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Chapter 27

Circadian Redox Regulation

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Abstract

Modern theory implicates damage and regulatory distortions associated with oxidation and energy shortfalls as primary causes of aging. Recognition that regulatory organization revolves around energy-redox cycles highlights the circadian clock. This chapter identifies and explores temporal compartments that serve to minimize conflicts and optimize synergism among associated functions. Wake-associated niche interfacing, resource acquisition and ATP production is the most obvious phase. A second anabolic phase in early sleep is associated with protein synthesis, growth and immune function. This is regulated by the growth hormone axis and the target of rapamycin (TOR) which is strongly implicated in aging. A third window in late sleep manages activities incompatible with oxidative conditions and coordinates recharging and upregulation of stress resistance systems in anticipation of waking. Associated functions include proteosome activity, autophagy and DNA repair. This window appears dominated by forkhead transcription factors (FOXO) associated with extension of longevity by dietary restriction. Thus, sleep may encompass both TOR and FOXO functions critical to aging even though these pathways are largely antagonistic to one another. The implications of this circadian framework are that distinct temporal compartments may be serially regulated to achieve and maintain youthful function. Current logic dictating that TOR and FOXO are necessarily antagonistic and that inhibiting TOR or upregulating FOXO will extend healthy lifespan reflect a temporally static perspective that may be misdirected (particularly if TOR supports stem cell function). Increasing the duration and quality of both sleep compartments could be a more viable strategy for extending the human lifespan.

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1. Introduction

Oxygen is highly reactive but cellular metabolism generates even stronger free radicals, more broadly defined as reactive oxygen species (ROS). ROS damage or alter membranes, organelles, nucleotides, proteins, lipids and extracellular elements (Vol. I, Chapters 2-9). ROS are generated by immunocytes, cytochrome P450 enzymes, oxidases, lipases, nitric oxide (NO) synthase, and particularly, mitochondria (Chapter 15). The reigning "*Free Radical Theory*" holds that aging involves accumulating ROS damage (i.e., biological rusting) [1,2] but ATP shortfalls also mediate stress and dysfunction associated with senescence [3]. Thus, metabolism and ROS are inseparably intermeshed via rhythmic respiration, ATP production and energy expenditure [4-6].

Although accumulating "damage" was the basis for the free radical theory [1], ROS were anciently harnessed as reliable signals indispensibly woven throughout the fabric of life [6-11]. These signals involve oxidation-reduction of proteins and lipids, the quintessential example being reversible oxidation of protein cysteine and methionine residues. Thus, tyrosine phosphatases are reversibly inhibited by oxidation of specific cysteine residues, freeing kinases to mediate intracellular signaling cascades [11-14]. This mechanism alone highlights that signal transduction toggles between oxidative and reduced phases of redox cycles. Reversal of oxidative modifications in protein signaling largely involves glutathione, thioredoxin, and glutaredoxin [6,15,16]; see also Chapters 1 and 9. Nor do modifications need to be reversible. Removal and replacement (e.g., protein degradation and synthesis) commonly contribute to regulation (e.g. as in the cell cycle). Oxidative modifications of lipids also mediate diverse signaling.

Redox switches pervasively regulate cellular functions including neurotransmitter systems, ion channels, antioxidant and stress response systems, heat shock and chaperone proteins, apoptosis, growth, metabolism, mitochondria, detoxification, immunity and protein metabolism. Binding activity of critical transcription factors is also regulated by redox-sensitive cysteine residues (e.g., AP-1, NF-κB, nuclear factor-1, Sp-1, HIF-1α and p53) [6, 7, 11, 15, 17-20]. Aging is associated with increasingly oxidative conditions that may distort signaling networks, a circumstance as important to aging as is ROS damage itself [6, 11, 21]; see also Chapter 23.

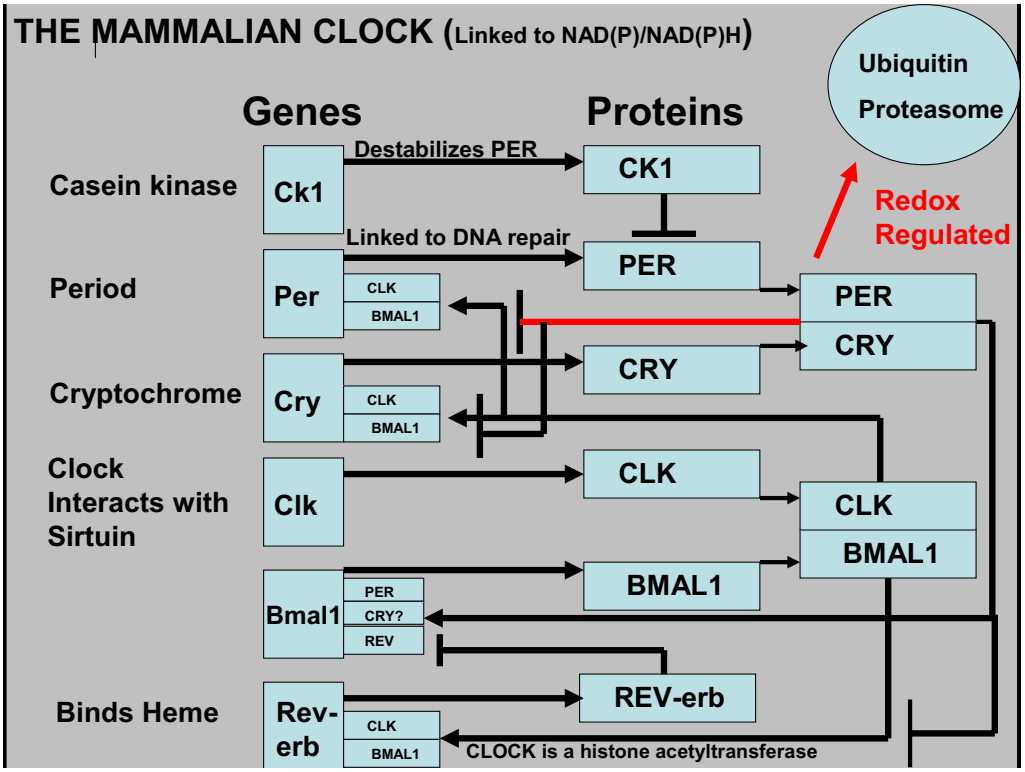

Figure 1. Major components of the mammalian circadian clock. Core timing derives from transcription of *Period* and *Cryptochrome* by CLOCK:BMAL1 dimers. Translated PERIOD and CRYPTOCHROME form dimers that move to the nucleus where they inhibit their own transcription. This completes one clock transcriptional cycle. REV-ERB (and likely other nuclear receptors) provides feedback according to heme status relevant to redox. Antagonism of CLOCK acetyltransferase activity by the SIRTUIN deacetylase links the clock to redox, energy and aging modulators expressed in dietary restriction. Period is also regulated by redox and its degradation (mediated by casein kinase) is linked to ubiquitin-proteasome function that may also be impacted by oxidative conditions.

Like any good computer, complex regulation of biological functions and metabolism are coordinated by sophisticated timekeeping (Figure 1). Clocks are particularly valuable for interfacing to circadian day-night cycles and seasonal change. A temporal theory of regulatory evolution emerged from our observations that transgenic growth hormone (GH) mice express accelerated aging and ROS, low ATP, reduced motor activity (2.9 hours/day below normal), hypothermia, vastly increased sleep (3.4 hours/day longer than normal) and remarkably superior maze learning [22-24]. We postulated that sleep regulates a circadian tradeoff between activity and other physiological demands like growth [22]. Thus, growth is relegated to sleep when energy can be diverted to this sink. Cycles of activity, feeding and sleep were tightly integrated and regulation of glucose and lipid metabolism by GH suggested metabolic underpinnings [22, 25].

Increasing dietary carbohydrate restored sleep-wake balance and activity in GH transgenic mice, confirming a metabolic aspect of sleep. We envisioned that sleep serves as an umbrella for a suite of anabolic functions (e.g., growth, immunity, memory consolidation, recharging, repair, replacement, detoxification). Relegating anabolism to sleep in a secure insulated nest allows full dedication of waking resources to niche interfacing (resource

acquisition, risk avoidance, stress resistance, competition for mates and parental care). Any impairment of competitive, niche-related waking performance by drains to housekeeping would compromise fitness [23,25]. Futhermore, sleep-associated enhancement of reserves, functional enzymes and stress resistance may derive even higher waking fitness than otherwise possible.

Consider an example elaborated from Darwin [26]. An arctic hare pursued by a fox across a snowfield is running for its life. Crisp perception, cognition, coordination and cardio-muscular performance determine survival. Such are equally critical for the fox's dinner. Endurance and thermogenesis also contribute. Muscular strength and metabolism indeed show significant time-of-day effects [27]. Neither the fox nor rabbit can afford to have critical functions compromised by diversion of resources to anabolism. As elaborated here, neither would be well served by sleep deprivation either.

This theory of circadian regulatory structure envisioned two antagonistic watersheds regulated by the hypothalamic-pituitary-adrenal axis in waking (catabolic niche interfacing, resource acquisition and energy production) and the GH axis in sleep (anabolic growth, replacement, repair and recharging) [23-25,28]. Such tradeoffs are rooted in the evolutionary "Principle of Allocation" [23] but telescope downward to reveal conserved regulatory integration spanning neuroendocrine to genomic levels. This regulatory "Bauplan" (blueprint) involves temporal compartmentalization of functions that maximize synergy and minimize interference [25, 29, 30].

Oxidative stress is a key target of sleep homeostasis [31] *and oxidized glutathione induces sleep* [32, 33]. *This links circadian sleep to metabolism and redox.* ROS processes proved greatly accentuated in sleepy transgenic GH mice [3, 34-36]. Further expansion of theory forged ion channels, redox, metabolism and electrical activity into a unified "Electroplasmic Cycle" reflecting a basic circadian organization of life [6].

Electroplasmic organization is exemplified by elegant studies of metabolic-redox cycles in yeast. Yeast express intermeshed redox-metabolic rhythms and functional compartmentalization that are largely conserved in vertebrates. Expression of more than half of the genome is entrained to yeast cycles in association with distinct temporal windows of respiration/mitochondrial membrane potential, mitochondrial biogenesis, ribosome production, sulfur metabolism, autophagy, fatty acid oxidation, glycolysis, replenishing NAD(P)H and antioxidant pools, cell division, DNA transcription, and pH [37-42]. Nucleotides, amino acids and carbohydrates also cycle [41]. Circadian regulation of heme biosynthesis, energy metabolism, neurotransmitter activity, P450 functions and lipid metabolism show conservation spanning 600 million years [43]. Yeast cycles, vertebrate circadian rhythms, sleep-wake cycles and even hibernation are highly analogous [14, 29, 40, 41, 44].

Remarkably, metabolic-redox cycles of yeast display three distinct phases of functional organization rather than two. These consist of an oxidative *energy-producing* phase, a reducing *building* phase and a reducing *charging* phase [41]. In plants, four clock promoter elements suggest there may be even finer temporal differentiation [45]. It is worth considering whether such refined organization extends to vertebrates. Thus, the yeast oxidative phase maps onto mammalian waking associated with high mitochondrial respiration and locomotor activity. Numerous genes related to energy metabolism, arousal and waking are upregulated during early waking and initiation of feeding (including glucose transporters and genes regulating mitochondrial complexes I to IV and tyrosine hydroxylase) [4, 43]. Feeding (and

induction of hormones like insulin) would further impact liver function. Rhythms in body temperature, ventilation and oxygen consumption are strongly elevated during waking whereas O_2 consumption declines markedly with sleep [46].

The reductive-building phase of yeast maps to early sleep in mammals characterized by large pulses of GH secretion. Regulation of protein synthesis, growth and IGF-1 by GH involves target of rapamycin (TOR) pathways. TOR signaling may promote ROS and accelerate aging (see also Chapter 23). Crucial functions restricted to reducing conditions in yeast included heme and sulfur metabolism, replenishing NAD(P)H reducing equivalents and recharging antioxidant pools [41]. The reductive-charging phase of yeast is congruent with late sleep when antioxidants, stress resistance elements, detoxification systems and gluconeogenesis rise in anticipation of waking [the realm of HPA activation, and forkhead transcription factors (FOXOs)]. Such a framework has numerous implications, particularly since TOR and FOXO are antagonists residing on different branches of the phosphoinositide 3-kinase (PI3K) pathway (and they oppositely impact redox and aging).

Here I extrapolate the yeast triumvirate of redox-metabolism to the vertebrate Electroplasmic Cycle [6, 47]. This includes clocks, neuroendocrine systems, sleeping-waking, the PI3K pathway, growth (GH-TOR), and stress resistance (SIRT1-FOXO). Aging provides a background foil. One might envision that global regulatory structure should be readily apparent but there are many complications. Rhythmic elements strongly differ among tissues in multicellular organisms. Circadian rhythms are overlaid by 3-4 hour “ultradian” cycles of waking-feeding-sleep that are to some extent mini-versions of the daily rhythm. Rodents show more prominent ultradian organization than humans, complicating interpretation and measurements because most studies ignore them.

Gene arrays do not capture dynamics of proteins or their activity. Thus, the clock gene *Period* (*Per)* shows maximal expression in sleeping rodents (light) but protein levels (that inhibit *Per* gene expression) peak in the night (waking) [48]. Another clock element *Clock* (circadian locomotor output cycles kaput) does not show circadian rhythmicity but its acetyltransferase activity does [49]. The clock gene *Cryptochrome2 (Cry2)* does not show strong transcriptional rhythmicity but its protein does [50]. Regulation of systems like the clock or GH axis involves transcription, translation, secretion, and degradation. Activity of transcription factors like FOXOs depends on cytoplasmic-nuclear shuttling. Such complexity and scope limit the current vision of the electroplasmic cycle to a caricature outlined with broad strokes.

2. The Neuroendocrine System and Temporal Signaling

Multicellular organisms coordinate differentiated tissues and organs via a neuroendocrine overlay that regulates metabolism, redox and function. Sleep is homeostatic and its timing and duration can be regulated independently and dissociate from that of the clock. The clock and arousal states show independent but reinforcing regulation of associated functions. To limit the scope of this synthesis, I consider that clock and arousal states are congruently aligned as in normal entrainment so they can be treated as simply circadian time.

There is a circadian march of hormones and neurotransmitters associated with circadian and ultradian wake-feeding-sleep cycles. The standard interpretation of hormone axes as auto-regulatory feedback loops resolves into a domino-like regulatory circuit from a temporal perspective. Essentially, each regulatory element tandemly upregulates the next player in the circular parade while specifically downregulating the preceding element that induced it. Thus, GHRH induces GH and GH in turn suppresses GHRH while upregulating the GH inhibitor, SRIF. Inhibition of general GH axis function also involves negative feedback from IGF-1. Similarly the HPA axis successively deploys corticotrophin releasing hormone (CRH), ACTH and glucocorticoids. This successively activates neuropeptide Y-driven hunger, the orexin arousal system and motor activity associated with catecholamines. Inhibitory glucocorticoid feedback to central CRH brakes HPA activity. Meals are anticipated inputs to the system, as they inhibit orexigenic but stimulate anorexigenic pathways. In particular, meal-induced insulin spikes largely dominate waking PI3K activity. Such "domino" organization likely derives temporal patterns independently of the clock, and ultradian cycles may emerge from such structure. There is no need for the clock to regulate each step if the entire system expresses fundamental temporal organization.

Other complexities are also resolved by a temporal perspective. Considering that the HPA and GH axis are counter-regulatory (day-night) it is not surprising that the effector of the HPA axis (corticosterone or cortisol: CORT) inhibits GH secretion. Rhythms of CORT and GH cycle 180^{o} out of phase. Consequently, it is considered "peculiar" that glucocorticoids strongly stimulate GH transcription [51]. Such organization, however, is exactly what is required for the HPA axis to regulate the amounts of GH released, thus linking the degree of stress during waking to sleep-associated anabolism (perhaps including FOXO-mediated investments in stress-resistance). Thus, despite being antagonistic in function, timing and redox status, mean daily concentration and the number and height of GH peaks positively correlate with mean daily cortisol [52].

2.1. Hypothalamic-Pituitary-Adrenal Axis

The HPA axis is closely tied to the clock. Sleep and associated GH secretion are homeostatic and are more directly responsive to HPA control than clock signals. This undoubtedly reflects that waking and niche interfacing have priority over sleep. Rather than engaging complex signaling cascades like those of GH and IGF-1, glucocorticoids bind cytosolic receptors and directly induce nuclear translocation and transcription. Sleep may be deferred to ensure acquisition of resources or to manage risk (e.g., wake up to deal with that sound of breaking glass) and it is significant that hearing is not downregulated in sleep as are vision and motor functions. Gene arrays distinguish genes separately regulated by the clock or arousal states [53, 54]. Regardless, sleep deprivation incurs costs (particularly in aging animals) and is ultimately lethal.

Clock regulation of the HPA axis may provide a circadian boot to rhythmicity that ensures proper phasing of waking to the day-night cycle. Lesions of the suprachiasmatic nuclei (SCN) housing the central clock abolish feeding, locomotor and glucocorticoid rhythms. Mice lacking *Per2* express no rhythmicity in corticosterone although responses to feeding, ACTH and stress remain intact [55]. SCN innervation targets the paraventricular (HPA axis) and arcuate (GH axis) nuclei, medial preoptic area, dorsomedial hypothalamus

and the orexin system in the lateral hypothalamus [56-59]. The HPA axis is upregulated in late sleep associated with increasing REMS, corticotrophin releasing hormone (functions of which include early-waking grooming and defecation), ACTH and glucocorticoids. Production of pituitary ACTH in response to CRH from the paraventricular nucleus is circadian. A clock in the adrenal cortex governs a window of ACTH sensitivity crucial to adrenal steroid production. Neural signals also communicate photic information from the SCN to adrenal *Per* [58-60]. The SCN signal to the HPA axis may actually be dis-inhibitory, but regardless, ablation of the SCN abolishes HPA rhythmicity [58, 59]. In mice plasma ACTH rises during the photophase (resting) to peak ~2 hours prior to the scotophase (wake-activity) transition. This elicits a steep rises in corticosteroid (CORT) in the late photophase peaking ~2 hours later than ACTH and in phase with the light-dark transition [60]. In mice with a defective clock, CORT was arrhythmic and levels were consistently low [60].

Although the SCN does not possess glucocorticoid receptors, glucocorticoids synchronize many peripheral clocks [60]. A single glucocorticoid treatment synchronized about 60% of the hepatic circadian transcriptome [58] and likely initiates activity in rhythmic skeletal muscle genes [61]. A glucocorticoid response element activates *Per* and a negative response element occurs in *Rev-erbα*. Glucocorticoid regulation of gluconeogenesis during sleep crucially contributes to metabolism and glucocorticoids also respond to the timing of meals. Adrenergic signals (usually wake-associated) may also synchronize circadian clocks in heart and liver [62].

2.2. Phosphoinositide 3-Kinase Pathway

Insulin and IGF-1 signal via the phosphoinositide 3-kinase pathway (PI3K) that regulates energy, oxidative metabolism and aging [63] (Figure 2). Decreased PI3K signaling is associated with many models of extended longevity. PI3K activity requires ROS generated via NAD(P)H oxidase [NOX] [63]. This inhibits tyrosine phosphatases via redox-sensitive cysteine residues. Growth factors (particularly insulin and IGF-1) mediate PI3K signaling and ROS generation. Catalase and peroxiredoxin are inhibitory [63].

A critical PI3K crux point centers on three protein kinase B isoforms (PKB/Akt), particularly Akt1. Akt1 is generally distributed, Akt2 is mainly expressed in insulin-dependent tissues, and Akt3 is expressed in testes and brain. Akt1 is highlighted in growth (including cell size and cell cycle progression), protein and glycogen synthesis, immunity and aging (i.e., TOR functions). Akt2 is highlighted in insulin-mediated glucose transport and fat deposition [64-66]. Akt acts as a switch dichotomously activating either TOR (phosphorylation and activation of Akt) or FOXO (low PI3K-Akt signaling and inactive Akt). This bifurcating regulatory structure confers mutual antagonism between stress resistance (FOXO) and growth (TOR) that is phylogenetically conserved from yeast to vertebrates.

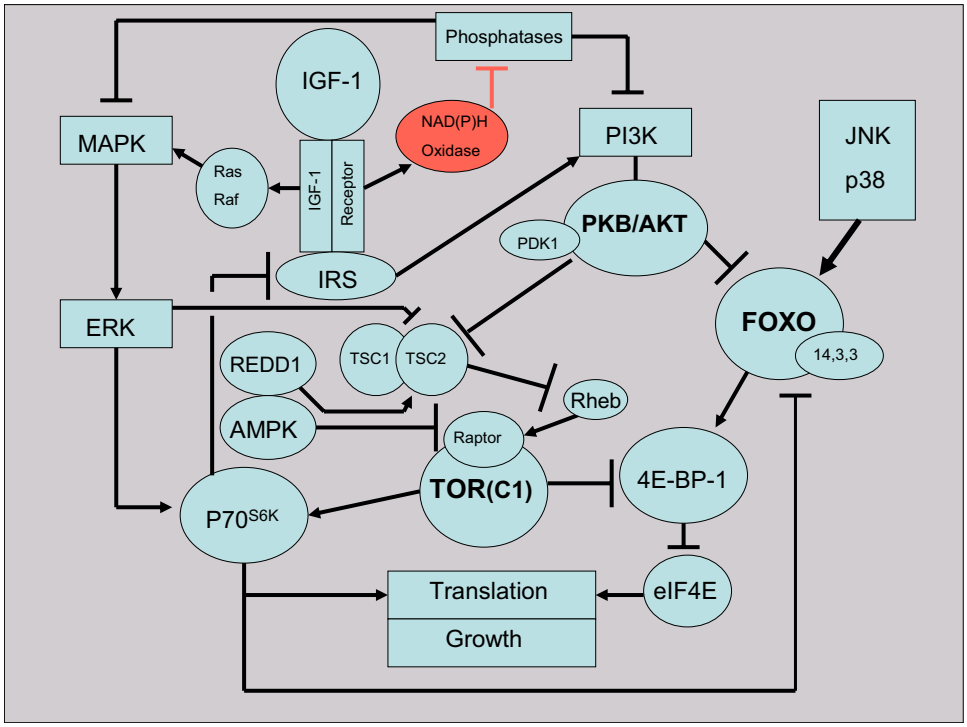

Figure 2. Aspects of sleep-associated IGF-1 signaling relevant to the target of rapamycin (TOR) and forkhead transcription factors (FOXO). IGF-1 activates two main pathways (PI3K-PKB and MAPK ERK) that are both involved in protein synthesis and growth. Note that IGF-1 activity is strongly associated with free radical generation by membrane bound NAD(P)H oxidases. Oxidation of phosphatases disinhibits the IGF-1 signaling pathways. The crux point regulating TOR-FOXO antagonism is bifurcation of PI3K signaling at Protein Kinase B (PKB). PKB inhibits FOXO activity and activates TOR via disinhibitory signaling to Tuberous Sclerosis Factor 2 (TSC2). Inhibition of TSC2 releases activity of Rheb which in turn activates TOR. TOR signaling then activates eIF4E and $P70^{S6K}$ activity required for protein synthesis. Numerous stressors, amino acid availability and enegy levels strongly modulate TOR activity. REDD1 signals status of diverse stressors and AMPK reflects energy associated with AMP/ATP status.

Thus, activation of PI3K-Akt by insulin or IGF-1 activates TOR but inhibits FOXOs (Figure 2). Similarly, inhibition of phosphatases upregulates TOR [67]. Alternatively, low Akt activity disinhibits FOXOs but downregulates TOR. As explored below, *TOR-FOXO antagonism dichotomously regulates protein synthesis, proteasome function, autophagy, cell proliferation, apoptosis, gluconeogenesis, redox status, and stress resistance (including antioxidants)*. All of these are highly relevant to protein turnover, growth, cancer, stem cell function, immune activity, and ultimately, aging rates. TOR-FOXO antagonism is likely involved in two phenomena associated with slower aging – dietary restriction (DR) and increased lifespan of larger species [68, 69].

2.3. The Growth Hormone Axis

Insulin and IGF-1 both signal via PI3K but their roles are rarely differentiated. A temporal framework suggests that insulin is mainly meal-associated whereas IGF-1 functions

independently during a specifically designated window. GH is secreted in large spikes during early sleep. IGF-1 is the key effector of GH but does not exhibit strong rhythmicity. Plasma IGF-1 may signal protein/amino acid availability much as leptin reflects general fat reserves. IGF-1-TOR signaling is essential for cellular growth, including cancers. IGF-1-TOR signaling is associated with upregulation of the mitochondrial pyrimidine nucleotide carrier (PNC1) that functions in growth control. Reduced expression of PNC1 reduces cell size and proliferation rates. IGF-1 critically regulates growth and permissively regulates other growth factors, thus coordinating growth and TOR activity [70]. PI3K activity in *Drosophila* also depends on dietary protein [71]. Coordination of growth involves the fat body. Downregulation of the amino acid transporter "slimfast" in fat body produced flies resembling those with nutrient deprivation and downregulated PI3K-TOR signaling in other tissues [72]. FOXO expression in fat body also extended longevity, reduced fecundity and increased paraquat resistance [73].

The MAPK-ERK pathway that mediates growth factor signaling in conjunction with PI3K is active in sleep [74] and sleep-associated IGF-1 signaling is modulated via binding proteins (IGFBPs). IGFBP-3 is the dominant chaperone of circulating IGF-1 whereas IGFBP-1 negatively regulates the bioactive pool. *Igfbp-1* expression showed the highest amplitude changes in liver of any gene, exceeding even core clock genes (22-fold) [75]. Expression of *Igfbp-1* was low during the early sleep/light phase as predicted here. GH suppresses IGFBP-1 function independently of insulin, which is also inhibitory [76]. IGF-1-Akt signaling is well documented to activate TOR via impacts on TSC2 (see "Target of Rapamycin") and IGF-1, p53 and TOR cooperatively regulate cell proliferation and apoptosis [77]. Exogenous IGFBP-1 also inhibits proliferation as in breast cancer [78].

GH impacts on IGFBP-1 would activate MAPK-ERK and PI3K-TOR via IGF-1 receptors and insulin response substrates. Significantly, IGFBP-1 is also negatively regulated by TOR and upregulated by amino acid deficiency [79, 80]. Relevant to TOR signaling outlined below, Ames dwarf mice showed elevated levels but reduced phosphorylation of S6 kinase 1 isoforms in muscle and liver and reduced phosphorylation and increased activity of the translation repressor binding protein, 4E-BP1 in liver. Both indicate downregulation of TOR [81]. GH induction of the PI3K-Akt-TORC1 pathway stimulated protein synthesis and phosphorylation of TSC2, S6 kinase I and 4E-BP1 in hepatoma cells [82]. Furthermore, GH transgenic mice express elevated levels and phosphorylation of hepatic ERK, Akt, and TOR [83]. Thus, GH/IGF-1 clearly regulates protein synthesis and growth via TOR and a dedicated temporal window occurs during the first few hours of sleep.

Protein synthesis in brain was strongly restricted to slow wave sleep in rats [84]. Deep slow wave sleep is greatest in the first few hours of sleep in association with peaks in GH secretion. Sleep favours Ras pathway functioning and mitogen-activated protein kinase activity [74]. Further, inhibition of protein synthesis specifically stimulated slow-wave sleep. Endoplasmic reticulum stress inhibits protein synthesis via phosphorylation of p-eIF2α. A p-eIF2α agonist elevated slow-wave sleep by 255%, suggesting a strong linkage between endoplasmic reticulum stress, increased demand for protein synthesis and sleep [85]. Such associations are consistent with GH/IGF-1 signaling and mTOR function in early sleep.

Waking was associated with negative regulators of protein translation (*PEK:* gene encoding heme-regulated EIF2a kinase) whereas sleep expressed positive associates (eEf2, eIF4AII) [48, 53]. The largest categories of genes showing enhanced expression in sleep regulated synthesis (protein, cholesterol, lipids and heme) and transport of proteins and lipids

[74]. Categories of genes associated with protein synthesis identified those regulating transfer RNA, ribosomal assembly and biogenesis (both cellular and mitochondrial). Sleep-associated translation was indicated by upregulation of subunits for translation initiation factors *eIF4b, eIF5, eIF3* and the translation elongation factor *eEF2*. Sleep deprivation reduces protein synthesis and sleep restoration was offsetting [74].

SRIF specifically induces IGFBP-1 so rising SRIF following GH peaks in early sleep terminates GH-IGF-1-TOR signaling at multiple levels [76, 86]. Concurrently, reduced PI3K-Akt phosphorylation would disinhibit FOXO activation later in sleep. IGFBP-1 protein and cortisol levels are tightly linked whereas insulin and glucose are negatively associated with IGFBP-1 [87-89]. Insulin displays meal-associated peaks during waking but low levels in sleep. However, corticosteroid and GH rhythms are opposite in phase such that the circadian nadir of IGFBP-1 and cortisol (and maximal release of IGF-1) occur during GH secretory peaks (early sleep). Following low levels in early sleep, IGFBP-1 steadily rises across the late sleep-associated fast to peak in early waking.

Interestingly, SRIF peaks about 4 hours into the sleep period of mice, even in the suprachiasmatic nucleus [43]. GHRH induced phase advances of the rat SCN during the resting-photophase [90] and the hypothalamic SRIF system projects to the clock [91]. Significantly, IGFBP-1 is generally upregulated by fasting, amino acid deprivation and by FOXO itself [79, 92]. The *Igfbp-1* promoter contains a glucocorticoid receptor (GR) response element and FOXO1 and FOXO3 interact with the p300/CBP acetyltransferase and GR to enhance transcription of *Igfbp-1* [87, 93, 94]. IGFBP-2 was also upregulated by ~3-fold by additive effects of DR and dwarfism whereas IGFBP-1 was upregulated by > 7-fold in dwarf mice [95]. Low insulin, combined with upregulated SRIF, glucocorticoids (and FOXO itself) carve out a temporal nadir of PI3K-Akt signaling by either insulin or IGF-1. This represents a window ideally permissive of FOXO in mid- to late-sleep associated with low oxidative conditions. Such conditions are associated with localization of GRs and FOXO in the nucleus. Like *Igfbp-1*, pyruvate dehydrogenase kinase 4 (*Pdk-4*) transcription is induced by cooperation of GR, FOXO and p300/CBP. PDK4 upregulation during fasting has a glucose sparing function. Insulin suppresses this glucocorticoid action by inhibiting FOXO [93]. Marshalled evidence is congruent with a dedicated window of FOXO-mediated stress resistance and glucose metabolism in late sleep.

2.4. Insulin

Insulin is particularly stimulated by feeding and consequently remains low across the sleep-associated fast. During waking, plasma insulin shows prominent 3-4 hour ultradian peaks closely associated with meals [96]. In humans three such peaks are usually expressed across the waking photophase. Rodents also express ultradian rhythms of waking-foraging-eating and sleep, and ultradian patterns may remain prominent across the diurnal resting phase [25]. Ultradian rhythms in GH secretion also occur, particularly in rodents [25]. This reinforces that although TOR may be strongly activated by GH-IGF-1 in sleep, utradian activation by insulin-PI3K activity is also likely in waking. Since insulin downregulates IGFBP-1, it may also recruit reinforcing IGF-1 signaling associated with meals. Of equal significance, ultradian cycles of insulin have very deep troughs that would suppress PI3K and disinhibit SIRT/FOXO. Such aspects appear largely unexplored. Differential PI3K signaling

via insulin or IGF-1 across the wake-sleep cycle suggests that these hormones could have both quantitatively or qualitatively different impacts (particularly in combination with other temporally distinct signals). Thus, TOR-mediated functions such as growth, protein synthesis or mitochondrial activity likely differ with IGF-1 versus insulin signaling (e.g., differential activation of Akt1 versus Akt2).

2.5. Melatonin

Melatonin production in the pineal gland involves a neural connection from the SCN and melatonin may interact with key clock genes [97]. Thus, SCN cells implanted in permeable capsules restore behavioural rhythmicity in SCN-ablated animals, but not the pineal melatonin rhythm [98]. SCN signaling restricts melatonin production to the dark, thus providing both a circulating signal and a measure of seasonal daylength [97]. Adenosine receptors A_{2B} interact with melatonin to entrain *Per1* expression in the pituitary [99]. Besides neuroendocrine implications this suggests that melatonin could generally coordinate peripheral clocks. Melatonin is also an effective antioxidant and may impact both NAD(P)H oxidases and sirtuins [97, 100].

2.6. Neuroendocrine Aging

Aging is associated with profound neuroendocrine alterations including rising HPA axis function and radical declines in the GH axis and melatonin. Detection and signaling of increasing ROS by the immune system could centrally upregulate the stress axis (a normally appropriate response) [68]. In aging this would chronically suppress GH. Elevations in ROS with age could also chronically activate PI3K (see Figure 2), thus downregulating the SIRT/FOXO pathway associated with longevity [11]. Either condition could be normalized by reducing ROS. Gene expression patterns among aging organisms suggest common mechanisms of aging [101]. Genes upregulated in aging mainly fell into categories of stress responses, immune/inflammation responses, cell cycle, DNA damage, FOXOs, and apoptosis. Those downregulated involved ribosomes, mitochondria, metabolism, oxidative phosphorylation, growth factors, IGF-1, growth, reproduction and synaptic activity [101]. Such features suggest that some responses to aging are in seemingly adaptive directions.

3. The Clock, Sirtuins, Resveratrol, PGC-1 and Heme

Clocks are ubiquitous from unicells to vertebrates and higher plants. In cyanobacteria, a clock regulates the entire genome, genomic compaction, metabolic rhythms and the cell cycle. In *Synechococcus elongatus*, the clock is based on phosphorylation and ATP [4]. In cyanobacteria, nitrogen fixation is incompatible with the oxidative photosynthetic milieu and is compartmentalized to the scotophase [29]. In mammals, paired hypothalamic suprachiasmatic nuclei (SCN) function as the master clock. The hypothalamus represents a

central microprocessor that evaluates biological status and accordingly allocates resources to functional demands. Thus, this clock is strategically placed. Mammalian genes identified as circadian usually amount to < 15% of the genome in a given tissue but digital signal algorithms suggest that most genes may be rhythmic [102].

SCN lesions abolish circadian rhythms in sleeping-waking, locomotion, feeding and drinking (replaced by ultradian cycles) as well as glucocorticoids, leptin, and glucose tolerance [103]. Similarly mice lacking the clock gene *Bmal1* showed loss of circadian rhythmicity while maintaining ultradian activity and feeding rhythms. This did not occur with liver-specific loss of *Bmal1* function [104]. Peripheral tissues have their own clocks that employ similar genes as the SCN. Peripheral clocks allow local phase adjustments and tissue-specific intracellular signaling but are ultimately coordinated by the SCN [75]. The retina conveys a light signal to the SCN allowing the clock to entrain to daily light-dark cycles.

Diverse mechanisms derive clock function, including transcription, translation, phosphorylation, dimerization, cytoplasmic-nuclear transport, acetylation, chromatin alterations, redox modifications and ubiquitination-proteolysis. Translation of *Clock* is regulated by the miRNA, *Bantam* in *Drosophila* [105] and inhibition of the miRNA biogenic pathway alters behavioural rhythms. Core clock mechanisms (Figure 1) involve feedback among gene transcription loops, and movement of translated transcription factors to the nucleus [30, 106-108]. The mainspring involves induction of *Cryptochrome (Cry)* and *Period (Per)* by CLOCK:BMAL1 dimers. CRY:PER dimers consequently inhibit their own transcription, establishing a negative feedback loop and timekeeping mechanism.

Other clock elements such as REV-ERBα reinforce and accentuate amplitude, ensure proper phase and integrate the clock to metabolic and redox processes [107, 108]. Clocks are not ubiquitously connected to all genes but signal strategically. Master controllers, rate-limiting enzymes and switch elements are predominant targets. This coordinates key functions while leaving downstream flexibility. Clock targets vary markedly among tissues, highlighting that integration of clock signaling is a critical aspect of tissue differentiation [43, 109].

3.1. Clocks, Redox and Oxidative Stress

Fusion of clocks to redox confers temporal coordination to diverse functions including kinase signaling, *neuropeptide activity, chromatin structure, gene transcription, protein metabolism, the cell cycle, transport, chaperones, ion channels, energy metabolism, and stress resistance* [6]. Remarkably, H_2O_2 phase shifts the yeast metabolic cycle and timing of cell division [110]. Monoamine oxidase A inhibitors also alter yeast rhythms [111]. In zebra fish embryonic cells, H_2O_2 or light equivalently shift the clock [112, 113]. H_2O_2 induced Cry1a and Per2 and circadian oscillations in Per1. H_2O_2 was induced by light, linking redox to clock-associated signaling. Catalase oscillated in opposite phase to Cry1a and Per2 and modulated their photosensitivity [113]. Rhythmic NO synthase activity also contributes to SCN rhythmicity [114]. Oxidative stress also alters clock function in Drosophila [115]. *Drosophila* lacking *Per* function expressed greater oxidative damage with age and accelerated functional decline. Null mutant flies showed reduced longevity, increased ROS damage, declining locomotor activity and neuronal degeneration in response to hyperoxia [116]. Irradiation was associated with restriction of Per-1 to the nucleus suggesting redox-sensitive

localization [117]. γ-Irradiation induced many hepatic clock genes, including mPer1, mPer2, Clock, Cyr1, and Bmal1. Mice deficient for mPer2 were radiosensitive and lacked rhythmic c-myc expression (likely to dysregulate p53). Results suggested a role for the clock in regulation of DNA repair [118].

Bmal1$^{-/-}$ mice show disrupted circadian behavior and transcription and reduced growth and lifespan (~ one year). Accelerated aging involved wasting, cataracts, reduced subcutaneous fat, and slow hair growth [119]. Mice also expressed radiation sensitivity and differential ROS elevations among tissues. Impacts of BMAL1 on insulin sensitivity implicated the PI3K pathway. N-acetyl-L-cysteine ameliorated accelerated aging of *Bmal1*-defective mice as indicated by reduced cataracts and extended longevity but it did not affect hair growth or wasting [120]. Several key antioxidants have E-boxes in their promoters suggesting that BMAL1 regulates antioxidant expression [120]. Mouse and rat express E-boxes in *Sod1,* and humans additionally show an E-box in *Sod3*. Catalase expressed E-boxes in all three species. Various GSH peroxidases, peroxiredoxins and thioredoxin have promoter E boxes [120]. Catalase expresses circadian rhythmicity in yeast and *Drosophila* [41]. Knockout of *Per1* and *Per2* in mice eliminated circadian rhythmicity and exacerbated aging, radiosensitivity and cancer. *Per2* mutant mice also showed hyperplasia of the salivary glands and kidney [121, 122].

Aging of the clock may contribute to general aging. Besides accelerated aging with defective *Bmal1, Per1 or Per2* [119, 121] mutant or aged hamsters had defective clocks and reduced longevity that was rescued by transplantated normal fetal SCNs [123]. *Per1* and *Per2* mutant mice showed reduced age-related fertility consistent with accelerated aging [124] and acceleration of season photoperiodic cycles induced aging of the clock in mouse lemurs [125]. In mice, impaired circadian vascular rhythmicity was associated with declines in NO with age. This impacted *Per* activity, and blood pressure. A NO donor upregulated *Per* via a CREB pathway and ameliorated dysregulation of blood pressure [126]. Young mice showed peak *Per2* expression at ZT:12 (ZT = zeitgeber time where 0 = lights on, and 12 usually = lights off).

This was delayed in old mice until ~ ZT:18. Although *Per 2* expression declined with age that of *Per 1* did not [127]. Weekly phase-shifting of the clock strongly increased mortality in old mice [128]. In *Drosophila* several strains with defective clocks had elevated protein carbonyls [129]. Like mammals, *Drosophila* express age-related sleep fragmentation that could reflect ROS damage [130].

Understanding linkages of redox and energy metabolism to the clock has rapidly expanded to recognize silent information regulators (sirtuin: SIRT1), heme, peroxisome proliferator-activated receptor-γ coactivator 1 (PGC-1), PPARα, PPARγ and forkhead transcription factors (FOXOs) as critical elements. Interactions among a plethora of nuclear receptors may provide a combinatorial code linking the clock to tissue-specific metabolic and redox processes [59, 131-135].

3.2. SIRT1, Nicotinamide Adenine Dinucleotide Phosphate and the Clock

Mammals have seven sirtuins, three of which are mitochondrial [136, 137]. SIRT1 is an NAD^+-dependent deacetylase strongly associated with redox and energy metabolism via the

nicotinamide adenine dinucleotide phosphate [NAD(P)/NAD(P)H] couple. SIRT1 antagonizes acetyltransferase activity of CLOCK, forging linkage of the clock to aging, stress resistance, DR and FOXOs [49, 138-140]. Elevated sirtuin increases longevity in yeast, nematodes and flies [141]. Sirtuins impact stress resistance via deacetylation of p53, FOXOs, NF-κB and Ku70 [136, 142, 143]. SIRT1 is induced by the grape flavinoid, resveratrol and sirtuin, p53 and resveratrol function in the same pathway extending longevity of *Drosophila* via DR [144]. Stress resistance in mammalian DR may particularly involve SIRT1 deacetylation of FOXO1 [142, 143, 145]. SIRT1 transgenic mice were lean with elevated metabolism and insulin sensitivity, reminiscent of DR [141].

NAD(P)H is the main source of cellular reducing equivalents and the NAD(P)/NAD(P)H ratio is a reliable biomarker of energy and redox linkages to ATP production, PARP-associated DNA repair, GSH, thioredoxin and dopamine (DA) synthesis. NOX responds to melatonin and may contribute to the clock via impacts on redox, ROS, NAD(P)H and disulfide-thiol interchange [6, 100]. Moreover, NAD(P)H is itself an antioxidant [146].

Yeast cycles encompass NAD(P)/NAD(P)H and GSH rhythms [4, 147]. Strongly rhythmic yeast gene expression includes 60 nuclear genes involved in mitochondrial functions relevant to metabolic and redox processes [44]. Genes in the pentose-phosphate pathway and production of NAD(P)H (the main source of reducing equivalents in cells) are compartmentalized to the yeast reductive/charging phase [26, 41, 148] and vertebrate sleep [74]. NAD(P)H levels of yeast rise rapidly as respiration falls [41, 148]. Glucose-6-phosphate 1-dehydrogenase (G6PD) cycling (the initial enzyme in NAD(P)H production via the pentose-phosphate pathway) is also important in *Drosophila* [149]. *Transketolase*, another gene in the pentose-phosphate pathway is also elevated in sleep [53]. Mutation of G6PD ablates yeast rhythmicity, indicating a strong role of NADPH in the cycle [29]. A linkage of the clock to redox as indicated by the NAD(P)/NAD(P)H ratio was established even before a role of SIRT1 in clock function was known. Binding activity of CLOCK:BMAL1 and NPAS2:BMAL1 was associated with redox. Reduced forms (NADH, NADPH) strongly enhanced dimerization and DNA binding whereas oxidative conditions were inhibitory. This confers a redox-sensing function to CLOCK and NPAS2 [8, 106, 135, 138, 150-152].

Clock-driven transcription involves alterations in chromatin mediated by acetyltransferase activity of CLOCK and deacetylase activity of SIRT1 [135, 140, 152]. Cyanobacteria express rhythmic coregulation of transcription and chromosomal supercoiling suggesting ancient linkage of clocks to DNA structure [153]. Acetyltransferase-deacetylase antagonism likely derives euchromatin-heterochromatin cycles. These may involve targeted delivery of chromatin-regulating complexes to particular targets (e.g., REV-erbα interaction with Bmal1) or more general patterns. Human buccal epithelium showed circadian chromatin alterations with more condensation at night than in the morning (following antioxidant recharging?). Physical activity increased condensation, likely via actions of catecholamines and cortisol [154].

Acetylation extends to clock and metabolic proteins [135]. Other acetyltransferases (e.g., p300/CBP) also interact with CLOCK:BMAL1 complexes [152, 155]. SIRT1 contributes to high-amplitude transcription of *Bmal1, Per2* and *Cry1*. Acetylation of BMAL1 and histones by CLOCK and CBP/p300 promotes suppressive binding by the PER/CRY complex. SIRT1 deacetylates histones, BMAL1 and PER2, leading to PER destabilization and degradation. Establishment of repressive heterochromatin by SIRT1 starts a new cycle [49, 59, 138, 139].

NAD^+ levels reflect synthesis (e.g., from tryptophan) and activity of the NAD^+ salvage pathway [156]. NAD^+ contributes to mono- and poly-ADP ribosylation of proteins, deacetylase activity and Ca^{2+} regulation. NAD^+ is a donor of ADP ribose, yielding nicotinamide as a byproduct. Nicotinamide inhibits sirtuin activity and reduces yeast replicative lifespan [157]. The key enzyme in the NAD^+ salvage pathway, nicotinamide phosphoribosyltransferase (NAMPT), increases in response to stress and DR and increases NAD^+, SIRT1 and stress resistance. Variation in NAMPT and NAD^+ impact transcription of diverse genes involved in SIRT1 effects on stress resistance, antioxidants and aging (e.g., p53, *Foxo3*, *NFκB*, *Ku70*, *CBP/p300*, estrogen receptor-α and *Pgc-1α*) [27]. SIRT1 recruits nicotinamide mononucleotide adenylyltransferase 1 (NMNAT-1), an element of the NAD^+ salvage pathway, to targeted promoters where NMNAT-1 regulates SIRT deacetylase activity [27].

NAD^+ displays a clock-driven circadian rhythm in phase with SIRT1, but opposite to nicotinamide and acetylation of histone H3 and BMAL1. CLOCK/BMAL1 confers circadian rhythmicity to nicotinamide phosphoribosyltransferase gene (*Nampt*) [encoding the rate-limiting enzyme salvaging NAD^+ from nicotinamide] via binding to three E-boxes in the *Nampt* promoter. NAD^+ circadian rhythmicity is abolished in mouse embryonic fibroblasts with *clock* or *cry1/cry2* mutations [156]. NAMPT is highlighted in senescence, detoxification and cell metabolism. NAMPT activity declined with age in vascular smooth muscle cells. Reducing NAMPT resulted in premature senescence in these cells whereas overexpression of NAMPT delayed senescence, increased oxidative stress resistance and offset age-related increases in p53 expression [158]. Extension of yeast lifespan by DR requires Sir2. Overexpression of the yeast NAD^+ salvage pathway enzyme, nicotinate phosphoribosyltransferase (NPT1) extended lifespan by ~60% with no change in NAD^+ [157]. Knockout of NPT1 decreases NAD^+ and abolished life extension by DR. NPT1 and Sir2 also increased resistance to heat shock [159].

SIRT1 contributes to *Nampt* transcription and rhythmicity, complexing with CLOCK/BMAL1 to bind *Nampt* E-boxes [156]. Alternatively, SIRT1 is inhibited by NADH and the byproduct of NAD^+ metabolism, nicotinamide. Lactate, acting via lactate dehydrogenase, may reduce the NAD^+/NADH ratio which is coupled to $NADP^+$/NADPH via nicotinamide nucleotide transhydrogenases [106]. Lactate dehydrogenase is regulated by NPAS2:BMAL1 [106, 150]. These various feedback loops closely link cellular metabolism and redox to the circadian clock [49, 156]. Indeed, inhibition of NAMPT decreases expression of *Per2* and acetylation of *Bmal1*. Linkage of GSH and thioredoxin to NAD(P)H [160] even extends clock impacts to redox signaling via protein cysteine residues.

NAD^+ precursors include tryptophan, nicotinic acid and nicotinamide. Both NAMPT and NAD^+ express clock-driven circadian rhythmicity. Rhythmicity in *Nampt* mRNA driven by CLOCK:BMAL1, peaks at the beginning of the scotophase in mouse liver and adipose tissue. NAMPT rhythmicity in turn drives NAD^+ rhythms. SIRT1 binds BMAL1 and reduces its activity at a *Per2* promoter E-box. Inhibition of NAMPT reduces SIRT1 suppression of CLOCK:BMAL1, thus promoting *Per2*. Thus, feedback loops related to metabolism and redox involve NAMPT/NAD^+ and SIRT1-CLOCK/BMAL1 [132, 161].

SIRT1 and resveratrol inhibited *Per2* whereas nicotinamide (that inhibits SIRT1) increased *Per2* expression. Synthesis of NAD^+ also uses the AMP moiety of ATP suggesting linkage to the energy sensor, AMPK [156]. Resveratrol induces activation of AMPK which supports *Nampt* transcription [135]. Inhibition of NAD^+ reduced levels of key targets of

SIRT1, phosphoenolpyruvate carboxykinase (*Pepck*) and glucose-6-phosphatase [161]. Besides histones, SIRT1 deacetylases FOXOs, PCG-1α, PPARγ proteins [138] and integratively links them to the clock. NAD^+ serves as a cofactor in diverse processes (including DNA repair, antioxidant functions, SIRT activity) so circadian rhythmicity at this juncture propagates to cellular metabolism, organismal activity and aging rates. The fact that SIRT1 is strongly connected to FOXO, and that gluconeogenic enzymes are FOXO targets suggests a clock connection to FOXO. Indeed, the promoter of hepatic *Foxa3* contains an E-box [162].

Hepatic *Nampt* mRNA rises in the late photophase to peak at ~ZT:12 [161]. In adipose tissue *Nampt* expression peaked at ~ ZT:15. Mice with dysfunctional CLOCK showed no rhythmicity in NAMPT expression or protein. Significantly, free-running rhythms of hepatic NAD^+ expressed bimodal peaks at ~ZT:07-08 (mid resting photophase) and ZT:18-19 (active scotophase). Further, NAMPT protein showed corresponding peaks at ~ZT:06-07 and ZT:17-18, the photophase (resting) peak being lower. A bimodal pattern of NAD^+ suggests the possibility of four redox phases: 1) Rising NAD^+ (oxidatitive conditions) in waking (rising physical activity), 2) Rising NAD^+ indicative of oxidation in sleep associated GH-IGF-1-TOR anabolism, 3) falling NAD^+ (reducing conditions) in late waking and 4) falling NAD^+ associated with heme, FOXO and reducing conditions in late sleep [161]. Many animals anticipate the dawn or dusk and return home to sleep hours before. It would not be surprising if late waking represents a distinct circadian compartment that could even anticipate pending TOR-associated ROS and energy consumption.

Sleep association of sirtuins has been proposed, based on the idea that sleep is associated with low insulin signaling and consequently must express reduced TOR [11]. In fact, IGF-1-PI3K signaling in early sleep may define the main TOR window. This shifts likely SIRT activity to mid sleep, possibly preceding activity of PGC-1 and FOXO. One mechanism of resveratrol associated with SIRT1 activation was inhibition of PI3K [163]. SRIF attenuation of GH axis signaling in early sleep could similarly promote sirtuin activity. Based on the known association of insulin with waking it has been suggested that this would preclude sirtuin/PGC-1/FOXO activity [11]. However, insulin expresses high ultradian rhythmicity with feeding peaks followed by deep troughs [6] that could derive post-meal expression of stress resistance pathways. This would be of particular advantage for P450 detoxification of plant defensive chemicals and could explain reports of stress resistance elements upregulated other than in late sleep. The P450 system may well be differentially associated with the post-feeding period relative to other stress response elements.

Resveratrol-induced SIRT extended longevity of yeast, nematodes, flies and fish and ameliorated impacts of high-caloric diets in mice. Resveratrol ameliorated loss of motor and cognitive functions with age [164, 165, 166] and increased activity and aerobic capacity of mice. Furthermore, it upregulated genes for oxidative phosphorylation and mitochondrial biogenesis, reduced IGF-1 signaling and increased activity of AMPK and PGC-1α [164, 167]. Resveratrol impacted vascular elasticity, inflammation, motor coordination, cataracts and bone mineral density in mice, although longevity was not extended [166]. In liver, resveratrol suppressed glucose-6-phosphatase activity crucial to gluconeogenesis. Immunological alterations in adipose tissue indicated reduced inflammation and greater anti-microbial activity [166]. However, GSH metabolism was not upregulated as occurs in DR.

Remarkably, resveratrol mediates vascular relaxation by downregulating the $gp91^{PHOX}$ catalytic subunit of vascular NOX and upregulating eNOS [166]. Thus, resveratrol could

benefit health and lifespan via effects on angiotensin II (AT) and perhaps NOX in general [168, 169]. AT is but one of numerous growth factors (including insulin and IGF-1) that require NOX for signaling (Figure 2). NOX contributes to many aging pathologies including neurodegeneration, artherosclerosis, diabetes, cancer and inflammation [12, 170-176]. NOX also modulates signaling via impacts on GSH, NAD(P)H and redox-sensitive cysteine residues [6, 12, 100]. Besides cell division, NOX activity drives cellular enlargement (a TOR-associated process). NOX transfers electrons from cytosolic NAD(P)H to the cell membrane [177] via a CoQ10 couple. This could alter NAD(P)H/NAD(P) balance, redox and mitochondria function [178, 179]. Angiotensin II significantly elevated mitochondrial H_2O_2 generation and depleted mitochondrial GSH. This was mediated by PKC, increased NOX activity, reduced NO and opening of mitochondrial K_{ATP} channels [180]. Besides direct damage NOX may exacerbate other stressors. Thus, loss of dopamine neurons via 6-hydroxydopamine was ameliorated by inhibition of NOX in rats [181].

Although excessive ROS generation by NOX may induce harm, moderate activity could upregulate antioxidant defense via kinase signaling to factors like Nrf2-Keap1 [182] (see also Chapter 13). Alternatively, sustained activation may deplete NADPH required as a cofactor for eNOS and several antioxidants [182]. Under other circumstances, NOX could elevate NAD^+, thus favouring PARP and SIRT activity. Activity of NOX may contribute to NAD(P)H/NAD(P) balance under normal circumstances, and could toggle cells between redox states. In this regard, early sleep associated with GH and TOR could reflect NOX-derived ROS. If so, lowest oxidation may be restricted to mid-to-late sleep. This is consistent with elevated ROS and aging by GH overexpression [34].

There is potentially strong linkage of ROS generation by mitochondria and NOX. Mitochondrial ROS also influence intracellular transduction cascades relevant to cell survival and aging rates. Factors modulating mitochondrial ROS include p53, FOXO, MnSOD, cMyc, TOR and $p66^{shc}$ [183]. Mitochondrial ROS may promote phosphorylation of Akt, thus suppressing FOXO. Association of UCP-2 with FOXO is interesting [184] as knockout of UCP-2 increases the oxidative burst generated by macrophage NOX by 80% [185].

AT activates NOX via the AT_{r1} receptor. AT was among the first growth factors shown to generate ROS via membrane-bound NOX [186, 187] (see also Vol. III, Chapter 8). Aging is associated with elevated cardiovascular ROS that dysregulates vasodilation in mice. This was mediated by AT_{r1} and ameliorated by a superoxide scavenger [188]. AT increased nuclear ROS in sheep and this was abolished by a AT_{r1} antagonist [189]. Cardiovascular damage by AT is also linked to $p66^{shc}$ [190]. Increasing ROS stress and disrupted control of relaxation in the aging vasculature of rats was ameliorated by inhibition of NOX [191].

AT regulates growth, immunity, inflammation and blood pressure via type I receptors but also mediates hypertension, cardiovascular disease, type II diabetes and vascular-associated brain aging [137, 169]. AT_{r1A} regulates blood pressure and its disruption significantly improved cardiovascular health, reduced ROS damage and extended life span of mice by ~ 26% (~ 6 mo). Feeding, body size and motor function were unaffected, although first year growth was slower. Mitochondrial density declined in aging control kidney but in receptor knockouts mitochondrial number increased and the *Nampt* and *Sirt3* (but not *Sirt1*) genes were upregulated. SIRT3 localizes to mitochondria and can reduce ROS and mitochondrial membrane potential while increasing cellular respiration [137, 169, 192]. AT inhibited SIRT3 mRNA in epithelial cells and this was reversed by a AT_{r1A} antagonist [169]. Nitrotyrosine (a biomarker of peroxynitrite) increased with age in control heart and kidney but receptor

knockout mice maintained levels equivalent to 2 month old controls. Reduced ROS by AT_{r1A} blockade likely protects mitochondria [137, 169].

AT_{r1} receptor blockers and AT converting enzyme (ACE) inhibitors are prescribed for hypertension. Resveratrol activated SIRT1 and reduced AT_{r1} mRNA and protein in vascular smooth muscle cells. Resveratrol ameliorated AT-induced hypertension in mice whereas nicotinamide was antagonistic [168]. Downregulation of the renin-AT system with ACE or AT_{r1} inhibitors protected against structural and functional deterioration in aging kidney, cardiovasculature and brain [193, 194]. Inhibition of AT in the cardiovasculature diminished blood pressure and prevented age-related loss of NOS activity (see also Vol. III, Chapter 8). Suppression of AT with either an ACE inhibitor or receptor blocker reduced emotionality, increased locomotion, and improved learning. Whereas all control rats died by 26 months, treated rats lived up to 37 months [195]. A key mechanism was reduced oxidative stress. Old rats treated with ACE inhibitor or AT_{r1} resceptor blocker showed lower mitochondrial NOS activity, UCP-2 levels, and GSH/GSSG ratio in kidney compared to age-matched rats [193]. MnSOD activity and H_2O_2 were elevated by ~70% and > 40%, respectively in old rats [193]. Old rats showed reduced numbers of mitochondria and energy production compared to youth but an ACE inhibitor and AT_{r1} blocker improved responses to energy demand [193]. Hypertension may represent a general aging mechanism in which case AT_{r1} blockers and ACE inhibitors (e.g., resveratrol, ramipril) may slow general aging [137, 168, 193, 194]. Reduction of ROS and increased energy supply associated with downregulated AT signaling would also contribute.

SIRT1 is linked to GH axis function relevant to PI3K signaling and aging. Alterations in GH axis signaling are crucial to extended longevity in dwarf animals, foreshortened longevity of giant transgenic GH mice and life extension via dietary restriction. GHRH elevated ROS generation by 36% in human prostate cancer cells [196]. Free radical-associated signaling of IGF-1 regulates PI3K-Akt-TOR (see above). Alternatively, low PI3K-Akt signaling activates FOXO-associated pathways. Dwarfism and DR can be envisioned as forms of cellular stress requiring reduced proliferative signaling. In DR, upregulated *Socs2* likely inhibits GH-JAK-STAT signaling [95]. Dwarf mice obtained further extension of longevity via DR [95] but mice lacking the GH receptor (that display greatly extended longevity) obtained little further benefit from DR [197]. Mice with knockout of SIRT1 in brain, however, showed altered GH axis function and failed to reduce IGF-1 in response to DR. This forges SIRT1 to both the GH axis and DR responses associated with alterations in aging rates [140]; see also Chapter 23.

3.3. Peroxisome Proliferator-Activated Receptor Γ Coactivator 1 (PGC-1)

Any attempt at a simple unified vision is particularly discombobulated by nuclear factors like PGC-1. Both upstream controllers and downstream targets of PGC-1 vary remarkably among tissues [134]. PGC-1α and β are preferentially expressed in highly respiring tissues where they facilitate mitochondrial utilization of lipids and mitochondrial biogenesis. In muscle, PCG-1 activity is linked to TOR [198]. mTOR critically regulates muscle mitochondrial oxidative metabolism via controlling the interaction between yin-yang 1 (YY1) and PGC-1α [198]. Rapamycin reduced gene transcription of estrogen-related receptor α, nuclear respiration factors and PGC-1α. This was associated with inhibition of YY1, 12% reduction of mitochondrial respiration and a 32% decrease in mitochondrial DNA [198].

SIRT1 protein, NAD^+ and PGC-1α activity increase under fasting in liver and PGC-1α activity is reduced in type II diabetes [167, 199]. Target genes of PGC-1 in various tissues include nuclear respiratory factor-1 (*Nrf-1*), GR, thyroid hormone receptor, *Ppars*, estrogen receptor, myocyte enhancer factor-2 (*Mef-2*) and *Foxo1*. PGC-1α is induced by exercise in skeletal muscle and by fasting in heart and liver. Transcription involves association with acetyltransferases like p300 and thyroid hormone receptor associated protein (TRAP). For our purposes, the association of PGC-1 with SIRT and FOXO is best exemplified in liver. Hepatic PGC-1 is suppressed by PI3K-Akt signaling and is favoured by SIRT, FOXO1, cAMP and glucocorticoids [134].

SIRT1 activation of PGC-1α promotes insulin sensitivity and and hepatic gluconeogenesis while down-regulating fat deposition and glycolysis [141, 167, 199]. Resveratrol also upregulated PGC-1α activity via SIRT1 (likely via deacetylation of PGC-1α). PGC-1α, but not SIRT1, was importantly involved in mitochondrial activity and biogenesis under fasting [199]. Others have observed increased expression of mitochondrial genes by resveratrol [166]. The thyroid hormone axis might be expected to show greatest elevation in waking (when metabolism is greatest) but paradoxically, thyroid stimulating hormone displays high sleep-associated plasma levels. Thyroid receptor α expression rose through the early sleep period, prominently peaked in mouse liver at ~ZT: 06 and fell after ZT: 08 [200]. This may reflect cooperation of the GH and thyroid axes in regulation of growth but perhaps more importantly, T3 and another one of its targets, PGC-1α, are involved in mitochondrial biogenesis [201, 202]. *Pgc-1* is required for proper clock function and potentially impacts aging and redox via connections to mitochondrial biogenesis, energy supply (gluconeogenesis), stress resistance (including ROS stress), respiration and heme production. PGC-1α also regulates UCP-1 and thermogenesis [203]. Mitochondrial biogenesis is particularly important to redox and aging given the role of mitochondria in respiration and ROS generation and mitochondrial vulnerability to ROS (see also Chapter 15).

DR of mice induced SIRT1, eNOS, mitochondrial biogenesis, elevated respiration and ATP production, cytochrome c and COX IV in various tissues. This was attenuated in eNOS KO mice suggesting a role of NO and mitochondrial biogenesis in dietary restriction [145]. PGC-1α and NRF-1 were also upregulated. In yeast, mitochondrial biogenesis is constrained to a narrow window in the reductive-building phase following cessation of oxidative respiration and processes associated with amino acid synthesis and ribosomal metabolism. Mitochondrial transcripts showed exceptional temporal amplitude suggesting strong temporal control and compartmentalization [44]. Thus, mitochondrial biogenesis might be relegated to periods of lower respiration. Indeed, PI3K-Akt suppressed mitochondrial biogenesis in mice but inhibition of PI3K during the sleep period enhanced both mitochondrial biogenesis and autophagy [204]. Autophagy is generally upregulated in sleep and fasting and suppressed by feeding [205] (see also Chapter 23), consistent with regulation during sleep associated with FOXO. PGC-1α overexpression in heart or muscle elevated mitochondrial biogenesis and could mediate responses to exercise [203].

Pgc-1α and *Pgc-1β* are rhythmically expressed in liver and muscle. In brain *Pgc-1* was required for induction of ROS-resistance genes including GSH-peroxidase1 and *Sod2*. PGC-1 protected neurons in the striatum and hippocampus from ROS-mediated loss [206]. PGC-1α interacts with diverse transcription factors and enzymes (e.g., FOXO, glucocorticoid receptor (GR), thyroid hormone receptor-β (THRβ), PCK-1/PEPCK, fructose-1, 6-biphosphatase, glucose-6-phosphatase and proliferator-activated receptors (PPARs). Activation of glucose 6

phosphatase and phosphoenolpyruvate carboxykinase-1 by PGC-1α requires FOXO1 [94]. In mouse liver *Pparγ* expression peaked at ZT:08 whereas *Pparα* rose throughout the photophase (resting) to peak ~ZT:12 [131]. PPARα is associated with fasting and is low when food supplies sugars. *Pgc-1α* is particularly induced by fasting and stress. Day or night feeding shifted plasma glucose rhythms by 12 hours and reversed morning versus evening expression of clock-associated genes (*Bmal1, Cry1, Cry2,Pgc-1α*) [207]. *Bmal1* expression in the SCN, however, does not respond to day-versus-nighttime feeding.

PGC-1α stimulates transcription of *Bmal1* and *Rev-erbα* via coactivation of RORα [59, 135, 203, 208]. Alternatively, REV-ERBα regulation of *Bmal1* may inhibit PGC-1α activation of *Bmal1* [207]. PGC-1α associates with the SIRT1 deacetylase complex suggesting linkage to chromatin structure and NAD(P)H/NAD(P) couples [207]. Lack of *Pgc*-1α results in disruption of clock gene expression, thermoregulation and behavioral activity. *Pgc-1α* knockout mice are lean and hyperactive with elevated metabolic rate and high insulin sensitivity. Alternatively, *Clock* mutant mice develop obesity [207]. PGC-1 appears essential for peripheral clock function [27, 207]. Many rhythmic genes are stimulated by PGC-1α near the light-dark (rest-wake) transition or shortly thereafter in mice. PGC-1α peaks in the early scotophase in rodents (i.e., initiated in late sleep) in association with BMAL1.

Although plasma fatty acid levels are lowest during the scotophase-activity period, genes regulating fatty acid metabolism and responses of the rat heart to fatty acids peaked at this time [62]. Fatty acids directly induce transcriptional activity of the three *ppar* genes (α, β/δ, and γ). Increased sensitivity of PPARs appears clock-controlled, partly by activators like *Pgc-1* [62]. Further, Rev-erbα antagonizes PPAR transcriptional activity by interfering with binding at promoter response element sequences and Rev-erbα and PPARα cycle in reverse phase. UCP3, CD36 and pyruvate dehydrogenase kinase 4 (Pdk4) also showed circadian expression in rat heart [62]. Pdk4, which inhibits the pyruvate dehydrogenase complex (PDC) and carbohydrate oxidation, exhibited a trough just before the light-dark transition. This is then associated with greater amounts of active PDC proteins near the light-dark transition. This, combined with late photophase peaking of REV-ERBα in the light phase, could serve to shift the energy substrate balance of the heart from lipid to carbohydrate in early waking [62].

Fibroblast growth factor (FGF21) regulates peripheral insulin sensitivity and is repressed by PGC-1α. PGC-1α induction by fasting coactivates FOXO1, the glucocorticoid receptor, nuclear respiratory factor-1 (NRF-1) and PPARα. Reduced Fgf21 expression is mediated by REV-ERBα. Induction of the rate limiting enzyme for heme synthesis, aminolevulinate synthase 1 (ALAS-1) is negatively regulated by insulin and positively regulated by PGC-1α, FOXO1 and NRF-1 [209]. Maximal expression of *Alas* mRNA occurs between ZT:08-ZT:12 in mice (late resting photophase) [210]. Heme consequently varies BMAL1-REV-ERBα and indirectly regulates FGF21 [211]. Thus, PGC-1α signaling includes BMAL1, REV- ERBα and their targets. PGC-1α also binds and co-activates FOXO1 whereas phosphorylation of FOXO by Akt is inhibitory. FOXO1 is required for PGC-1α induction of gluconeogenesis [212] and FOXO may mediate stress resistance impacts of PGC-1.

PGC-1 function varies among tissues. For example, brain does not engage gluconeogenesis but this is a mandate for liver. Regulation of UCP-1 is largely limited to brown adipose tissue. Regulation of mitochondrial biogenesis and gluconeogenesis can also be dissociated. Gluconeogenesis and heme production compete for metabolic resources which is thought to explain antagonistic regulation of gluconeogenesis by heme [213]. It seems likely that mitochondrial biogenesis, heme synthesis and gluconeogenesis may be relegated to

distinct temporal windows to avoid competition or incompatabilities. This could involve interactions of PGC-1s with other key regulators like sirtuins and FOXOs.

3.4. Heme Metabolism and the Clock

Regulation of ALAS-1 by PGC-1 links heme to the clock and diverse aspects of signaling. Heme is essential for O_2 transport and activity of enzymes involved in respiration, hormone synthesis, detoxification, signaling and NO production [210, 213, 214]; see also Chapter 19. Interestingly, intracellular heme in muscle correlates with mitochondrial biogenesis [132]. Heme synthesis and regeneration of heme prosthetic groups on enzymes involved in oxidative metabolism are incompatible with oxidative conditions. Consequently, heme synthesis is relegated to the reductive-building phase in yeast [41]. This represents the post-TOR window of sleep in vertebrates. In mammals, heme metabolism and the clock reciprocally interact. NPAS2 regulates ALAS-1 and alternatively, heme binds PAS domains of NPAS2 and mPER2. Heme enhances *mPer1* expression but inhibits *mPer2* [215]. Binding of heme allows CO to inhibit DNA binding by NPAS2. This favours formation of inactive BMAL1 homodimers over active NPAS2/BMAL1 heterodimers [151].

Heme is a specific ligand for REV-ERBα and REV-ERBβ. REV-ERBα effects on transcription are mediated by recruiting the NCoR-HDAC3 corepressor complex to targeted promoters, and heme is required for association of REV-ERBα and this inhibitory complex [132, 213]. Suppression of target genes also involves deacetylation and chromatin condensation. REV-ERBα activity is stimulated by reversible heme binding providing a mechanism for sensing of redox/metabolic status by the clock. REV-ERBα suppresses *Bmal1* transcription, further linking circadian rhythms in heme to the core clock [213]. The expression profile of *Rev-erbα* in mouse muscle, fat and liver shows a large mid-sleep peak (ZT: 06-07) [200]. Heme/REV-ERBα particularly inhibits genes contributing to gluconeogenesis (e.g., glucose 6-phosphatase) and lipid metabolism [132, 213]. Since glucogenesis is associated with FOXO and GR in late sleep, heme-REV-ERBα may occupy a distinct window.

4. Forkhead Transcription Factors (FOXOs), Stress and Energy Metabolism

Forkhead transcription factors regulate energy metabolism (e.g., gluconeogenesis), DNA repair and stress resistance and inhibit TOR-associated IGF-1 activity, protein synthesis, growth, ROS generation and immunological activity. Mammalian FOXOs (Foxo1, Foxo3a, Foxo4, Foxo6) are differentially expressed among tissues [183] and have disparate functions [216]. FOXOs are highlighted in fasting and DR, perhaps accounting for the mutual association of restricted nutrient supply, stress resistance and reduced aging rates. Involvement of SIRT1 in clock function links FOXO to the clock. Froy [59] suggests that life extension and stress resistance imparted by intermittent feeding may not trace to DR so much as upregulation of stress pathways via altered clock function. Sleep is a fasting state and FOXO is upregulated by fasts [217]. Thus, known responses of FOXO to DR reflect

extension of normal sleep functioning. *Foxo* upregulation in starvation also improves survivorship [218, 219].

Insulin is largely controlled by feeding [220] so insulin is reduced during sleep, DR and starvation (see also Chapter 23). FOXO proteins contain nuclear localization and export motifs and Akt phosphorylation mediates nuclear export. FOXO association with 14-3-3 proteins facilitates localization and SIRT binding. In mammals SIRT1 deacetylates FOXO and can alter transcriptional targets [94, 136, 221]. Under low-insulin signaling, reducing conditions, and in quiescent cells, FOXOs localize to the nucleus where they upregulate gluconeogenesis, lipid catabolism and stress resistance [219, 222, 223]. Glucagon positively contributes [219]. ROS stress, however, can also be activating [136, 222]. Specificity of FOXO actions depends on associated cofactors [221, 223], some of which may be specific to stressors, tissues or time. FOXOs interact with numerous nuclear receptors including those for estrogen, androgen, progesterone, thyroid hormone, glucocorticoids, retinoic acid, and PPAR [94]. SIRT1 is mainly nuclear so FOXO3 and SIRT1 are colocalized in the nucleus under oxidative stress [139].

Interaction of SIRT1 and FOXO3 was stimulated by ROS and heat shock, but not growth factors or radiation. SIRT1-FOXO3 interaction was inhibited by nicotinamide. In *C. elegans*, *Daf-16/FoxO* preferentially responds to stressors other than DR [216, 224]. Long-lived *C. elegans* with PI3K mutations require *Daf-16/FoxO* [224]. The *FoxA* othologue *PHA-4* that regulates glucose metabolism was required for life extension in a slow-eating mutant. Interestingly, activation of *Daf-16/FoxO* by low insulin upregulated *Sod1*, *3* and *5* but *PHA-4/FoxA* induced by DR induced *Sod1, 2 ,4* and *5*. Thus, *Sod3* was specific to low insulin but *Sods 2* and *4* were specific to DR [224]. Results suggest that multiple *Foxo* genes differentially contribute to DR and low PI3K signaling.

FOXO also contributes to extended longevity in *Drosophila* [225]. Besides evidence that FOXO critically modulates longevity, increased life extension via resveratrol and SIRT may also involve FOXO [164-166]. A *Foxo3a* variant was associated with both genders in centenarian cohorts from several countries and a *Foxo1a* variant was particularly associated with female centenarians [226, 227].

Oxidative stress reduces clock gene cycling in peripheral tissues of *Drosophila.* Flies lacking *Foxo* show loss of behavioural rhythmicity and central clock function whereas expression of *Foxo* in the fat body restores normal rhythmicity [115]. This reinforces that FOXOs functions in normal circadian regulation. PI3K pathway elements impacting FOXO modulate sensitivity of the clock to oxidative stress. *Foxo* mutants showed rapid age-related decline in behavioural rhythmicity suggesting that ROS stress may damage the clock [115].

4.1. Stress Resistance and TOR-FOXO Balance

Growth and TOR signaling suppress stress resistance whereas stress and reduced resources not only reduce growth and TOR signaling, they upregulate stress resistance via pathways associated with FOXO. This pertains to life extension via both caloric and amino acid restriction. The DR paradigm is yielding to a “nutritional geometry” interpretation that envisions high ratios of protein versus energy as supporting production processes (growth and reproduction) whereas reduced food or low protein/energy ratios promote longevity. This framework is consistent with antagonistic TOR-FOXO balance.

Variation in aging rates trace to defense, repair and replacement systems that resist somatic and genotypic degradation and cancer [23, 228]. Diverse stressors invoke coordinated upregulation of a multifaceted defensive system [47, 68, 229-231]. This includes antioxidants, metal chelators, heat shock proteins, DNA repair systems, protein degradation systems, autophagy, P450 monooxigenases, glutathione-S-transferases, apoptotic pathways and multiple drug resistance [efflux] proteins. Exceptional longevity is associated with generalized stress-resistance across broad phylogenies. Conversely, selection for stress resistance can yield extended lifespans [228, 232-235].

Specificity and fine tuning of stress responses also occur [222,236]. Thus, different methods of DR extend longevity of *Caenorhabditis elegans* via different mechanisms. *Clk1* (ubiquinone biosynthesis), AMPK and *FoxO/daf-16* were involved in two approaches (serial dilution and peptone dilution). Despite being implicated in other forms of DR, *Sir-2.1* (and *hsf-1*) was dispensable for life extension by serial dilution [223]. Combined methods of DR (a slow eating mutant + serial dilution) had additive impacts on longevity similar to additive gains associated with DR and dwarfism in mice [237].

Autophagy involves enveloping cytoplasmic elements in a double membrane and linking the resulting vacuole to the lysosome for degradation and recycling of released substrates (see also Chapter 16). This is valuable both for removing damage and recycling resources under metabolic stress or energy shortfalls (fasting). Rat mitochondria are recycled every 2-4 days [5]. Like other stress elements, autophagy is generally inhibited by PI3K-Akt-TOR and upregulated by SIRT-FOXO [238, 239]. Nutrient import into cells is reduced by lack of growth factor signaling. Consequently, autophagy is upregulated when growth factor signaling falls (e.g., low PI3K-Akt). Alternatively, growth factors specifically downregulate autophagy. TOR is central to integrating growth factors, resource supply (amino acids and energy) and autophagy in diverse tissues [238]. FOXO3a promotes both autophagy and proteasome function [239]. Alternatively, Akt activation was required, but TOR was dispensible, for inhibiting autophagy in skeletal muscle. A single day of fasting was sufficient to elevate autophagy-associated genes, suggesting that autophagy is a normal circadian function regulated by circadian FOXO [239].

DR and stimulation of autophagy extend longevity in mice, flies and nematodes. *Sirt-1* overexpression induced autophagy in *C. elegans* and human cells. Under DR, SIRT1 responses to increasing NAD^+ likely induce autophagy. Autophagy was required for life extension in human cells or *C. elegans* as demonstrated by SIRT1-resveratrol, DR, rapamycin or inhibition of p53. SIRT1 was required for autophagy induced by resveratrol or DR, but not that induced by rapamycin or inhibition of p53 [240].

Besides mitochondrial renewal and recycling of resources, autophagy regulates respiration by reducing the mitochondrial complement. This can be adaptive in hypoxia, by reducing high ROS generation that would otherwise occur [5]. Balance between mitochondrial biogenesis (post-TOR?) and autophagy (FOXO) provides a mechanism regulating optimal respiratory balance. Autophagy involved interaction of regulatory elements with Bcl2, and Bcl2 overexpression reduced mitochondrial autophagy [5].

Yeast show specific subsets of genes that are either induced or suppressed by stress. Stress-resistance genes showed strong negative association with growth [42, 241]. Rapamycin downregulated those genes induced by growth and upregulated those inhibited by growth attesting to TOR- mediated signaling of growth status [241]. The strong negative regulation of stress resistance by growth led Castrillo et al. [241] to conclude that:

> "….a large part of what others have termed a "generalized stress response" may more properly be viewed as a slow-growth response." Brauer et al. [42] arrived at virtually identical conclusions, and consistent with GH transgenic mice, further suggested that growth itself might represent an endogenous stressor.

Many genes downregulated by growth-TOR fall within the sphere of FOXO. These included genes involved in extracellular stress signals, stress and free radical management, autophagy, peroxisome functions, ATPase activity and metabolism of carbohydrates and lipids [42, 241]. Sixteen percent were oxidoreductases [42]. Interestingly, chaperone proteins were not among genes induced by low growth in yeast [42] despite their strong representation in mammalian stress responses. Exceptions notwithstanding, growth rate is negatively correlated to intra-specific longevity [23, 34, 68, 69] and models of extended longevity are generally more stress resistant. Increasing longevity with larger size of species is associated with slower, more protracted growth rates [69]. Slower growth may also confer increased stress resistance. Yeast are similar to animal cells and many growth-associated genes are highly conserved, even in humans [241].

For multicellular species there is also a strong negative association between growth and stress-resistance. Significantly, administering GH to dwarf mice downregulated their otherwise elevated antioxidant defenses [228]. Both DR and dwarfism were associated with upregulated gluconeogenesis and stress resistance in association with altered induction of *Foxa2* and *Foxa3* [95]. Akt phosphorylation was also reduced in GH receptor knockout mice, but elevated in progeroid GH transgenics. GH receptor knockouts had exceptional longevity associated with elevated *Foxo1* and *Pgc1-α* mRNA and protein in association with undetectable IGF-1 [197, 242]. These mice also expressed phosphorylated CREB, active p38, and elevations in adiponectin, AMPK and SOD2. Serum from calorically restricted animals induced SIRT1 in cell cultures implicating serum factors in DR responses. Insulin and IGF-1 attenuated this response [142]. Transgenic GH mice with elevated IGF-1 showed greatly diminished levels of phosphorylated CREB, active p38 and PGC-1α. Unexpectedly, SIRT1 was upregulated. Although *Foxo1* showed no change in GH transgenics, protein activity was likely reduced [242]. Such findings are consistent with modulation of aging rates by GH axis linkages to TOR and FOXO via PI3K-Akt signaling.

Particular drug dosages may kill rodents at one time of day but not another [50]. The clock regulates xenobiotics (largely associated with food) via influences on hepatic, intestinal and kidney detoxification systems [50]; see Vol. III, Chapter 16. Three PAR-domain basic leucine zipper transcription factors (PAR bZIP) show strong circadian rhythmicity and regulate diverse xenobiotic metabolism genes. Circadian rhythms occur in cytochrome P450 enzymes, carboxylesterases, constitutive androstane receptor [CAR], ALAS1, and P450 oxidoreductase [POR]). PPARα is considered as a coordinating sensor for xenobiotics [50]. Cytochrome P450 enzymes have monooxygenase activity. They are crucially involved in xenobiotic detoxification but some also function in lipid, steroid, cholesterol, arachidonic acid and bile metabolism. P450 degradation of melatonin has important implications for the clock [243] and antioxidant protection. POR is required to mediate electron transfer for P450 enzyme function and may confer circadian rhythmicity on clients [243]. Heme is the prosthetic group for all cytochrome P450 monooxygenases, critically forging ALAS1 to detoxification [50]. Rodents show 3-4 hour ultradian cycles of foraging-feeding and sleep.

Thus, sleep-associated contributions to detoxification may be under-appreciated from a strictly circadian perspective.

Knockout of PAR bZIP transcription factors confers xenobiotic hypersensitivity and accelerated aging [210]. Although stress responses may be acutely induced, there is phylogenetic conservation of a particular temporal window regulating stress resistance. Most models of extended longevity show increased stress resistance (particularly to ROS) suggesting that this is a key mechanism impacting aging rates [47]. In mice, dwarfism and DR both increased expression genes involved in xenobiotic metabolism including multiple drug resistance [95]. A crucial value of clocks is mediation of feedforward-anticipation and preparation for reliable alterations in endogenous or exogenous environments. Indeed, the clock may have originally evolved to avoid UV light exposure in unicells and regulation of stress resistance and detoxification are paramount [244].

For animals, elevations in metabolic rate and activity in waking are reliably associated with oxidative stress and greater exposure to environmental insults and toxins (e.g., plant secondary substances in food). Respiration may require subsequent recharging and detoxification. Regulation of stress resistance genes by the clock suggests that anticipation and preparation are important aspects of fitness [245]. In mice, *Nfe2*, a gene that coordinates antioxidant responses, is upregulated in sleep. Antioxidants induced in sleep included glutathione-S-transferase, glutathione peroxidase, thioredoxin, glutathione reductase, catalase, superoxide dismutase and methionine sulfoxide reductase. Some cytochrome P450 enzymes and nitric oxide synthase 3 also showed enhanced expression in sleep [74]. Null *Per* flies have impaired stress responses [116]. The aryl hydrocarbon receptor (AHR) and AhR nuclear translocator (ARNT) proteins are involved in xenobiotic detoxification and hypoxia responses. Circadian rhythmicity of these proteins were similar in rat liver, peaking ~ 5 hours into the mid photoperiod (resting) phase. Following decline, levels began to rise again in the scotophase [246].

In *Drosophila* many genes associated with sleep were involved in detoxification processes (including P450s, catalase, tocopherol, tocopherol-binding proteins, glutathione transferases and thioredoxin) [149, 214]. Protein processing genes highly relevant to senescence were also strongly circadian in *Drosophila* (protein stability, degradation, proteasome function and ubiquitin-related enzymes) [214]. *Per* function increased resistance to oxidative stress (H_2O_2) whereas loss increased susceptibility and abolished rhythmicity in susceptibility [247]. Stress susceptibility was associated with degree of protein carbonylation and loss of *Per* generally increased carbonylation. Flies were more resistant to oxidative stress during the sleep phase but the mechanism was not identified. Catalase expression and protein showed little circadian rhythmicity [247]. Similalrly, catalase expression was arrhythmic in mouse liver and protein was mostly elevated in waking [248]. Alternatively, several antioxidants showed rising levels during the light (sleep) period in rat plasma, some peaking in the photophase (e.g., catalase, ZT: 7:31, SOD, ZT: 12:01, GSH peroxidase, ZT: 8:00). GSH-S-transferase peaked at ZT: 13:39 but was already rising in the late photophase. Lipid peroxidation peaked at ZT: 18:53 (nocturnal activity) [249].

Recharging of antioxidant systems, stress-resistance elements and detoxification capacities appear relegated to the late reductive phase in anticipation of ensuing oxidative conditions. In animals this represents late-sleep immediately preceeding waking. This is particularly well documented in yeast where stress-resistance mechanisms (including autophagy, the ubiquitin-proteasome system and heat shock proteins) are temporally

compartmentalized to the reductive charging phase [41]. The plant clock also compartmentalizes metabolic activities into optimal temporal windows. Production of phenylpropanoids that provide resistance to microbial and UV damage occur just before dawn and the oxidative/photosynthetic phase. Nitrate and sulfur metabolism may also be restricted to the end of the night [4]. Six genes involved in synthesis of vitamin E, and others involved in carotenoid production and heat tolerance, are relegated to the dark-light transition in Arabidopsis [45]. Depriving rats of sleep for 5-10 days reduced hepatic catalase and GSH by 23%-36% [250]. This strongly supports FOXO functioning in sleep. Liver-derived antioxidants buffer tissues throughout the body, so compromised hepatic production is immensely significant.

Plant signaling elements (KIN10/11) homologous to vertebrate AMPK integrate plant stress and metabolic signaling. Many dark-induced genes are responsive to stress and are repressed by sucrose, glucose and light. Extension of the night in plants rapidly induces a starvation-like stress response [251] as plants activate broad-spectrum stress resistance in response to low energy. Gene expression patterns under energy starvation (including hypoxia) resemble those induced by KIN10, whereas these genes were repressed by sugars. Genes induced by KIN10/11 included those involved in amino acid and protein degradation, trehalose metabolism, gluconeogenesis (e.g., PEPCK), stress resistance and autophagy, whereas genes involved in ribosome biogenesis, transport processes, calcium metabolism and especially protein synthesis (i.e., TOR) are repressed [251, 252]. Overexpression of *Kin10/Kin11* extended plant longevity and starvation tolerance, whereas loss was associated with biomarkers of accelerated senescence. Deficiency also impaired dark-associated starch mobilization [252]. Organization of plants resembles antagonistic organization of synthesis versus stress resistance mediated by AMPK and TOR-FOXO signaling in animals.

C. elegans expresses circadian rhythmicity in locomotor responses to osmotic and H_2O_2 shocks as well as nocturnal expression of the glutathione peroxidase and glycerol-3-phosphate dehydrogenase genes [253]. *Drosophila* also shows circadian variation is susceptibility to oxidative stress [115, 116]. Circadian rhythms in glutathione-S-transferase and some cytochrome P450 enzymes involved in detoxification peak at dawn and into the morning, providing protection and resistance for pending environmental interactions in flies [4]. P450 oxidoreductase (*Por*) expression increased across the photoperiod (resting in rodents) to peak at the light-dark transition. Protein accumulated in the late dark-activity phase even though gene expression fell across the scotophase [210].

In *Neurospora*, trehalose synthase shows circadian expression. Trehalose is an energy source but also protects cells from environmental stress (including free radicals, heat, osmotic pressure, nutrient deprivation and desiccation). It is induced by heat, osmotic stress and glucose shortfalls. Trehalose also serves in stress responses in yeast. Metallothionein and glyceraldehyde-3-phosphate dehydrogenase are also circadian in *Neurospora*. Circadian expression of trehalose synthase anticipates stress associated with spore development [245]. Remarkably, trehalose confers general stress resistance via chaperone properties and also enhances autophagy and clearance of substrates that include mutant huntingtin [254].

Rhythmic clock output in *Neurospora* activates the highly conserved p38 "stress-activated" pathway thus anticipating and preparing for hyperosmotic and desiccation stress associated with daybreak. Catalase was also regulated in this pathway [255]. FOXO1 contains 15 MAPK phosphorylation sites and was phosphorylated by both ERK (MAPK-ERK pathway) and p38. These authors did not observe binding by Jun but others suggest JNK may

phosphorylate FOXO and mediate nuclear localization [221]. Thus, MAPK stress pathways regulate phosphorylation and activity of FOXO1 in addition to the well established role of PI3K [256].

In breast cancer cells, induction of apoptosis was associated with upregulation of p38, ERK, JNK and FOXO3a. In this case JNK facilitated transfer of FOXO3a to the nucleus and enhanced its activity (including induction of pro-apoptotic *Bim*) probably via inhibition of Akt [257]. ERK and p38 were not required, suggesting that MAPK pathways may differentially impinge on various FOXOs. Overall, evidence suggests FOXO activity may also be associated with p38 and JNK stress signaling pathways in late sleep (associated with rising glucocorticoids). Although SIRT-FOXO stress resistance pathways can be upregulated during waking, particularly those associated with detoxification (e.g., p450 enzymes, glutathione-S-transferase) [75], p38 signaling may also contribute to sleep and clock-regulated anticipation of waking stress. Significantly, transgenic GH mice expressing accelerated aging (likely dominated by TOR) show negligible p38 activity [242]. There is a relatively rich and complex literature linking FOXO and p38 that cannot be fully explored further here.

Given the role of FOXO in DNA repair, it is highly significant that excision repair in mice shows a single large circadian peak centered near the light-dark transition (late sleep-wake) [258]. Three distinct periods of gene transcription correspond to functional states identified in yeast [41, 44, 147]. Only 12% of gene transcription occurred in the oxidative phase with the remainder about equally distributed between the building and recharging reductive phases. *Restriction of transcription and DNA replication to reducing conditions in yeast likely functions to avoid oxidative damage* [41, 110, 147] *Mouse DNA repair is also associated with reducing conditions* [258].

Waking is associated with stress as rapidly rising blood pressure, elevated heart rate and muscular activity are engaged. Excised rat hearts showed higher oxidative stress (~1.4 times greater lipid peroxidation) in early waking than in the early light (inactive) phase despite ~1.5-fold greater expression of GSH in early waking [259]. Elevated GSH in early waking is consistent with elevated stress resistance. Many mammalian xenobiotic detoxification processes show circadian partitioning to the inactive (sleep) [244]. In birds and mammals rhythms of glutathione peroxidase and glutathione reductase activity were in phase with melatonin, a linkage that could optimize compensation for wake-associated oxidative stress [260]. Induction of mammalian detoxification genes (particularly via coordinating PARbZip transcription factors) was strongly associated with the photophase (sleep) in mice. CLOCK/BMAL1 heterodimers bind and activate the *Pparα* promoter, but PAR bZip factors that regulate detoxification also contribute [59, 243, 244]. Sleep may particularly serve to protect neurons in the brain from stress and apoptosis [48, 261].

Control of detoxification pathways in rats by dimers of the aryl hydrocarbon receptor (AHR) and the Ahr nuclear translocator (ARNT) are compartmentalized to the rodent photophase and GSH-S-transferase expression was also higher in sleep [53]. Nuclear receptors regulating detoxification, the pregnane X receptor (PXR) and constitutive androstane receptor (CAR) were strongly expressed in late sleep (ZT:08-09) with second peaks in late waking (ZT:18-22) [131, 200]. AHR and PPARα variously impact several members of the core clock machinery (e.g., *Bmal1, Per,* Rev-erbα, *Cry*). Retinoic acid also downregulates CLOCK/BMAL1 and NPAS2/BMAL1 heterodimers via interaction of the retinoic acid receptors RAR and RXR with CLOCK or NPAS2. RXR further interacts with

PPARα, CAR and PXR, thus affecting detoxification [244]. PPARα can also bind and positively regulate the *Bmal1* and Rev-erbα promoters. When dimered with the retinoid X receptor α (RXRα), it inhibits transcriptional activity of CLOCK/BMAL1 [59, 135]. PPARγ may play a strong role in the cardiovasculature. Knockout specific to vasculature altered blood pressure and heart rate and reduced sympathetic neuronal rhythmicity [133].

Growth factors, including IGF-1, are anti-apoptotic and their activation of PI3K-Akt not only mediates growth, but may ensure that growth-associated ROS are not interpreted as dysregulation that could engage apoptosis. FOXOs are antagonistic to growth but crucially induce stress resistance (e.g. FOXO3a upregulates MnSOD and catalase). FOXO activation can induce cell-cycle arrest, protect non-proliferating cells by upregulating stress resistance, and with sufficient dysregulation, facilitate apoptosis [262, 263]. FOXOs regulate several apoptotic pathways.

These include the cell death receptor Fas, the Bcl2 interacting mediator of cell death (Bim) and the tumor necrosis factor apoptotic pathway. In NIH 3T3 cells, phosphorylation of FOXO1 by cyclin-dependent kinase 2 regulated FOXO1 localization and activity and apoptotic responses to DNA damage. Silencing FOXO1 decreases DNA damage-mediated apoptosis [264]. In HIV-infected macrophages, PI3K-Akt signaling was reduced, resulting in dephosphorylation of FOXO3a and translocation to the nucleus. Constitutive FOXO3a activity promoted apoptosis whereas downregulation reduced apoptosis [263].

Akt promotes proliferation and survival in cell cultures but cellular senescence and growth arrest increase with time as potential cell divisions reach their “Hayflick” limit. Akt-induced senescence involves p53/p21 pathways and requires inhibition of FOXO3a. A mutated *Foxo3a* unresponsive to Akt inhibition extended replicative lifespan of human cells [265]. Akt inhibition of FOXO3a reduced MnSOD, increased ROS stress, and induced p53/p21, cell senescence and growth arrest [265]. Cells from human progerias have reduced proliferative capacity [265], as do cells from giant GH transgenic mice with accelerated aging [266].

FOXOs induce catalase, *MnSod* and *Gadd45*. Deletion of *Foxo*1, 3 and 4 in the hematopoietic system impacted immunological cell cycles and decreased the hematopoietic stem cell compartment. This involved increased cell cycling, elevated ROS and apoptosis. Note that FOXO contributions to stress resistance do not need to be marshaled at the same time as cell cycle progression. The stem cell defect was ameliorated by N-acetyl-L-cysteine suggesting that FOXO-associated amelioration of oxidative stress can contribute to stem cell maintenance. Contributions of FOXO to stem cell quiescence may have also been a factor [267]. It should be considered that growth (and TOR) are also inhibited by reducing conditions so effects may not have been exclusively related to ROS.

5. Growth, TOR, Redox and Clocks

Key aspects of growth and synthetic processes related to redox and temporal organization include the GH axis, growth factor kinase signaling via ROS, NAD(P)H oxidases, the cell cycle, sleep, the target of rapamycin (TOR), downregulation of stress resistance and accelerated aging. Changes in size are not necessarily associated with longevity but growth is

negatively correlated with intra-specific longevity as documented for otherwise healthy rats, mice, domestic animals and humans [68, 268, 269]. Dwarf mice and those with reduced IGF-1 [268, 270] or defective GH receptor signaling [197] are long-lived, whereas giant GH transgenic mice express accelerated aging [23, 24, 34, 271]. Life extension by DR is also generally associated with reduced growth rates and adult size [68]. Hepatic antioxidants show a parabolic pattern with age, young and older mice having the lowest levels [272]. Reduced antioxidant activity in young mice may facilitate oxidative conditions favourable to rapid growth. Mutations that reduce PI3K signaling by insulin, IGF-1 and other growth factors are generally associated with extended longevity and increased stress resistance.

Growth signaling involves NAD(P)H oxidase (NOX) production of superoxide radical (Figure 2). This extends to the MAPK-ERK and PI3K pathways (utilized by insulin, IGF-1, other growth factors, and many neurotransmitters) as well as the JAK-STAT pathway utilized by growth hormone and cytokines [13, 24, 63, 68, 170, 273-275]. GHRH also elevated ROS by 36% in human prostate cancer cells [196] and radiation activates growth factor pathways [68]. ROS-associated signaling by IGF-1 activates TOR via both MAPK-ERK and PI3K-Akt pathways [277, 278]. Superoxide dismutase functions in this context to generate sufficient H_2O_2 to oxidize redox-sensitive cysteine residues of inhibitory tyrosine phosphatases, thus promoting kinase activity [16, 279, 280]. NAD(P)H oxidase activity can also significantly elevate mitochondrial H_2O_2 and deplete mitochondrial GSH [180]. H_2O_2 also enhances PLC-mediated phospholipid breakdown and accumulation of inositol phosphate.

We previously proposed that the GH axis increases mitochondrial coupling. Faster growth could be achieved by more efficient ATP production (at the expense of elevated ROS production) even if metabolic rate did not change or even declined (as in sleep). Indeed, gender dichotomy in body size of livestock and more efficient feed utilization by males has been attributed to coupling actions of testosterone [281, 282]. The juxtaposition of accelerated aging, elevated free radical processes, thermogenic dysfunction and high production (growth) efficiency in transgenic growth hormone mice, as well as a general negative relationship between longevity and intra-specific growth rates, is consistent with GH axis upregulation of mitochondrial coupling [34, 68, 283]. Such linkage is now supported by upregulation of mitochondrial activity and coupling by TORC1 [284, 285]. Rapamycin reduced membrane potential, O_2 consumption and ATP production in mammalian cells [284]. Further, membrane potential was downregulated with decreased TOR function in association with increased lifespan in yeast (although increased respiration maintained ATP) [286].

5.1. The Target of Rapamycin (TOR), Protein Synthesis and Growth

The target of rapamycin (TOR: a serine/threonine kinase) reflects a highly conserved linkage between growth and aging that likely reflects oxidative influences associated with growth [3, 68] or continued TOR signaling beyond the normal growth period [287]. Increasing cell size associated with cellular senenescence may reflect TOR signaling [287]. Inhibition of TOR extends longevity of yeast, *Drosophila*, nematodes and mice [287, 288]. TOR acts as a nutrient and energy sensor that coordinates cell enlargement, the cell cycle, amino acid and glucose transport, ribosomal production, transcription, protein translation and mitochondrial function. Alternatively TOR antagonizes protein degradation, apoptosis, autophagy and stress resistance [285, 287, 289-292]. Inhibition of TOR is linked to DR [223]

largely via antagonism to sirtuin, FOXOs and stress resistance. TOR inhibition in yeast increased longevity via mechanisms observed in DR: increased sirtuin activity (Sir2p) and stabilization of rDNA [286, 293]. Reduced nutrients during DR extend longevity of *C. elegans* by reducing TOR and elevating *FoxA,* whereas *FoxO* may preferentially respond to stressors other than DR [216].

TOR integrates signals from growth factors, branch-chain amino acids (e.g., leucine), energy status and stress and is highly conserved from unicells to vertebrates (Figure 2). Association of TOR with the GH axis, the cell cycle, redox state and SIRT/FOXO antagonism supports temporal dynamics. TOR signaling pathways are activated by light pulses (rest phase) in the mouse SCN [294]. Much of what we know about TOR we owe to the highly specific inhibitor of TOR complex-1, rapamycin. Rapamycin inhibits mammalian cellular growth and immune functions (both upregulated in sleep by the GH axis). TOR inhibition holds promise for ameliorating cancer, cardiovascular disease, autoimmunity, metabolic dysregulation and aging [291].

The yeast oxidative phase is associated with synthesis in support of growth and cell division and this is reflected by induction of genes for ribosomal and amino acid production. These processes require high levels of ATP and by the end of the oxidative phase transcripts reach low levels (possibly reflecting degradation). Unlike multicellular organisms, yeast have few higher-order functions so relegating synthesis to the oxidative phase is not constrained by waking behavioural activity and other functions seen in higher animals. Ribosomal production, protein translation and growth appear shifted to sleep with other anabolic processes in vertebrates, although ribosomal genes were already activated during the late dark period preceding sleep in the SCN [43].

Protein translation in vertebrates is strongly downregulated by sleep deprivation and is restored by sleep [295]. The oxidative phase of yeast is intense and short whereas waking is a substantial portion of the vertebrate day. A metabolic basis for vertebrate sleep, however, is reflected in inter-specific allometric relationships documenting that total sleep duration increases with metabolic rate (i.e., smaller size) while sleep bouts are shorter. Thus, shrews sleep more than larger mammals but their wake-sleep activity is dominated by 3-4 hour ultradian cycles. Growth rates of smaller species are also faster, larger species attaining increased size by prolonged but slower growth [68, 69].

About 27% of yeast genes are strongly linked to growth. Ribosomal genes are particularly associated with growth whereas peroxisomal functions show a negative relationship [42]. The building-reductive phase of yeast is associated with lower O_2 consumption and transcription of genes associated with mitochondrial biosynthesis [41]. This may reflect that turnover of mitochondria requires low mitochondrial activity (low oxygen consumption, low ATP production). In endotherms the reductive phase (sleep) additionally includes reduced heat generation. The yeast reductive phase is also associated with gene expression associated with cell division. About 45% of cells undergo cell division in any given cycle, and this is never initiated in the oxidative phase [44]. Artificially inducing DNA replication in the oxidative phase increases mutation rates suggesting that the reductive phase is protective [41, 44]. Control of cell replication in yeast is analogous to the mammalian cell cycle which is also linked to the circadian clock [41, 296, 297]. Although cell cycle timing varies among tissues in multicellular animals, the growth/building phase associated with TOR activity likely corresponds most closely to GH secretion and IGF-1-PI3K-Akt activity in early sleep.

Growth regulation by TOR mainly involves post-transcriptional mechanisms. This is relevant to vertebrates where transcription is predominantly wake-associated. The succinate dehydrogenase complex (linking transfer of electrons from oxidation of succinate to ubiquinone) is upregulated by growth [241]. TOR contains redox-sensitive cysteines likely to impact activity and degradation rates [67]. The MAPK/ERK, PI3K/Akt pathways and TOR activation involve free radical-mediated signaling but oxidative stress has variable impacts on TOR depending on tissues, levels and duration [290]. Thus, physiological levels of oxidation are stimulatory, but stressful or chronic exposure is inhibitory (as is true for the PI3K pathway itself) (Figure 2).

The metabolome of yeast cycles in tight phase with peaks of NAD(P)H. A transcriptional complex contributing to biosynthetic, reductive and cell cycle functions is linked to TOR and rapamycin induces a reduced redox state lasting 60 hours. A distributed network of interacting elements rather than a central regulatory oscillator may derive yeast cycles [14, 148]. A "domino" regulatory structure as described above would likely suffice. However, when subjected to 24-hour temperature cycles, *Saccharomyces cerevisiae* can express circadian rhythmicity [298]. More than 70% of yeast genes regulated by growth were associated with TOR (i.e., impacted by rapamycin). Associated functions included ribosomes, metabolism (of nucleic acids, amino acids, sulfur and RNA), protein synthesis and nuclear transport. Sulfur metabolism was highly associated with functions such as GSH metabolism contributing to reduced states [148].

Yeast has two distinct TOR proteins but in mammals a single protein forms two complexes (mTORC1 and mTORC2). In vertebrates, the GH axis and associated growth factors signal via the MAPK/ERK and PI3K pathways that both impinge on TOR. ERK1/2 phosphorylates and inactivates tuberous sclerosis factor 2 (TSC2), leading to dissociation of TSC1 and TSC2, disinhibition of RHEB and activation of mTOR1. mTOR1 stimulates protein translation via activation of ribosomal protein S6K and the initiation factor eIF4E. TSC2 inhibits RHEB which when activated, promotes activity of mTOR and ribosomal protein S6K. TSC2 mediates hydrolysis of GTP on the small G-protein RHEB, inactivating it (Figure 2).

PI3K signaling activates Akt which phosphorylates and inhibits TSC2, thus disinhibiting RHEB and activating mTOR1 [290]. TOR2 activation feeds back to reinforce Akt activity and full activation of the TOR pathway. TOR signaling may be terminated by negative feedback of $p70^{S6K}$ on insulin substrate 1 [290]. Such feedback may induce insulin resistance and promote type II diabetes [291]; see Vol. III, Chapter 9). Ribosomal biogenesis utilizes a substantial portion of cellular energy. TOR controls ribosomal production by downregulating transcription of RNA polymerase I rRNA, RNA polymerase II ribosomal protein, and RNA polymerase III tRNA [291].

Association of the small ribosomal unit with mRNA requires establishment of a translational initiation complex. This involves binding of initiation factors (eIF4A, eIF4E and eIF4G) to the 5' cap of the mRNA. The protein synthesis inhibitor, 4E-BP-1 binds eIF4E and prevents assembly of a functional complex. Phosphorylation of 4E-BP-1 frees bound eIF4E to form active complexes [292]. Transcription of *4E-BP* is stimulated by FOXO whereas 4E-BP binding is inhibited by TOR phosphorylation. Thus, Akt phosphorylation-inhibition of FOXO suppresses 4E-BP and disinhibits eIF4E and protein translation (Figure 2). Concomitantly, Akt activity disinhibits TOR by downregulating TSC2. Downregulation of 4E-BP binding activity by TOR phosphorylation releases eIF4E and protein translation. eIF4B is responsive

to $p70^{S6K}$ and rapamycin. It is also phosphorylated by $p90^{S6K}$ (RSK), a target of MAPK/ERK. Reduced amino acid supply lowers activity of S6K and increases binding of the translation suppressor 4EBP1 to eIF4E [72].

Oxidative conditions prevent rapamycin-FKBP12 from binding and inhibiting TORC1. CuZnSOD defects mediate such a state, implicating superoxide/H_2O_2 in maintaining TOR function. Insulin and oxidative conditions increased growth of ovarian theca-intersticial cells. Inhibition of MAPK-ERK and PI3K signaling reduced proliferation, but rapamycin was more effective, implicating oxidation and TOR in growth signaling [299]. The serine/threonine kinase $p70^{S6K}$ is one effector of growth factors and TOR in mediating cell growth and cell cycle control. TORC1 phosphorylation of S6K allows binding and activation by phosphoinositide-dependent kinase (PDK). S6Ks regulate function of translation initiation factors but also ribosomal production [292]. Phosphorylation of $p70^{S6K}$ was promoted by UV and inhibited by antioxidants specific to H_2O_2 and by rapamycin. H_2O_2 effectively mediated $p70^{S6K}$ phosphorylation [300]. This reinforces the association of TOR, protein translation and growth with oxidative conditions. PI3K, MAPK/ERK and free radicals converge on activation of $p70^{S6K}$ and eIF4B [277, 292, 299, 300]. Alternatively, reduced environments inhibit mTOR1 [291]. Oxidative states may partly explain the disappointing results obtained with rapamycin in cancer therapy [301]. Combining rapamycin with an effective antioxidant cocktail (that might itself reduce TOR) could synergize effectiveness of rapamycin in pathological states associated with oxidative conditions, including aging [3].

Growth and anabolic processes require abundant nutrients, energy and conditions of otherwise low stress. Ribosomal biogenesis and protein translation are particularly costly, protein synthesis consuming perhaps ~ 20% of cellular energy [289]. Consequently, growth and reproduction linked to TOR are impaired by resource and energy shortfalls and diverse stressors (e.g., osmotic stress, viral infections, ROS) [289, 300]. Activation of p53 by DNA damage also inhibited TORC1 [291] and TOR inhibition may precede apoptosis [289].

Suppression of TOR, protein translation or ribosomal production extended longevity of yeast, nematodes and flies [72, 287] acting via pathways common to DR including increased sirtuin activity [293]. In yeast this involved translocation of the stress factors MSN2p and MSN4p to the nucleus where they induced expression of a nicotinamidase gene (PNC1) and SOD2. Nicotinamidase acts on nicotinamide to increase sirtuin activity and is induced by stressors other than DR [293]. PI3K-TOR signaling and protein translation pathways are downregulated in long-lived Ames dwarf mice [81] and normal mouse longevity was extended by rapamycin even when treated at 600 days of age [288, 302]. Rapamycin treatment of organ transplants also reduced cancer and fat deposition [287]. Diet-induced obesity is associated with chronic Akt activation and Akt-deficient mice were resistant to obesity-associated vascular senescence. Rapamycin protected obese mice from vascular senescence and ameliorated limb necrosis and ischemic stroke, implicating TOR in obesity and cardiovascular disease [303]. TOR inhibits stress-resistance genes, macroautophagy (degradation of cellular organelles), mRNA degradation and ubiquitin-dependent protein degradation that are all implicated in aging [287, 290, 291]. Rapamycin can reduce protein aggregates via autophagy, suggesting benefits for neurodegenerative diseases [304, 305].

Elevated mitochondrial and NOX generation of ROS associated with TOR could exacerbate cancer and aging although specific downregulation of stress resistance may particularly accelerate aging in rapidly growing animals (see “stress resistance”). This may be masked by association of rapid growth with youth better able to tolerate such insults. Indeed,

for progeroid transgenic GH mice the greatest elevations in ROS may occur in youth rather than old age [3]. Alternatively, costs of TOR functions such as protein synthesis may exacebate aging independently of downregulated stress resistance. Thus, inhibition of TOR-mediated protein synthesis by 4E-BP binding of eIF4E is critical to FOXO-mediated regulation of stress resistance [306]. 4E-BP null flies showed reduced longevity and induction of 4E-BP in starved larvae was critical to survival. Flies lacking FOXO or 4E-BP function were hypersensitive to H_2O_2 but this was normalized by elevated 4E-BP binding activity [306].

Growth signals associated with TOR not only downregulate stress resistance (see above); TOR is inhibited by diverse stressors (Figure 2). The stress-signaling protein REDD1 competes with TSC2 for 14-3-3 binding. 14-3-3 inhibits TSC2 GAP function so diversion by REDD1 inhibits TORC1 [292]. REDD1 conveys stress signaling from glucocorticoids, energy shortfalls, hypoxia inducible factor-1α, DNA damage, toxins, H_2O_2 and other stressors. Such convergence of multiple stressors on particular signaling pathways may contribute to generality of stress responses [289, 290, 292, 307, 308]. An 18-hour fast repressed TORC1 activity in rat skeletal muscle. This recovered within 45 minutes of feeding, demonstrating high sensitivity of TOR to nutrients. *REDD1* mRNA and protein rose markedly with fasting in association with hallmarks of DR - low serum insulin and elevated glucocorticoids. Glucocorticoid inhibition of TORC1 is likely mediated by REDD1 [308]. REDD1 negatively correlated with muscle growth and positively correlated with atrophy. REDD1 and 2 were circadian in skeletal muscle and likely modulate TOR activity according to energy availability [109].

Conclusions

The juxtaposition of information brought to bear supports the reality of three temporal windows associated with distinct functionality. Finer resolution is likely possible. Declines in detoxification, stress resistance and protein metabolism exacerbate aging and may involve deterioration of the clock [231, 244]. Rhythmic dynamics and compartmentalization means that alterations in one phase will cycle impacts forward to other interdependent windows. Thus, interventions to reduce wake-associated ROS could hypothetically interfere with ATP or nutrient supply. This in turn could impact sleep-asociated synthesis, growth, recharging, detoxification and stress resistance. Defects in these compartments could then increase stress and decrease function in the next waking phase.

Functions of FOXO in the late sleep, fast and contributions that extend longevity under DR (stress resistance, autophagy, detoxification, DNA repair) share regulatory congruence. Age-related reduction in amounts and quality of sleep associated with increasing HPA axis and reduced GH axis activity may reduce repair and replacement systems and production of redox-sensitive products like heme, NAD(P)H and antioxidants. If FOXO-mediated longevity assurance is mainly marshaled in late sleep, then losses in that compartment may particularly impact aging and pathologies. This also delineates a specific circadian time to target aging and health interventions aimed at stress resistance and antioxidants. Alarm clocks and shift work deserve even stronger scrutiny as sources of real potential harm.

This framework suggests that our goal should be to maintain the high-highs and low-lows expressed in youth into older ages [309]. This is complicated if temporal structure is more than a simple wake-sleep dichotomy, but it also holds unexpected promise. Thus, a static temporal perspective envisions that interventions to induce FOXO-associated stress resistance are necessarily suppressive of TOR. Displacement of TOR and FOXO in time means that both can theoretically be increased on any given day. The value and necessity of this perspective is immediately apparent if TOR-associated proliferation extends to stem cells and immune function whereas FOXO-associated (anti-proliferative) stress resistance upregulates longevity-assurance mechanisms. FOXO may protect quiescent cells but loss of TOR could ultimately cause us to age like terminally differentiated insects or die from infectious disease. Rather than single-focused interventions such as mimicking DR, we need gobal approaches to restore and maintain youthful balance. Effective intervention might entail time-released enhancers and inhibitors to channel elecroplasmic cycles to trajectories consistent with youth. Any such approach must face the complications associated with tissue-specificity. Beyond that, temporal phase relationships among tissues represent another evolutionary dimension that is undoubtedly tuned to maximize finess. Consider that muscular activity is centrally blocked during sleep which normally prevents activation even by dreams. Clearly we need a consortium dedicated to a systems approach and computer modeling to understand the emerging complexity. The fact that most aspects of life are mutually organized around a circadian clock provides a firm, albeit dynamic, foundation that should simplify capturing the core structure.

Finally, we must consider that we should not strive to mimic youth if we wish to live extraordinary lifetimes. That is because evolution maximizes youthful function to yield the greatest early fitness, and this invariably precludes evolution of longevity in post-reproductive ages [310]. *The framework described here suggests that other than for mitochondrial activity and coupling, most processes determining longevity occur in sleep*. It has long been known that duration of sleep is inversely associated with body size and metabolic rate interspecifically, presumably because high-rate species require more sleep to offset waking stress [311]. Larger species have lower mass-specific metabolic rates allowing daily sleep investments to be reduced. This suggests that sleep duration may be a feature related to aging (which may be obscured by sleep quality intra-specifically). In that case aging theory would suggest that evolved sleep durations are shorter than required to prevent aging. Manipulating sleep to maximize and extend both TOR and FOXO functions certainly appears possible. Could extended lifespan be achieved at the cost of an extra hour or two of sleep, artificially enhanced to elevate longevity assurance mechanisms? Offsetting an evolutionary deficiency irrelevant to modern human societies may not be that difficult as the machinery to modulate aging is likely already onboard. The hands of the clock may need only to be reset to “lifespan savings time.”

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