Review

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Lessons from mammalian hibernators: molecular insights into striated muscle plasticity and remodeling

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Abstract: Striated muscle shows an amazing ability to adapt its structural apparatus based on contractile activity, loading conditions, fuel supply, or environmental factors. Studies with mammalian hibernators have identified a variety of molecular pathways which are strategically regulated and allow animals to endure multiple stresses associated with the hibernating season. Of particular interest is the observation that hibernators show little skeletal muscle atrophy despite the profound metabolic rate depression and mechanical unloading that they experience during long weeks of torpor. Additionally, the cardiac muscle of hibernators must adjust to low temperature and reduced perfusion, while the strength of contraction increases in order to pump cold, viscous blood. Consequently, hibernators hold a wealth of knowledge as it pertains to understanding the natural capacity of myocytes to alter structural, contractile and metabolic properties in response to environmental stimuli. The present review outlines the molecular and biochemical mechanisms which play a role in muscular atrophy, hypertrophy, and remodeling. In this capacity, four main networks are highlighted: (1) antioxidant defenses, (2) the regulation of structural, contractile and metabolic proteins, (3) ubiquitin proteosomal machinery, and (4) macroautophagy pathways. Subsequently, we discuss the role of transcription factors nuclear factor (erythroid-derived 2)-like 2 (Nrf2), Myocyte enhancer factor 2 (MEF2), and Forkhead box (FOXO) and their associated posttranslational modifications as it pertains to regulating each of these networks. Finally, we propose that comparing and contrasting these concepts to data collected from model organisms able to withstand dramatic changes in muscular function without injury will allow researchers to delineate physiological versus pathological responses.

Keywords: antioxidant pathways; macroautophagy; mammalian hibernation; muscle mass; structural proteins; ubiquitin proteosomal machinery.

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Introduction

Mammalian striated muscle is composed of a heterogeneous mixture of fiber types which are classified based on oxidative capacity, contraction time, fatigue resistance, and power production. The diversity of myofibers allows human muscle to perform a wide range of tasks including standing upright, long distance running, short bursts of speed in sprinting, and fine motor control of fingers. In 1960, Buller and his colleagues were the first to reveal the alterability of muscle properties through cross-innervation experiments which demonstrated the complete reversal of muscle phenotype from fast-contracting to slow-contracting myofibers and *vice versa* (1). This cellular plasticity is an enormous advantage giving mammals the ability to adapt the muscular apparatus based on phenotypic stimuli. However, pathological conditions exist which transition these positive adaptations into ones which may be deleterious to the health and fitness of the organism. Muscular atrophy signaling pathways may be initiated as a result of prolonged periods of inactivity, unloading, or may be exacerbated as a result of disease states such as muscular dystrophy, cancer, and renal dysfunction (2–5). In contrast, muscular hypertrophy may be promoted during resistance training, following myocardial infarction and other forms of myocardial injury, hypertension, and neurohumoral activation (6, 7).

Recent insights into the signaling pathways that regulate muscle remodeling and myocyte cell size have refined

our view of the composite nature of striated muscle as well as its capacity for change. Many studies have focused on the regulation of single and multi-complex transcription factors (TF) which change the intracellular environment by controlling the amount and types of cellular transcripts made (8–10). Revealing mechanisms of transcription factor regulation has isolated molecular pathways responsible for a wide range of myopathies and sparked a whole new realm of intervention strategies as well as research initiatives. Consequently, there is an overwhelming interest in highlighting specific networks which transition muscle towards pathological conditions such as muscular atrophy and hypertrophy as well as those associated with a multitude of disease states. Many of these studies focus on the dysregulation of central muscle-specific pathways failing to address the natural capacity of myocytes to alter structural, contractile and metabolic properties. Perhaps by highlighting the different dimensions of striated muscle plasticity in mammals able to withstand dramatic changes in muscular function without injury, we can elucidate further mechanisms and principles that contribute to mammalian muscle plasticity.

In this review we first outline current knowledge in muscle research with a particular focus on muscular atrophy, hypertrophy and striated muscle remodeling. We then identify four main networks critical to muscle health: [1] antioxidant defenses and cellular preservation strategies, [2] the regulation of key structural, contractile and metabolic proteins, [3] the ubiquitin proteosomal machinery responsible for the selective degradation of myofibrillar proteins, and [4] macroautophagy pathways which control turnover of long-lived proteins and organelles. Finally, we discuss the role of transcription factor regulation by means of protein-protein interactions and post-translational modifications (e.g. phosphorylation, acetylation, sumoylation, ubiquitination, etc.), as it pertains to these four main networks. We focus in particular on these networks in mammalian hibernators (e.g. thirteen-lined, Richardson's, and golden-mantled ground squirrels) that are known champions of muscle plasticity.

Defining muscle atrophy, hypertrophy, and remodeling

Muscle atrophy and hypertrophy

Atrophy is defined as the wasting or loss of muscle tissue, a decline in force/power output, and the stimulation of muscle catabolism. Muscle atrophy is a common occurrence in a wide range of conditions such as chronic bed rest, casting, denervation, limb suspension and immobilization, and may be amplified as a result of aging (sarcopenia) (11, 12). Atrophy also occurs in association with diseases. One such case is duchene muscular dystrophy and becker muscular dystrophy (DMD and BMD). Although these are genetic disorders caused by mutations in the dystrophin gene, ultimately they cause severe symptoms of muscle weakness and degeneration, often also including weakening of the heart. Common to human DMD, X-linked muscular dystrophy mice (*mdx*) and canine X-linked muscular dystrophy (CXMD) both reveal infiltration of inflammatory cells as a central mechanism for the induction of muscle wasting (13). The upstream signals that trigger muscle atrophy in other pathological conditions such as cancer cachexia, AIDS, sepsis, and uremia involve marked increases in cortisol secretion and cytokines (12).

Hypertrophy is defined as an increase in cell size, enhanced protein synthesis, and the re-expression of genes that are important during early development (i.e. the fetal gene program) (8, 14). Following the first few weeks after birth, cardiac myocytes generally withdraw from the cell cycle, and subsequently rely on increases in cell size rather than proliferation for growth of cardiac cells. While cardiac hypertrophy may occur as a result of various stresses, hypertrophy also occurs to accommodate an increase in workload. At first, hypertrophy serves as an adaptive response, allowing the enlarged cardiomyocytes to compensate for increased demands placed on the heart. However, over time the response is decompensated and may result in heart failure (15). No single intracellular transduction cascade regulates cardiomyocyte hypertrophy in isolation. Instead, each pathway works in concert with extensive crosstalk resulting in the interdependence of several networks. This feature of cardiac hypertrophy has resulted in much confusion over how these signals are first integrated in the cell and subsequently bring about changes in gene expression. Nonetheless, evidence has placed the spotlight on several pathways which are important for the induction of cardiac hypertrophy.

Although the triggers that cause atrophy/hypertrophy are different, the loss/gain of muscle mass in each case involves the disregulation of some common programs. Recent strides to elucidate molecular attributes involved in the regulation of muscle mass have isolated pathways such as antioxidant defenses, muscle remodeling, and muscle proteolysis by both the ubiquitin proteasomal pathway and macroautophagy as molecular crossroads common to both skeletal and cardiac cells (4, 11, 16). To date, culpable targets include proteins involved in the detoxification

of harmful reactive oxygen species (ROS) [e.g. superoxide dismutase (SOD), catalase (CAT), heme-oxygenase-1 (HO-1)], the altered expression of sarcomeric, contractile, and metabolic proteins [e.g. myosin heavy chain (MyHC), myosin light chain (MLC), troponin (Tn) complex, creatine kinase), ubiquitin ligases which actively degrade cellular proteins [e.g. muscle atrophy F box/atrogin-1 (MAFbx); muscle RING finger 1 (MuRF1)], and the autophagy pathway which recycles cellular compounds (e.g. Beclin1, microtubule-associated protein 1A/1B-light chain 3 (LC3), autophagy-related proteins Atg12, Atg5, and Atg9). The upstream signaling pathways which regulate these targets also play a pivotal role in the regulation of muscle mass. For example, myokines (e.g. myostatin), insulin signaling [e.g. protein kinase B (Akt/PKB); mammalian target of rapamycin (mTOR)], the calcium-calmodulin pathway (e.g. calcineurin), and stress activated protein kinases [e.g. p38; extracellular-signal-regulated kinase (ERK1/2); c-Jun N-terminal kinase (JNK)] have all been shown to direct the changes in muscle mass (3, 17).

Muscle remodeling

In response to changing functional demands, striated muscle tissue demonstrates a remarkable ability to adjust its structural, contractile, and metabolic profile. These changes may be collectively known as muscle remodeling whereby some aspect of the cellular environment is altered via directed changes in gene expression (18). Muscle remodeling provides the basis for muscle plasticity and the malleability of myocytes. These mechanisms can provide striated muscle with the optimal combination of cellular proteins to adapt to changing conditions (e.g. mechanical loading, metabolic requirements, etc.), although much literature discusses 'muscle remodeling' in the context of pathological states. For the purpose of the present review muscle remodeling is considered in its roles as both a positive survival strategy (e.g. exercise induced transformation of muscle fibers) as well as maladaptive changes in the muscle apparatus (e.g. overgrowth of connective tissue and extracellular matrix (ECM) proteins). These changes, regardless of their classification as a physiological or pathological response, need not be dramatic or result in massive cellular reorganization, instead may involve discrete components which alter the cellular environment. Therefore, the desire to delineate the mechanisms of muscle remodeling is a topic of great interest because we gain increased understanding of the mechanisms of adaptation and compensation that are available to mammalian muscle.

Skeletal/cardiac muscle is highly organized and shows a remarkable ability to modify physiological parameters based on extrinsic demands. This requires a high level of integration between neuromuscular signaling, cellular morphology, contractile factors, and intracellular organelles (Figure 1). The coordination of a wide range of contractile proteins [e.g. Tn, tropomyosin (Tm)], sarcomeric proteins (e.g. myomesin, titin), intermediate filaments (IF) (e.g. desmin, vimentin), myofibrilar proteins (e.g. myosin heavy/light chain isoforms), structural proteins vital to signaling cascades (e.g. integrins, dystrophin, utrophin), proteins that improve ATP availability (e.g. glucose transporters, myoglobin, creatine kinase), and ECM proteins (e.g. fibronectin, decorin) are at the heart of muscle remodeling. Each muscle-specific protein may undergo alterations that result in changes in their sensitivity, relative expression level, differential abundance of a given isoform, interacting proteins, and post-translational modifications. Finally, the recycling of sarcomeric proteins due to damage, oxidative stress, or proteolysis may also be utilized as a muscle remodeling tool (19).

Muscle networks associated with muscle health

Structural, contractile and metabolic proteins

The main constituents of the sarcomere are thin and thick filaments, which are composed of actin and myosin, respectively (Figure 1). Actin polymers are associated with actin-binding proteins and act as the platform for myosin motors that bind and move along actin filaments. Tn and Tm bind to actin thin filaments playing a pivotal role in calcium regulation and striated muscle contraction. The Tn complex has three subunits: troponin I (TnI) inhibits the actomyosin Mg^{2+}-ATPase, troponin C (TnC) acts as the intracellular Ca^{2+} sensor, and troponin T (TnT) links the complex to Tm (20). Under conditions of low calcium (Ca^{2+}), TnI drives Tm to the outer domain of thin filaments and blocks the myosin binding sites on actin to prevent contraction (21). Once Ca^{2+} is released from the sarcoplasmic reticulum and binds to TnC, Tm moves away from its blocking position and thereby facilitates myosin binding on actin thin filaments. In this capacity, Tm oscillates between the inner actin domain (open state) and the outer actin domain (closed state) (22). Accordingly, changes in calcium uptake and release as well as sensitivity of the

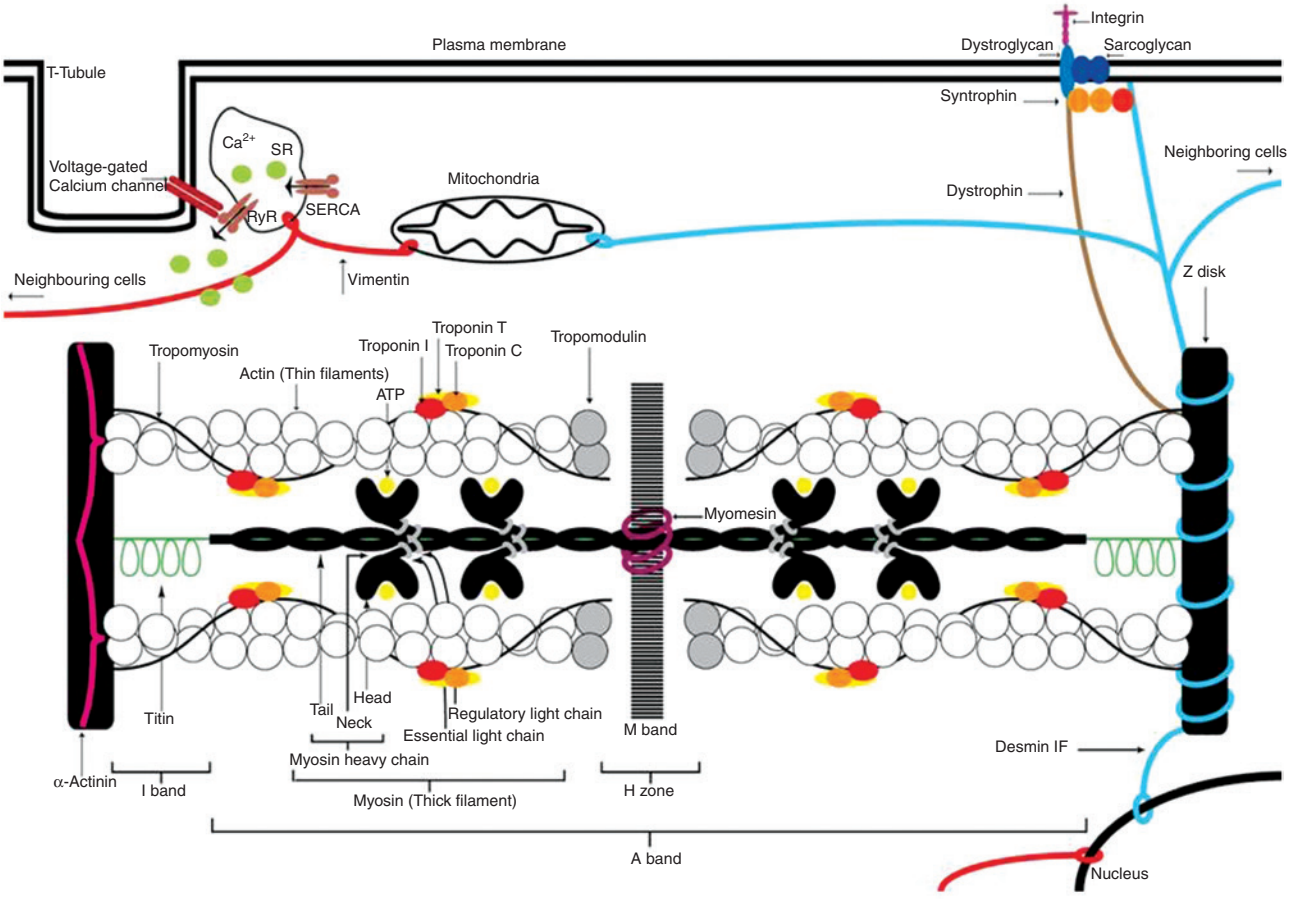

Figure 1: A striated muscle cell highlighting key sarcomeric, cytoskeletal, and sarcolemmal proteins as well as intracellular organelles. The main constituents of the sarcomere are thin and thick filaments, which are composed of actin and myosin, respectively. Troponin and tropomyosin associate with actin polymers and play an integral role in striated muscle contraction. The M-line and Z-disk are connected via titin and together constitute the framework of the sarcomere. Muscle cells contain several intermediate filaments (IF) including desmin, vimentin, nestin, synemin, syncoilin. IFs also participate in signaling cascades that are linked to both integrin and dystroglycan complex (DGC) signaling. Integrins have a structural role as extracellular matrix (ECM) proteins mediating attachment between the cell and the surrounding tissue.

Tn complex may all be critical candidates that alter the cellular environment to meet changing demands (18, 23, 24). The importance of Tm and Tn in normal muscle is demonstrated by the fact that mutations in cardiac TnI, TnT, α-tropomyosin (amongst others) are associated with hypertrophic cardiomyopathy (24, 25).

Myosin II is the myosin type responsible for muscle contraction and is composed of two heavy chains and four light chains (including the regulatory and essential light chain). Myosin comes from a multigene family containing several isoforms with various properties (26). The myofibers that make up muscle cells are classified based on the MyHC isoforms they contain; type I and type IIa MyHC isoforms are associated with muscle fibers that contain more capillaries, myoglobin, and mitochondria, making them well-suited for aerobic activities (slow twitch). In contrast, type IIb and type IIx MyHC isoforms are associated with glycolytic muscle fibers that are more appropriate for short bursts of speed and power (fast twitch). Consequently, fiber type transitions are central to the adaptability of the muscular apparatus. For example, fiber-type transitions from slow to fast twitch muscle have been implicated in situations of disuse atrophy, while exercise training results in fiber type transitions from fast to slow (27).

The M-line and Z-disk are connected via titin and together constitute the framework of the sarcomere. Myomesin is the major M-line protein expressed from three isogenes (MYOM1-3) (28). Myomesin functions as a titin-stabilizing protein, provides elasticity to the sarcomere, and has also been shown to be regulated by post-translational modifications such as phosphorylation (28, 29). The C-terminus of titin is embedded into the M-band

forming direct contact with myomesin (28). Titin is the largest known polypeptide which spans the length of the sarcomere, forms important protein-protein interactions the full length of the sarcomere, and is responsible for the resting elasticity of muscle (28). Using a titin knock-out model, it was observed that loss of the titin kinase domain and myomesin binding site resulted in muscle wasting as the sarcomere disassembled from the M line to the Z-disk (30). Therefore, the loss of titin and myomesin is directly related to reduced stability of the Z-disk and M line during atrophy.

IFs interconnect myofibrils through the Z-disk and link the contractile apparatus to the sarcolemma as well as to the nucleus. These play roles in cellular integrity, force transmission, and integration of organelle structure (31). Moreover, IFs also participate in signaling cascades that are linked to both integrin and dystroglycan complex (DGC) signaling (31, 32). Muscle cells contain several IF including desmin, vimentin, nestin, synemin, and syncoilin; however, desmin is the major muscle specific IF protein and is essential for the proper development of both cardiac and skeletal muscle (33, 34). Desmin filaments connect adjacent Z disks maintaining the lateral alignment of myofibrils, make important connections with subsarcolemmal proteins and the plasma membrane, form connections with organelles such as the nucleus and mitochondria, and also bridge connections between cadiomyocytes at intercalated disks (35). The extensive desmin network implicates desmin in a wide range of functions including enhancing cell-to-cell communication, mediating signal transduction, and organization of the sarcomere. For example, desmin has been shown to be upregulated in many cardiac diseases and the hearts of desmin null mice develop hypertrophy (36). Indeed, a wide range of desmin-related myopathies exist in both skeletal and cardiac muscle collectively referred to as desminopathies [reviewed in 34]. Vimentin is another type III intermediate filament which is responsible for the organization of proteins involved in adhesion, migration, and cell signaling (37). Vimentin filaments are connected to integrins via connector proteins and may play a role in integrin signaling (37). Additionally, many studies have linked vimentin to signal transduction pathways such as ERK signaling (37).

Integrins have a structural role as ECM proteins mediating attachments between the cell and the surrounding tissue. When integrins are activated they cluster into focal adhesion sites along with cytoskeletal proteins and signaling molecules implicating integrins as extracellular to intracellular communicators (38). At the extracellular side of the plasma membrane integrins form important connections with proteins such as collagen, elastin, laminin, and fibronectin and can stimulate changes in the synthesis and types of ECM proteins having diverse effects on cardiac/skeletal muscle cells (38). The accumulation of ECM proteins plays a central role in the transition from hypertrophy to heart failure. As collagen is deposited into the cardiac interstitium it increases the wall stiffness and, ultimately, less blood is available to be pumped to the body during each beat (39). Intracellular counterparts (e.g. α-actinin, vinculin, talin, dytrophin, etc) are also activated by integrins which are associated with kinases that transduce signals through the cytosol including focal adhesion kinase, ERK, JNK, and protein kinase C (38).

Antioxidant defenses

Attack by ROS is a major cause of damage to cellular macromolecules (e.g. DNA, protein, lipids) and therefore, all organisms maintain robust antioxidant defenses which prevent, minimize or repair damage done by ROS. Nonetheless cells can still be overwhelmed by ROS attack under a variety of stress and disease states. However, some ROS, such as hydrogen peroxide, also serve positive roles as signaling molecules and help to regulate the expression of proteins/enzymes that play a vital role in normal myocyte function (40). Therefore, muscle is consistently harmonizing levels of ROS where redox-sensitive pathways are functional yet the amounts and nature of these species do not overwhelm their cellular capacity. This cellular homeostasis is critical to cell preservation and its dysregulation accompanies a multitude of myopathies. For example, a key feature of muscle wasting is heightened levels of oxidative stress which is tightly linked with activation of proteosomal networks and the resultant degradation of myofibrillar proteins (4, 41). Indeed, improved mechanisms of damage limitation, such as enhanced antioxidant defenses, provides one means to reduce the effects of muscle wasting in patients with neuromuscular disorders (42). Redox-sensitive mechanisms have also been implicated in cardiomyocyte health. For example, studies have indicated that redox modifications at critical positions of MyHC in cardiac muscle may alter myosin ATPase activity possibly leading to myofilament dysfunction (43). Similarly, other structural, sarcomeric, and regulatory proteins may be altered in an analogous fashion in both skeletal and cardiac muscle (44).

Critical intracellular enzymes/proteins, such as superoxide dismutases (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR), peroxiredoxins (PRDX), thioredoxin (TXN) and thioredoxin

reductase (TXNRD), heme oxygenase-1 (HO-1), and aflatoxin aldehyde reductase (AFAR), are responsible for the primary antioxidant defense system of mammals (Figure 2). SOD are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. They are regulated by metal co-factors with copper/zinc SOD (Cu/Zn SOD) functioning in the cytoplasm and manganese SOD (MnSOD) in the mitochondria (45). Highlighting the importance of SOD enzymes, some individuals with hereditary amyotrophic lateral sclerosis (ALS) have a mutant Cu/Zn SOD enzyme where loss of function causes the symptoms of the disorder (46). While SOD is considered to be a main line of defense against ROS, the cellular levels of its product, hydrogen peroxide, must be closely monitored. Hydrogen peroxide serves an important function as a second messenger signaling molecule for various cellular events (47) but it is also readily converted into hydroxyl radicals (in the presence of Fe^{2+} or Cu^{+}) that are the primary agents of ROS damage to biomolecules. As a result, in unloaded muscle rising levels of hydrogen peroxide can stimulate degradation reactions by increasing the expression of ubiquitin ligases including MAFbx and MuRF1 (4). The conversion of hydrogen peroxide to the highly reactive hydroxyl radical is guarded by CAT which degrades hydrogen peroxide into oxygen and water (48).

GPX performs a similar reaction to CAT; however, its reaction involves reduced glutathione that is converted to glutathione disulfide (49). The enzyme GR then restores the glutathione back to its reduced state. PRDX are intracellular enzymes which also reduce and detoxify a wide range of hydroperoxides in cells, typically in the presence of TXN and TXNRD which act as the electron donor (38). There are six known PRDX family members in mammals which are divided into two classes: 1-Cys and 2-Cys PRDX,

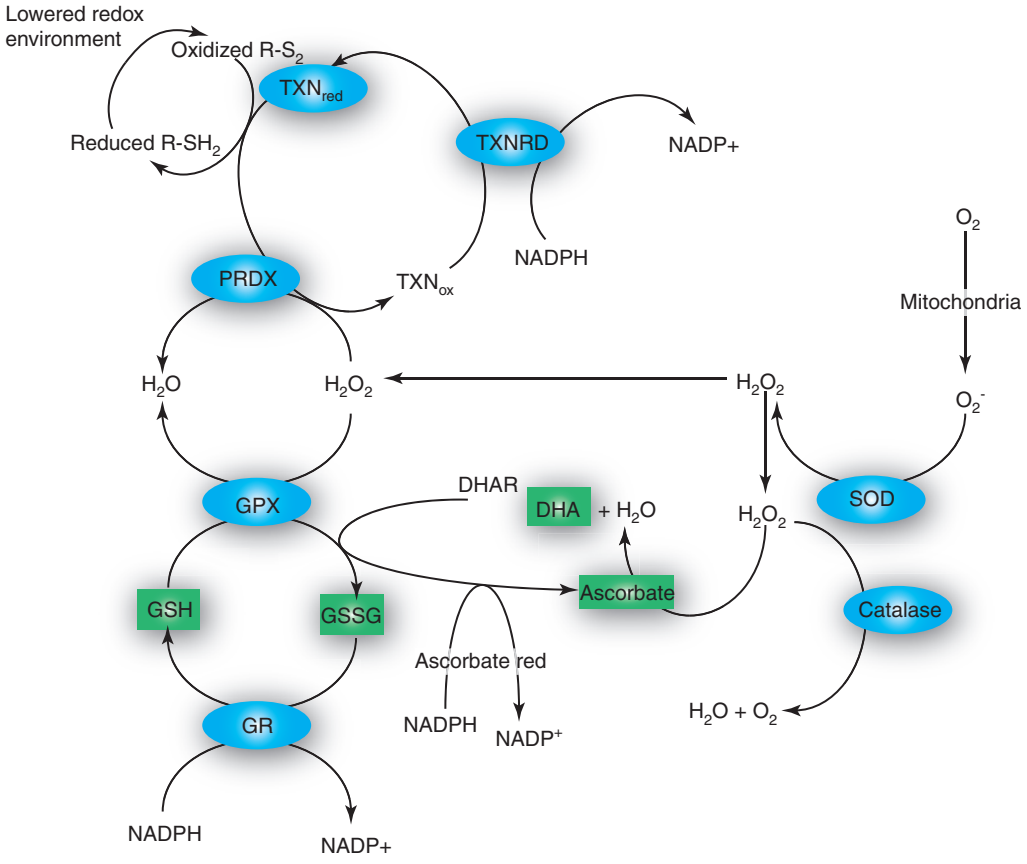

Figure 2: Intracellular antioxidant enzymes critical to the maintenance of muscle and cardiac health.
Superoxide dismutase (SOD) is considered one of the main line of defense against ROS, converting the superoxide ($O_2^{\cdot}$) radical into oxygen and hydrogen peroxide (H_2O_2). In turn, the cellular levels of hydrogen peroxide are closely monitored by catalase (CAT). Glutathione peroxidase (GPX) utilizes reduced glutathione to detoxify peroxide and hydroperoxide substrates and glutathione reductase (GR) reconverts glutathione disulfide back to the reduced form using NADPH as substrate. Peroxiredoxins (PRDX) reduce and detoxify hydroperoxides in cells in the presence of thioredoxin (TXN) and thioredoxin reductase (TXNRD) restores oxidized TXN to the reduced state. Antioxidant enzymes are shown as blue circles whereas associated antioxidant metabolites are shown as green squares. Arrowheads denote the forward reaction of antioxidant enzymes.

depending on the number of cysteine residues involved in catalyzing the reaction (50). Importantly, the expression of PRDX genes is increased in many pathological states that are characterized by high levels of oxidative stress and muscle atrophy (50, 51). Evidence in rodent models showed decreases in the levels of thioredoxin-1 and peroxiredoxin-3 protein during the late phase of disuse atrophy, suggesting possible culpability in increased oxidative stress (51). HO-1 is an inducible enzyme that is involved in heme degradation and subsequent production of biliverdin which acts as a known antioxidant. This enzyme has been shown to be activated by situations that generate ROS such as anoxia, hypoxia, and ischemia (50). The enzyme AFAR1 is an aldo-keto reductase that is involved in the detoxification of various aldehydes and ketones. ROS also generate reactive aldehydes that inhibit plasma membrane Ca^{2+}-ATPase activity which inhibits removal of calcium ions from the cytosol (4). As a result, intracellular calcium levels climb and stimulate calpain activity and calpain-dependent proteolysis in skeletal muscle (4).

Ubiquitin proteasome pathways

Harmonizing the relative rates of catabolic pathways with anabolic pathways of protein synthesis and growth is central to metabolic regulation in all tissues and, in muscle, controls muscle mass and remodeling. Proteolytic pathways, including the ubiquitin proteasome pathway (UPP), are highly regulated networks partly responsible for muscle catabolism (52). In the simplest of terms, skeletal muscle atrophy is the result of greater activities of catabolic versus anabolic pathways resulting in the net degradation of structural, metabolic, and muscle regulatory proteins (53). For example, the pathology of neuromuscular disorders frequently includes degradation of sarcomeric proteins which ultimately results in a gradual decline in total skeletal muscle fibers and a decrement in contractile capacity (54). Indeed, inhibition of the ubiquitin proteasome system (UPS) seems to have some beneficial effect on muscle mass and metabolism during periods of atrophy (55). Such global indiscriminate control is not the only important regulatory mechanism of the proteasome system, however. More targeted regulatory mechanisms are also important to muscle health. For example, degradation of calcineurin, a common marker of pathological cardiac hypertrophy, may be specifically targeted by MAFbx ubiquitin ligases thereby partly diminishing cardiomyocyte growth (56). Another level of regulation involves control by the ubiquitin proteosomal network in the regulation of TFs. An ever-growing number of cardiac and skeletal muscle specific TFs may be regulated through post-translational modifications such as ubiquitination (57). As such, by tagging proteins with ubiquitin to mark them for destruction, the cell can modify the amount and types of regulatory proteins, such as TFs, that are crucial to the up/down regulation of genes.

The UPS is conserved throughout eukaryotes with a similar modification system in prokaryotes (58). At its base, the regulated pathway consists of a sequential enzyme cascade of three ubiquitin ligases E1, E2, and E3 (Figure 3). Firstly, a ubiquitin-activation enzyme (E1) utilizes energy from ATP hydrolysis to generate a thioester bond between the carboxy-terminal glycine residue of ubiquitin and a cysteine residue within the enzyme itself (58). This activated ubiquitin is then successively transferred to the active cysteine residue of E2 (ubiquitin-conjugating enzyme) and finally to the target protein. The final step requires one of the many E3 ubiquitin ligases which interacts with E2 and transfers ubiquitin to the ε-amino group of a lysine residue of the target protein (59). The initial ubiquitin then serves as an acceptor for repeated cycles of ubiquitination ultimately generating a target which is poly-ubiquitinated. The ubiquitinated proteins are then shuttled to a multi-subunit, barrel-shaped cellular protease containing chymotrypsin-like, trypsin-like, and caspase-like activities. This 26S proteasome contains a 19S gated entrance which first recognizes targeted proteins and subsequently degrades them in the 20S chamber (59). Depending on the topology of the growing ubiquitinated chain, different cellular fates have been established (58). For example, chains linked through ubiquitin Lys48 are associated with localization to the proteasome whereas chains linked through Lys63 are not generally thought to be selected for degradation (58, 60). Similarly, evidence suggests that monoubiquitination may serve directed functions which do not include the canonical proteasomal fate.

Two critical genes play a pivotal role in the control of the UPP: MAFbx and MuRF1. Multiple lines of evidence have indicated that MuRF1 is significantly upregulated under atrophy conditions [reviewed in (61)]. For example, combined MAFbx/MuRF1 null mice (MAFbx -/-; MuRF1-/-) show significantly lower loss of muscle mass under denervation-induced muscle atrophy (62). As an E3 ubiquitin ligase, MuRF1 has been shown to bind to the myofibrillar protein titin at the M line, myosin heavy chain, myosin light chain1/2, and myosin binding protein C, implicating these sarcomeric proteins as MuRF1 substrates (62–64). Similarly, substrates of MAFbx, an F-box containing E3 ligase, include creatine kinase, myogenic differentiation (MyoD), myogenin, and the translation initiation factor eIF3f (65–68). According to their targeted substrates it

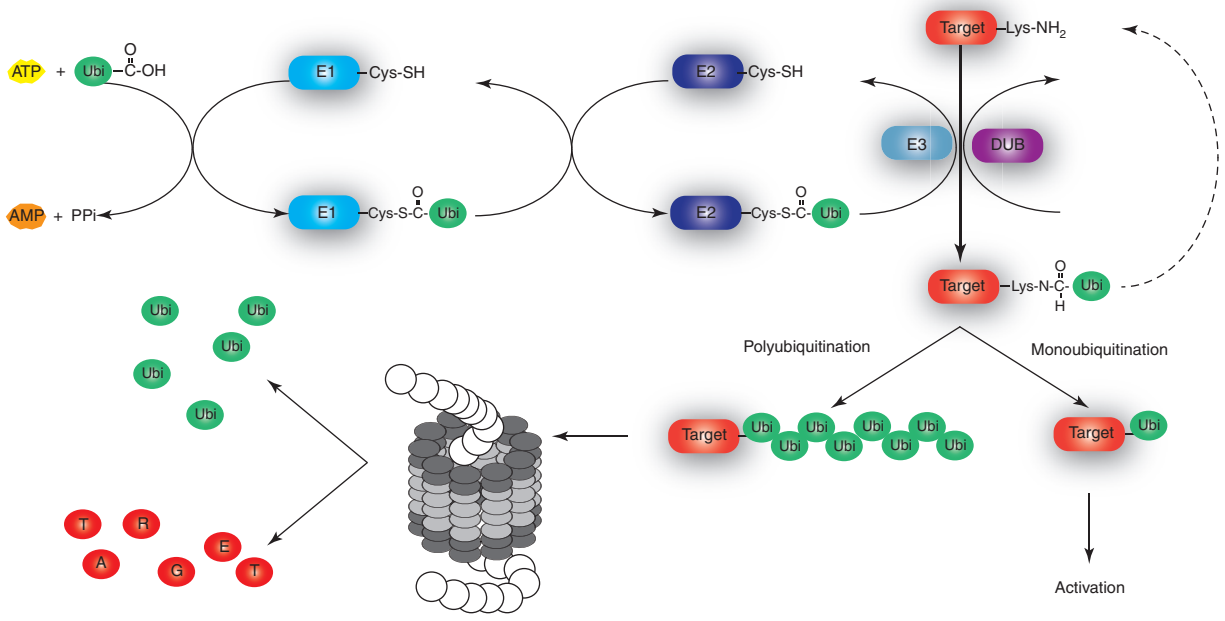

Figure 3: The ubiquitin proteasome system (UPS) is a highly regulated network which plays an important role in muscle catabolism. Conjugation of ubiquitin (Ubi) to substrate proteins involves three classes of enzymes: ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligases (E3). The cleavage of ubiquitin from proteins and other molecules is catalyzed by deubiquitinating enzymes (DUB). Ubi conjugation may occur in different modes (poly-Ubi or mono-Ubi) and a subset is recognized by the 26S proteasome. Modified from ref. (58).

appears that MAFbx primarily controls muscle mass and metabolism whereas MuRF1 may be more critically involved with muscle remodeling. In cardiac muscle, during conditions of stress, ischemia/reperfusion, and/or hypertrophy the activation of 5′ AMP-activated protein kinase (AMPK), which is intimately involved in energy homeostasis, results in the enhanced expression of ubiquitin ligases such as MAFBx/Atrogin-1 (69). This response, in turn, controls MAFBx/Atrogin-1 and MuRF1 which act downstream on key cardiac TFs that control cardiomyocyte mass and remodeling (69).

Autophagy

While the ubiquitin proteasome controls the majority of myofibrillar and short-lived proteins, the lysosomal machinery is thought to control long-lived proteins and cellular organelles (i.e. macroautophagy) [reviewed by 70, 71]. Autophagy is a tightly regulated, catabolic process which plays an essential role in cellular homeostasis (72). The mechanism involves targeting a region of the cell, separating its contents from the rest of cytoplasm via the formation of an autophagosome, transport to lysosomes, degradation, and utilization of the recycled products. The extent of cellular degradation must be balanced between too much or too little destruction, both of which would be harmful for the cell. In response to environmental cues, such as starvation, low oxygen, osmotic stress, and other intracellular stresses (e.g. damaged organelles, mutant proteins, microbial invasion) an enhanced autophagic response is thought to regenerate necessary amino acids and substrates for intermediary metabolism by recycling existing cellular compounds (9). These recycled compounds may then be utilized by other pathways until the stress passes. For example, amino acids may be turned into glucose through the glucose-alanine cycle, used directly as an energy source through the tricarboxylic acid cycle (TCA), or to synthesize proteins which are important for adaptation to the stressful environment (72). Moreover, lysosomal lipases have been shown to contribute to intracellular lipolysis through the macroautophagy pathway (71). While the process is not completely understood, lipid droplets are commonly sequestered by the autophagosomes and may even be favored during prolonged starvation (71).

The key players involved in the autophagy pathway are highlighted in Figure 4 and thoroughly described in select reviews (72, 73). The process of autophagosome formation is highly conserved from yeast to mammals and involves specific autophagy-related genes (Atg). Briefly, Beclin1 forms important protein-protein interactions

which together form a class III phosphoinositide 3-kinase complex (PtdIns3K complex) and results in the formation of the pre-autophagosomal membrane. These protein-protein interactions include positive regulators such as UV radiation resistance-associated gene protein (UVRAG), Atg14 (autophagy-related protein 14), vacuolar protein sorting 34 (Vps34), p150, Bax-interacting factor 1 (Bif-1), and activating molecule in Beclin1-regulated autophagy

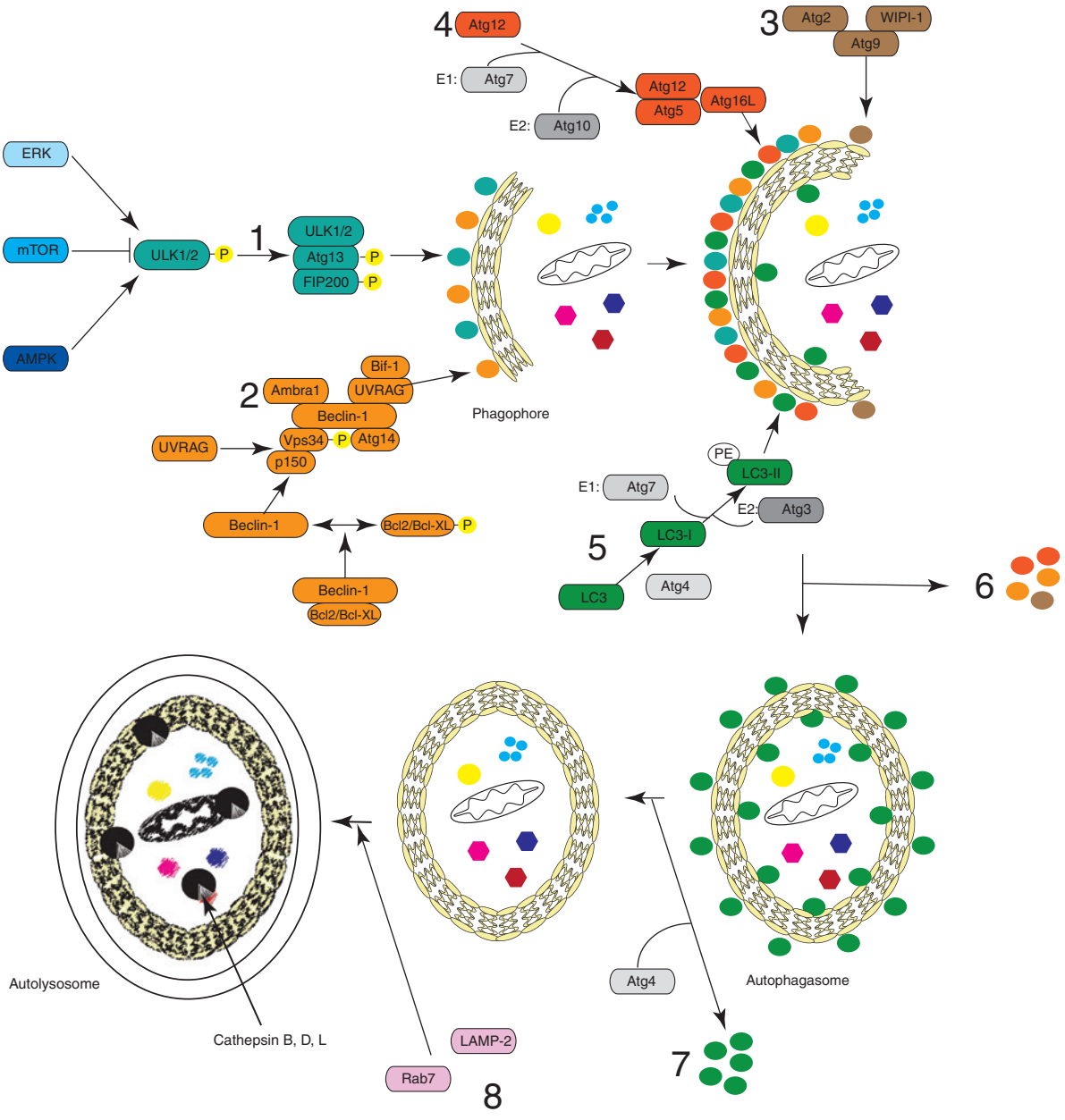

Figure 4: Autophagy is a tightly regulated, catabolic process which plays an essential role in cellular homeostasis and muscle health. Beclin1 forms important interactions with several proteins: UVRAG (UV radiation resistance-associated gene protein); Atg14 (autophagy-related protein 14); Vps34 (vacuolar protein sorting 34); p150; Bif-1 (Bax-interacting factor 1); Ambra1 (activating molecule in Beclin1-regulated autophagy). Together these form a class III phosphoinositide 3-kinase complex (PtdIns3K complex) and results in the formation of the pre-autophagosomal membrane. The formation of the autophagosomal membrane is mediated by the formation of the Atg12-Atg5 complex (via Atg7 and Atg10) followed by binding to Atg16L. The processing of LC3 (microtubule-associated protein 1A/1B-light chain 3) to LC3-I (by Atg4), LC3-I to LC3-II (by Atg7 and Atg3), and the conjugation of LC3-II with phosphatidylethanolamine (PE) allows binding to the growing membrane. Atg9 plays a role as a carrier supplying membrane to the growing autophagosome.

(Ambra1) as well as negative regulators such as Rubicon, B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma-extra large (Bcl-XL) (73–75). The formation of the autophagosomal membrane is mediated by the formation of the Atg12-Atg5 complex (via Atg7 and Atg10) followed by binding to Atg16L (76). In addition, the processing of LC3 to LC3-I (by Atg4), LC3-I to LC3-II (by Atg7 and Atg3), and the conjugation of LC3-II with phosphatidylethanolamine (PE) allows binding to the growing membrane (77). A final component, Atg9, plays a role as a carrier supplying membrane to the growing autophagosome. Common markers used to measure the level of autophagy in mammalian cells include Beclin1, Atg12, Atg5, LC3, and Atg9 (77).

While altered autophagy has been observed in various myopathies, the direct implication of a mismanaged autophagy network remains elusive (78, 79). Recent insight into its involvement in regulating muscle mass and health have uncovered some of these enigmas. In atrophying muscle, a knock-down of LC3 (called Atg8 in lower eukaryotes) using RNA interference (RNAi) has been shown to partially prevent muscle loss (70). Conversely, deletion of Atg7 in mouse muscle resulted in profound atrophy and a decrease in force (78). In addition, when Atg7 null mice were subjected to inducers of muscle atrophy (e.g. fasting and denervation) both groups demonstrated enhanced expression of atrophy-related genes (e.g. MuRF1) as compared to controls (78). These results suggest that basal levels of autophagy are beneficial in regulating muscle mass and that either over or under activity of autophagy may be harmful to the cell. A similar principle is seen in cardiomyocytes whereby the down regulation of protein turnover can lead to the promotion of endoplasmic reticulum stress, apoptosis, and cardiac dysfunction (79) whereas enhanced autophagy may help meet the metabolic challenges associated with the stressful state (71). Perhaps having a compensatory function, autophagy in the heart is upregulated in response to stress (e.g. ischemia/ reperfusion injury, cardiac hypertrophy, and heart failure) (80). RNAi mediated knockdown of Beclin 1 (called Vps30/Atg6 in lower eukaryotes) and Atg5 was capable of attenuating pathological cardiac hypertrophy (81). Conversely, complete abrogation of Atg5 in adult animals resulted in disorganization of the sarcomere, aggregation of mitochondria, and contractile dysfunction (79). In general, autophagy leads to improved cellular fitness by balancing synthesis, degradation and recycling of cellular components when new nutrients run low. However, when the stress is severe autophagy may enhance pro-apoptotic signals leading to maladaptive changes in the autophagy network (82).

Transcription factor regulation

TFs regulate the expression of a group of genes/proteins that serve common cell functions and, as a result, identification of the regulatory mechanisms available to muscle-specific TFs holds immense insight into their important cell functions as it pertains to muscle health. There are many mechanisms which regulate TFs, many of which are just beginning to be uncovered. Post-translational modification (PTM) is one such mechanism (83). The majority of PTMs are readily reversible and do not require a large input of ATP to accomplish. Thus, cells may modify the intracellular environment in a very cost effective manner and ensure quick transitions under changing environmental conditions. While protein phosphorylation plays a pivotal role in TF regulation, other understudied PTMs, such as acetylation, methylation, sumoylation, ubiquitination, etc., are recently proving to play important regulatory roles. Over 80 TF(and counting) have now been shown to possess at least one of these PTMs, aside from phosphorylation, demonstrating the importance of these regulatory mechanisms in the cell (84). In addition, at any given time, a transcription factor may exhibit multiple PTMs, ever expanding the range of cellular control. PTM modification of TFs may [1] stimulate localization of TFs to the nucleus or other cellular compartments, [2] increase/decrease binding capacity to DNA, [3] increase/decrease association with accessory proteins which aid in regulation, and [4] increase/decrease the stability of the TF/protein complex. The role of an individual PTM in TF regulation can often be delineated by the location of the PTM within functional domains. For example, acetylation of FoxO within the conserved DNA-binding domain is likely to alter the DNA binding capacity of the TF to target sequences within the nucleus (85).

Another TF regulatory layer in addition to PTMs are protein-protein interactions. Similar to PTMs, protein-protein interactions are a cost-effective mechanism of altering the activity of a given TF (provided the binding partner need not be synthesized). In addition, they are easily reversible and highly effective, often acting as a virtual on-off switch. TFs may be negatively regulated (decreased transcriptional capacity) or positively regulated (increased transcription) depending on their binding partner. The majority of TFs are now known to function in complexes rather than individually (85–87). This not only extends their range of cellular functions but also means that there is an added dimension of cross-talk between pathways. Protein-protein interactions may have similar effects on TF regulation as PTMs albeit in a slightly different mechanism. For example, protein-protein interactions may [1] block the nuclear export/

import signal to affect movement between the cytosol and the nucleus, [2] direct the expression of only a subset of genes under the control of a particular TF, [3] increase/decrease the efficiency of DNA binding, and/or [4] act as a docking station for PTMs. It is apparent that protein-protein interactions and PTMs are not mutually exclusive but often may either promote or inhibit one another.

Nrf2

The expression of antioxidant enzymes at the optimal time, concentration and in the optimal cellular compartment is the key to the maintenance of redox homeostasis. Regulation of antioxidant genes is under the primary control of redox-sensitive TFs which rescue cells by upregulation of key antioxidant enzymes (88, 89). One such redox-sensitive TF is nuclear factor (erythroid-derived 2)-like 2 (Nrf2) also known as NFE2L2. Nrf2 is a basic leucine zipper transcription factor responsible for regulating phase II antioxidant enzymes (90). Studies with Nrf2 knockout mice showed that expression levels of important detoxifying enzymes, such as CAT, SOD, GST, HO-1, and others, is strongly reduced implicating Nrf2 as a central regulator of the antioxidant defense system in mammals (88). Nrf2 binds to the cytoplasmic repressor Kelch-like ECH-associated protein 1 (Keap1), a component of the Cul3-Keap1 ubiquitin ligase complex (91). The high affinity of Nrf2 for Keap1 under normal conditions means that Nrf2 is a relatively short-lived TF which is readily polyubiquitinated and subsequently degraded by the 26S proteasome (91). Under conditions of oxidative stress Nrf2 is phosphorylated at S40 thereby releasing Nrf2 from its negative regulator and allows it to move into the nucleus (92). Another mechanism for Nrf2-KEAP1 dissociation involves the modification of reactive cysteine residues of Keap1, leading to a decline in E3 ligase activity and aggravation of the Nrf2 docking site (91).

Once released from Keap1, Nrf2 may dimerize with other basic leucine zipper proteins such as small Mafs (sMafF, sMafG, sMafK; small musculoaponeurotic fibrosarcoma), JunD, activating transcription factor-4 (ATF4), and polyamine-modulated factor-1 protein (PMF-1) (90). This activates Nrf and promotes binding to the antioxidant response element (ARE) that is present in the promoter region of genes that respond to oxidative stress (90). In addition, PTMs such as acetylation by CREB-binding protein (CBP) increase transcription of Nrf2 while sirtuin 1 (SIRT1) antagonizes this interaction by deacetylating and thereby reducing DNA binding/nuclear retention (93, 94). Other inhibiting signals also play a role in Nrf2-ARE mediated transcription such as the NFκB subunit p65 (95). In cells that co-expressed the p65 subunit and Nrf2, the transcriptional activity of Nrf2 was inhibited and correlated with a strong suppression of HO-1 (95). In this mechanism a phosphorylated p65 (S276) competes with CBP for binding to Nrf2 thereby reducing Nrf2 interaction with its transcriptional coactivator (95). In addition, it was shown that p65 can promote the recruitment of histone deacetylase 3 (HDAC3) to the ARE by facilitating a protein-protein interaction with either CBP or MafK (95). HDAC3 causes histone modifications close to the ARE and thus ensures the positive charge on histone tails forms a tight association with the negatively charged phosphate group on the DNA backbone (95).

Nrf2 has received much attention for its role in the regulation of crucial antioxidant genes as it pertains to the maintenance of skeletal and cardiac muscle health. Within the heart, oxidative stress inducers are thought to influence pathological progression toward cardiac dysfunction and disease (96). Indeed, Nrf2 downstream genes/enzymes such as SOD (97), HO-1 (98), and GPX (51) have been shown to play a critical role in maladaptive cardiac remodeling and hypertrophy (99). In a mouse model of chronic transverse aortic constriction, Nrf2 null mice developed greater cardiac dysfunction resulting from increased cardiac hypertrophy and interstitial fibrosis (99). Another study showed that persistent increases in ROS levels and a decrease in the activity of Nrf2-regulated antioxidant enzymes were associated with failing human hearts (100). Nrf2 also plays a role in skeletal muscle disuse atrophy, although it is not clear whether redox imbalances are a cause or simply a consequence of muscle disuse. Narasimhan et al. (101) discovered decreased stem cell population and MyoD (a muscle-specific transcription factor) expression which were matched with the activation of ubiquitin and apoptotic pathways in Nrf2-null vs. wild-type mice upon acute endurance exercise stress.

MEF2

The myocyte enhancer factor-2 (MEF2) family of TFs has recently attracted much interest and focus in the field as a vital regulator of muscle plasticity (102). MEF2 TFs are extremely dynamic, imposing positive controls on a wide range of muscle proteins and remodeling muscle to meet physiological demands (102). Four isoforms of MEF2 (MEF2A, -B, -C, -D) are known in mammals and all are regulated by reversible phosphorylation (102). MyoD, Myf5, myogenin, and myogenic regulatory factor 4 (MRF4) are all members of a family of proteins known as myogenic

regulatory factors (MRFs). MRFs are part of the basic helix-loop-helix (bHLH) family of TFs which bind to a DNA consensus sequence called an E box (CANNTG) (103). MEF2 and MRFs interact directly, as evidenced by the close proximity of MEF2 DNA binding sites and E boxes in the promoter and enhancer regions of muscle specific genes (102). Several studies suggest that MEF2 factors function as essential myogenic coregulators, alongside the bHLH proteins, to initiate myogenesis via a combinatorial mechanism (104, 105). For example, a synergy between MEF2 and MRF4 has been shown to be a critical regulator of the gene expression of desmin IF (106). In addition, a critical response which depends on MEF2 and MyoD synergy is a documented phenomenon known as MyHC restructuring (107). MEF2 and MyoD have been shown to act synergistically to promote MyHC profile transitions and improve aerobic capacity through a preference of type I versus type II myofibers (102). While MEF2 association with MRFs in skeletal muscle is well established, this synergy may be more important in skeletal muscle rather than in cardiomyocytes. In cardiac cells MEF2 and GATA4 act synergistically to activate the transcription of several cardiac genes such as atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), α-MyHC, and cardiac α-actin indicating a significant cooperative role of MEF2 and GATA4 in the transcription of these cardiac genes (108).

MEF2 has also been implicated in calcium signaling forming important protein-protein interactions with Nuclear factor of activated T-cells (NFAT) TF and being phosphorylated by Ca^{2+}/CaM-dependent protein kinases (CAMKs). Similar to the MEF2-MyoD association, other studies have shown that inhibition of calcineurin activity also promotes slow-to-fast fiber transformation via a MEF2-NFAT synergy (109). Increased intracellular calcium activates calcium binding proteins such as calmodulin (CaM) which, in turn, regulate calcineurin and CaMKs (110). CaMK mediates MEF2-dependent gene transcription through the phosphorylation of the class II histone deacetylase (HDAC) transcriptional repressors (HDAC-4, -5, -7, -9) which physically associate with MEF2 to repress transcription (111). Phosphorylation of HDACs by CaMKs results in recruitment of 14-3-3 protein which dissociates HDAC-MEF2 complexes and sequesters them from the nucleus. In turn, this promotes MEF2 association with p300/CBP and NFAT (112). Additionally, calcium-calmodulin protein kinase IV has been shown to directly phosphorylate MEF2 acting as a potent inducer of activity (113). MEF2 also acts as a coregulator for the peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α) which together regulates genes/proteins involved in glucose homeostasis and oxidative metabolism (114). Using a rodent model of atrophy, PGC-1α protein and mRNA were decreased, expression of calcineurin (Cn) was depressed, and MEF2 and NFATc activity was reduced (114).

Above and beyond the capacity for MEF2 to act as a pivotal coregulator, these TFs are also regulated by multiple post-translational events. For example, phosphorylation by casein kinase II (CKII) within the MADS-box domain enhances MEF2A transcriptional activity, likely by inducing a conformational change which enhances MEF2A contact with DNA (115). Multiple MAPK phosphorylation sites exist on both MEF2A and MEF2C. Phosphorylation of three amino acids in the C-terminal domain of MEF2C by p38 (116) and ERK5 phosphorylation at Ser-387 in the transactivation domain of MEF2C enhance transcriptional capacity (117). Similarly, MEF2A has been shown to contain multiple p38 phosphorylation sites such as S89, S192, S289, T312, T319 as well as ERK5 phosphorylation at S255 (118). In other studies, the p38 binding site was traced to the transactivation domain (119), while the ERK5 binding site was located at the N-terminal domain spanning somewhere between amino acid 1–87 (120). Acetylation of MEF2 also plays a regulatory role in transcription factor-DNA binding. Ma et al., 2005 demonstrated that MEF2C is acetylated by p300 at six lysine sites and this acetylation is antagonized by class II HDACs. Other acetylation sites (Lys 4) have also been reported by Angelelli et al. (121) which correlate with increased DNA-binding. It has been proposed that acetylation of MEF2C may promote myogenin-MEF2 protein-protein interactions and thus selectively upregulate genes under their cooperative control (122). Sumoylation has also been shown to regulate MEF2 via a priming step which requires phosphorylation of MEF2C at S396. This phosphorylation event enhances the sumoylation of K391 which presumably inhibits MEF2-dependent transcription by the recruitment of corepressors (119).

MEF2 TFs have consistently been shown to act as key transcriptional regulators for cardiac hypertrophy [(123); reviewed by 6, 8]. For example, MEF2 DNA-binding activity is increased in the hearts of rats subjected to pressure and/or volume overload (124). In addition, upstream signals such as the activation of p38 MAPK induces hypertrophic growth in cultured cardiomyocytes and p38 MAPK phosphorylates MEF2 in the hypertrophied heart (125). Similarly, ERK5 is activated under hypertrophy-stimulating conditions such as oxidative and osmotic stress in cultured cardiomyocytes (126). Calcium signaling is also pivotal to the hypertrophic response. Activation of either calcineurin (127) or CaMKs induces cardiac hypertrophy and CaMKIV stimulates MEF2 activity (110).

FOXO

Expression of FoxO3 is sufficient as well as necessary to activate proteins responsible for lysosomal-dependent protein breakdown through the expression of genes involved in the core machinery (e.g. Beclin 1, LC3, Atg12) (70, 128). The FOXO family of TFs are also pivotal in the regulation of ubiquitin ligases, MAFbx and MuRF, which play a critical role in muscle wasting. At the core of FoxO regulatory mechanisms lies the shuttling of these TF to the cytosol (hence decreasing transcriptional capacitance) versus their localization to the nucleus (to increase transcription) (128). The main regulator of FoxO function is the phosphoinositide 3-kinase (PI3K) pathway and its downstream mediator Akt (also called PKB) which is a serine/threonine protein kinase (53). FoxO1 and FoxO3 contain three highly conserved PKB recognition motifs (129). Akt phosphorylation within the forkhead domain (S253 for FoxO1; S252 for FoxO3) disrupts DNA-binding as well as the nuclear localization signal (NLS) (128). This first phosphorylation site also enhances the accessibility of the N- and C-terminal PKB sites which may be phosphorylated by either Akt or SGK (128, 130). Priming FoxO TF with multiple phosphorylation sites results in high affinity binding of 14-3-3 protein as well as other binding partners, such as Ran and CRM1, leading to transport through the nuclear pore to the cytosol (131). Once in the cytosol the E3 ligase, Skp2, binds directly to FoxO1 promoting its ubiquitination and subsequent degradation (132). Similarly, FoxO3 phosphorylation by ERK leads to its degradation via an MDM2-mediated UPP (133). Proteolysis may also be controlled by an Akt-independent mechanism involving IκK (a central regulator of NFκB). IκK phosphorylates FoxO3 through a physical interaction targeting the transcription factor for degradation by the ubiquitin-dependent proteasome pathway (134). Above and beyond phosphorylation and ubiquitination, FoxO TFs are also regulated by acetylation, however, the direct implications of this are an area of debate (85, 135). For example, evidence suggest that acetylated FoxO1 is more readily phosphorylated by Akt (85) whereas others have demonstrated enhanced transactivation of target genes via p300/CBP-mediated acetylation (135). Perhaps isolated acetylation sites have the capacity to alter activity differently.

FoxO1 and FoxO3 are also regulated in the inverse direction to positively influence transcription. Dephosphorylation of FoxO by the protein phosphatase 2A or phosphorylation by mammalian Ste20-like kinase (MST1) results in dissociation of 14-3-3 protein from FoxO TFs (136, 137). Similarly, FoxO nuclear retention may also be achieved by protein arginine N-methyltransferase 1 (PRMT1) methylation (138). JNK phosphorylation may also antagonize Akt signaling, however, this effect is mediated via phosphorylation of 14-3-3, rather than FoxO, to release its inhibitory affects (139). Importantly, phosphorylation and protein-protein interactions allow FoxO TFs to direct the transcription of a subgroup of the genes they influence. For example, phosphorylation of FoxO3 at six sites by AMPK, activates transcription of genes involved in autophagy (60). In the same capacity, glycosylation of both FoxO1/3 results in upregulation of gluconeogenic genes (140). Moreover, deacetylation and protein-protein interactions by sirtuins (NAD-dependent class III HDACs) at the LXXLL motif increases the ability to induce cell cycle arrest genes but inhibits cell death (60). Other sirtuins also play a role in FoxO regulation. For example, FoxO1 once dissociated from Sirt2 becomes acetylated, binds to Atg7 and influences autophagy in a transcription-independent manner (141). Furthermore, Sirt3 plays a role in decreasing cellular levels of ROS and blocking cardiac hypertrophy through a physical interaction that activates FoxO3 (142).

FOXO TFs play a critical role in striated muscle health. For example, Sandri et al., 2004 showed FOXO TFs were activated in cultured myotubes undergoing atrophy and this was associated with MAFbx (atrogin-1) induction. Additionally, others showed that inhibition of FoxO-DNA binding in the muscles of mice inhibited increased mRNA levels of MAFbx, MuRF1, cathepsin L, and/or Bnip3, resulting in the inhibition of muscle fiber atrophy during cancer cachexia and sepsis (143). Some have suggested FoxO1 as the main MAFbx regulator (144), while Foxo3a has also shown to be a critical regulator (145). In the same capacity, FoxO3 and many autophagy-related genes show coordinated expression/activation in mouse muscle atrophy caused by denervation or fasting (68). In cardiac muscle, overexpression of FoxO1 in cultured cardiomyocytes significantly reduced cell size and this effect was attenuated upon inhibition of autophagy (146). It is also thought that FOXOs reduce cardiac hypertrophy through the inhibition of the calcineurin/NFAT pathway, which is a critical contributor to cardiac hypertrophy (147).

Expert opinion: mammalian hibernation and muscle plasticity

Mammalian hibernation

A common feature of organismal response to severe environmental stress is metabolic rate depression, entry into

a hypometabolic state that minimizes energy expenditures and preserves life until resources are replenished. The molecular mechanisms of hypometabolism include global suppression of energy-expensive cell functions (e.g. protein synthesis, gene transcription, ATP-dependent ion pumps) and reprioritization of ATP use by vital cell functions, including enhanced expression of multiple preservation mechanisms (e.g. antioxidants, chaperones) that protect and stabilize cellular macromolecules (89, 148, 149). Common biochemical themes amongst organisms facing chronic food shortages include: [1] reversible protein phosphorylation of enzymes/proteins as a critical mechanism that coordinates the suppression of metabolic processes (148, 150–152), [2] the widespread enhancement of antioxidant defenses as a means of viability extension in the hypometabolic state and of providing defense against a rapid production of ROS when animals returned to an active state (89, 153, 154), [3] the selected upregulation of genes and proteins when animals enter hypometabolism and stress-responsive TFs, including multiple studies of hibernation (50, 155–159), [4] global controls on gene suppression (e.g. histone modification) [(160); reviewed in (161)] and mRNA translation via differential expression of microRNAs (162, 163), that all contribute to resculpting metabolism for long term survival in a hypometabolic state.

For small mammals, long-term hibernation can conserve huge amounts of energy that would otherwise be needed to maintain active euthermic life and a body temperature (T_b) of ~37°C under cold winter conditions where food availability is greatly reduced (164). By letting T_b cool to ambient (sometimes as low as 0–5°C) and strongly suppressing energy-expensive cell functions, metabolic rate can often be reduced to <5% of normal resting values in euthermia. Hibernation consists of repeated long periods of deep torpor (T_b falls to <5°C) followed by short periods of arousal where the animal returns to euthermic T_b (~37°C; Figure 5) (89, 164). Furthermore, hibernators must deal with major changes in organ perfusion rates (<10% of normal) and respiration rates (~2.5% of normal) which have made these animals interesting models for ischemia research (149, 165). In anticipation for the winter season, ground squirrels enter a phase of hyperphagia where excessive eating results in weight gains of up to 40% (89, 166). This is matched with a sharp shift in their metabolic profile which moves from a heavy reliance on carbohydrate catabolism to dependence on β-oxidation of lipids (167). The central nervous system also requires distinct adaptations where overall activity is reduced with only specific regions of the brain demonstrating active neuronal firing (168).

Striated muscle plasticity of the hibernating ground squirrel

Using ground squirrels (e.g. thirteen-lined, Richardson's, and golden-mantled ground squirrels) as model organisms, studies in our lab and others have examined many aspects of hypometabolism in hibernators [for review see refs. (148), (169–171)]. While there are many important molecular controls which ensure safe transitions between periods of torpor and euthermia, we will focus here on recent discoveries in the area of striated muscle plasticity and remodeling during hibernation. During hibernation, ground squirrel cardiac muscle becomes hypertrophic and this is linked to the need to pump cold, viscous blood whereas skeletal muscle must resist atrophy under extended periods of disuse (172). Importantly, ground squirrels represent a natural model system that displays physiological changes in striated muscle that do not directly lead to pathological conditions despite

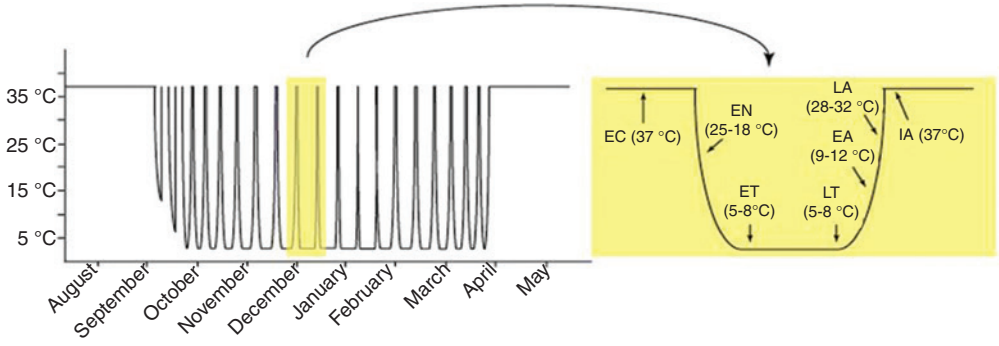

Figure 5: Body temperature (T_b) of a thirteen-lined ground squirrel as a function of time over one hibernation season. Highlighted in yellow is one torpor-arousal cycle. The different stages are: (1) euthermic, cold room (EC), (2) entrance (EN), (3) early torpor (ET), (4) late torpor (LT), (5) early arousal (EA), and (6) interbout arousal (IA). Modified from ref. (201).

enduring stresses known to stimulate these conditions in non-hibernators. Specifically, hibernating mammals experience minimal loss of muscle mass despite long periods of inactivity (107, 173–176) and hibernator hearts display physiological contraction-relaxation cycles and maintain a homeostatic intracellular calcium environment despite deep hypothermia and hypertrophy (177–180). As such, hibernating ground squirrels are a powerful model system to explore the genetic and regulatory mechanisms that are crucial to the atrophy/hypertrophy response and remodeling as they demonstrate improved natural muscle plasticity.

While complete cellular reorganization is not conducive to conserving energy during hypometabolism, myocytes strategically alter key pathways and reprioritize cellular demands depending on the particular needs of the organism. For example, the functional and structural parameters of skeletal and cardiac muscle rely on a balance between those pathways which are activated to protect the cell from damage [e.g. initiating antioxidant defenses (181–184), selectively altering muscle structural proteins (107, 173–175, 177, 178, 185)] and in contrast, modulating signals which promote the degradation of cellular machinery [e.g. the regulation of proteosomal networks and autophagy (186–188)]. TFs have been shown to play an important role in metabolic reorganization to support the hypometabolic state because they regulate suites of downstream genes to coordinate specific modifications to cell structure/functions. During hibernation, TFs have been shown to play a prominent role in skeletal and cardiac muscle in each of these themes including antioxidant defenses, regulation of sarcomeric/structural proteins, proteosomal networks and autophagy, each of which is described in greater detail in the following sections (178, 181–183, 185, 187–190).

Antioxidant defenses during mammalian hibernation

Adaptation of antioxidant defenses is widespread as a response to physiological or environmental challenges. Hibernating mammals show a strong reduction of heart rate and blood flow in deep torpor which can last for weeks at a time (89). These conditions would undeniably be considered highly ischemic for non-hibernating mammals. Additionally, in order to rapidly elevate core body temperature by 30°C or more during arousal, skeletal muscle must undergo the metabolically demanding process of shivering thermogenesis whereby major increases in oxygen levels and oxygen consumption occur over a time frame of minutes to hours (89). The natural cycle of torpor-arousal; therefore, parallels other well-known systems, such as heart attack and stroke, where ischemia-reperfusion cycles can lead to damaging levels of oxidative stress. In addition, another factor that may lead to the generation of ROS is the altered composition of lipid reserves in the hibernator. In preparation for hibernation, body fat depots are adjusted to contain high levels of polyunsaturated fatty acids (PUFAs) that keep fat depots more fluid so that they can be metabolized at low T_b. PUFAs are highly susceptible to free radical attack leading to autoxidation and the generation of lipid peroxide radicals (181). The antioxidant response in hibernators, therefore, must have improved regulatory mechanisms to combat the deleterious effects of oxidative damage during long periods of inactivity, especially when replacement of damaged macromolecules is not an option.

Hibernation-responsive changes in antioxidant enzymes and the role of the Nrf2 transcription factor have been studied in thirteen-lined ground squirrel skeletal muscle, heart, and other tissues (181, 182). In the heart, Nrf2 relative expression was elevated by 1.4–1.5 fold during entry, long term torpor, and early arousal as compared to euthermic controls (181) and the levels of the associated co-activator MafG were also enhanced (182). Additionally, the protein levels of Nrf2 downstream target genes Cu/Zn SOD, AFAR, and HO-1 increased significantly during entry into torpor and then gradually declined as animals aroused from torpor. Further to this, *Nrf2* sequenced from cardiac tissue revealed high identity with sequences from other mammals, although select substitutions were detected. The authors suggested that amino acid substitutions might be important for conformational stability of the protein at T_b values of 0°C in the torpid state – a concept which may extend to other proteins critical to cardiac health during hibernation. Overall, these data suggest a role for Nrf2 in regulating the antioxidant defenses of cardiomyocytes needed for hibernation success. However, data collected for skeletal muscle in the same study show a different trend. Nrf2 mRNA/protein expression did not change nor did the expression of MafG in skeletal muscle comparing control and deep torpor (181, 182).

One possible explanation for this is other antioxidant regulators are critical for maintaining muscle redox homeostasis during hibernation (such as NFκB) (183). Analysis of the relative expression levels of NFκB revealed activation at select time points throughout the torpor-arousal cycle, possibly providing an alternate mechanism for activation of the antioxidant response in hibernator muscle (183). In the same study, levels of

MnSOD increased significantly during deep torpor and arousal and HO-1 increased during early arousal, as compared to controls (183). Additionally, the expression of SOD1, CAT, and GPX were significantly enhanced comparing control and hibernating European ground squirrels (184). Alternatively, Nrf2 may be more important during the transitory periods (entry and arousal) which were not evaluated or while total protein levels did not change, post-translational modifications or protein-protein interactions may modify Nrf2 activity in muscle during hibernation. While further data is required in order to ascertain the specific effect of post-translational modification on Nrf2 activity, other papers from our lab have begun to describe the general role of acetylation during hibernation by analyzing sirtuins (SIRTs) (191). As described above, Nrf2 DNA binding/nuclear retention is antagonized by SIRT1-mediated deacetylation (93, 94). While SIRT1 relative expression did not change throughout the torpor-arousal cycle in muscle, total SIRT activity did increase, suggesting acetylation may play an important regulatory role during torpor, although no specific link was described between SIRT1 and Nrf2 (191).

Muscle regulatory factors during hibernation

Hibernating mammals are able to alter skeletal/cardiac muscle gene/protein expression and thereby shape the structural apparatus of myocytes to meet rapid oscillations in functional demands (185). One proposed method of adapting to long bouts of inactivity during hibernation is altered expression of MyHC isoforms including greater expression of slow isoforms, which presumably provides a more optimal mix of isoforms to adapt the muscles to the reduced work load and thermal conditions of the torpid state (107, 173–175). This response has been observed in ground squirrels, bears, and prairie dogs which show muscular resistance to inactivity as well as the maintenance of slower MyHC isoforms in select muscles (e.g. soleus, biceps femoris, gastrocnemius, and extensor digitorum longus, and diaphragm) (186, 192–195). While hibernators show relative resilience to changes in muscle size and demonstrate different MyHC isoform profiles in comparison to nonhibernators, there are nonetheless discrepancies in this trend (175). Some muscle groups still show some atrophy, others completely maintain mass, and both can occur with shifts in slower MyHC isoforms (175), suggesting MyHC expression alone is not sufficient to explain atrophy resistance during hibernation. Similarly, MyHC restructuring may also play a role in adapting cardiac muscle to both cold temperature and the contractile demands of the torpid state (155, 177, 196). Different ratios of MyHC isoforms can contribute to the differing contractile properties of cardiac muscles (177). Indeed, hibernating mammals show a preference for α-MyHC proteins in heart during hibernation which confer a higher shortening velocity and greater ATPase activity than β-MHC proteins (177).

While MyHC restructuring/remodeling are central to adapting the muscular apparatus to hibernation-specific conditions, other proteins have also been shown to play a role. In the muscle, glucose transporter isoform 4 (GLUT4) increased during torpor and in heart the protein expression of GLUT4 was elevated over five sampling points (entrance, early and late torpor, early and late arousal) of the torpor-arousal cycle (178, 185). Furthermore, Myomesin 1 protein levels increased during entrance and deep torpor in the heart. Other structural proteins have also been shown to fluctuate during cycles of torpor/arousal including members of the Tn complex and ANP. In this capacity, Luu et al. (189) showed TnI, TnC, and ANP levels increased significantly during entrance, while TnI levels returned to levels comparable to controls during torpor before decreasing during arousal. These data suggest that the transitory phases of torpor and arousal are critical for the regulation of these structural proteins and that these proteins may play a role in the adaptation of the contractile machinery, calcium sensitivity and salt-water balance.

While a case has been made for the importance of structural proteins as they pertain to hibernation, the transcriptional regulation of their genes by TFs has also been intimately described. Indeed, MEF2 has been at the frontline of these studies and evidence suggests its direct importance during hibernation. A recent study evaluated global changes in DNA methylation as a possible mechanism for mediating cycles of torpor-arousal and reported changes in the promoter of the *mef2c* gene in skeletal muscle (197). In this study, a large increase in the methylation state occurred in the *mef2c* promoter during entrance into torpor and this correlated with a reduction in gene expression, providing a possible mechanism for regulating gene expression during hibernation [(197); reviewed in (161)]. Despite decreases in *mef2c* gene expression during entrance, data from our lab shows an elevation of both MEF2A and C total protein expression from entrance into torpor through periods of deep torpor (early hibernation and late hibernation) in skeletal muscle (185), suggesting other regulatory mechanisms must also be involved. Furthermore, enhanced levels of phosphorylation of MEF2A Thr-312 and MEF2C Ser-387 indicated activation of these TFs. Hence, signals that control MEF2 were clearly triggering a two-fold response

– both enhanced MEF2 protein levels and increased phosphorylation (activation) of the proteins (185). In the same study, a coordinated upregulation of MEF2 and MyoD mRNA transcripts during arousal from torpor and a corresponding upregulation of MyoD protein levels in early arousal suggest crosstalk between MEF2 and MyoD. In the heart, MEF2A protein levels increased throughout the torpor-arousal bout and levels of phosphorylated, activated MEF2A (Thr312) correlated with increases in MEF2A-DNA binding (178). MEF2C transcript/protein levels were significantly elevated over controls at selected sampling points whereas phosphorylated/activated MEF2C (Ser387) levels rose during torpor and DNA binding was most prominent during entrance into torpor.

Other MRF TFs have also been studied in the thirteen-lined ground squirrel in the context of understanding the involvement of skeletal muscle satellite cells which are involved in muscle growth and repair (190). Satellite cells are normally in a quiescent state and express the transcription factor Pax7; however, alterations in the environment may result in an exit from quiescence resulting in entrance into the cell cycle and proliferation. In this capacity, Myf5 and MyoD may be expressed during proliferation, with Myf5 playing a particular role in promoting self-renewal of satellite cells and to promote the return of at least one cell to the pool of quiescent satellite cells (198). Using histological analysis, satellite cells were analyzed in the muscle of thirteen-lined ground squirrel whereby statistically significant increases in the percentage of satellite cells were observed during early torpor, but returned to control values during late torpor and arousal (190). While this data suggests satellite cells are not dormant during hibernation and may provide another mechanism to limit muscle atrophy, other studies suggest satellite cells are not involved in protection against muscle atrophy during hibernation. Andreas-Mateos et al. (199) exposed the right hind-limb of hibernating 13-lined ground squirrels to radiation in order to inactive the satellite cell population in the quadriceps muscle. After descending into torpor, morphological and morphometric analyses showed no difference between irradiated and non-irradiated quadriceps (199). Furthermore, elevated levels of the cell cycle inhibitor, p21, was observed in early torpor, possibly linking cell cycle control to the subsequent halting of satellite cell proliferation in deep torpor (190). Similarly, Myf5 showed stable expression protein levels as compared to controls during late torpor with decreased expression during interbout arousal (190), providing another possible regulatory mechanism for satellite cell quiescence throughout the torpor-arousal cycle.

The regulation of proteolysis during hibernation

The regulation of catabolic pathways such as the UPS have been well described in hibernators, while much remains to be learned about autophagy. Metabolic rate depression must include coordinated suppression of both protein synthesis and proteolysis to support survival over the winter months when fuel stores cannot be replenished by feeding and when fuel reserves must be stretched to sustain energy production and selected necessary biosynthesis over many months. In hibernating bats (*Murina leucogaster*), immunoblot analysis revealed the involvement of FOXO TFs, as well as proteolytic proteins, MAFbx/atrogin-1 and MuRF1, in the response to torpor and arousal (187). Comparing summer active animals and deep 3-month hibernation, phospho-FoxO1 levels remained unchanged and MAFbx/MuRF1 remained constant during torpor to arousal transitions (187). The authors suggest that this maintenance of proteolytic capacity, despite dramatic changes in muscle use, may be a key control which counters atrophy in bat muscle (187). Data for MAFbx mRNA in golden-mantled ground squirrels showed slightly different results (186). MAFBx expression was two-fold and four-fold higher during hibernation as compared to active ground squirrels in the gastrocnemius and plantaris, respectively (186). In contrast, there were no observed changes in MAFbx expression in the soleus and diaphragm (186). While a consistent response was not observed, these changes suggest some level of protein degradation still occurs during torpor and the level of degradation differs between specific muscle groups. Nonetheless, the atrophy observed in hibernator muscles represents a reduction in the disuse atrophy commonly seen in other mammalian models, although they may reflect the requirement for shivering thermogenesis.

Eloquent studies using thirteen-lined grounds squirrels have also shown the relationship between hibernation, muscle mass, and proteolysis (188). The authors analyzed the relative phosphorylation state of Foxo3a at serine-253, demonstrating increased phosphorylation (associated with the inactivation of Foxo3a) during hibernation. Additionally, analysis of the relative mRNA expression of ubiquitin ligases and autophagy genes such as MAFbx, MuRF1, and LC3B, revealed no significant increase in expression during hibernation, as compared to summer active animals. While an elevation of ubiquitinated proteins was observed during hibernation, proteasome activity was not increased. This increase in ubiquitination without an associated increase in protesomal activity is very interesting and may suggest that this

post-translational modification serves another function during hibernation or may be linked with lower T_b which would slow rates of proteolytic enzymes. Furthermore, the same study observed increased levels of Sequestosome 1 (p62/SQSTM1) and a decreased ratio of LC3B-II/LC3B-I during hibernation, suggesting suppression of autophagy. In a follow-up paper, the authors point out that although Akt has an established role in skeletal muscle maintenance, activated Akt was decreased in skeletal muscle of hibernating squirrels (176), a finding which our lab has also corroborated (200). Instead, the authors suggest serum- and glucocorticoid-regulated kinase 1 (SGK1) is a central contributor to protection from loss of muscle mass via downregulation of proteolysis and autophagy and an increase in protein synthesis.

Highlights

The biological processes regulating atrophy, hypertrophy, and remodeling are multidimensional and further research is required in order to delineate pathological versus physiological phenotypes. In this review, we discuss recent literature as it pertains to antioxidant defenses, muscle regulatory factors, and proteolytic networks in the regulation of muscle health and function with a particular focus on mammalian hibernators, a model organism with the ability to maintain striated muscle functionality in the face of dramatic physiological changes. An increased understanding of the mechanisms of adaptation and compensation that are available to mammalian muscle will be of central importance in the field of muscle health as it pertains to atrophy, hypertrophy, remodeling and disease.

List of abbreviations

AFAR	aflatoxin aldehyde reductase
ALS	amyotrophic lateral sclerosis
Ambra1	activating molecule in Beclin1-regulated autophagy
AMPK	AMP-activated protein kinase
ANP	atrial natriuretic peptide
ARE	antioxidant response element
ATF4	activating transcription factor-4
Atg	autophagy-related proteins
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
bHLH	basic helix-loop-helix
Bif-1	Bax-interacting factor 1
BMD	Becker muscular dystrophy
BNP	brain natriuretic peptide
CAT	catalase
CaM	calmodulin
CAMK	Ca^{2+}/CaM-dependent protein kinases
CBP	CREB-binding protein
CKII	casein kinase II
CXMD	canine X-linked muscular dystrophy
DGC	dystroglycan complex
DMD	Duchene muscular dystrophy
ECM	extracellular matrix
ERK	extracellular-signal-regulated kinase
GLUT4	glucose transporter isoform 4
GPX	glutathione peroxidise
GR	glutathione reductase
HDAC3	histone deacetylase 3
HO-1	heme oxygenase-1
IF	intermediate filaments
JNK	c-Jun N-terminal kinase
Keap1	kelch-like ECH-associated protein 1
LC3	microtubule-associated protein 1A/1B-light chain 3
MAFbx	muscle atrophy F box/atrogin-1
Mafs	small musculoaponeurotic fibrosarcoma
MEF2	myocyte enhancer factor-2
MRF	myogenic regulatory factor
MST1	mammalian Ste20-like kinase
mTOR	mammalian target of rapamycin
MuRF1	muscle RING finger 1
MyHC	myosin heavy chain
MyoD	myogenic differentiation
NFAT	nuclear factor of activated T-cells
Nrf2	nuclear factor (erythroid-derived 2)-like 2, also known as NFE2L2
PE	phosphatidylethanolamine
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-α
PI3K	phosphoinositide 3-kinase
PKB	protein kinase B (Akt)
PMF-1	polyamine-modulated factor-1 protein
PRDX	peroxiredoxins
PRMT1	protein arginine N-methyltransferase 1
PtdIns3K	phosphoinositide 3-kinase
PTM	post-translational modification
PUFA	polyunsaturated fatty acids
ROS	reactive oxygen species
SIRT1	Sirtuin 1
SOD	superoxide dismutases
TCA	tricarboxylic acid cycle
TF	transcription factors
Tm	tropomyosin
Tn	troponin
TnC	troponin C
TnI	troponin I
TnT	troponin T
TXN	thioredoxin
TXNRD	thioredoxin reductase
UPP	ubiquitin proteasome pathway
UPS	ubiquitin proteasome system
UVRAG	UV radiation resistance-associated gene protein
Vps34	vacuolar protein sorting 34.

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