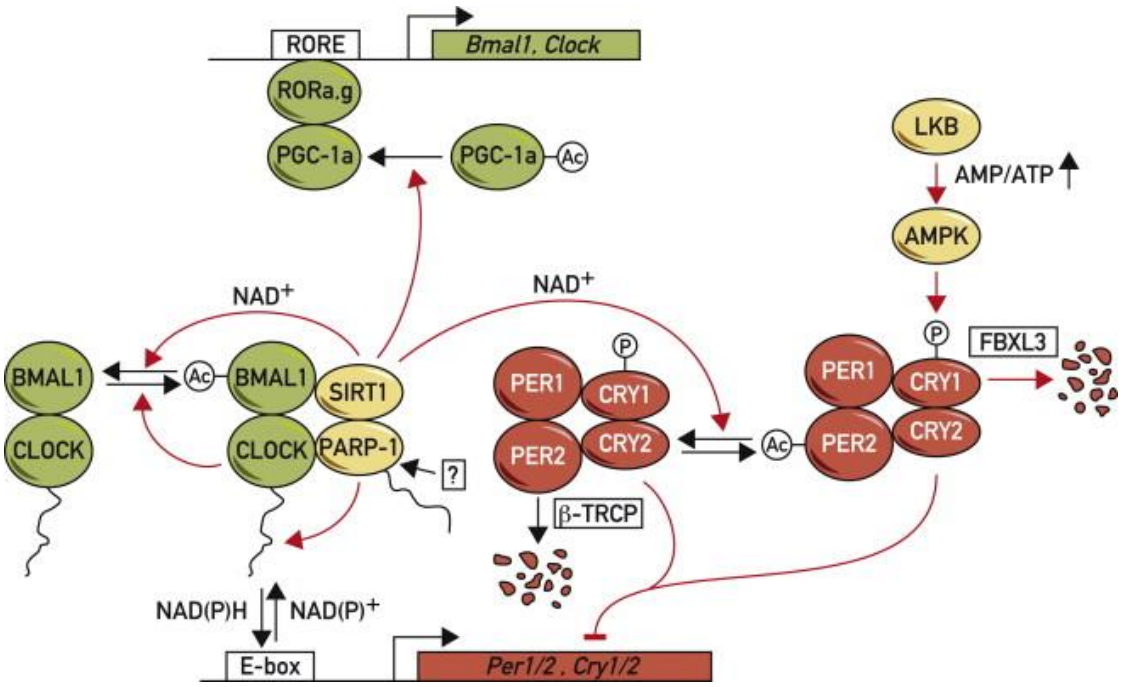


Figure 1.7 The above figure (Ascher, Schibler 2011), shows that the metabolic regulators [marked in yellow] can interact with the core clock negative limb [marked in red] as well as the core clock positive limb [marked in green]. Ubiquitin ligase family proteins FBXL3 and beta-TRCP mark the negative limb proteins CRY and PER for degradation. Another factor LKB is a phosphorylation sensor that targets CRY1 for degradation through AMPK [when AMP/ATP ration increases]. Another sensor of metabolism is Sirtuin-1 which can deacetylate BMAL1and PER2-thereby affording post-translational modification control. Such deacetylation diminishes PER2 half-life and enhances PGC1 alpha activity. The enhanced PGC1 alpha activity acts in concert with ROR alpha, gamma activity to activate *Bmal1* transcription.

1.5 Biological Clock and the mTOR system

Time-keeping is a cornerstone of life on earth. Across different branches of the kingdom of life and across various evolutionary time-scales, it has arisen to confer the evolutionary advantage of various kinds. It has been shown that the biological clock can be of various time-scales -viz day-long [circadian], less than a day [ultradian], more than

a day [infradian] etc. Among the different types, the most common rhythm observed on the planet is the circadian rhythm.

Research has shown that circadian time-keeping evolved three different times – prokaryotic cyanobacteria, cells that gave rise to higher plants and a third branch that evolved into fungi and animals (Dunlap & Loros, 2017). In simple systems like the fungi the clock enables time-oriented sporulation, stress-response, growth-rate variation as well as aspects of metabolism.

In the cyanobacteria, *S. elongatus* – there is a circadian time-keeping system constituted of the three proteins –Kai A, Kai B and Kai C. It has been found that a mixture of the three proteins in vitro- while supplied with ATP-can constitute an oscillator. It is an example of a Post-Transcriptional Oscillator (Egli, 2017). In the fungus, we can notice a timing system with two transcription factors at its core- namely the WC-1 and WC-2. They form the so-called White-Collar-Complex. This conjugate complex regulates the expression of the frq gene. The FRQ protein, in turn, regulates other clock-controlled genes. The degree of phosphorylation of the FRQ protein serves to control the period of this clock. Similarly, all across the animal kingdom, there are variations of the clock to tackle environmental variation. Geophysical conditions cycle over time and this causes abiotic changes like variations in temperature, humidity circulation of wind and precipitation. Connected to the abiotic cycles we have biological changes like oscillations of food availability, predator activity, tree cover etc. The internal biological clock serves as a reference to the animals to keep track of all these external factors.

Studies in yeast in which mTOR complex component was knocked out found that this increased the lifespan of yeast (Kaeberlein et al., 2005). In another study mTORC-1

deletion caused extension of lifespan in *C. elegans* (Vellai et al., 2003). Mutations of mTORC-1 pathway established that this manipulation can increase lifespan in *Drosophila melanogaster* (Kapahi et al., 2004).

It is assumed that the beneficial longevity effects of dietary restriction are mediated through the mTOR complex 1 pathway. In systems with mutations in the mTOR complex 1 signaling, dietary restriction fails to provide longevity advantages.

Low levels of insulin IGF signaling results in enhanced longevity in fruit flies and mice. The IS system activates mTOR complex 1through AKT. There is a crosstalk from mTOR complex 1 to IIS through S6K, IRS1, and TSC1.

AMPK overexpression correlates with extended lifespan in *Caenorhabditis elegans* Pharmacological activation of AMPK can confer longevity to cancer-prone strains of mice. AMPK can inhibit mTOR complex 1 through 2 separate mechanisms

1. Phosphorylation of TSC 2 and activation of TSC 2 which downregulates mTORC-1.
2. Direct phosphorylation of raptor which inhibits mTORC-1 signaling.

In a comprehensive study by National Institute of Aging called interventions testing program (ITP), the chemical rapamycin was utilized to artificially inhibit mTOR. It showed age-related degeneration in heart, liver, adrenal glands, tendon etc. and a decrease of spontaneous physical activity all occurred more slowly in rapamycin-treated mice (Harrison et al., 2009). In another study C57, BL/6 mice treated with rapamycin showed decreased tumor formation and oncogenesis. While reduced mTOR complex 1 signaling is associated with extended lifespan. It is known that increased mTOR complex 1 signaling leads to disease. Those diseases include- cancer as well as early aging.

mTOR activity is organized around 2 distinct complexes-mTOR complex 1 and mTOR complex 2. In a study mTOR complex, 2 was shown to localize to mitochondria-associated endoplasmic reticulum which is enriched in ribosomes. Since ribosomal activity is at least partially controlled by mTOR complex 1. This phenomenon links the 2 complexes. Studies determined that the beneficial effects of rapamycin treatment on insulin sensitivity are at least partially mediated through modulation of mTOR complex 2 signaling.

There are reports where mice heterozygous for AKT1 (within mTOR complex 2 signaling) show enhanced lifespan (Nojima et al., 2013). In long-lived Snell mice, mTOR complex 2 signaling is high. The same mTOR complex 2 signaling is also high in growth receptor knockout mice (Dominick et al., 2015). To summarize current research indicates that attenuated mTOR complex 1 signaling and enhanced mTOR complex 2 signaling is good for longevity.

In our lab, an earlier study has established that the circadian clock can inhibit mTOR signaling to prevent wasteful use of resources in anabolic processes (Khapre, Kondratova, et al., 2014). Such processes can severely limit available nutrients and energy leaving the organism with bad homeostasis and poor fitness. Thus, the core clock mechanism has confirmed the link with the mTOR signaling system in mediating longevity.

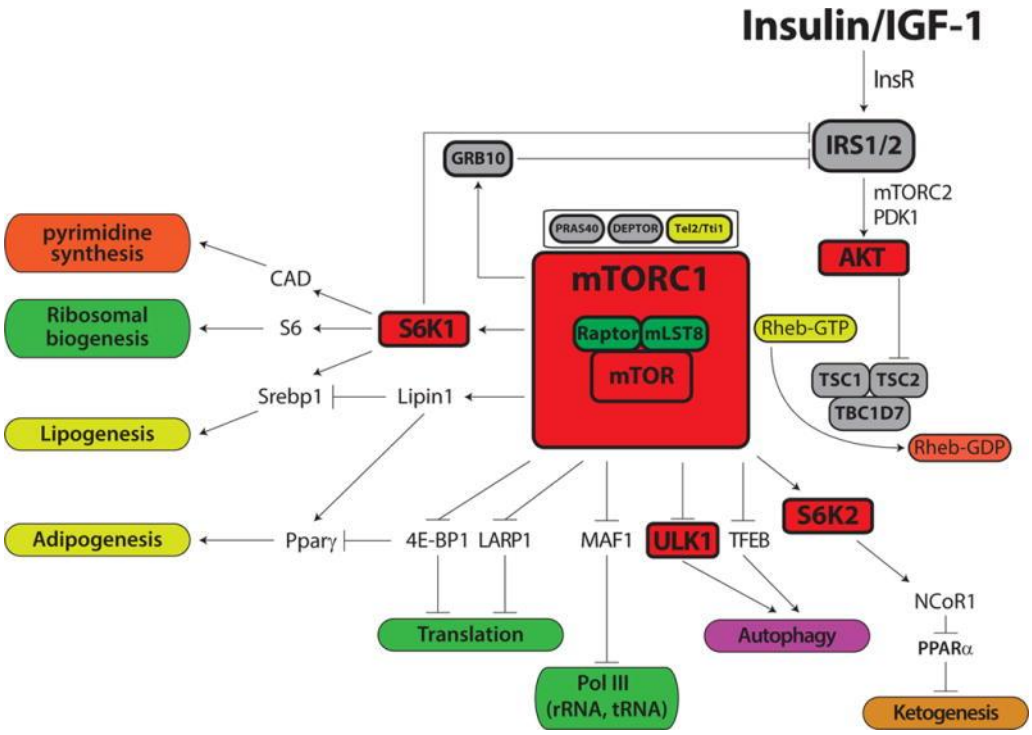


Figure-2-1 mTORC-1 regulates many metabolic processes. A major portion of its downstream effects comprises of anabolic actions whereby pyrimidine, ribosomes, lipids are synthesized as a result of activated mTORC-1. (Figure reproduced from Lamming et al)

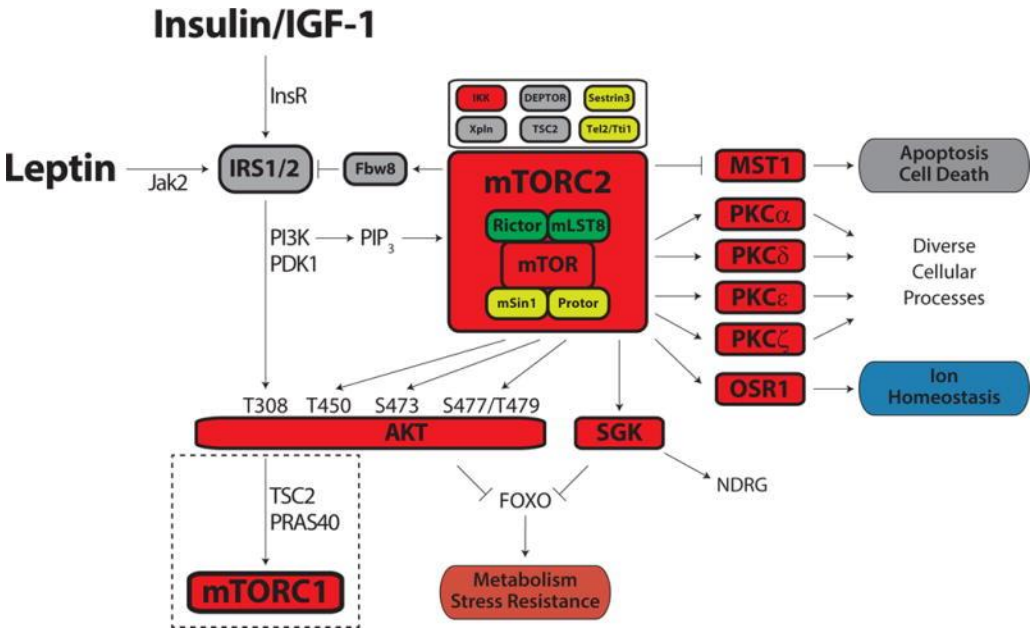


Figure 2-2 mTOR-C2 regulates various aspects of cellular homeostasis. (Figure reproduced from Lamming et al)

1.6 Comparative biology of aging

Mammals show the maximum spectrum of longevity among all animal groups. Thus, it is possible to use a pair of mammals with widely differing mean lifespans to carry out studies in comparative biology related to aging. Let us now evaluate some promising candidate mammalian species for aging studies. Bats(myotis) also known as little brown bats show size diversity of 3-30 grams. Up to 22 species have been studied of which 10 species live for 20 years. 2 species live 30 years and 1 species can survive up to 41 years. It is thought that bats of genus myotis have outstanding abilities to hunt, avoid predators and fight infectious diseases (Austad, 2010). Another interesting mammal is the warm-blooded bowhead whale *Balaena mysticetus* can live up to 200 years. These animals have low fecundity and long development time. It is thought that given large size, the bowhead

whale has a strong anti-tumor mechanism. Bowhead whale cells have a lower probability of neoplastic transformation compared to humans (Keane et al., 2015).

In the search for enhanced longevity – a useful tool is to compare peer species. As investigator Austad has pointed out -there are several criteria for selecting species for longevity studies. An important yardstick is a longevity to body-size ratio. It has long been observed that animals show a positive and linear correlation between body-size and mean lifetime. A few species show deviation from this rule wherein they are much longer lived compared to their peer species. Such species would be examples of maintenance of the integrity of physical functions well into advanced age- a key defining feature of enhanced health-span (Austad, 2009). A second criterion is the proximity of two species in the tree of life where one has exceptionally longer life-span compared to the other species. Pairwise comparisons between such peer species are, according to Austad, useful for identifying biological pathways for enhanced longevity (Austad, 2009). A third criterion -proposed by Austad – is the similarity of a model organism to the humans. Such similarities would make new findings more easy to apply in developing the anti-aging technology (Austad, 2009).

The naked mole rat fulfills these criteria. It is found in Eastern African Savanna in xeric habitats. It lives a strictly subterranean life in sealed burrow systems. It has a body-size not much larger than common mouse [Mus musculus]. Yet compared to its peer species- it lives up to 10 times longer. [it lives 17 years in the wild and 30 years in captivity compared to the 3 years found in a lab mouse] (Buffenstein, 2005). The mouse and the naked mole rat are separated by a few million years of evolution and yet the naked mole rat has developed an unusual life-span. The naked mole rat has social similarity to humans in that they live in communities and participate in the collective care of the young. Also,

they participate in collective foraging for food- mirroring behaviors found in early human societies. Thus, the naked mole rat is an interesting candidate for comparative biology of aging research. With that in mind, we may take a closer look at the lifestyle of the naked mole rat in its subterranean habitat.

The habitat of the naked mole rat can be classified as part of the dark biosphere. The closed burrow system is effectively arrhythmic with respect to exposure to sunlight and ambient sound. In this respect, it is similar to polar habitats and deep-sea habitat. Polar animals like the Svalbard reindeer and Ptarmigan loose rhythms of behavior as well as melatonin secretion. Transgenic reindeer fibroblasts carrying mouse Bmal1 or Per2 gene reporter constructs showed non-circadian rhythms. Ptarmigan birds of polar regions show the daily cycle of melatonin. It is thought that environmental zeitgebers like daylight in polar regions are unusual because of their arrhythmic nature. However, animals can respond to internal zeitgebers like sexual maturity, stage in life history etc. and show various activity pattern in constant light (Beale, Whitmore, & Moran, 2016).

Deep-sea habitats deeper than 1000 meter are outside the reach of sunlight. However circadian rhythms of feeding behavior have been noted in several fish species from this region. Pineal melatonin content in fish caught across the day (e.g.: the grenadier and deep-sea eel) show zeitgeber influence of lunar period and tidal effect at depths more than 1000 meter.

It is thought that in both arrhythmic environments such as the above an internal 24-hour clock continues to function. However, the output of that clock is masked by various physiological mechanisms. This helps the animals to use the internal clock to continue to anticipate environmental conditions and thrive.

The naked mole rat lives in a habitat which is similarly arrhythmic. The average naked mole rat is never exposed to day-night transitions. They live, move, forage in sealed burrow systems where sunlight does not show appreciable variation over 24-hour periods, yet strong activity rhythms have been noticed in various species of mole rats. There is evidence that the more social species show less activity rhythm and the solitary species show more rhythmicity of activity. However, just it is possible that just like other arrhythmic environments the mole rat has a functional clock and the variation in activity rhythms reflect a weak coupling between the endogenous clock and behavioral activity.

The naked mole rat -while phylogenetically close to mouse -has a mean lifespan several times that of the mouse. Moreover, it has a eusocial lifestyle which is comparable to the social behavior of humans. It lives in an underground environment where it is shielded from day-night transitions on the surface. This is comparable to modern humans who stay active long beyond daylight times owing to artificial lighting. All these traits together make the naked mole rat an interesting model organism for studies related to the biology of aging.

1.7 Naked mole rat as a model of aging

Among mammals, the rodents are the most abundant (about 40% of all mammals). They have extremely diverse lifespans with 3-4 years in mice to over 20 years in mole rats and over 30 years in naked mole rats. This degree of variation is bigger than that found in other branches of mammals. This naturally evolved difference is greater than anything achieved through artificial interventions. The naked mole rat lives in a protected environment and displays extreme longevity. It also shows no increase in the risk of death

with advancing age-thereby defying Gompertzian rule. It shows an unusual resistance to cancer development (Ruby, Smith, & Buffenstein, 2018).

The naked mole rat has very high levels of resistance to lack of oxygen, high carbon-di-oxide and as well as heavy metals. In a recent study, it was discovered that the naked mole rat could survive for up to 18 minutes without oxygen and could switch back to normal activity when oxygen was reintroduced (Larson & Park, 2009).

The naked mole rat genome has been sequenced with the explicit purpose of explaining its unusual lifespan and cancer resistance. Several features have been discovered from this (Kim et al., 2011). The naked mole rats have evolved, modified p16 ^{INK4A}. This may contribute to cancer resistance through early contact inhibition. The naked mole rat has also evolved a modified thermogenesis regulator UCP1. They have modified melatonin receptors and modified sodium channels. The latter is supposed to be an adaptation of high carbon-di-oxide environment serving to block pain reception through tissue acidosis. Unusually most genes showed similar expression between four-year-old and 20-year-old naked mole rats. A number of genes -through unusual expression pattern in the naked mole rat are supposed to play key roles in their anti-aging ability. CYP46A1 gene is a mediator of amyloid beta aggregation in mammal brain. This is down-regulated in naked mole rat brain. The SMAD3 regulates tgf beta signaling and helps control cancer. This gene shows high-level expression in naked mole rat. Genes encoding mitochondrial proteins were expressed in a stable manner with advancing age, thereby indicating strong mitochondrial function (NDUFB11, ATP5G3, UQCRQ) telomerase maintenance proteins such as TERT showed strong and stable expression even in advanced age. Transcript analysis of naked mole rat shows decreased expression of insulin IGF1 signaling in liver

compared to mice. A literature survey indicates that the naked mole rat has evolved altered oxygen affinity and altered metabolism to cope with low oxygen conditions. Taken together these clues offer various possible explanations towards the enhanced longevity of the naked mole rat.

In the field of aging, the onset of senescence has 3 markers. Firstly, there is a change in reproductive ability. Secondly, there is a change in physiological function and thirdly there is increased mortality with advancing age. Within the naked mole rat, there is the maintenance of physiological composition between 2-24 years. The breeding females within the species maintain fecundity till the end of their lives. There is no increase in mortality risk from cancer with advancing age. In brief, the naked mole rat has evolved to counter senescence as we know it. As such the naked mole rat an inhabitant of the xeric landscapes of East Africa has emerged as a very important species in the field of biogerontology.

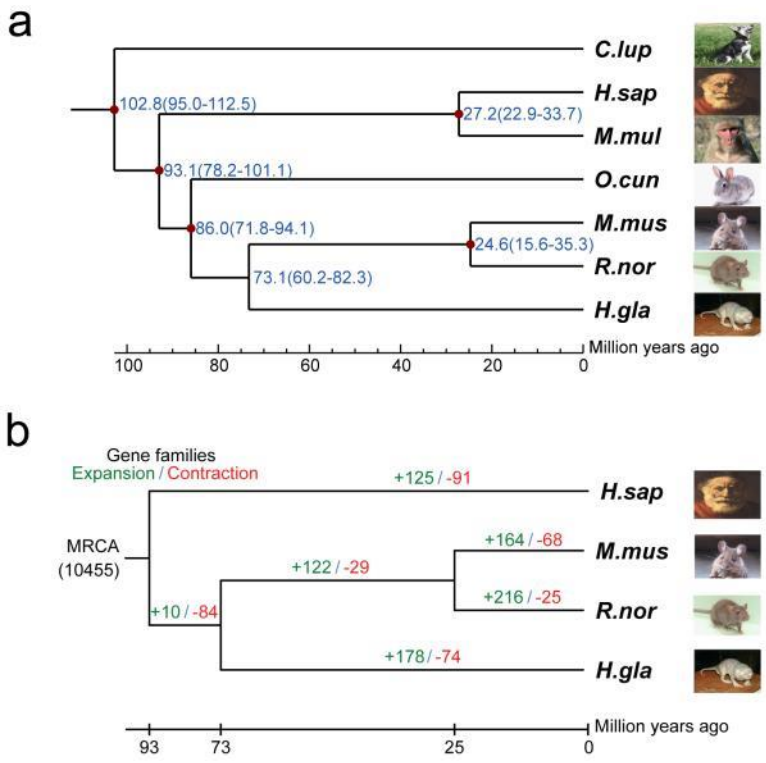


Figure 3-1 Naked Mole Rat evolutionary tree

The above figure (Kim, Gladyshev et al 2011) shows

a. the evolutionary relationship of the naked mole rat with multiple other mammals. The distances shown are in millions of years.

b. The changes in gene families. Green shows the number of gene families gained and red shows a number of gene families lost since the last common ancestor evolution.

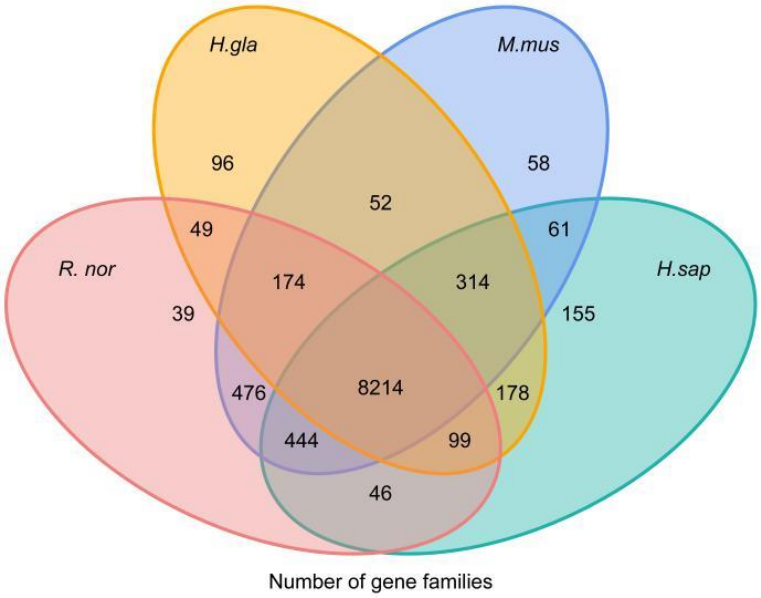


Figure-3-2 The above figure (Kim, Gladyshev et al 2011)shows through a Venn diagram the number of genes that are common among various groups pf rodents that include the naked mole rat [“H.gla” in the figure] and humans. [“H.sap”, in the figure].

1.8 Eusocial species

The naked mole rat is a highly interesting animal in that it is the only known perfectly eusocial mammal. In the animal, kingdom eusociality is defined as comprising of three main parameters. There is collective care of the young by all members of a group. There is a reproductive division of labor among the members of a group. There are overlapping generations living within a single group. This trait is usually found among honey bees, bumble bees, as well as various species of ants. Within mammals, it is found within some species of mole rats that live in underground burrow systems.

There is considerable work regarding the evolutionary value of circadian rhythms within such eusocial species, we note here that within insect colonies, nursing workers are

active throughout the day. In contrast foraging workers that venture out of the nest show strongly entrained 24-hour rhythms and consolidated periods of sleep at night. In parallel social mole rats show weak daily rhythms in behavior, but individual members isolated from the colony and observed show distinct 24-hour behavior rhythms.

The above observations indicate that consolidation of 24-hour rhythms in behavior can be dependent on the social context and life stage. Within a eusocial species-*Bombus terrestris* - the solitary reproductive female displays round the clock activity. In contrast, virgin queens show sharp 24-hour rhythms (Bloch, Barnes, Gerkema, & Helm, 2013). The collective living of the naked mole rat is supposed to confer a benefit to the colony with foragers repair workers and defenders active in non- circadian format. It is assumed that continuous nursing of the young without interruption from environmental cues helps the colony gain new members at a faster rate.

In a study daily activity was observed within mole rats under constant temperature in 3 species with different degrees of sociality. In the solitary species *G. capensis*, 9 animals showed dark phase activity and 2 animals showed light phase activity. In the social species, *C. hottentotus* 5 animals showed dark phase activity and 2 showed light phase activity. In the eusocial species *C damarensis*, 6 showed light phase activity and 2 showed dark phase activity. These observations indicate that even in an arrhythmic environment, a circadian behavior pattern can evolve even within eusocial species (Oosthuizen, Cooper, & Bennett, 2003).

In another study, parallel studies on solitary, social and eusocial species of mole rats demonstrated several sociality related genes undergoing selection. The photoreceptor gene RP1 and visual transduction gene SLC24A2 have been selected out. 3 genes related

to circadian rhythms NAGLU, HCRTR2, NPY2R are under positive selection within the naked mole rat species (Davies, Bennett, Tsagkogeorga, Rossiter, & Faulkes, 2015).

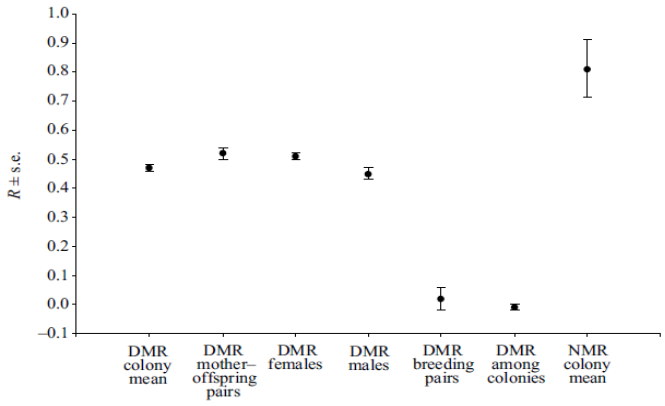


Figure-3-3 The above figure shows the degree of relatedness among the members of a Damaraland Mole Rat colony and within the members of the Naked Mole Rat colony. As shown in the graph, the relatedness among the Naked Mole Rat individuals is much more -thereby confirming their eusocial character in colonies. (Jarvis,2000)

CHAPTER II

MATERIALS AND METHODS

2.1 Experimental Animals

Young male naked mole-rats (2 years; n=4) and C57BL6/J mice (5month; n=?) were used in this study. The ages selected yielded young, healthy individuals that were approximately physiologically age-matched (~15-20% of their observed maximum lifespan. All animal studies were performed with an approval from the Institutional Animal Care and Use Committee (IACUC) of the Cleveland State University and that of the UTHSCSA (IACUC # Protocol No. 21124-KON-S). All the animal studies were performed with approval from the Institutional Animal Care and Use Committee (IACUC) of Cleveland State University (Protocol No. 21124-KON-S). The care and use of mice were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Cleveland State University. All animals were wild-type (WT) mice of C57BL/6J inbred strain background, housed under standard conditions of 12 hr. light/dark cycle (LD 12:12) with lights turned on at 7:00 am and turned off at 7:00 pm.

Mice were purchased from The Jackson Laboratories (Bar Harbor, Maine, USA) and maintained in the vivarium at CSU for at least one month prior to use on the 12:12 light: dark cycle with lights on at 7:00 am with unrestricted access to the standard 18% protein rodent diet (Harlan catalogue no) and water. The naked mole-rats were part of the well-characterized Buffenstein colony housed at the University of Texas Health Science Center at San Antonio. The selected ages yielded young, healthy individuals that were approximately physiologically age-matched (~15-20% of their observed maximum lifespan). Both species were maintained on a 12-hour light-dark cycle. In the naked mole-rat vivaria, the lights came on at 6.00am.

Naked mole-rats were housed in family groups in interconnected systems consisting of tubes and cages of varying sizes to simulate the multi-chambered burrow and tunnel systems that the species inhabits in the wild. Climatic conditions also approximated those found in their native habitat (30°C; 50% relative humidity), although atmospheric oxygen was ~21%. Naked mole-rats met all their nutrient and water needs through an *ad libitum* supply of fruit and vegetables (bananas, apples, oranges, butternut squash, red bell pepper, romaine lettuce, cucumber, green beans, corn, carrots, and red garnet yams).

Animals were euthanized at set times (four hourly intervals over a 24-hour period) using isoflurane followed by cardiac exsanguination and the liver tissue rapidly harvested, flash frozen in liquid nitrogen and stored at -80oC until analyses. We chose to use liver tissue since the liver is a crucial organ involved in metabolic regulation, responding to both food intake and regulating fuel substrate levels throughout the day. Moreover, in other species it is well documented that liver physiology is under robust circadian clock control (Bozek, Rosahl, Gaub, Lorenzen, & Herzel, 2010).

Liver samples collected from naked mole-rats at the various time points were shipped to Cleveland on dry ice, overnight. All liver samples were used in gene expression studies.

The experimental design is further described in Figure 4-1.

Experimental set-up

	Mouse	NMR
Number of animals	Total 4 animals	Total 4 animals
Age	Young adults ,Females	Young adults ,Females
Feeding	Ad-libitum , provided at zt14	Ad-libitum ,provided at zt6
Housing	Single in cage	Communal artificial burrows
Light-Dark cycles	12 h day/night	12h day/night
Quantification	Standardized to daily average of mouse	Standardized to daily average of NMR
Read-out	mRNA expression -clock and gluc. met. Protein activity – mTOR	mRNA expression -clock and gluc. met. Protein activity – mTOR

Figure 4-1 Experimental design

2.2 RNA isolation and analysis of mRNA expression.

For gene expression studies, liver tissues from 4 female mice on Ad-Libitum diet and for the Wild Type genotype were collected every four hours throughout the day and stored at −80 °C. Similar liver tissue was obtained from Dr. Rochelle Buffenstein's lab in Texas for the naked mole rat. The samplings were spaced 4 hours apart. Both the species were life-stage matched-namely Young Adults.

Total RNA was isolated using TriZol reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's protocol. Briefly, the frozen liver piece was minced in 1 ml TriZol reagent with pestle on ice. Following the chloroform extraction step, total RNA was precipitated

with isopropanol by centrifugation and pellet obtained was washed with 70% Ethanol. RNA pellet was diluted in 30 µl of RNase-free water and quantified on Nanodrop. RNA integrity was checked on 1% agarose gel run at 90 V for 30 minutes. 20 µl of RT mix was prepared using 1 µg of RNA, 50 ng of 50 uM random hexamer (N8080127, Invitrogen), 10 mM dNTP (DD0058, Biobasic), 0.1 M DTT.

Complementary DNA synthesis.

It was then reverse transcribed by qPCR machine using 200 ug/µl of SuperScript® III Reverse Transcriptase (18080-044, Invitrogen) as per the manufacturer's instructions. Incubation conditions used were: 65 °C for 10 minutes, 4 °C for 1 minute followed by incubation on ice for 1 minute; 23 °C for 10 minutes; 50 °C for 10 minutes and 80 °C for 10 minutes.

Quantification of RNA expression

RNA quantification was performed using qPCR with Universal Syber Green mix (1725125, BioRad). The reaction was carried out in triplicates for the gene of interest and in duplicates for the normalizing control using CFX96 qPCR Detection System (BioRad) with 50 ng of cDNA. Thermal cycling conditions used were according to the instruction of the SYBR Green mix protocol relative mRNA abundance was calculated using the comparative delta-Ct method with ribosomal 18S rRNA as reference genes. Water was used as the negative control for the qPCR analysis.

Table I: Primers for qPCR

Naked Mole Rat Gene Name	Number	Primers (forward)	Primers (reverse)
Bmal 1 -Arntl	XM_004851452.3	5'-GTC TGT GAA GCT TAC TGG ATA GG -3'	5' -GAG CAG GCT TAG TTC CAC TT -3'
Per 1	XM_013076854.2	5' -CCC TCC AGG GAC ATG GCT T -3'	5'- TGC TAC CAC AGT CCA CAC AG -3'
Per2	XM_013066976.2	5' -CTA CCT GGT CAA GGT GCA GG -3'	5' – GGA CTG CCC TTT CAT CCA CA -3'
Per 3	XM_021243444.1	5' – ACA GAC TTC TCT TGC AGC CG -3'	5' – TGC AAG GTC ATC AGC TCT GG -3'
Cry 1	XM_004844795.2	5'- CTG AAC ATC GAG CGG ATG AA -3'	5' -CAT GAG ACC TCC ATT CCC ATT AG -3'
Cry 2	XM_021264296.1	5' – CGC CTG GAT AAG CAC TTG GA -3'	5' – GCT GTT CCG CTT CAC CTT TTT -3'
Dbp	XM_004866998.3	5'- GAG GTG CTG ATG ACC TTT GA – 3'	5'- TGG CAT GGT GCT ACT GTG TAA GG-3'
Nr1D1 -Rev-erb alpha	XM_021240369.1	5'-CTT CAT CCT CCT CCT CCT TCT A-3'	5'-GTA ATG TTG CTT GTG CCC TTG-3'
Naked Mole Rat Gene Name	Number	Primers (forward)	Primers (reverse)

Naked Mole Rat Gene Name	Number	Primers (forward)	Primers (reverse)
Ror-gamma	XM_021265966.1	5’ – GTG GAG TGG GTG GGA TAT AAA G -3’	5’ – GAA GGC CTC TTC TTA GCT TCC -3’
Pfk	XM_021251240.1	5’ – GCA CAT GAC GGA GAA GAT GAA -3’	5’ – CTC CGA CGA GTA GAG GTT GTA -3’
Gck	XM_021254283.1	5’ – CTT CTT CGA GAC GCC ATC AA -3’	5’ -CTC GCA TTG GTG GTC TTC ATA -3’
Pck	XM_004840930.3	5’ – CTG GTG TCC CTC TTG TCT ATG -3’	5’ -GAC TTT GCC TTT GTG CTC TG -3’
G6pc	XM_004859296.3	5’- GCT GGA GTC TTG TCA GGT ATT G -3’	5’ – GGA AGG TAA CCA GGA GGT ACT -3’
FbP1	XM_021251240.1	5’ – CAA CAT GGA GGC CCT ACT AAT C -3’	5’ - GAC CTT CCT CAT GGC TGA AAT A -3’
Hxk1	XM_004846184.2	5’ -GGA CGC TCT ACA AGC TTC AT -3’	5’ – GAC AGG AGG AAG GAC ACA TTA C -3’
Slc2A5	Park, T. J. *et al.* (2017) ‘.	5’ – GTG CCC CAG CTC TTC ATC -3’	5’ – GTT CCG AAA ACC GAA CAG C -3’
Khk-C	Park, T. J. *et al.* (2017) doi: 10.1126/science.aab3896.	5’- CGT GGA TGT GTC TCA GGT GT -3’	5’ – AGA TGT TGA CGA TGC AGC AG -3’
Khk-A	Park, T. J. *et al.* (2017)	5’ – TCC GTG GAC CTA CGC TAC TT -3’	5’ – CTC GCT GAT GAT GAC TGT GG -3’

Mouse Gene Name	Number	Primers (forward)	Primers (reverse)
Bmal 1 -Arntl	NM_001357070.1	5’- CAC TGT CCC AGG CAT TCC A-3’	5’- TTC CTC CGC GAT CAT TCG- 3’
Per 1	NM_001159367.2	5’- AGG TGG CTT TCG TGT TGG-3’	5’- CAA TCG ATG GAT CTG CTC TGA G-3’
Per2	XM_006529249.3	5’- AAT CTT CCA ACA CTC ACC CC- 3’	5’- CCT TCA GGG TCC TTA TCA GTTC-3’
Per3	XM_017320036.1	5’- GGT CGA CAT AAA GTC CGA ACG A- 3’	5’- TCG TTA CTG GCT GCC TTT TTT ATT- 3’
Cry 1	XM_011243349.1	5’- CGT CTG TTT GTG ATT CGG GG- 3’	5’- ATT CAC GCC ACA GGA GTT GC- 3’
Cry2	NM_009963.4	5’- GGC AGA CCG AGA CCC AGT CCA- 3’	5’- ATC GAT TGC GCG GGG ACC G- 3’
Dbp	XM_006540596.2	5’- CCT GAG GAA CAG AAG GAT GA- 3’	5’- ATC TGG TTC TCC TTG AGT CTT- 3’
Nr1D1 -Rev-erb alpha	NM_145434.4	5’- TGG CAT GGT GCT ACT GTG TAA GG- 3’	5’- ATA TTC TGT TGG ATG CTC CGG CG- 3’
Nr1D2 -Rev-erb beta	NM_011584.4	5’- GGA GTT CAT GCT TGT GAA GGC TGT- 3’	5’- CAG ACA CTT CTT AAA GCG GCA CTG- 3’
Ror-gamma	XM_006501164.3	5’- ACT ACG GGG TTA TCA CCT GTG AG- 3’	5’- GTG CAG GAG TAG GCC ACA TTA C- 3’

Mouse Gene Name	Number	Primers (forward)	Primers (reverse)
Pfk	NM_008826.5	5’- AGA GGA CCT TTG TTT TGG AG -3’	5’- TCT GCG ATG ATG ATG ATG TT-3’
Gck	XM_006514443.3	5’- CAC AAT GAT CTC CTG CTA CT -3’	5’- TTC TGC ATC TCC TCC ATG TA -3’
Hxk-1	NM_001146100.1	5’- TAG TGA GCC ATT GTC GTA TG -3’	5’- CTG TCA CCA TAT GCT AAG CC-3’
Pck	NM_011044.2	5’ -TGA GAT CTA GGA GAA AGC CA-3’	5’ – CCT TGA AGT GGA ACC AAA AC-3’
G6pc	NM_008061.4	5’- CTA AAG CCT CTG AAA CCC AT -3’	5’- ATG ACT CAG TTT CCA GCA TT -3’
FbP1	XM_011244488.2	5’- TGA CCT GGT GAT CAA TAT GC-3’	5’- CAA AAA TGG TTC CGA TGG AC-3’
Slc2A5	Park, T. J. *et al.* (2017) ‘	5’- AGA GCA ACG ATG GAG GAA AA -3’	5’ – CCA GAG CAA GGA CCA ATG TC-3’
Khk-C	Park, T. J. *et al.* (2017) ‘	5’- CGT GGA TGT GTC TCA GGT GT -3’	5’ – AGA TGT TGA CGA TGC AGC AG -3’
Khk-A	Park, T. J. *et al.* (2017) ‘	5’ – TCC GTG GAC CTA CGC TAC TT -3’	5’ – CTC GCT GAT GAT GAC TGT GG -3’

2.3 Immunoblot Analysis

Protein extraction

For gene expression studies, tissues were collected every four hours throughout the day and stored at -80^{o}C. Total RNA was isolated from the liver using TriZol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA quantification was performed using real-time RT-PCR with Universal Syber Green mix (BioRad, Hercules, CA) as described in (Khapre, Patel, et al., 2014). Primers used for the analysis of mRNA expression are listed in the Supplementary Materials.

Quantification of protein expression

For the analysis of protein phosphorylation and expression, tissues were collected every four hours throughout the day and stored at -80^{o}C. For a representative Western blotting, samples from four different animals were pooled together at each time point. For quantitative analysis, the samples were run individually to estimate variability between biological replicates. Tissues were lysed in the Cell Signaling lysis buffer with Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology, Beverly, MA, USA). The Bradford protein assay kit was used to determine protein concentration. Protein (37 μg) was loaded into 3–8% Tris-acetate and 4–12% Bis-Tris gels (Invitrogen). After SDS–PAGE, the protein was transferred to PVDF membrane. The loading of proteins was checked by Ponceau staining. The membranes were probed with following primary antibodies AKT (#4691), p-AKT (S473) (#4060), PRAS40 (#2691), p-PRAS40 (246) (#13175), S6K1 (#2708), p-S6K1 (T389) (#9206), S6 (#2317), p-S6 (S235/236) (#2211), from Cell Signaling Technology. Probing the same membranes with anti-beta-actin antibodies was used for normalization of the signal. Images were obtained with Odyssey

FC imaging system (LI-COR, Lincoln, NE), quantification was performed with the Odyssey FC imaging system version 3.0 software.

Blots were incubated overnight on a shaker at 4°C with specific primary antibodies listed in the table below (Table II).

Table 0 List of antibodies for immunoblot analysis

Reagent or Resource	Source	Identifier
pAKT (s473)	Cell Signaling Technology	Cat #4060
PRAS 40	Cell Signaling Technology	Cat #2691
pPRAS40 (246)	Cell Signaling Technology	Cat#13175
S6K1	Cell Signaling Technology	Cat #2708
S6K1 (t389)	Cell Signaling Technology	Cat #9206
S6	Cell Signaling Technology	Cat #2317
pS6 (s235/236)	Cell Signaling Technology	Cat #2211

2.4 Statistical Analysis

For all experiments, four female mice and four female NMR for every time point were used. Data are shown as average +/− standard error of the mean. IBM SPSS Statistics 20 software package was used for statistical analysis. For the analysis of Circadian Rhythms in gene expression "R," Version 3.2.5 software – JTK Analysis package was used. To assay the effects of species on the gene expression, the analysis was performed using two-way repeated ANOVA.$P < 0.05$ was considered as a statistically significant difference.

Pubmed BLASTp program was used for sequence alignment .The naked mole rat sequences were obtained from PUBMED and analyzed for the presence of CCE motifs. The transcription start site was identified and few thousand base pairs upstream and downstream of the site was selected. This stretch was manually screened for the presence of the CCE motifs.

CHAPTER III

RESULT

3.1 Overall Findings

Circadian clocks are master regulators of metabolism and they generate circadian rhythms in behavior and physiology in different organisms. These circadian rhythms are impaired with age and it was hypothesized that clock decay contributes to many age-associated, chronic diseases including cancer and Alzheimer's disease. (Musiek, Xiong, & Holtzman, 2015; Wegrzyn et al., 2017). We initially questioned if a subterranean species, known to have led a strictly subterranean existence since the early Miocene would retain a robust circadian clock gene expression in keeping with their circadian patterns of body temperature, metabolism, and running activity (Riccio & Goldman, n.d.). We also asked if a clear pattern was evident for the clock genes how this rhythm would differ from that of the well-characterized nocturnally active mouse. First, we found that circadian clock mechanisms are preserved in the liver of naked mole-rat and all known core circadian clock genes are conserved in naked mole-rat and expressed rhythmically. We also found significant differences between circadian rhythms in gene expression and cellular signaling between mice and naked mole-rats.

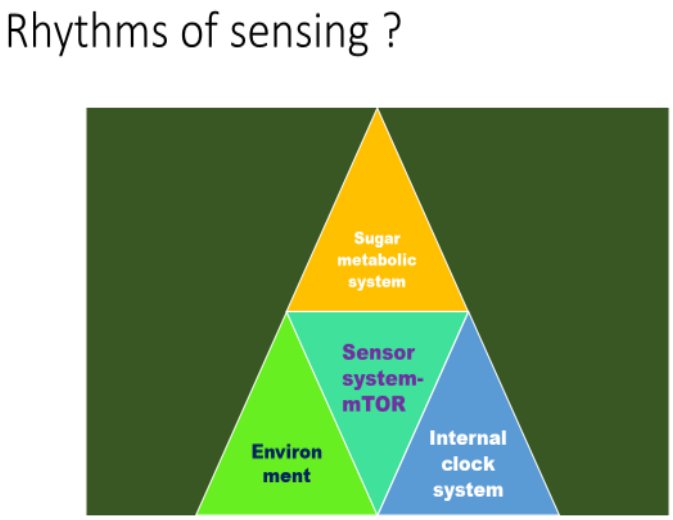


Figure 4-2: Summary of parameters checked

3.2 Circadian clock proteins are conserved

Several circadian genes have been found to be necessary for circadian rhythm generation in vivo: Clock, Bmal1, Per1 and Per2, Cry1, Cry2, NrD1 (Rev-Erb alpha), NrD2 (Rev-erb beta), casein kinases delta and epsilon and three Ror genes. We identified all these genes in the naked mole-rat reference genome and compared protein homologies between the naked mole-rat, mouse, and human use -www. blast.ncbi.nlm.nih.gov Circadian clock proteins were highly conserved between species with >90% homology (Table 3). It appears that despite having evolved to live in an environment devoid of light, all major components of the molecular circadian oscillator are present in the naked mole-rat genome and overall, the sequences of these genes are conserved suggesting that the circadian clock may still operate in naked mole-rat.

The liver is a crucial organ involved in metabolic regulation. Because circadian rhythms are intimately wired with the metabolism, the liver physiology is under strong circadian clock control. Expression of the circadian clock genes in the liver demonstrates robust circadian rhythms. Therefore, we decided to compare circadian rhythms in expression and metabolism between naked mole-rat and mouse in the liver tissue.

Table-III : Conservation of core clock proteins

Core Clock Components Protein	Naked Mole Rat	Mouse	Conservation
BMAL1	XP_ 004851503.1	NP_031515.1	98 %
PER1	XP_ 004857534.1	NP_035195.2	90 %
CRY1	XP_ 004844851.1	NP_035196.2	87 %
PER2	XP_ 004868464.1	NP_031797.1	79 %
CRY2	XP_004852021.1	NP_ 034093.1	97 %
PER3	XP_ 012920518.1	NP_001276806.1	58 %
DBP	XP_ 004867055.1	NP_058670.2	92 %
NR1D1	XP_ 004859514.1	NP_663409.2	94 %
ROR-GAMMA	XP_ 021121614.1	AAH_14804.1	87 %
NR1D2	EHB 08111.1	NP_035714.3	89 %

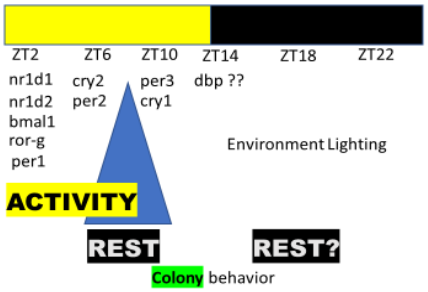
Clock and Food cue

Food as zeitgeber		
Gene	NMR peak time	Mouse peak time
NR1D1	2 H before food	8 H before food
NR1D2	2 H before food	6 H before food
BMAL1	4 H before food	8 H after food
ROR-GAMMA	6 H before food	4 H after food
PER 1	4 H before food	2 H before food
PER 2	2 H before food	AT food
CRY 1	2 H after food	4 H after food
CRY 2	2 H before food	2 H before food
PER 3	2 H after food	4 H before food
DBP	10 H after food [no 24 h rhythm]	6 H before food

Figure 4-3: Comparison of the core clock gene expression peak times between the mouse and the naked mole rat when considering food as the primary zeitgeber

Clock and Light cue

In the large majority of animals more activity occurred during the light phase than during the dark.



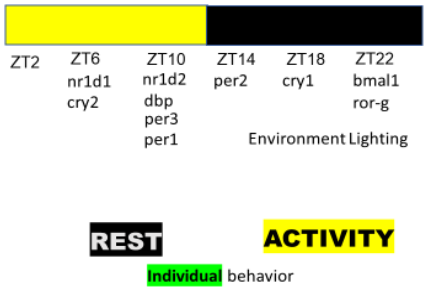


Figure-4-4 : Comparison of the core clock gene expressions between mouse and the naked mole rat when considering light as the major zeitgeber

3.3 Expression of mRNA for circadian clock genes

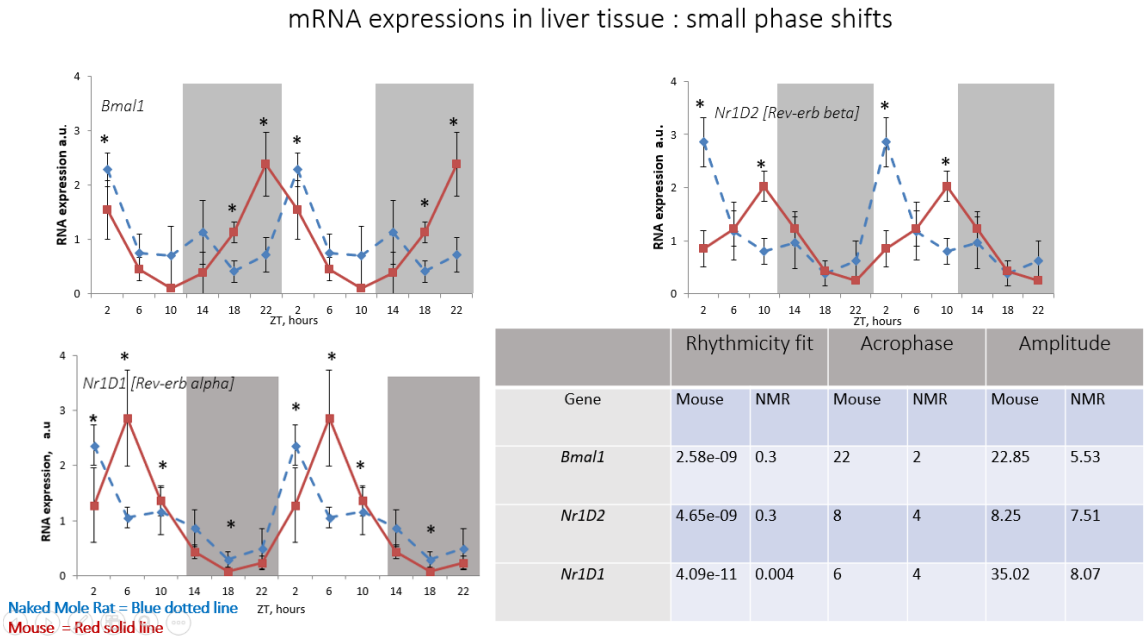
The mRNA expression was analyzed over a 24-hour period at 6 different time-points. We observed that the expression level for 9 out of 10 circadian clock genes analyzed was significantly lower (in some cases more than 10-fold lower) in the naked mole-rat liver than the expression of the same genes in mouse liver (Figure: 4-6). The only gene with on the same level in both species was the *Cry2*). The above represent absolute values pf expression and they were obtained by normalizing the expression in both animals to the average daily expression of the mouse. Currently, we do not know why the absolute expression was lower in naked mole-rat liver. For the convenience of result presentation, the data on the expression of clock genes were normalized independently for mouse and naked mole-rat and we compared not absolute but relative expression.

Results are presented in Figure 1 and JTK analysis the Table 2. As expected, in the mouse liver the expression of all tested circadian clock genes was rhythmic also in agreement with previous reports. In the naked mole-rat liver, the expression of all tested genes was also circadian, except for *Dbp*. Rhythms were less robust; for mouse, the rhythmic fits were about 0.9 while for most naked mole-rat genes the rhythmic fits were between 0.5 and 0.7. We observed the difference in the amplitudes and acrophases of clock gene expression between species (Table 4).

We did not observe any significant difference in the amplitudes of *Per1* and *Per2* mRNA expression. The amplitude of *Bmal1* expression rhythms was less in naked mole-rat than in mouse but it was still high amplitude rhythms. Finally, *Cry1* mRNA expression rhythms demonstrated very low amplitude in the naked mole-rat. The phase shift observed between the species was not the same for all genes, they can be subdivided into two groups

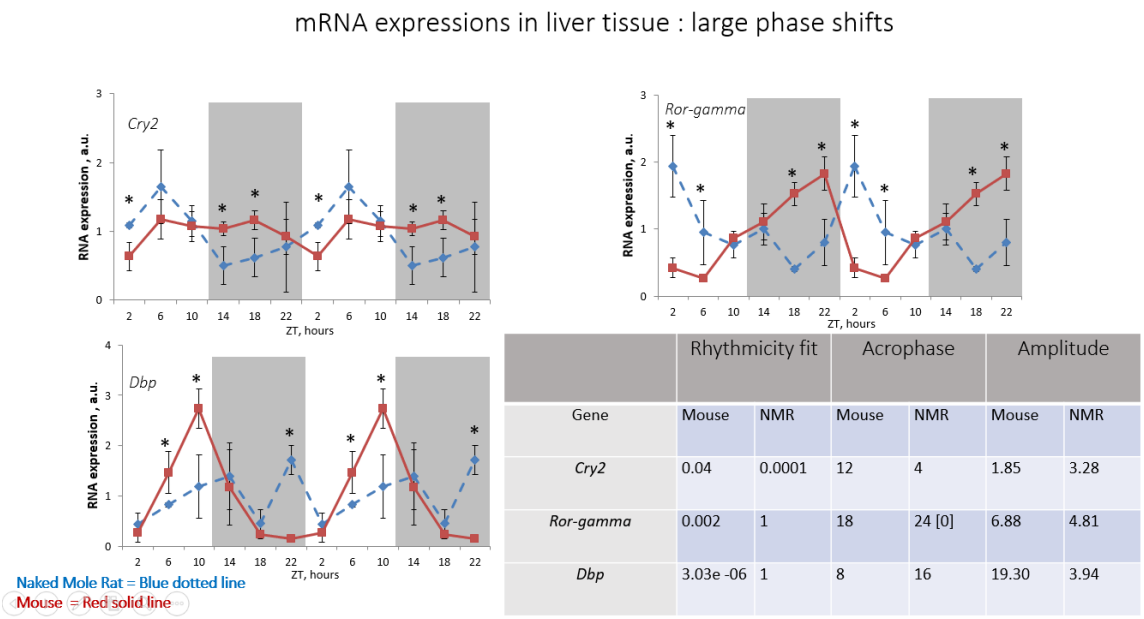
based on the magnitude of the shift. The first group has a relatively small phase shift between naked mole-rats and mouse: these genes are *Bmal1* (4-hour phase delay in naked mole-rats), *Nr1D1* (2-hour phase advance in naked mole-rats) *and Nr1D2* (6-hour phase advance in naked mole-rats) genes (Table 4). The phase shifts in the second group were significantly higher: about 10-hour phase advance in naked mole-rats for *Cry1*, *Per1,* and *Per2* gene expressions and about 10-hour phase delay for *Ror gamma* expression (Table 4). Interestingly, the expression of *Cry2* gene was arrhythmic in the mouse liver and demonstrated robust rhythms in naked mole-rat. *Cry1* and *Cry2* have a partially redundant function in the clock, however, there is more recent evidence that they might have different physiological functions in the control of glucose metabolism and cancer. If the improved circadian rhythms in *Cry2* expression in the naked mole-rat liver help to maintain good health needs to be studied in future.

We also analyzed the expression of two circadian clock-controlled genes. The mRNA expression for *Dbp* and *Per3* genes were rhythmic with high amplitudes in the mouse liver (Table 4). In the naked mole-rat, the mRNA for *Per3* was still rhythmic across the day with high amplitude and no phase shift between species, while the expression of *Dbp* did not show circadian rhythms (Table 4). Thus, the observed differences between mice and naked mole-rats in phases and amplitudes of rhythms were gene-specific.



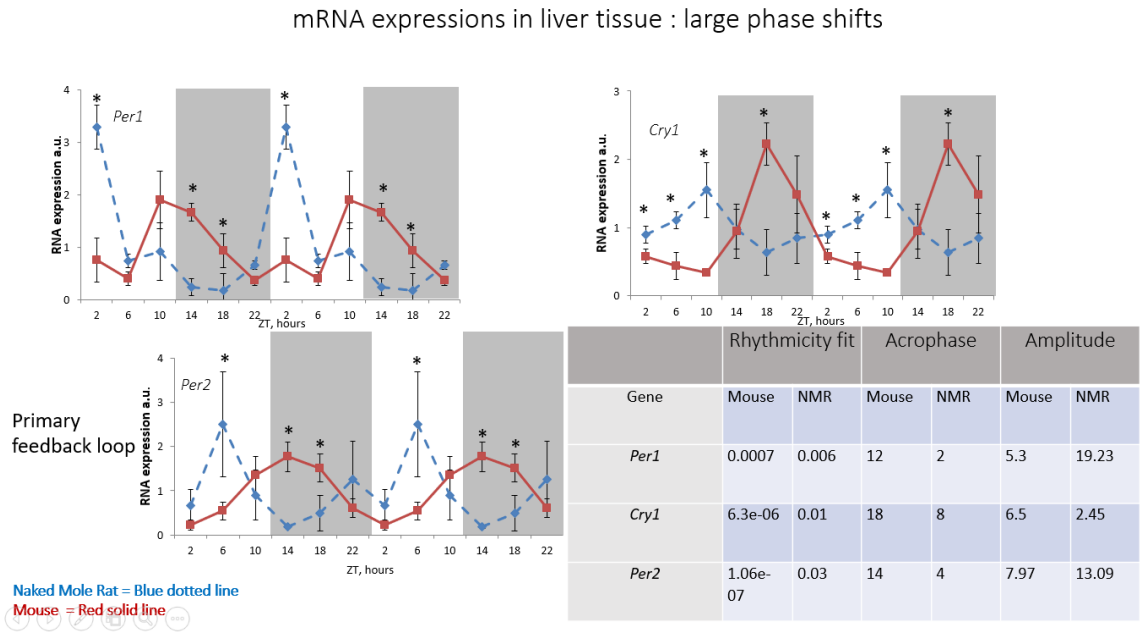
	Rhythmicity fit		Acrophase		Amplitude	
Gene	Mouse	NMR	Mouse	NMR	Mouse	NMR
Bmal1	2.58e-09	0.3	22	2	22.85	5.53
Nr1D2	4.65e-09	0.3	8	4	8.25	7.51
Nr1D1	4.09e-11	0.004	6	4	35.02	8.07

Fig -4-5-1: The above figure shows two examples of small phase shifts in gene expression in core clock of naked mole rat compared to that in the mouse.



	Rhythmicity fit		Acrophase		Amplitude	
Gene	Mouse	NMR	Mouse	NMR	Mouse	NMR
Cry2	0.04	0.0001	12	4	1.85	3.28
Ror-gamma	0.002	1	18	24 [0]	6.88	4.81
Dbp	3.03e -06	1	8	16	19.30	3.94

Figure 4.5.2: The above figure shows more examples of large phase shifts in gene expression within the naked mole rat compared to that in mouse



	Rhythmicity fit		Acrophase		Amplitude	
Gene	Mouse	NMR	Mouse	NMR	Mouse	NMR
Per1	0.0007	0.006	12	2	5.3	19.23
Cry1	6.3e-06	0.01	18	8	6.5	2.45
Per2	1.06e-07	0.03	14	4	7.97	13.09

Figure 4.5.3: The above figure shows large phase shifts in gene expression in the core clock system within the naked mole rat compared to that in the mouse

Table-IV: Expression rhythms of core clock genes

	Rhythmicity fit		Acrophase		Amplitude	
Gene	Mouse	NMR	Mouse	NMR	Mouse	NMR
Bmal1	2.58E-09	0.3	22	2	22.85	5.53
Per1	0.0007	0.006	12	2	5.30	19.23
Per2	1.06E-07	0.03	14	4	7.97	13.09
Per3	1.26E-05	0.04	10	8	5.66	2.16
Cry1	6.3E-06	0.01	18	8	6.50	2.45
Cry2	0.04	0.0001	12	4	1.85	3.28
Dbp	3.03E-06	1	8	16	19.30	3.94
Nr1D1 (Rev-erb alpha)	4.09E-11	0.004	6	4	35.02	8.07
Nr1D2 (Rev-erb beta)	4.65E-09	0.3	8	4	8.25	7.51
Ror-gamma	0.002	1	18	0	6.88	4.81

3.4 The expression of mRNA for glucose metabolism genes

One of the hallmarks of delayed aging is improved glucose homeostasis. Glucose metabolism is also under strong circadian clock control. We observed the circadian rhythms in the liver of naked mole-rat and, therefore, we decided to explore if glucose metabolism is also circadian in these rodents. We analyzed the expression of mRNA for several glucose metabolism genes, we focused on those encoding enzymes catalyzing rate-limiting steps in glycolysis and gluconeogenesis (Table-6). Most of these proteins were highly conserved between the species (Table 7). The expression of mRNAs for three glycolytic enzymes – Phosphofructokinase (*Pfk*), Glucokinase (*Gck*) and Hexokinase (*Hxk*) was rhythmic in both species. In the naked mole-rat liver all three mRNAs peaks around ZT2 (Figure 5-1 and Table 7). The feeding behavior was not monitored in naked mole rat, the fresh food was provided to animals around ZT6, therefore, they most likely start to eat around this time and the expression of mRNAs for glycolytic enzymes peaked 4 hours before that. Mice are nocturnal animals and under *ad libitum* conditions they will consume most of the food during the dark phase of the day. In agreement with the expression of *Pfk* and *Hxk* have peaked around ZT6-ZT10 (again a few hours before the start of feeding), at the same time *Gck* expression peaked at ZT14 demonstrating significantly delayed phase. Interestingly, in the naked mole-rat liver, the rhythms of the expression for glycolytic enzymes have significantly higher amplitude than in the mouse liver.

mRNA expression for *Pepck*, *Fbpase*, and *G6pase* (the genes encoding rate-limiting enzymes in gluconeogenesis) was rhythmic in the liver of naked mole-rat (Figure 5-2 and Table 7). The expressions of *Pepck* and *G6pase* were well synchronized and peaked at

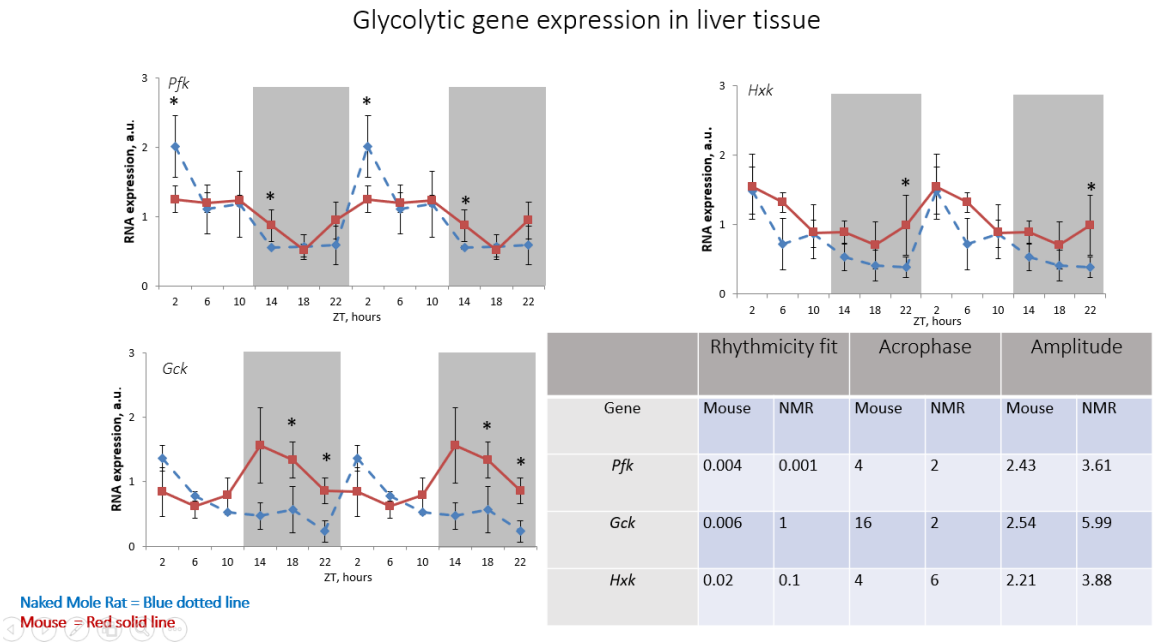
ZT10 (4 hours post-prandial period), while *Fbpase* peaked at ZT2, with a secondary peak around ZT10 (Figure 5-3 and Table 7). In mouse, the expressions of *Pepck* and *G6pase* were also rhythmic but in contrast to the naked mole-rat the expressions were not synchronized, they peaked at different times: ZT10 for *Pepck* and ZT18 for *G6pase*. The expression of *Fbpase* was arrhythmic in mouse liver (Table 7). Similar with the expressions of glycolytic genes, rhythms in the expression of gluconeogenesis genes were more robust with higher amplitude in naked mole-rat.

It was recently reported that the expression of enzymes involved in anaerobic metabolism is different between the naked mole-rat and mouse We investigated the circadian rhythms in the expression of *Khk-A*, *Glut-5,* and *Khk-C*. In the naked mole-rat liver, mRNAs for all three genes peaked at ZT10. In the mouse liver only *Khk-A*, *Glut-5* were rhythmic (Figure 5-3 and Table 7).

Thus, the expression for all investigated glucose metabolism genes was rhythmic in naked mole-rat liver, the peaks of the expressions were closely packed for the genes encoding enzymes in the same pathways. Not all these genes were rhythmic in mouse and time-synchronization of the peak phase was not so well-organized.

Table-V : Conservation of the components of the mTOR complex

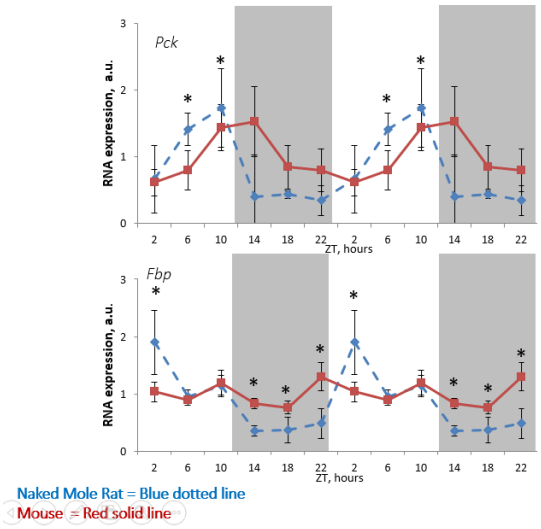
mTOR complex components Protein	Naked Mole Rat	Mouse	Conservation
mTOR	XP_004863641.1	NP_064393.2	98 %
RAPTOR	EHB09315.1	NP_083174.2	98 %
DEPTOR	XP_012929541.1	NP_663445.2	88 %
mLST8	XP_021106983.1	NP_001239393.1	99%
PROTOR	JAN98042.1	NP_666173.4	89%
RICTOR	XP_021117136.1	NP_084444.3	93%
mSIN1	EHB05016.1	NP_796319.1	97%
Downstream targets of mTOR complex Protein	Naked Mole Rat	Mouse	Conservation
AKT	EHB01398.1	NP_001318036.1	91 %
S6	EHB15621.1	NP_033122.1	84 %
PRAS40	EHB05673.1	NP_001277623.1	94 %
S6K1	XP_004850711.1	AAA50300.1	96 %



	Rhythmicity fit		Acrophase		Amplitude	
Gene	Mouse	NMR	Mouse	NMR	Mouse	NMR
Pfk	0.004	0.001	4	2	2.43	3.61
Gck	0.006	1	16	2	2.54	5.99
Hxk	0.02	0.1	4	6	2.21	3.88

Fig 5-1: Glycolytic gene expression rhythms compared between the naked mole rat and mouse

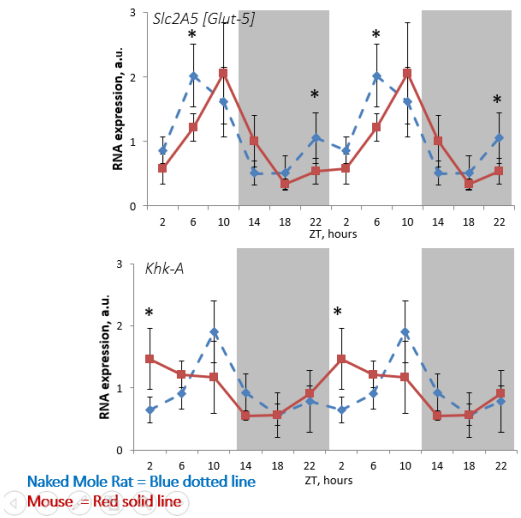
Gluconeogenesis gene expression in liver tissue



	Rhythmicity fit		Acrophase		Amplitude	
Gene	Mouse	NMR	Mouse	NMR	Mouse	NMR
Pck	0.01	0.03	12	8	2.49	5.07
G6pc	0.82	0.09	16	8	2.12	4.20
Fbp	0.3	0.01	22	4	1.70	5.34

Figure 5.2 Gluconeogenic gene expression compared between the naked mole rat and the mouse

Hypoxic glucose metabolism gene expression in liver tissue



	Rhythmicity fit		Acrophase		Amplitude	
Gene	Mouse	NMR	Mouse	NMR	Mouse	NMR
Glut-5 /Slc2A5	2.45e-05	0.03	8	4	6.09	3.93
Khk-C	0.3	0.1	22	6	1.86	2.36
Khk-A	0.0001	0.003	4	8	2.67	3.41

Figure 5.3 Hypoxic metabolism genes expression compared between the naked mole rat and the mouse

Table-VI: Conservation of the glucose metabolism enzymes

Glucose metabolic enzymes Protein	Naked Mole Rat	Mouse	Conservation
PFK	XP_004842542.1	NP_032852.2	98 %
GCK	XP_004839982.1	NP_001274315.1	94%
HXK	XP_004846239.1	NP_001139572.1	71 %
PCK	XP_004840987.1	NP_035174.1	92%
G6PC	XP_004859352.1	NP_032087.2	81%
FBP	XP_004846136.1	NP_062268.1	86%
SlC2A5	XP_012920668.1	NP_062715.2	79%
KHK-C	XP_004839226.1	NP_032465.2	91%
KHK-A	XP_021109265.1	NP_001335995.1	90%

Table VII: The comparison of glycolytic gene expression and gluconeogenic gene expression between the naked mole rat and the mouse

	Rhythmicity fit		Acrophase		Amplitude	
Gene	Mouse	NMR	Mouse	NMR	Mouse	NMR
Pfk	0.004	0.001	4	2	2.43	3.61
Gck	0.006	1	16	22	2.54	5.99
Hxk	0.02	0.1	4	6	2.21	3.88
Pck	0.01	0.03	12	8	2.49	5.07
G6pc	0.82	0.005	16	8	2.12	4.20
Fbp	0.3	0.01	0	4	1.70	5.34
Slc2a5	2.45E-05	0.03	8	4	6.09	3.93
Khk-C	0.3	0.1	22	6	1.86	2.36
Khk-A	0.0001	0.003	4	8	2.67	3.41

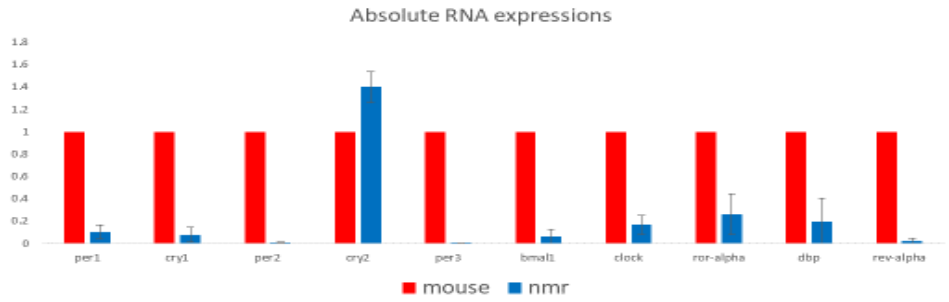


Figure-5.4 Absolute levels of expression of the core clock genes, compared between the naked mole rat and the mouse.

3.5 Activity rhythms in mTOR complex signaling

Recently, we and others demonstrated the circadian rhythms of mammalian/mechanistic Target of Rapamycin (mTOR) activity in different mouse tissues, and we further proposed that mTOR signaling is under control of biological clock. The mTOR signaling pathway is one of the major pathways linked with aging and metabolism. There are two mTOR complexes: mTORC1 and mTORC2. Complexes differ in their composition, downstream substrates, and biological functions. mTORC1 phosphorylates downstream targets such as S6 protein kinase 1 (S6K1) and eukaryotic translation initiation

factor 4EBP1 to execute protein translation, ribosome biogenesis, cell growth, and proliferation. Major targets of mTORC2 include AKT, SGK, and PKC-alpha. mTORC2 is mainly involved in the maintenance of glucose metabolism, cell survival, and actin cytoskeleton rearrangement. We analyzed the conservation of the components of mTORC1, mTORC2, and their major downstream targets. As illustrated in Table 5 all these proteins are highly conserved between mice and naked mole-rats. We analyzed the activity of both complexes in mouse and naked mole-rat's livers.

mTORC1 activity was measured by assaying the phosphorylation of S6K1-T389 (Figure 6-1 and Figure 6-3), as it was previously reported mTORC1 activity oscillated across the day with maximum activity at the dark phase and minimum at light phase. In naked mole-rats, phosphorylation of S6K1 was showing some low amplitude oscillation but it was not circadian (Table 8). The most striking observation was about the significantly higher level of S6K1 phosphorylation in mice than in naked mole-rats across the day. The total protein level was lower in naked mole-rats only by 20% and cannot be the only cause of the observed 5-fold reduction in the phosphorylation. The T389 phosphorylation site is identical between mouse and naked mole-rat (Figure 6-5), therefore, we did not expect the differential abilities of antibodies to recognize the site. However, to further confirm the different activity of mTORC1 between mouse and NMR livers we assayed the phosphorylation of ribosomal protein S6 on Ser235/237, these sites were also identical between mouse and naked mole-rat. S6 is a direct target of S6K1 and it is frequently used as a surrogate marker of mTORC1 activity. Phosphorylation of S6K1 on T389 increases the kinase activity, in agreement with low phosphorylation of S6K1 in the naked mole-rat liver the phosphorylation of S6 on S235 was also significantly lower at multiple times of the day (ZT10, ZT14, ZT18). All these data together argue that the activity of the mTORC1

complex is low in naked mole-rat compared with mouse. Interestingly, there were circadian rhythms in the phosphorylation of S6 with a maximum at ZT2, thus it was in antiphase with the mouse. Interestingly, S6 phosphorylation is induced by feeding and in mouse liver, it followed the anticipated feeding time, but in the naked mole-rat liver, the peak was before the anticipated feeding time at ZT6. This suggests that there is an additional level of regulation of mTORC1 activity in naked mole-rat.

To measure mTORC2 we assayed AKT phosphorylation at S473. In both species daily rhythms in activity were circadian and rhythms were more robust in naked mole-rats. We observed that mTORC2 activity is significantly upregulated in naked mole-rats at ZT10 and ZT14 when compared with mice (Figure 6-2 and Table 8). The sequence around the S473 site is identical between mice and naked mole-rats and there was no significant difference at the total AKT protein levels. The S473 phosphorylation causes an increase in AKT kinase activity toward its downstream targets such as phosphorylation of PRAS40 at T246. To further confirm that activation of AKT is higher in the naked mole-rat we assayed PRAS40 phosphorylation at T246 (the sequence around this site is also identical between two species). In agreement with rhythms in AKT phosphorylation, the phosphorylation of PRAS40 oscillated across the day in both species and it was significantly higher in naked mole-rats at ZT2, ZT10, ZT14, ZT18, and ZT22. Thus, the activities of mTORC1 and mTORC2 complexes in mice and naked mole-rats are very different: mTORC1 is highly active with robust daily rhythms in mice, while mTORC2 is more active and rhythmic in naked mole-rats.

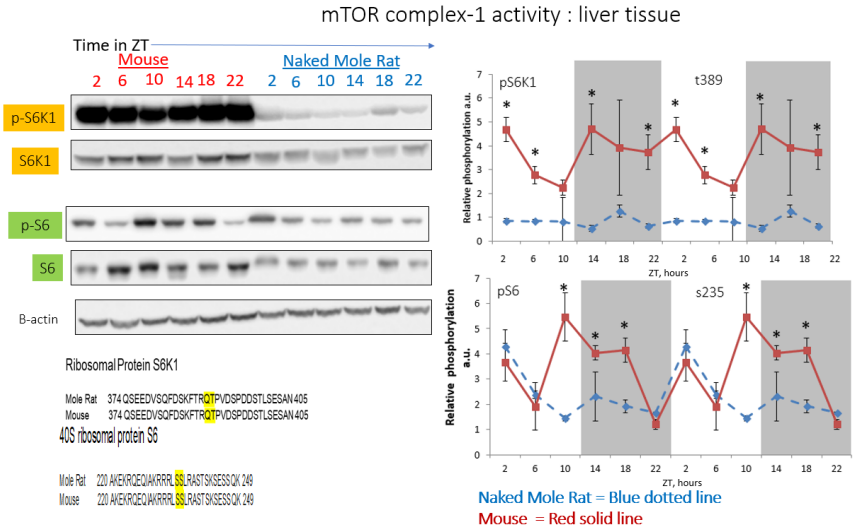


Figure 6.1. The comparison of mTOR complex 1 activity between the mouse and the naked mole rat

Antibody recognition sites are conserved and marked in yellow

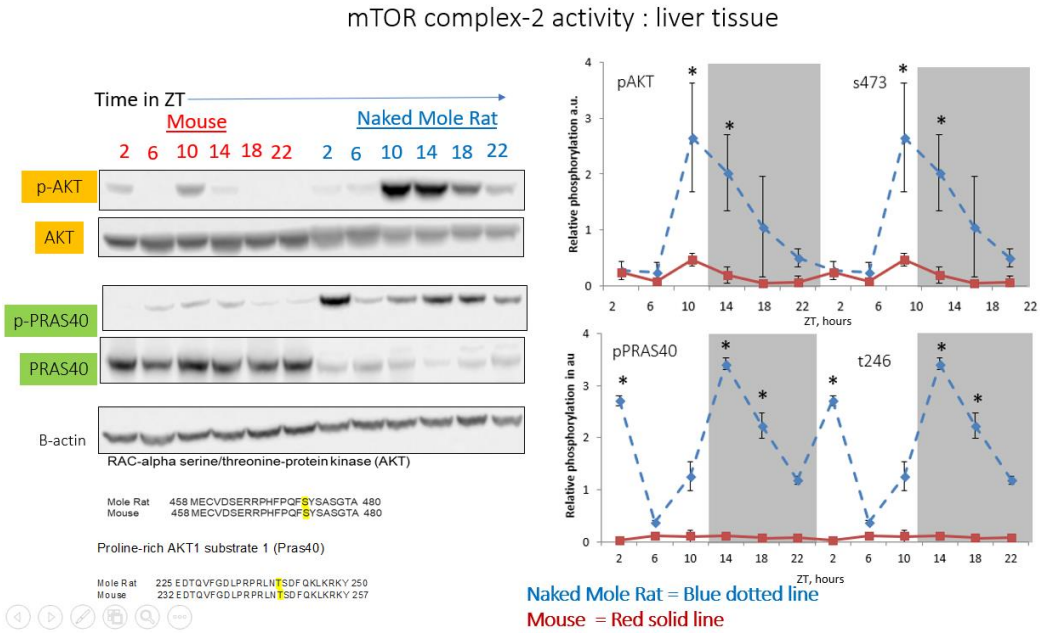


Figure 6.2 mTOR complex 2 activity in the liver tissues of the naked mole rat and the mouse.

Antibody recognition sites are conserved and marked in yellow

Table VIII The rhythms in the activity of the mTOR complexes

	Rhythmicity fit		Acrophase		Amplitude	
Site of phosphorylation	Mouse	NMR	Mouse	NMR	Mouse	NMR
AKT-S 473	0.005	0.001	8	12	8.22	9.63
PRAS -40 T426	0.044	6.27E-05	10	18	3.53	8.95
S6K1 -T389	0.46	0.46	18	16	2.09	2.35
S6-S235	0.17	0.81	16	22	4.56	2.99

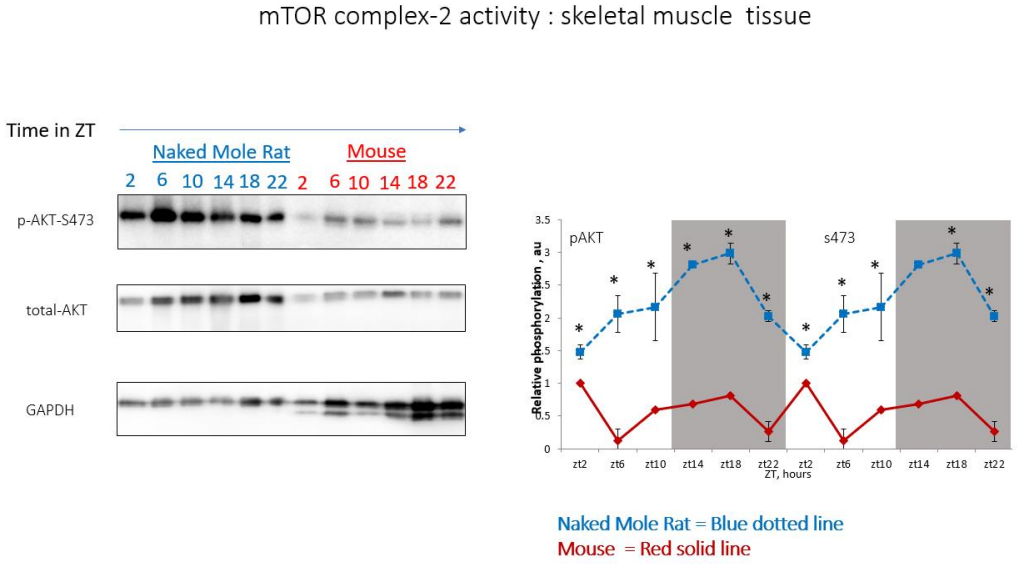


Figure 6.3 Comparison of the mTOR complex 2 activity between mouse and the naked mole rat in the skeletal muscle tissue

CHAPTER IV

DISCUSSION

4.1 Summary of results

We identified the core clock genes to be present in the naked mole rat. Then we compared the protein sequences of the core clock genes between the mice and the naked mole rat and found that there is a high degree of sequence homology between the two. We have observed that the naked mole rat liver expresses the core clock mRNAs. Most of these core clock mRNAs oscillate with a periodicity of 24 h. We have observed that the core clock genes within the liver of the naked mole rat express with a phase different from that in the mouse.

We have also observed that the expression of the glucose metabolism genes within the liver of the naked mole rat is circadian. However, the phase of expression of the genes in the naked mole rat liver is closer to each other in time than that in the liver of the mouse. Intriguingly we observed that the activity of the mTOR complex-1 in the liver of the naked mole rat is quite low compared to that in the mouse. In contrast, the activity of mTOR complex-2 in the liver of the naked mole rat is higher compared to that in the mouse.

4.2 Possible mechanism : the different rhythms of the core clock

Clock gene expressions oscillate over a 24 h period. It is postulated that the negative arms and the positive arms need to be shifted with respect to each other for the maintenance of the circadian rhythms (Korenčič et al., 2012). We observed that in the naked mole rat, the clock genes have different phase relationships. For example, the *Bmal1* and *Per1* mRNAs expressed always in anti-phase in mouse liver (Mukherji et al., 2015) , whereas within the naked mole-rat liver they peaked almost at the same time (Figure 1 and Table 2). Thus, the postulated phase shift rule on circadian clock gene expression is violated in the liver of the naked mole-rat which suggest that the rhythms can exist in different settings. The phasing of the circadian clock has been experimentally shown to have connections with metabolism, hibernation, aging and food availability. However, the models used in these studies were rats, European hamster, Golden Hamsters and Voles. None of these species is known to have a particularly long life-span like the naked mole rat. Therefore, we speculate that the existence of a functionally active but mildly oscillating circadian clock -especially one that has complex phasing of gene expressions-may be a contributory factor in the long life-span of the species.

There can be different possible mechanisms behind this. One possibility is the difference in the hierarchical relationship of central and peripheral clock regulation between the species. The second source of difference may be there in the promoter organization and positioning of the regulatory elements i.e. the CCE motifs.

We will first examine the possibility of the hierarchical relationship of central and peripheral difference in clock regulation between the mouse and the naked mole rat.

The current concept is that within a tissue, the output of the clock is a product of both the central signaling and the signals received by the peripheral clock, based on different cues.

These “cues” may be humoral as well as non-humoral. Also, there are metabolic feed-back systems that convey the metabolic status of the local tissue to the central clock. Thus, peripheral tissues such as the intestine, the liver, the pancreas, the skeletal muscle as well as the adipose tissue all exert feedback influence on the central clock. Thus, the circadian system – far from being a strictly hierarchical one- is based upon “mutual feedback” between the SCN clock and the “peripheral clocks”. We can discuss this through the example of the “feeding-process” -a behavior common to all mammals. The feeding process can independently regulate the peripheral clocks also. There are studies where the feeding tie has been experimentally perturbed to be at odds with the SCN dominated signaling. It is now known that the liver based peripheral clock is especially sensitive to a strong feeding-based cue. *Liver based* clock gene and clock protein rhythms quickly readjust to feeding cues independent of the SCN cue.

Let us look at some experiments. In a set of experiments, the tissue-clock in liver cells of mice was ablated. However, the SCN clock was kept intact (Kornmann, Schaad, Bujard, Takahashi, & Schibler, 2007). In this work, the investigators devised a mouse-strain with a doxycycline-dependent conditionally active liver clock. When the liver clock was in an arrested state, the investigators found that most of the liver’s own circadian transcriptome stopped their oscillation. That said, a small subset of transcripts continued to show robust rhythms. One of those transcripts was the *Per2*. However, those transcripts were rhythmic in liver explants only when the central i.e. the SCN clock was intact. This indicated that at least part of the circadian transcription -even in a peripheral tissue-the liver- is primarily sustained by the central clock SCN through possible systemic cues.

This indicated that the rhythm in a tissue may be driven by both the localized tissue-based clock and the signal coming from the central clock through systemic cues. In mice,

with ablated *Cry1/Cry2* the maintenance of a regular feeding schedule can sustain rhythmicity of many transcripts. (Vollmers et al., 2009) Thus it is thought that if "food supply" is the zeitgeber- that can bypass or over-ride the core circadian feedback loop.

Other receptors like the PPAR alpha and the Glucocorticoid receptors -all with metabolic activity-can potentially influence the clock-based rhythmicity. The Glucocorticoid receptors have been shown to induce the transcription of the Period genes. The Glucocorticoids are especially interesting as they could sustain the oscillation of a large number of the liver-based transcripts in mice even when the mouse had been SCN lesioned. Circadian clocks in peripheral tissues are synchronized by the central signal from SCN and by local signals such as the food. The daytime feeding resets clock gene expression in the liver and uncouples it from SCN (Damiola et al., 2000), but in the heart-tissue, the rhythms are still controlled by SCN. *All the above show that the food-cue is a strong zeitgeber, that the peripheral tissue-clocks may be regulated independently of the SCN clock in certain circumstances and that the liver-based clock is especially amenable to a local food-based regulation.*

Let us apply the above principles to our observations. We may notice that our results show a liver-based food-dominant cue in the naked mole rat. Mice and naked mole-rat have different feeding pattern, which might contribute to the observed effect on phases. Mice are nocturnal animals and *ad libitum* fed mice consume most of the daily food intake between ZT12 and ZT18 (Vollmers et al., 2009). For naked mole-rats, fresh food is provided around ZT6 and they will eat most of the food during the light phase of the day. However, the temporal organization of clock gene expression in the naked mole-rat liver cannot simply be explained by the different feeding times. Indeed, in the mouse liver, the daytime feeding results in a similar phase shift for each clock gene (Damiola et al., 2000).

The clock genes in the naked mole-rat can be subdivided into discrete groups according to the phases of their mRNA expression. It is possible that the different gene groups are controlled to a varying extent by different cues. The peaks of *Per1*, *Per2*, *Cry1*, and *Ror-gamma* expression followed the feeding time, most likely, these genes are regulated predominantly by the feeding derived signals. The expressions of the second group: *Per3, Nr1d1,* and *Bmal1* were in synchrony with light/dark cycle and therefore it is most likely, that the phase of these genes is dictated by the light cue signal from the SCN. Finally, the expressions of *Cry2* and *Nr1D2* s were affected by both feeding and light.

The availability of food and its metabolism can possibly act independently of the light signal. It is possible that the evolution of the naked mole rat as a strictly subterranean species has uncoupled the light signal and the food availability signal.

Let us now consider the second possible mechanism behind the observed differences-namely the differences in the promoter organization and position of the regulatory elements i.e. the CCE motifs, within the naked mole rat compared to that in the mouse.

Let us consider the classical control elements of the circadian gene expressions - the clock control elements [CCE motifs] and their distribution within the naked mole rat clock promoter sites. The clock control elements are short sequences found in the promoter regions of the circadian genes. They can be classified mainly into three categories – D-box, E-box, RORE elements. Studies have shown that the CCE motifs – E-box, D-box, RORE – have high conservation among human, mouse, and rat. Thus, it is reasonable to assume that such motifs are in play within the naked mole rat also-which is phylogenetically close to the above mammals.

When we manually scanned the genome -specifically the putative promoter regions of the core clock genes – we found instances of each of the CCE motifs. The location and frequency of each motif had correspondence between the naked mole rat and the mouse. This indicated a common regulatory strategy between the species in sustaining a rhythmic core clock mechanism.

The presence of these elements serves as a cis-factor to enable the rhythmic expression of genes. The E-box -found in the promoter regions of many clock-controlled genes as well as the *period* and *cryptochrome* genes -is bound by the BMAL1-CLOCK heterodimer. The canonical E-box is CACGTG. But variations of this sequence are also effective in enabling periodic transcription. The presence of a detectable E-box is not sufficient to assume its role in the core circadian clock. There are instances when potential motifs detected in-vitro are not occupied by the transcription factors in-vivo. But the presence of CCE motifs in combination with transcriptional rhythms is a strong indicator of the core clock mechanism being active.

Studies have established that in mice, all three Period genes [*per1, per2, per 3*] hold E-boxes and they serve as anchors to the Bmal1-Arntl-Clock heterodimer. Thus, the central importance of this motif in rodents is well established. In our studies, we found that the E-box elements are similarly present in the promoter regions of *period* genes in the naked mole rat. Given that the expression of the period genes shows 24-hour rhythmicity in both the species, we may assume that the core clock machinery is active in the naked mole rat. We find similar 24-hour rhythms in the *Cryptochrome* genes within the naked mole rat also. The E-box *distribution* and *number* also have a close correspondence between the species. Yet there is the difference in the phasing and amplitude. *We may thus speculate that while the CCE motifs are sufficient to impart 24 h rhythmicity to these genes,*

the nature of those rhythms is influenced by other cis and trans factors. Further investigations are needed to uncover those factors. Meanwhile, the CCE motifs prove the persistence of the primitive TTFL within the liver tissue of the naked mole rat.

Studies have established that the RORE motifs found in the *Bmal1* promoter region are competitively bound by the NR1D1/2 and ROR proteins. The competitive balance causes the repression and activation of transcription of the *Bmal1* transcription. -thereby maintaining a second rhythmic component in the core clock. In our studies, we delineated the presence of a similar RORE motif within the naked mole rat. Given the phylogenetic closeness of the two-rodent species, it is reasonable to assume that the central regulatory strategy of gene expression between the two species has not diverged. The *Bmal1* transcription shows a similar profile of expression between the two species. It also shows a 24-hour rhythm in both species although it has a much weaker rhythm within the naked mole rat.

The D-box element is assessed to be imparting a delay function to the central oscillator to bring the rhythm in line with the 24-hour terrestrial time. The importance of the presence of this motif may thus correlate with the centrality of a 24-hour transition in the life-style/behavior of a given species. In mouse, the presence of this motif is found in the *Nr1D* and *Ror* genes. In naked mole rat, we found the presence of this motif in the components of the secondary loop. However, the trans-factor of this loop is DBP whose 24 h rhythmic binding to the motif marks the 24 h periodicity. We did not detect a 24-hour rhythm of the *Dbp* expression within the naked mole rat. *Perhaps this particular CCE motif offers a leeway to the naked mole rat to adapt to the terrestrial day-night transitions under specific circumstances through its presence in the promoter region. Example of such*

circumstance may be when solitary animals must venture outside the tunnels for extended periods to establish new colonies.

The circadian rhythms present in different genes within a species may be appraised on several parameters. The first criterion may be the presence or absence of the 24-hour rhythms. The second criterion may be the phase of the expression or the time of peak expression of the mRNA transcripts of the genes. The third criterion may be the amplitude or the ratio between the maximum and the minimum expression across the day. The different species exhibit circadian rhythms as the most optimized strategy that balances the rest/activity, feeding/fasting, and sleep/wake cycles. These, in turn, are affected by ecological constraints such as exposure to sunlight, food availability, predator pressure and socialization opportunities. An evolutionary stable strategy [ESS] is one where the cost and pay-off ratio from each such consideration is maximized. The presence and distribution of the Clock Control Elements within the naked mole rat may thus reflect common ecological and ethological origins shared with its mammalian-rodent ancestors. *The CCE motifs thereby acquired have maintained the core clock ticking within the liver tissues of the naked mole rat. However, given its specialization within a special, sequestered-from-sunlight habitat as well as its eusocial adaptation-the naked mole rat has undergone considerable behavioral changes.*

The naked mole rat emerged in the open grasslands of Eastern Africa. It had to adapt to its xeric habitat with discontinuous food distribution and high predator pressure. It colonized the underground niche. Faced with the potential difficulty of finding mates over-ground they started eusocial living where colony members most commonly find mating among other colony members. The peculiar dependence on underground food supply meant that the best way to forage was to burrow in co-operative groups underground

to move from one tree-root system to another tree-root system. Thus, it was no longer optimal for the naked mole rat to persist with a sharp tracking system to detect day-night transitions. The depth of the burrow system ensured that sunlight penetration would be minimal, and a rest-activity cycle tethered to geophysical time would be ecologically unsound.

It was also not optimal for the eusocial species to have a rest-activity cycle where collective colony defense, collective care of the young, collective colony maintenance, collective foraging etc. would be compromised by the whole colony following monophasic behavior linked to external environments. As such observations of group behavior under artificial burrow systems have shown evidence of polyphasic activities. However, when individual colony members are isolated under experimental conditions, they do still display circadian behavior. [under light-dark cycles and ad-libitum food access]. This indicates that the CCE motifs are functional within the naked mole rat but somehow their phenotype is attenuated by eusocial living. Had these become vestigial the naked mole rat would probably not have been able to show the behavioral plasticity it exhibits.

We want to state here that we have examined only the presence of CCE motifs in the promoter regions of the clock genes in the naked mole rat genome. This has confirmed the presence of such elements in the naked mole rat. This also shows that the CCE motifs have number and distribution within the naked mole rat that is comparable to that in the mouse. However, we recognize that the mere presence of such motifs does not confirm that they are functional within the naked mole rat in the circadian context. We want to specifically note that the location of some of these motifs with respect to the Transcription Start Site is appreciably different from that in the mouse. Under the circumstances, it is pertinent to question if these motifs are indeed occupied by the transcription factors in a

manner consistent with their circadian functions. For example, in various models, the BMAL1 transcription factor is known to bind thousands of E-boxes at mid-day. What would be a corresponding reading for the BMAL1 factor within the naked mole rat? A good approach to ascertain this would be the experimental approach. We propose to utilize the technology of Chromosome Immunoprecipitation [ChIP] to do this. In this technique the proteins physically associated with the DNA are cross-linked. Then the protein/transcription factor bound DNA elements are sheared into smaller pieces. The cross-linked protein factors are then specifically immunoprecipitated out of the solution. The protein-DNA complex is then treated to unlink the DNA and the protein. The associated DNA is then sequenced. If a specific region on the genome is over-represented it indicates that the DNA is a preferred site of binding for the protein factor. We propose to use naked mole rat tissue samples from specific circadian points and subject them to this analysis. If we notice that the CCE motifs within the context of the promoter regions are frequently bound by the transcription factors, we could infer with confidence that the functionality of the CCE motifs is preserved within the naked mole rat.

Promoters are the sequences immediately upstream of the transcription start site of genes. Promoter activity in eukaryotes depends on combinatorial binding of many different transcriptional factors at discrete promoter elements. Such TF binding sites show enormous variability among organisms. It is known that the Transcriptional regulation diverges very rapidly in mammals. Even at closely related evolutionary distances, such as closely related inbred strains of mice, TF binding diverges with surprisingly greater speed than does the underlying coding sequences (Stefflova et al., 2013). The factors driving TF binding may be the result of variability of genetic sequences, the types and number of marks left in the

histone proteins that package DNA (commonly thought of as an epigenetic code), or even diet or environmental differences between different species.

Enhancers are short sequences distant from the coding site. They help fine tune the gene expression from the promoters. Mammalian genes are most often controlled by groups of Enhancer regions that *may* be located up to tens to hundreds of kilobases away from the Transcription Start Site of the genes. A recent comparative analysis of up to 29 placental mammals (Lindblad-Toh et al., 2011), that included the naked mole rat- has putatively identified ***regulatory*** regions for many genes. It is estimated that such regions change rapidly during evolution especially compared to the standard coding regions (Villar et al., 2015). In another study, the focus was narrowed down to the evolution of regulatory regions in the mammalian liver tissue by mapping the genome-wide landscape of active promoters and enhancers from 20 diverse species (Villar et al., 2015). It revealed that enhancer regions evolve much more rapidly than promoter regions-a trend conserved even among species separated by up to 180 million years. It also showed that 10-15 thousand promoters are widely shared among mammalian species, but shared enhancers are much less common. Another significant finding was the fact that 20-25000 different enhancers in mammalian species have evolved in species-specific manner. We believe that the sequence conservation we observe among the naked mole rat promoter regions and mouse promoter regions is representative of this trend. In the same way, the observed expression differences may be more easily attributable to enhancer region differences between the species in terms of values. The active enhancer elements have a mean lifetime three times shorter than the active promoters, even among closely related species.

In a related framework, the epigenetic process of DNA methylation is identified as a strong regulatory feature of mammalian genomes. In the mammalian order, the 5-

methylcytosine-the 5mC is predominant and appears in the context of the Cytosine-Phosphate-Guanine dinucleotides. Many CpG clusters are found in close proximity to promoters and enhancers and stay under-methylated. CpG methylation events are correlated with various aspects of gene regulation such as alternative splicing, retro-transpositions and crucially with active enhancers.

We may speculate that within the naked mole rats there is the presence of novel enhancers and modified CpG island sites. Together they provide modified epigenetic control points to the naked mole rat.

Thus, a specialized ecology coupled with underground root-based diet may have given rise to special environmental parameters which in turn gave rise to specialized epigenetics within the naked mole rat. This regulatory difference may be driving the observed differences in the circadian clock gene expressions between the mouse and the naked mole rat.

4.3 Possible mechanism :altered glucose metabolism gene-expression

Since we observed that there was a phase difference in the core clock gene expression between the naked mole rat and the mouse, we were interested in checking if these differences manifested in metabolic systems and nutrient sensing systems. We indeed found a significant difference in the rhythms of the expression of the enzymes of glucose metabolism. Several of these genes were already reported as rhythmic in mouse liver. We found them to be showing daily rhythms in the naked mole rat liver also.

Intriguingly, there was in the naked mole rat liver, two special features in the expression of the glucose metabolism genes. Firstly, the enzymes of a given metabolic pathway had close peak expression times. To illustrate, the enzymes of glycolysis-Pfk,

Hxk, Gck-all peaked at ZT6.Correspondingly the expression of the gluconeogenic enzymes- Pepck and G6pc- peaked at ZT8. The second aspect where the naked mole rat shows a difference with the mouse is the anticipation of behavior through the expression of metabolic enzymes. To illustrate, within the naked mole rat the glycolytic enzymes peaked around ZT6- this is at or immediately after the food intake time of the naked mole rat. In other words, the process of catabolizing glucose is initiated immediately after a large amount of glucose is available in the system. Correspondingly the gluconeogenic enzymes peaked around ZT8. This is well after the active feeding time. In another instance of behavior- metabolism conformity we observed that enzymes of anaerobic metabolism peaked between ZT7 and ZT10.

A number of the core clock components have metabolic functions where they control the gene expression of enzymes involved in metabolism. As for example, the NR1D1 and NR1D2 are involved in lipid metabolism as well as gluconeogenesis. NR1D2 plays important role in muscle-based lipid metabolism as well as myogenesis. Similarly, ROR-GAMMA participates in steroid metabolism as well as glucose metabolism. The ROR-GAMMA can regulate the periodic expression of certain glucose and lipid metabolic genes in different tissues. Within the liver, it is known to specifically regulate glucose metabolism through the transcriptional regulation of G6PC and PCK1. PER1 plays important roles in Glucocorticoid metabolism. PER2 has functions in the metabolism of fatty acids, circulating insulin as well as lipids. CRY1 plays important role in gluconeogenesis by controlling the cAMP-CREB pathway. CRY2 plays important roles in lipid and glucose metabolism. The various metabolic functions of the circadian clock proteins are independent of their circadian function. The metabolic regulation is important but not necessarily connected to 24-hour light and darkness phases.

It is possible that the clock gene expression, altered in the colony-living eusocial naked mole rat can directly influence and optimize the expression rhythms of the genes which are so important in the central metabolic pathways of glucose metabolism. This mechanistic connection may indeed be one of the main drivers behind the naked mole rat having an altered clock even though the components of the clock are very similar to that of the mouse.

4.4 Possible mechanism : specialized mTOR complex activity

The mTOR complex enables the organism to sense various nutrients and growth factors. The interplay of the two complexes mTORC1 and mTORC2 helps the organism decide between anabolism and catabolism. mTOR complex 1 has many downstream biological effectors. A common theme among the effects of mTOR complex1 is that they promote anabolism. Similarly, mTOR complex 2 has a variety of effects and many of these effects are concerned with cellular homeostasis.

Given the “sensory” function of the mTOR complexes – it is expected that their activity will be modulated by the availability of nutrients-both type and quantity. In case of mouse, the diet in the wild consists of frequent meals of a variety of fruits and nuts. In case of the naked mole rat, the diet is more uniform over a given season. A colony of naked mole rats survives on finding a tree-root system and feeds on underground tubers available in the same system. The feeding pattern consists of the naked mole rats burrowing through the tuber in a way that preserves the outer layer of the tuber so that it can grow back. This kind of conservative feeding may limit the drive for anabolic activity. mTORC1 promotes energy-intensive processes such as protein synthesis, ribosomal biogenesis lipid synthesis etc.

Most anabolic processes require energy in the form of ATP to carry out enzymatic reactions to generate sufficient lipids, nucleotides, and proteins for cell growth and division. mTORC1 senses the energetic status of the cell to modulate such energy-consuming anabolic processes and is thus inhibited under energetic stress conditions to ensure cell survival.

It is known that active growth requires glucose as the main energy source. Glucose is catabolized into 2 molecules of Pyruvate. The Pyruvate molecules get metabolized through the TCA cycle to move towards mitochondrial respiration to produce 38 molecules of ATP. In absence of oxygen, the process stays limited to substrate level phosphorylation and only 2 molecules of ATP are produced. As such, if glycolysis is inhibited or if mitochondrial respiration is inhibited or if there is a deprivation of Glucose- there is a rapid reduction of intracellular ATP levels. This shifts the intracellular balance of ATP/ADP as well as ATP/AMP. These balances are sensed by an intracellular AMP-dependent protein kinase called AMPK.

This AMPK is tethered to the mTORC1. When there are fewer nutrients, AMPK is activated. This activated AMPK transmits stress signal to mTORC1 through complex components TSC2 and Raptor. This is achieved partly through a phosphorylation event at Ser 1345 of TSC2-leading to mTORC1 inhibition. In an independent axis, AMPK can inhibit mTOR C1 by directly phosphorylating Raptor. These pathways through AMPK can be utilized in presence of oxygen stress also.

We notice that the food-habit of naked mole rat is such that there is a chronic deficit of sugar because entire colonies feed conservatively on very limited supplies of underground tubers. The naked mole rat also experiences an ever-present hypoxic condition because of

its strictly subterranean habitats. Both these ecological parameters dictate that given what is known about the energy sensing molecular pathways of mTORC1, we may expect a muted activity of mTORC1. Indeed, that is what we observe.

In nature it was observed, that wild naked mole rats partake of a plant diet that is unusually rich in polyphenols. However, their diet is not particularly rich in proteins. It was demonstrated that the withdrawal of amino acids in animal cell assays led to inhibition of mTORC1. Branched-chain amino acids such as Leucine and Arginine are crucial in maintaining mTORC1 activity.

Work done in Drosophila systems has shown that Rag GTPases [Rag A/B/C/D] are the main signal transducers of amino acid sensing to the mTOR system. Given the low-level amino acids in naked mole rat diet, we may assume that the mTOR complex1 activity of naked mole rat will mirror that of amino acid withdrawal and mTORC1 will show low activity levels. Indeed, that is what we have observed.

The TSC1/2 complex is known to downregulate mTORC1. However, this same complex is also known to upregulate the activity of mTORC2. It is also known that the TOR complexes share a limited amount of mTOR core protein which they compete for within the cell. Based on our observation, the low activity of mTORC1 is accompanied by mTORC2 high activity. We may speculate that the mechanisms driving mTORC1 low activity serve to move the balance towards mTORC2 low activity.

Our data shows that within the naked mole rat metabolic tissues such as liver and skeletal muscle, there is a consistently high and rhythmic activity of mTOR complex 2. The canonical target of mTOR complex 2 is Akt and further downstream of that -the pPRAS40. A highly active mTOR complex 2 should show a surge in Akt-s473

phosphorylation and pPRAS40-t246 phosphorylation. Indeed, that is what our data shows. But here we face an apparent contradiction- in that the mTOR complex1 is showing low-level activity in naked mole rat. According to established ideas in the field, a phosphorylated PRAS40 should be dissociated from the mTOR complex1 and bind to its other intra-cellular partner 14-3-3 proteins. Our data shows that mTOR complex1 has very low activity within the naked mole rat. We propose to explain this contradiction through the following possible mechanism.

PRAS40 is also a substrate of phosphorylation by mTOR-complex1 albeit at a different site namely- Ser 183 (Oshiro, Yonezawa, JBC 2007). It is also known to be a permissive factor for signaling to propagate downstream of the mTOR-complex1 (Fonseca, Proud, JBC 2007). Now it is also known that the Ser183 phosphorylation event is exclusively regulated by mTOR complex-1. So, any mechanism that can putatively inhibit mTOR complex-1can interfere with the PRAS40 -Ser183 phosphorylation. It is known that availability of amino acids can act through the RAG-GTPase axis to tune the activity of mTOR complex-1 (Kim J, Guan KL. Amino acid signaling in TOR activation. Annu Rev Biochem 80: 1001–1032, 2011). It is also known that the energetic status of the cell, as well as the oxygen availability, can regulate mTOR complex-1 activity. Due to the predominantly tuber-based diet of the naked mole rat, it is pertinent to assume that there may be a lack of ready supply of amino acids to this animal. Due to the strictly subterranean burrow-based life-style, the naked mole rat also encounters a chronic shortage of oxygen. It feeds conservatively on its main food source as a behavioral adaptation to ensure that the colony has a steady supply of food as long as they are conservative in the rate of consumption. This might also make the naked mole rat have a low energetic status compared to other rodents of its size and metabolic rate. Thus, the low supply of amino

acids, low availability of oxygen and relatively low energetic status may contribute to the low mTOR complex-1 activity. This, in turn, should prevent mTOR complex-1 mediated phosphorylation of the pPRAS 40 at Ser183. In the absence of this second event, the pPRAS40 inhibition will not be lifted from mTOR complex-1 and the waves of activation signaling would not propagate towards other downstream targets of mTOR complex-1 such as S6K1, S6 etc. Indeed, that is the downstream effect we have observed.

4.5A. The physiological significance : core clock gene expression differences

We observed that within naked mole rat liver tissue the clock gene expressions retain their 24-hour rhythms but display significant phase difference with those in the mouse. We note here that the mice consume most of the daily food intake at the beginning of the dark phase. We also note that the naked mole rat is provided food during the light phase. However even accounting for this difference in food intake time, there is a significant shift in naked mole rat. Within the mouse, the expression peak of Bmal1 and Per1 are always anti-phasic. In the liver of the naked mole rat, the expressions of those 2 genes have almost the same phase. When we checked the mRNA expression rhythms between the liver tissue of the mouse and the naked mole rat we observed that the 24-hour rhythmicity is preserved in most of the core clock genes within the naked mole rat. It is a known paradigm that every clock gene has its own phase of expression. It is assumed that the phase difference contributes to the generation and sustenance of the daily rhythms.

The expression phase of the clock genes in the naked mole rat had variable relationships with the zeitgebers. We could classify the clock genes in naked mole rat into discrete groups. In the case of Per1, Per2, Cry1, and Ror-gamma genes the expression phase closely followed the feeding time. In this group, there was a big shift between the

mice and the naked mole rats of about 10 hours. It is possible that the collective metabolic functions of these genes are more important than their time-keeping functions. As such, given the different feeding schedules of the two species, the expression phase of the naked mole rat genes have diverged sharply from the phase of the same genes within the mouse. There have been studies in African mole rats that demonstrate that underground mole rats experience very little light penetration in their colonies. Indeed, within Artificial burrow systems, at the distance of 5 cm from the opening, light intensity was about three orders of magnitude lower than below the light source. On the other hand, within the natural burrow system- while about 2.5%of the visible solar radiation entered into the transected burrow via the vertical opening, less than 0.3% of the radiation entered the burrow via the horizontally oriented opening. Thus, at the distance of 5 cm from the vertical opening, light intensity was about one order of magnitude higher than at the same distance from the horizontal opening. (Kott, Nemec,2014). In another study from the same group it was demonstrated that the subterranean mole rats of Africa exhibit clear light avoidance behavior under laboratory conditions (Kott, Nemec,2010). In another study, it was proposed that in a related subterranean rodent, the circadian system is gated by changes in temperature than as opposed to light-driven cues (Bennett, Cooper 2005). Taken together, these studies show that the naked mole rat lives in a sealed burrow system which is shielded (for all practical purposes) from light-penetration or day-night transition signals. The degree of illumination at any time within the burrow system is very low. Thus, any zeitgeber it receives is primarily of a non-photic nature.

Within the purview of this study, the two zeitgebers may be understood as light and food. In a second group, the phase shift between mouse and naked mole rat was relatively less – *Bmal1* (about 3 hours), *Nr1D1*(about 2 hours), and *Per3* (0 hours). The expression

of these genes tracked the light-dark cycle more closely. Thus, it can be speculated that such genes are more evolutionarily important in tracking geophysical time. Maybe even in the absence of a sharp day-night transition among the naked mole rats, the colony -as a whole- needs to function with at least some relation to external conditions.

This could be the presence of *predation pressure* outside the colony, outside *temperature* or outside *oxygen-levels*. It is known that when isolated males very occasionally disperse to form new colonies, the event always happens at night-time. This may be an indication that the species has retained a genetic mechanism to faithfully track day-night transitions. This is reflected in the close overlap of expression times of at least a few core clock genes between the naked mole rat and the mouse.

There was a third group of genes *Cry2* and *Nr1D2* where the shift was 6-7 hours. We feel that the expression of these genes may be dependent on both feeding cue and light cue. This indicates that even within the naked mole rat-where the light signal is much less important than that in the mouse- the physiological need to integrate two separate zeitgebers-namely the food and the light- is still present. The evolutionary cause of this is open to interpretation. However, we speculate that being terrestrial species, the naked mole rat has still the need to maintain a connecting link between the sleep-wake cycle and feed-fast cycle so that it may achieve better internal homeostasis.

4.5B Physiological significance : preservation of a clock within the NMR

The naked mole rat has been observed to be overwhelmingly eusocial and strictly subterranean in behavior. However, there is a very rare type of colony member that has been described as the “disperser morph”. This individual is invariably a male and it shows significant morphological and behavioral specialization compared to the rest of the colony.

This individual leaves the colony to traverse open ground and enter a new colony or establish a new colony. Prior to dispersal, the male develops much more bulky body composition. During the dispersal, it always displays nocturnal movement. It may be assumed that this behavior achieves survival purpose. By migrating at night, the solitary male minimizes exposure to predators. It also minimizes exposure to the day-time sun which can be damaging to its morphology given that the naked mole rats lack the ability to maintain a body temperature in high sun. What makes it interesting to note is that to sustain such behavior even over relatively short times, the animal must be able to accurately track the 24-hour geophysical cycle. We speculate that the CCE motifs present within the cognate regions within the core clock system enable the solitary animal to achieve this behavior where temporarily it faces challenges very similar to any other solitary rodent that is periodically exposed to sharp day-night transitions.

Based on lab-based experiments on naked mole rats it is known that several physiological behavioral parameters of the species show a circadian component especially when the animals are housed as single individuals as opposed to within their eusocial colony. Studies were conducted by Riccio and Goldman [2000].They demonstrated that when naked mole rats were observed while they were in social setting-there was no circadian component in their locomotor activity. However, there was exception-a few males-the largest of their respective colonies-did show a clear preference for night-time activity. However, these males had the physical attributes of the rare "disperser morphs" which prepare for leaving the colony to find new colonies. In another study, the same group established that when naked mole rats were isolated from their colonies and observed they showed clear circadian behavior. They had circadian rhythms in body temperature, metabolism. Both kinds of circadian rhythms peaked during the night-time- in a setting

when the animals were entrained to 12 l-12 d days. Further, the elevation of body temperature closely tracked the availability of running wheels which were made available to the animals at specific points during the study. When the running wheels were removed, the animals still sustained circadian rhythms of elevated body temperature, but the period of elevation was less than when the wheels were removed.

These studies were done in lab. -bred animals -which were either member of a colony or individuals temporarily isolated from a colony. They showed that there is a light dependence in the elevation of body temperature and the onset of circadian behavior, but it was apparent only in those cases where the naked mole rats were housed individually. Observations of isolated members of the naked mole rat colonies have shown increasingly sharp circadian oscillations of behaviors. It is tempting to think that under a colony living environment such phased behavior is masked by social cues and that there exist molecular pathways that act as adaptors of such cues. We believe that at least part of this regulation may be epigenetic in origin. The isolated individual that is migrating from the colony may develop a circadian clock expression system that utilizes *alternative enhancers.* This usage will enable the isolated and migrating individual to differ from its colony-mates in how closely it can track circadian geophysical transitions. Maybe the altered social environment sends cues to the core clock mechanism to use alternative enhancer modules that in turn generates different circadian rhythms. The experimental study by Riccio et al did detect elevated body temperature and elevated metabolic rate and elevated activity. The three parameters existed in tandem. This was during the dark phase in a 12 l – 12 d setting. Presumably, the animals showed an inclination for food foraging and food intake at dark when housed singly. However similar consolidated data has not been seen in colony living naked mole rats. So, it is highly probable that such elevated activity during dark is not seen

in the colony-living naked mole rat and this is probably due to masking. Since no strict circadian rhythms were detected in animals in colony living- it can be inferred that no diurnal or nocturnal feeding is confirmed.

This brings us to the next question- do they feed in the wild as in the lab conditions? It is true in the lab they are given fresh food. In the wild, it takes considerable energy to forage for food. But once the food source-usually an underground root or tuber- is found, it is carefully preserved so that the animals can feed off it for months on end. So extra energetic investment for food-acquisition is not needed and we can assume that lab housing approximates a stable colony with a steady food supply.

4.5C Physiological significance: different expression system for glucose metabolic genes in the NMR

As already mentioned, the key enzymes of a given pathway showed time-synchronized gene expression within the naked mole rat. We need to consider the possible payoff to the organism for such an expression strategy. It is conceivable that the internal clock of the naked mole rat while disconnected from daily day-night cycles- still keeps faithful synchronization for given metabolic pathways. A scenario where glycolytic enzymes express in concert will better enable them to function in concert. A similar strategy for gluconeogenesis will help the better handling of glucose metabolites in a fasted condition.

The closely packed acrophases of expression of key metabolic genes may confer better flux of metabolites through these pathways. This different glucose gene expression strategy within the naked mole rat has possible linkage to the different clock gene expressions also. It is widely known that the expression rhythms of the core clock genes generate output in various physiological systems. Most of the circadian clock gene products function as transcription factors. They can drive the expression of clock-controlled genes

which in turn provide the physiological circadian output. Moreover, many enzymes of the glucose metabolic pathways are known to participate in substrate channeling whereby the products of one enzymatic step are directly fed to the next enzyme without diffusing in the cytosol. A synchronized expression pattern can enhance the substrate channeling effects. The second aspect where naked mole rat shows the difference with a mouse is the anticipation of behavior through the expression of metabolic enzymes. The process of catabolizing glucose is initiated immediately after a large amount of glucose is available in the system. Correspondingly the gluconeogenic enzymes peaked around ZT8. This is well after the active feeding time. It is logical to assume that the naked mole rat has optimized the anabolic process of rescuing dietary glucose. Soon after it enters the passive or resting stage.

In another instance of behavior- metabolism conformity we observed that enzymes of hypoxic metabolism peaked between ZT7 and ZT10. This was in good agreement with the resting behavior of the naked mole rats when they rest in piles creating hypoxic environments. While behavior and internal metabolism are two separate aspects of life, optimal balance of the two ensures high evolutionary fitness. We hypothesize that the naked mole rat achieves this fitness through a better behavior -metabolism overlap than that in a peer species. These differences point to improved glucose handling metabolism within the naked mole rat liver. When we consider that there have been significant phase shifts in the core clock mechanism within the naked mole rat liver, it is pertinent to propose that modified clock has at least partially shaped an improved metabolism.

4.5D Physiological significance: specialized mTOR complex activity in the NMR

We observed mTOR complex 1 within the naked mole rat liver showing quiescent activity. Its components show gentle to no 24 h expression rhythms. The mTOR system helps an organism sense various nutrients and growth factors. The activity of the mTOR complex 1 is most commonly associated with cell proliferation and tissue growth. We similarly observed the mTOR complex 2 showing high activity within the liver of the naked mole rat. The activity of the mTOR complex 2 is usually associated with tissue homeostasis. An important point to note here is that the antibody recognition sites are fully conserved. A literature survey indicates that suppression of the complex 1 activity enhances longevity in many different organisms. Literature also indicates that at least in some cases high mTOR complex 2 activities can be associated with higher longevity in some organisms. We, therefore, found the down-regulation of mTOR complex 1 activity and the upregulation of mTOR complex 2 activity within a long-lived species to be interesting. We want to state that the enhanced mTOR complex 2 activity and the high level of synchronization of glucose metabolism genes can together contribute to the high-level performance of glucose handling capacity in the naked mole rat. It is known that both the complexes share the same mTOR kinase as the core. Given that the quantity of the kinase is limited, and the complexes compete for their limited amount, it is reasonable to assume that the balance of activity shifts from complex 1 to complex 2 within the naked mole rat- based on our data. This can potentially contribute to the exceptional longevity of the naked mole rat.

Studies on the inter-connectedness of the circadian clock and the mTOR system have shown that BMAL1 is a potential regulator of the mTOR system. This can mean that a core

clock which has a gentle oscillation can have complex regulatory effects on the mTOR system. We speculate that the functional yet gently rhythmic circadian clock within the naked mole rat may have contributed to an altered and heavily skewed TOR complex signaling system. We have identified and analyzed three different molecular adaptations within the naked mole rat that have potential contributions to the longevity of the naked mole rat. However, we have to remember that the difference between the lifespan of the naked mole rat and the mouse is up to ten-fold. This difference cannot be accounted for by the few mechanisms we have identified. There must be other mechanisms that have evolved to contribute to this extraordinary longevity.

Prior work has shown that reproductive status is important in regulating longevity. In C. elegans, the suppression of germline cells causes a 60 % increase in longevity [Hsin and Kenyon, 1999]. Unusually long-lived species like the Greenland shark that live for three hundred years are known to attain reproductive maturity at 120 years of age. Within the naked mole rat, we find that there is a strong reproductive division of labor. Only one female and a maximum of three males within a colony of many individuals reproduce. It is interesting to speculate what kind of signaling pathways may have undergone modification in such a species and how it might contribute to longevity. It is also known that Ultraviolet radiation is a key factor in the development of cancer in terrestrial species.

Given that the naked mole rat spends its entire life in completely sealed off burrows that are up to 2 m in depth means they have minimal exposure to solar ultra-violet rays. This can be another contributory factor to their long life-span. The naked mole rat is also known to live underground in a microbe-rich environment. It is possible that such exposure has caused them to evolve specifically strong immune systems to fight the onset of microbial infections. Given that, it is known that they can deal with effects of heavy metal

toxicity through specialized mechanisms, it is possible that parallel systems have evolved to deal with particularly severe microbial challenges. Finally, the diet of the naked mole rat was shown to include large quantities of polyphenols. This nutrient is known to be particularly effective at combating oxidative-aging. It is possible that this specialized dietary preference has caused the naked mole rat to achieve a long life-span.

We recognize that a possible limitation of our study might be the choice of female animals for the comparison. Our study design included animals from the small-worker class. This was due to the availability of such animals in sufficient numbers so that a six-time point study could be built with at least 4 animals represented at every time-point. Initially, we utilized two female animals and two male animals to make our sets. However, there was significant variation between the genders in gene expression profiles. At that point, we decided to supplement two females with two more females. The expression data thus obtained was sufficiently alike to combine into graphs. We recognize that strictly speaking, our study can be gender-skewed due to this approach. However, we have explored the circadian system, glucose metabolic system and mTOR system in our study. None of these systems is known to be particularly affected by gender-specific variation. Moreover, both the female and male animals are sexually immature. So, any difference - where they exist- should be small. As such the general principles we have described should hold true in a gender-neutral context. That said future studies can probe gender-specific differences in these parameters at a finer level.

In a previous study [Reznick, Cooney 2013], - it was demonstrated that simple altering of feeding time had a profound effect on the energy metabolism in the liver and muscle of ***rats.*** This had a significant effect on the adiposity of the rats. A mismatch between the day-night cycle and feeding time [day-time feeding instead of the normal

night-time feeding for rats] caused the animals to be less healthy. However, the expression of circadian clock genes was not much influenced. We report a shifted clock gene expression and glucose metabolism gene expression in the naked mole rat. We believe that our study demonstrates that in a long-lived species, the circadian clock has to be altered to keep pace with the metabolic enzymes. Long health-span is thus potentially dependent on an altered clock interacting with an altered metabolic system. In another study [Yasumoto, Oishi ,2013], – involving daytime feeding of ***rats***-it was demonstrated that day-time feeding can misalign circadian clock and metabolic rhythms in a tissue-specific way. While the systems became aligned in the liver, they remained misaligned in the muscle, after several days of day-time feeding. It was proposed that this tissue-specific misalignment can potentially contribute to various metabolic disorders. In our study, we show that the liver-based clock system is not particularly aligned with food input as much as the glucose metabolic genes. Yet there is long health-span in our subjects. We believe this may point at complex interactions operating between the central and peripheral clocks in the ***naked mole rat,*** which is a particularly long-lived rodent, unlike the rat.

The importance of the circadian clock in helping an animal adjust to its special eco-system is well documented. In another species of rodent- the ***European hamster***-it was demonstrated that the circadian clock stops ticking during deep hibernation [Revel, Pevet ,2007]. It was demonstrated that in case of a seasonally induced period of slumber, the clock genes *Per1, Per2* and *Bmal1* show constant levels of expression. In other words, the molecular clock stops ticking. We show that in our study, even if the naked mole rat is inhabiting an environment which is sequestered from day-night transitions, its clock is still gently oscillating. We believe this demonstrates that even in a non-circadian environment -a gently oscillating clock can confer advantages beyond mere time-keeping that can

potentially increase the health-span of the organism. In another species of rodents- the ***Golden Hamsters*** - it has been demonstrated [Kolker, Huang,2003], that aging can contribute to the alteration of circadian gene expressions. So, the connection between clock gene rhythmicity and onset of aging is seen across rodent species.

Finally, it has been demonstrated in the common ***Vole*** [Daan, Schibler 2006], that timed feeding and timed availability of running wheels can induce circadian rhythms in a usually *ultradian* species. This hints at the ability of discrete rodent species to conditionally switch their clock gene expression between circadian mode and other modes based on environmental input. Our data shows the circadian clock genes to have a mixture of circadian and non-circadian rhythms with the components of the primary feedback loop all retaining a clear circadian profile. We believe that this hints at the possible contribution of the clock genes to metabolic regulation independent of their time-keeping function. Even in a non-rhythmic environment, keeping a regular pulse of activity may be beneficial to health-span. Just like the ***Vole***, the naked mole rat can also potentially switch to a different-stronger circadian gene expression according to the environmental input. Given the seasonal variation of food availability and burrowing ease in the wild habitat of the naked mole rat-such plasticity in clock gene expression may confer definite advantages to its health-span.

The naked mole-rats were part of the well-characterized Buffenstein colony housed at the University of Texas Health Science Center at San Antonio. The selected ages yielded young, healthy individuals that were approximately physiologically age-matched (~15-20% of their observed maximum lifespan). Both species were maintained on a 12-hour light-dark cycle. In the naked mole-rat vivaria, the lights came on at 6.00am. Naked mole-rats were housed in family groups in interconnected systems consisting of tubes and cages

of varying sizes to simulate the multi-chambered burrow and tunnel systems that the species inhabits in the wild. Climatic conditions also approximated those found in their native habitat (30°C; 50% relative humidity), although atmospheric oxygen was ~21%. Naked mole-rats met all their nutrient and water needs through an ad libitum supply of fruit and vegetables (bananas, apples, oranges, butternut squash, red bell pepper, romaine lettuce, cucumber, green beans, corn, carrots, and red garnet yams).Animals were euthanized at set times (four hourly intervals over a 24-hour period) using isoflurane followed by cardiac exsanguination and the liver tissue rapidly harvested, flash frozen in liquid nitrogen and stored at -80 ^{o}C until analyses. We chose to use liver tissue since the liver is a crucial organ involved in metabolic regulation, responding to both food intake and regulating fuel substrate levels throughout the day. Moreover, in other species, it is well documented that liver physiology is under robust circadian clock control (Bozek et al., 2010; Li and Lin, 2015). Liver samples collected from naked mole-rats at the various time points were shipped to Cleveland on dry ice, overnight. All liver samples were used in gene expression studies.

Based on inputs from our collaborators we would like to state the following about animal care- The naked mole rat colonies receive fresh food every day near ZT 6-which is the middle of the light period. The animals are given a mixture of various kinds of leafy vegetables with high fiber content that resemble the in-the-wild food intake of roots and tubers. Their food mix also invariably contains at least one yam. The population-size within the lab-based colonies is variable. However, it stays at an upper limit of 100 individuals. In the case of the current study, the animals were obtained from colonies where the smallest number at any time was 20 individuals.

As for the nutrition, Naked mole-rats met all their nutrient and water needs through an *ad libitum* supply of fruit and vegetables (bananas, apples, oranges, butternut squash, red bell pepper, romaine lettuce, cucumber, green beans, corn, carrots, and red garnet yams). They were not provided with water separately as they obtain their water supply from the food they get. Food supply therefore reflected natural conditions.

4.6AFuture direction: Is the liver clock systemically controlled or is it cell-autonomous?

Studies have established that central signaling can attenuate the biological rhythms in the liver clock, muscle clock as well as adipose tissue clock. The central theme in all of these is that the clock in the suprachiasmatic nucleus of the brain tissue is, conditionally hierarchical upon the clocks in the peripheral tissues. However, in our current study we have obtained data from a whole organism model and at present, we cannot conclusively say if the observed differences are cell-autonomous or if they are systemic. In case they are systemic, there will be intriguing avenues of research to identify any special endocrine or neuronal signaling involved in the brain-liver communication.

We specifically want to identify if the brain-liver axis is playing a role in establishing naked mole rat liver circadian rhythms. The approach for this is to uncouple any anatomic connection by having an in-vitro liver cell model. We will induce circadian rhythms in this cell-mode [fibroblast] by using Forskolin. We will follow that up by measuring the clock output as before. We will then transfer the cells in conditioned media and maintain them for a week. After that, we will re-assay them for similar clock output. We aim to then compare the two data. We hope to establish the existence of any brain-liver connection within the naked mole rat. If the phase of expression of the clock genes in the liver of the naked mole rat is different because of systemic cues- we should observe that in the cell-

culture system, the expressions are more closely similar to that in the mouse. If the phase of expressions of the core clock genes in the naked mole rat liver is cell-autonomous then we should see the phase differences persist in the cell culture system.

4.6B Future direction2 : What is the effect of a modified mTOR complex within the naked mole rat?

We have noticed in our study that a modified clock is accompanied by a modified mTOR system and a modified glucose metabolism system. This raises important questions about the possible beneficial effects of the clock on metabolism and particularly on nutrient sensing.

In future, we intend to check if the differences in mTOR signaling -with a strong mTORC2 activity- helps the naked mole rat to maintain better glucose homeostasis. We intend to use the Glucose Tolerance Test in the Naked Mole Rat to do that. It is known that mTORC1 promotes protein synthesis. It also inhibits autophagy. Both these molecular mechanisms have significant effects on aging. We want to check if a reduced mTORC1 activity within the naked mole rat indeed correlates with less autophagy.

4.6C Possible translation: public health policy

The naked mole rats are known to spend considerable energy in the wild searching for roots and tubers by burrowing through hard soil-layers. It has been estimated that foraging for food-which is patchily distributed in the arid environment-requires fivefold higher expenditure of energy metabolism than at rest.

In contrast, in the experimental set-up, our collaborators provided the lab-colony animals - a mixture of vegetables, with a similar fibrous content to the naked mole rat wild diet. Thus, a significant source of extra energy expenditure was removed. This can be one

of the extrinsic factors that make lab-colony based naked mole rats significantly more long-lived [30 years] compared to their wild counterparts [17 years].

Considering human life-span, there are specific geographical areas- termed Blue Zones- where people live unusually long lives with relatively good health. It is thought that good and easily available healthy diet plays a major role in that long life-span. Our collaborators report improved life-span in their captive animals and we show that these animals may have some unusual molecular mechanisms that aid in their health-span.

Considered together-the reported extra life-span of the naked mole rats within the artificial burrow systems and the unusual molecular mechanisms we report- it may be proper to speculate that -proper nutrition made easily accessible can interact with already existing physiological mechanisms to confer extra-longevity on a species. The ease of food access that captive mole rats have ensures they do not have to invest large amounts of energy foraging for food in an uncertain environment. Assuming we can implement proper food availability programs in human communities across the world- it is reasonable to assume that the so-called Blue Zones can expand in number across the world.

CHAPTER V

CONCLUSION

The current study has compared two mammals of the class Rodentia- the common lab mouse [*Mus musculus*] and the naked mole rat [*Heterocephalus glaber*]. We examined the 24-hour rhythms in three different biological systems- the circadian clock system, the glucose metabolism system and the mTOR system. The results point to significant differences between the phasing of the body-clock, the mode of glucose metabolism as well as the mode of sensing of environmental inputs between the two rodents. The results can be summarized as follows:

Presence of an oscillating clock in a non-rhythmic environment

The results demonstrated that all the circadian clock genes are expressed in the liver tissue of the naked mole rat. A PubMed search of the annotated genome showed a high degree of conservation of the protein products of these genes in the naked mole rat compared to that in the mouse. This showed that a time-tracking system persists in the liver of this species even if it lives in an environment of constant darkness. Further, there were clear rhythms in the expression of the clock genes which suggested they were responding to some environmental zeitgebers. Future studies can probe how a non-rhythmic environment has sustained a rhythmic clock in a strictly subterranean species.

Phasing of the clock genes

The phasing of the clock gene expressions in the naked mole rat displayed a bias towards the light-phase. They also show a mutual relationship of phasing that is significantly different from that of the mouse. This hints at a strong food based zeitgeber that can over-ride light-based zeitgebers in the naked mole rat liver. But further studies are needed to investigate if other peripheral tissues-which are also metabolically active-present a similar phasing of the clock genes.

Amplitude of the clock genes

The amplitude of oscillation was found to be gentler in the naked mole rat compared to that in the mouse in many of the clock genes. However, all the genes of the primary feedback loop showed strong 24-hour rhythmicity. This may be a cell-autonomous effect in the liver tissue of the naked mole rat or it may be a result of systemic attenuation upon cue from the central clock. Further studies are needed to resolve this question.

Both the rodent species have comparable body-weight and had ad-libitum access to food. However, the naked mole rat colony received fresh food at zt 6 -the middle of the light-period and the mouse colony received food at zt 14-the beginning of the dark period. The individual naked mole rats show polyphasic activity decoupled from light-dark transitions within their colony. The mouse, on the other hand, shows strict night-day activity-rest cycles. We report here that these behavioral differences are accompanied by the two species having significant circadian clock differences in liver tissue. It is thus possible that the interplay of ***central***, light-driven signaling with ***peripheral*** food-driven signaling takes place differently in the naked mole rat compared to that in the mouse. Whereas such modified feeding has been reported to drive metabolic disorders in other species like rats, we know ***that the naked mole rat has an outstanding lifespan***. Thus, we believe that our

finding sheds light on a sophisticated circadian clock within the naked mole rat which - through special phasing and specially modulated daily amplitudes- can compensate for any such decoupling between the zeitgebers. The clock can also potentially contribute to the unusually long health-span of the species. Further investigations are needed to understand the exact molecular mechanisms through which a modified clock can confer such health advantages.

Mode of regulation of the clock genes

The canonical regulation of circadian clock genes is governed by short consensus sequences called clock control elements (CCE) in the promoter regions of such genes. Analysis of the CCE motifs in promoter regions of the clock genes within the naked mole rat showed them to be similar in number and distribution to that in the mouse. The genes of the secondary feedback loop did show some differences in CCE motif distribution. However, the overall trend shows a conservation of CCE motifs (and thus regulatory strategy) between the two species. The driver of differences may be different feeding patterns or epigenetic regulations. Further work is needed to clarify this. Alternatively, ChIP studies may reveal differential utilization of the conserved CCE motifs between the two species-which may explain some of the difference in phase and amplitude of clock gene expressions.

Expression of the glucose metabolism genes

We have shown in our studies that the naked mole rat has a glucose metabolism gene expression pattern that shows remarkable synchronization for each given pathway compared to a more diffused expression program for the mouse. Our studies also strongly hint at a good anticipation of behavior at the metabolic level-where a fed state is immediately followed by genes of gluconeogenesis whereas a stage of active feeding is

preceded by genes of glycolysis. We believe that such synchronized gene expression has definite health-benefits and requires deeper investigation. Our studies thus provide the foundations for metabolite profiling of the naked mole rat at different points of the day.

Activity of the mTOR complexes

In our studies, a crucial discovery was how the mTOR complex activity differs between the naked mole rat and the mouse. In the naked mole rat, the mTOR complex-1 activity is significantly lower than the mouse. In contrast, the mTOR complex-2 activity shows a strong and strictly circadian activity profile within the naked mole rat whereas it is weak in the mouse. The mTOR complex-1 promotes anabolism and mTOR complex-2 promotes homeostasis. Our results indicate that the naked mole rat has evolved a strategy that is highly conservative towards expensive anabolism but has a strong emphasis on homeostasis of tissue and physiology. The implications of this finding are profound. We feel that a modified mTOR system can independently confer life-extending health benefits on the naked mole rat. But mTOR can also act in complex synergy with the circadian clock system and the glucose metabolism system to have beneficial effects for the net life-span of the naked mole rat.

We state that our findings highlight specific evolutionary adaptations that the naked mole rat developed to tackle a strictly subterranean and eusocial lifestyle. We suggest that these different evolutionary adaptations in the naked mole rat play key contributory roles in its enhanced life-span. Future studies need to focus on the exact mechanisms by which the above adaptations may contribute to the long health-span of the naked mole rat.

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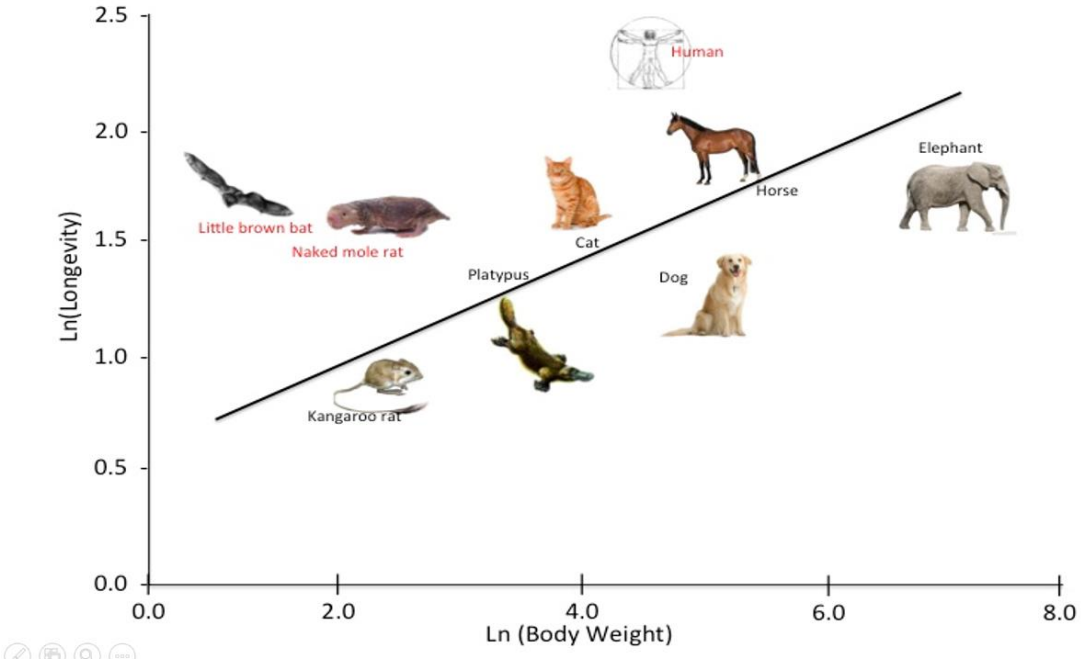
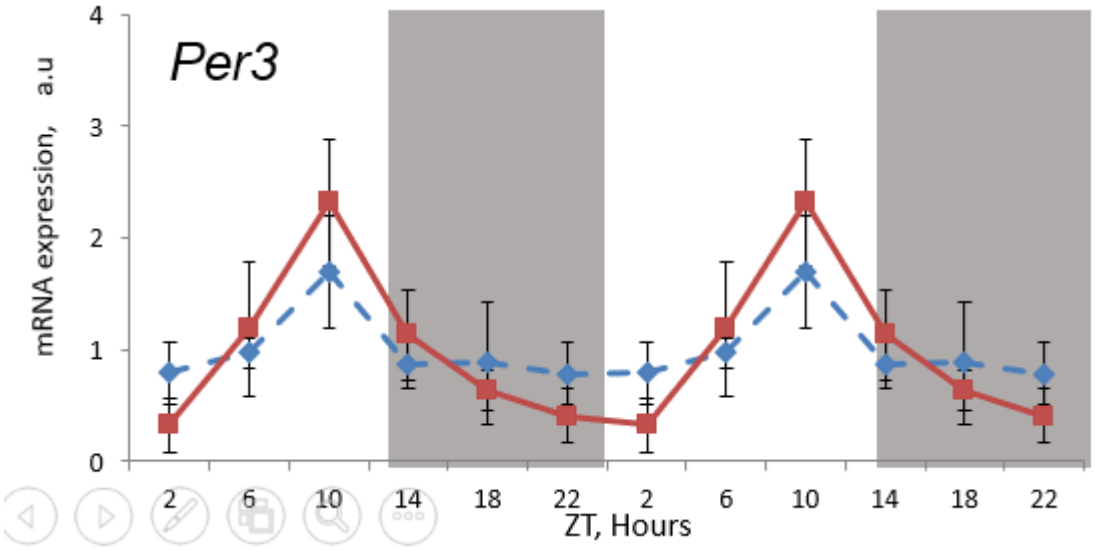
APPENDIX A

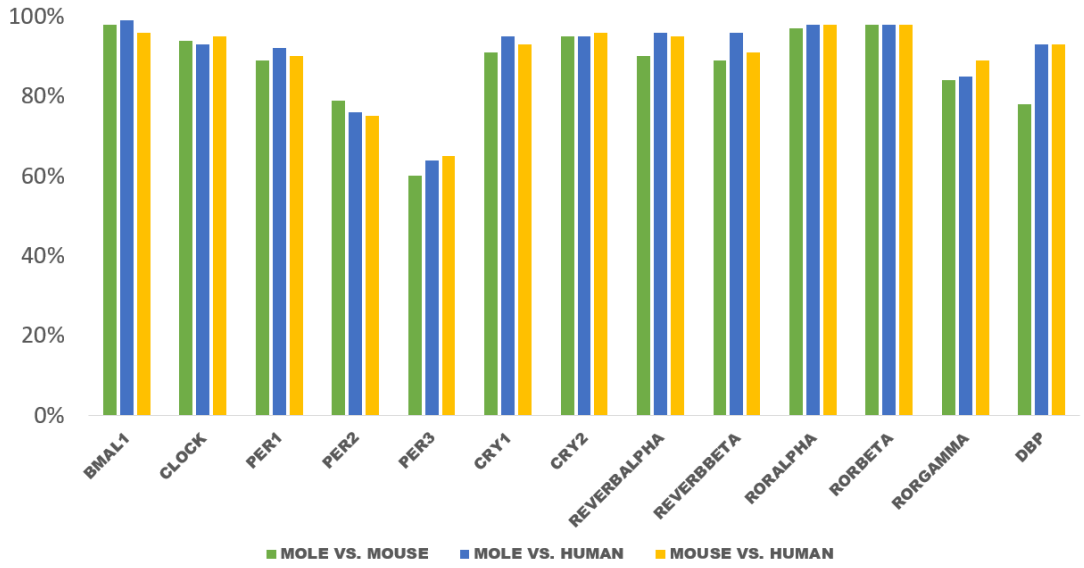


Relationships between mammalian body-weight and longevity: Data analyzed from the information available on the AnAge database.

Semi-log plot showing the outstanding longevity of the naked mole rat [NMR] among the rodents. Each data-point represents a separate rodent species.



Period 3 gene: An example of highly similar gene expression between the mouse and the naked mole rat.

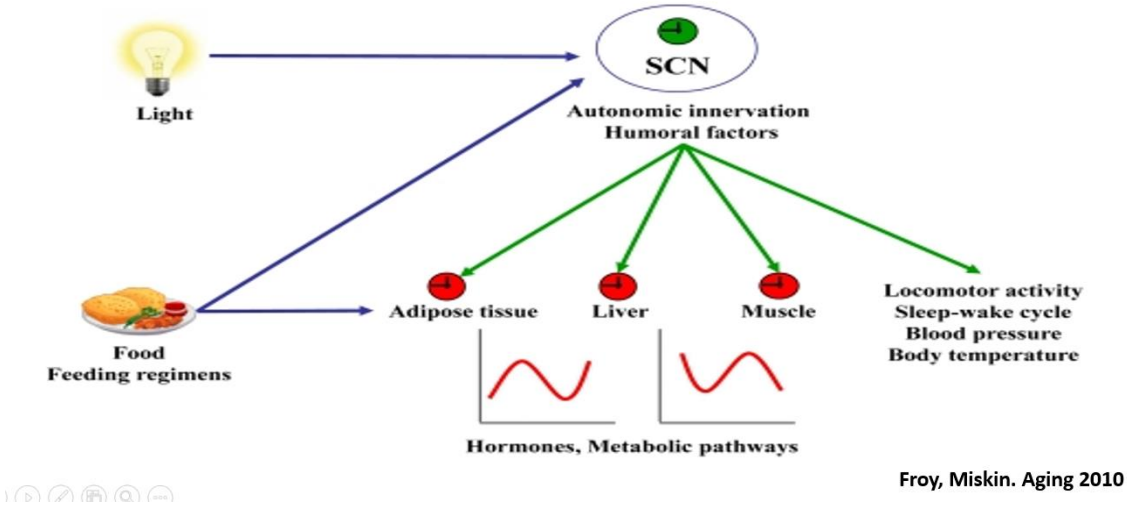


Protein sequence conservation among the species.

Conservation of protein sequence among the mouse, naked mole rat and human for the components of the circadian clock The analysis shows the high degree of similarity between the naked mole rat and the human.

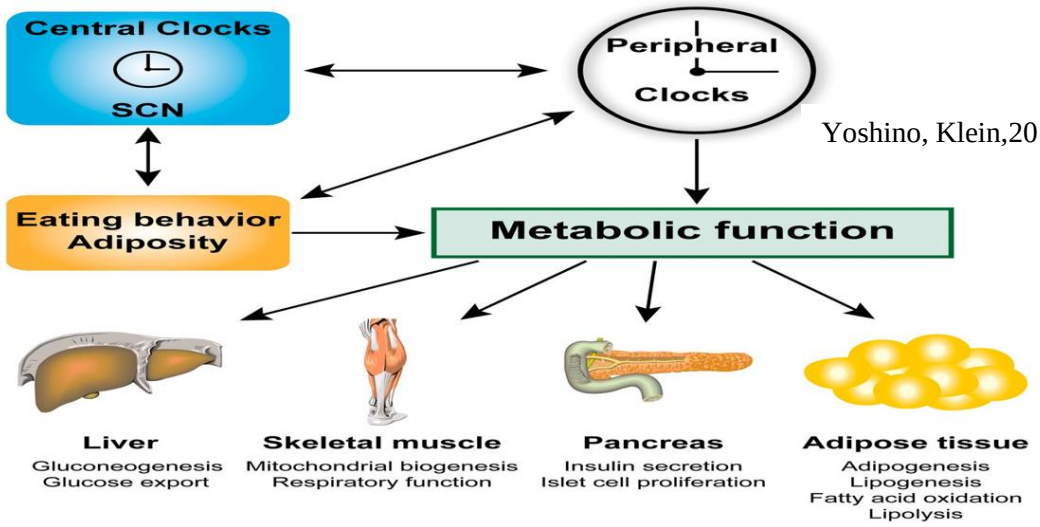
APPENDIX B

Central and Peripheral Circadian Clocks



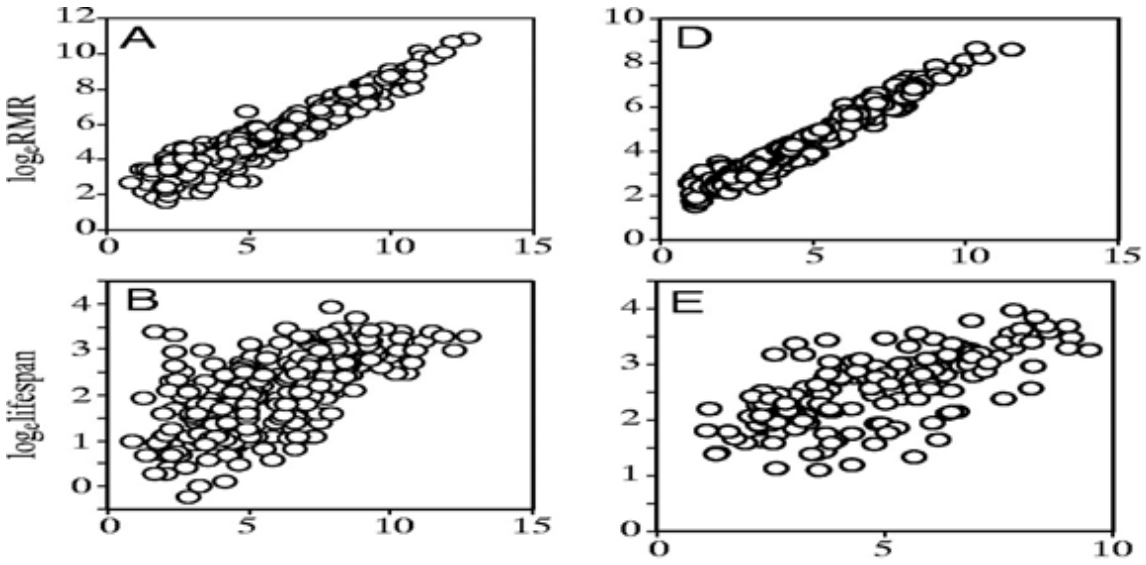
Circadian gene expression is a complex output of mutually interacting central and peripheral clocks.

Circadian clock regulates metabolism



Yoshino, Klein,2013.

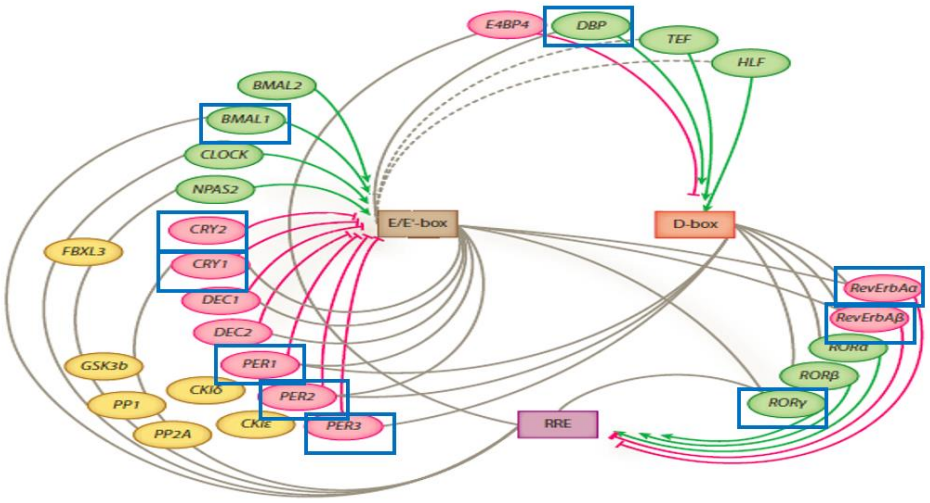
APPENDIX B [contd.]



The above figure [Speakman 2005] shows the relationship between metabolic rate and lifespan among animals . A and B : With *increasing body mass* ,mammals expend energy at progressively lower rates and tend to *live longer* A similar trend is present among birds where bigger birds expend energy at a lesser rate [D] and they tend to live longer lives. [E].

APPENDIX C

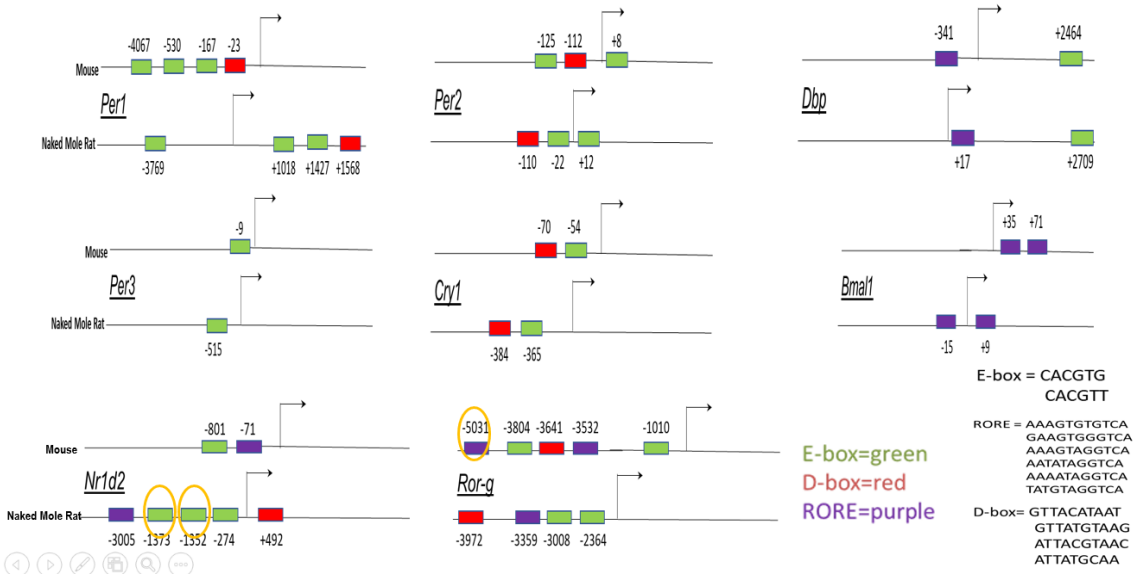
Clock control elements and their effect on the circadian clock



Ueda, Annual Review Physiology,2010.

Mutual interconnectedness of the Clock Control Elements (CCE motifs) in the circadian clock

CCE motifs : distribution in the promoters of mouse and naked mole rat



The Clock control elements (CCE motifs) in the promoter regions of the clock genes of the mouse and the naked mole rats. The analysis shows the similarity in CCE motif type and distribution between the two species.