

## COMMENTARY

### Aestivation: signaling and hypometabolism

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#### Summary

**Aestivation is a survival strategy used by many vertebrates and invertebrates to endure arid environmental conditions. Key features of aestivation include strong metabolic rate suppression, strategies to retain body water, conservation of energy and body fuel reserves, altered nitrogen metabolism, and mechanisms to preserve and stabilize organs, cells and macromolecules over many weeks or months of dormancy. Cell signaling is crucial to achieving both a hypometabolic state and reorganizing multiple metabolic pathways to optimize long-term viability during aestivation. This commentary examines the current knowledge about cell signaling pathways that participate in regulating aestivation, including signaling cascades mediated by the AMP-activated kinase, Akt, ERK, and FoxO1.**

Key words: antioxidant defense, metabolic rate depression, regulation of gene expression, reversible protein phosphorylation, signal transduction pathway, signaling cascade.

#### Introduction

Aestivation is typically defined as a summer or dry season dormancy. The word derives from the Latin for summer (*aestas*) or heat (*aestus*). Arid conditions that restrict water and food availability are the common trigger for aestivation, often but not always accompanied by hot summer temperatures. Aestivation is an ancient trait. The fossil record gives evidence of structures indicative of aestivation, including Pleistocene earthworm chambers, Devonian to Cretaceous lungfish burrows and Permian lysorophid amphibian burrows going back several hundred million years (Hembree, 2010). Indeed, the phenomenon is undoubtedly much older given the widespread use of hypometabolism (diapause, dauer, etc.) by some of the oldest metazoan life forms (e.g. sponges and nematodes) (Hu, 2007; Loomis, 2010).

Spurred on by ‘selfish genes’, all organisms are driven to grow, develop and reproduce. The primary inputs needed for this are water (the solvent of life), nutrients (both building blocks for biosynthesis and fuels for energy production) and energy (mainly ATP and reducing equivalents, mostly derived from oxygen-based respiration in animals). When one or more of these primary inputs for life is restricted or unavailable, organisms need a self-preservation strategy to help them avoid death. This frequently involves a strong suppression of metabolic rate and transition into a hypometabolic state (Storey and Storey, 1990; Guppy, 2004; Withers and Cooper, 2010). By strong global suppression of metabolic demands coupled with reprioritization of energy use to sustain a minimum suite of vital functions, organisms gain a proportional extension of the time that they can survive using the ‘on-board’ reserves in their bodies (Hochachka and Guppy, 1987). This time is hopefully sufficient to allow survival until conditions are once again favorable for active life. Hypometabolism is a central feature of phenomena including aestivation, hibernation, torpor, dormancy, dauer, diapause, anaerobiosis, freeze tolerance and anhydrobiosis (Storey and Storey, 1990; Storey and Storey, 2004). Some are facultative states (triggered by deteriorating

environmental conditions) whereas others may be seasonally or developmentally programmed obligate events (e.g. diapause and mammalian hibernation). Most involve a suppression of metabolic rate to at least 20–30%, and often as much as 1–10%, of normal resting rate for days to months, but multiple forms of cryptobiosis also occur (mostly among microfauna) where virtually ametabolic states are achieved that can remain viable for many years (Storey and Storey, 1990; Withers and Cooper, 2010).

Physiological and biochemical adaptations supporting aestivation have been studied in many species and are particularly well-researched in lungfish (e.g. *Protopterus* sp.), water-holding frogs (e.g. *Cyclorana* sp. and *Neobatrachus* sp.), spadefoot toads (*Scaphiopus* sp.) and several species of land snails (e.g. *Helix* sp. and *Otala lactea*). Current knowledge is excellently summarized in a recent book (Navas and Carvalho, 2010). Primary concerns for aestivators include mechanisms to conserve energy, retain body water, ration use of stored fuels, deal with nitrogenous end products, and stabilize organs, cells and macromolecules of over many weeks or months of dormancy. In general, aestivation appears to be a fairly ‘light’ dormancy involving no physiological changes that cannot be very rapidly reversed. Indeed, our studies of *O. lactea* show that arousal occurs within 10 min when aestivating snails are sprayed with water, as assessed both by enzymatic changes and emergence of the foot from the shell (Whitwam and Storey, 1990). Gut tissue may regress during aestivation (e.g. *Cyclorana alboguttata* showed reduced mass and absorptive surface area of the small intestine) (Cramp et al., 2009), but skeletal muscle largely resists disuse atrophy and maintains its contractile capacity (Symonds et al., 2007; Mantle et al., 2009). Strong metabolic rate depression during aestivation minimizes energy use to prolong total survival time, but this also means that the normal turnover of macromolecules (synthesis and degradation) is much reduced so that preservation strategies are needed to extend their functional lifespans. This is provided by mechanisms including enhanced antioxidant defenses and elevated

chaperone proteins, strategies that are well-known components of the stress response (Kültz, 2005) but are also widely used across all forms of natural hypometabolism to support viability and life extension (Storey and Storey, 2007; Storey and Storey, 2010; Storey and Storey, 2011).

As mentioned above, hypometabolism is an integral part of many animal survival strategies and, not surprisingly, many studies are now concluding that the core elements of hypometabolism are the same across the animal kingdom. These include a coordinated suppression (or even a near-complete shut-down) of virtually all cell functions (e.g. intermediary metabolism, membrane transport, protein synthesis, gene expression, etc.), a reprioritization of ATP use to favour vital functions, a transition to a reliance on stored reserves of body fuels, and the implementation of cell preservation mechanisms such as antioxidant defenses and chaperones (Storey and Storey, 2007; Storey and Storey, 2010; Storey and Storey, 2011). Overlaid on these are adaptations that deal with the specific issues and/or stresses surrounding each phenomenon. For example, hibernating mammals need to deal with wide changes in body temperature and have mechanisms to rewarm themselves during arousal (brown fat) whereas anoxia-tolerant animals need adaptations that optimize anaerobic ATP synthesis and deal with acid build-up associated with high rates of glycolysis. To our mind, aestivation is one of the 'purest' forms of hypometabolism in nature, needing relatively few adaptations layered onto the basic pattern of hypometabolism. The main 'overlays' needed for aestivation success involve water: conservation of body water, adjustments to deal with water restriction, and sometimes changes to the handling of nitrogenous end products. In addition, although aestivation is an aerobic dormancy, some improvements to hypoxia tolerance may be needed in some cases to support burrowing underground (for protection from hot, arid surface conditions) and/or apnoic breathing patterns that help to minimize respiratory water loss. Adaptations that improve physical methods for minimizing water loss are frequently employed, such as secretion of a mucous epiphragm by snails to seal the shell's opening, the mucous cocoon of lungfish, or the multiple layers of shed skin that form a cocoon for various frog species (Carvalho et al., 2010). Amphibian aestivators also typically enter aestivation with large water-filled bladders from which they can replenish body fluids that are lost over time due to evaporation across skin or respiratory epithelia. Metabolic strategies that elevate body fluid osmolality are also employed, such as the high urea levels accumulated by lungfish and amphibians. Elevated osmolality has traditionally been viewed as a means of resisting body water loss, but may equally function to promote water uptake from the soil across the skin. Urea accumulation is supported by a strong upregulation of genes for urea cycle enzymes, which in lungfish is also necessitated by the transition from aquatic life, where nitrogen is excreted as ammonia across gills, to terrestrial air-breathing life during aestivation, where a less toxic nitrogenous end product is required (Ip et al., 2010; Loong et al., 2011).

The focus of this commentary is on recent advances in our understanding of the intracellular biochemical regulation of aestivation, in particular concepts in signal transduction. There are two main options for regulating hypometabolism: (1) reversible controls that suppress cell functions (e.g. inhibiting activities of enzymes and functional proteins, and sequestering mRNA transcripts into stress granules), and (2) changes in the amounts of selected proteins due to differential transcription, translation or degradation. The latter typically include proteins that either address aestivation-specific issues (e.g. upregulation of urea cycle

enzymes) (Ip et al., 2010) or contribute to long-term life extension (e.g. antioxidant enzymes, iron-binding proteins and heat shock proteins are typically elevated) (Hermes-Lima and Storey, 1995; Ramnanan et al., 2009; Page et al., 2010; Storey and Storey 2007; Storey and Storey, 2011; Loong et al., 2011). Reversible protein phosphorylation (RPP) controls on enzymes are widely applied. For example, in foot muscle and hepatopancreas of aestivating land snails *O. lactea*, this mechanism achieves differential control of glycolysis (inhibits pyruvate kinase and phosphofructokinase) and the pentose phosphate cycle (activates glucose-6-phosphate dehydrogenase), suppresses activities of energy-expensive ion motive ATPases (NaK-ATPase and Ca-ATPase), inhibits enzymes of fuel biosynthesis [e.g. glycogen synthase (GS) and acetyl-CoA carboxylase], and inhibits ribosomal factors to suppress cap-dependent protein synthesis (Whitwam and Storey, 1990; Whitwam and Storey, 1991; Ramnanan and Storey, 2006a; Ramnanan and Storey, 2006b; Ramnanan and Storey, 2008; Ramnanan et al., 2009). However, RPP controls may not apply in all organs; for example, the metabolic rate depression seen in intestine of aestivating *C. alboguttata* was due to a reduction in tissue mass whereas the proportion of intestinal oxygen consumption associated with NaK-ATPase activity and protein synthesis were unchanged (Cramp et al., 2009). Selected enzyme-specific controls can also occur; for example, reduced expression of subunit I of cytochrome *c* oxidase (CCO) coupled with a strong reduction in the cardiolipin content of mitochondrial membranes in lungfish (*Protopterus dolloi*) contributed to a 67% decrease in CCO activity in liver mitochondria of aestivating fish (Frick et al., 2010).

Multiple competing needs and multiple inputs and/or signals from a huge number of environmental and internal factors have led to the development of complex webs of signal transduction pathways in cells that ultimately determine how fuel and energy needs are apportioned in good times and bad. These signaling pathways are ancient, most having analogues in prokaryotes and unicellular eukaryotes, and the fundamental regulatory principles for multicellular life have changed little between nematode (*Caenorhabditis elegans*) and human models. Indeed, the mechanisms for regulating the hypometabolic state of dauer in *C. elegans* provide a valuable framework for exploring signalling and regulation in aestivating species (Lant and Storey, 2010). Our goal here is to examine how selected signalling pathways and the cellular responses that they mediate allow organisms to reversibly transition to or from hypometabolic states with examples from specific recent studies with aestivating animals.

At the heart of intracellular signalling pathways are protein kinases and their oppositely directed protein phosphatases. Approximately 2% of the human genome codes for protein kinases, ~500 in total (Manning et al., 2002), and other species have similar numbers. At least 30% of all human proteins are substrates for protein kinases and RPP is crucial to the regulation of most cellular pathways and events. Among aestivators, RPP was first identified as the means of suppressing glycolytic enzyme activities in *O. lactea*, with protein kinase G (PKG) being a central player in both molluscan aestivation and anoxia tolerance (Brooks and Storey, 1997). More recent studies have shown that RPP is also responsible for the suppression of ATP-expensive NaK-ATPase and Ca-ATPase activities in *O. lactea* during aestivation (Ramnanan and Storey, 2006a; Ramnanan and Storey, 2008) and the differential regulation of glucose-6-phosphate dehydrogenase in its role as the supplier of NADPH for antioxidant defense (Ramnanan and Storey, 2006b). A central role for PKG in regulating these enzymatic responses during aestivation was again implicated.

### Energy signaling: the AMP-activated protein kinase

The AMP-activated protein kinase (AMPK) is often described as the fuel gauge or energy sensor of the cell (Hue and Rider, 2007; Hardie, 2011). It responds to the AMP:ATP ratio, with activity rising under conditions when AMP increases (e.g. hypoxia, intense muscle work or glucose deprivation). A need to conserve energy during aestivation suggested that AMPK could be involved in the reciprocal regulation of catabolic *versus* anabolic metabolism. Interestingly, however, relative adenylate levels (AMP:ATP ratio and/or the adenylate energy charge) typically do not change when animals transition into natural hypometabolism, although the total adenylate pool generally declines, e.g. for *O. lactea* (Churchill and Storey, 1989). Nonetheless, AMPK was activated during aestivation in *O. lactea*, as indicated by approximately twofold increases in measurable AMPK activity in foot muscle and hepatopancreas of 2 or 14 day aestivated snails compared with active controls, as well as 2.5- to 4.4-fold increases in the amount of active phosphorylated AMPK (Thr172) as assessed by immunoblotting (Ramnanan et al., 2010). AMPK is also activated in other forms of hypometabolism (Storey et al., 2010). The rise in phospho-AMPK content implicates upstream kinases (not allosteric action by AMP) as the primary factor in AMPK regulation during hypometabolism. AMPK can be phosphorylated by several upstream kinases (Hardie, 2011), and when three of these were analyzed in *O. lactea* tissues, LKB1 stood out: a twofold increase in phospho-LKB1 (Ser428) content occurred during aestivation that paralleled the rise in AMPK activity. Immunoprecipitation assays also documented enhanced association between LKB1 and AMPK during aestivation (Ramnanan et al., 2010). LKB1 is known as a tumor suppressor in mammals and the LKB1-AMPK pathway functions as an energy-sensing checkpoint that enables growth and proliferation of cells to be coupled to the availability of fuel supplies (Alessi et al., 2006). In the context of animals that enter hypometabolic states, this function of LKB1 is exactly what is needed to help shut down proliferation and growth responses. Hence, although first discovered as a tumor suppressor, this evidence from aestivators indicates that LKB1 clearly has a mainline natural role.

The functional significance of the LKB1-AMPK partnership to aestivation was confirmed with the demonstration that one of the best known AMPK actions was triggered in aestivating *O. lactea*; this is the phosphorylation of acetyl-CoA carboxylase (ACC) that leads to inhibition of lipid biosynthesis. ACC activity decreased by 30–60% in tissues of aestivating snails as a result of a threefold to fourfold increase in the amount of phospho-ACC (Ser79) (Ramnanan et al., 2010). Other targets of AMPK action were also suppressed during aestivation, including glycogen synthesis (the inactive phosphorylated form of GS increased by 2.5-fold) and markers of lipogenesis, gluconeogenesis and mitochondrial proliferation. In mammals, AMPK is also known to antagonize mTOR/p70S6K signaling to limit protein synthesis and cell size in skeletal muscle when nutrients are limiting (Lantier et al., 2010). Hence, in general, AMPK activation in aestivation facilitates a global inhibition of the synthesis and/or interconversion of metabolic fuels consistent with the lack of fresh input of nutrients from food and the need for an enhanced focus on catabolic (ATP-producing) metabolism to support long-term life extension in the hypometabolic state.

### Growth and/or development signaling: Akt and mTOR

All organisms spend huge amounts of energy on growth, proliferation, development and reproduction and, not surprisingly,

arrest of these activities makes a large contribution to energy savings in hypometabolic states. Central to these activities is protein synthesis, which consumes approximately five ATP per peptide bond formed. An early response to stress or nutrient and/or energy limitation by all cells is suppression of protein synthesis (DeGracia et al., 2002), and global suppression of protein synthesis is also crucial in all forms of hypometabolism (Storey and Storey, 2004). Specifically, cap-dependent protein synthesis is suppressed and most polysomes are dissociated, although typically stress-induced synthesis of selected mRNA transcripts is continued or induced, particularly proteins of the ‘stress proteome’ that are translated *via* the use of an internal ribosome entry site.

Strong suppression of protein synthesis has been clearly documented during aestivation with reductions of 50–80% measured in both vertebrate (desert frogs) and invertebrate (snails) models (Fig. 1A for *O. lactea*) (Fuery et al., 1998; Pakay et al., 2002; Ramnanan et al., 2009). Concomitantly, rates of proteolysis are also reduced, resulting in a strong net decrease in the rate of protein turnover and an effective extension of protein lifespan in the hypometabolic state. Key targets of translation inhibition are three ribosomal factors that are all regulated by RPP: the eukaryotic initiation factor 2 (eIF2), which brings the initiating methionine into the assembling ribosome; eIF4, which brings in the mRNA; and eukaryotic elongation factor 2 (eEF2). In aestivating snails, all of these are regulated, as seen in Fig. 1B for *O. lactea* hepatopancreas (Ramnanan et al., 2009). Relative phosphorylation of the alpha subunit of eIF2 (Ser51) soared by 15- to 20-fold in *O. lactea* tissues after 14 days aestivation. Phosphorylation of eIF2 $\alpha$  blocks the protein from being recharged with GTP and thereby inhibits its GTP-dependent function in the delivery and attachment of the initiating methionine residue to the assembling ribosome (Proud, 2007). Phosphorylated eIF2 $\alpha$  content also increased in liver of aestivating desert frogs (Pakay et al., 2003). The eIF4 complex is regulated at several points. Phosphorylation of eIF4GI and eIF4E enhances translation (Proud, 2007), so reduced phospho-eIF4GI (Ser1108) in hepatopancreas and phospho-eIF4E (Ser209) in foot muscle of *O. lactea* (Ramnanan et al., 2009) both indicate translational inhibition. Furthermore, phosphorylation of the eIF4E binding protein (4E-BP1) on Ser65 strongly decreased, so that the amount of eIF4E that could be bound to and inhibited by 4E-BP1 would increase greatly during aestivation. Phosphorylation of eEF2 (Thr56) also soared by approximately fivefold to 10-fold in *O. lactea*, demonstrating that peptide elongation is also strongly inhibited (Ramnanan et al., 2009). Notably, eEF2 phosphorylation is catalyzed by a dedicated eEF2 kinase that is, in turn, activated by AMPK, providing a direct route for low energy and/or nutrient signals to suppress protein synthesis (Proud, 2007; Storey et al., 2010).

The signaling cascade composed of phosphoinositide 3 kinase (PI3K), 3-phosphoinositide-dependent kinase (PDK1) and Akt (also known as protein kinase B) is a central pathway that stimulates multiple survival, growth and proliferation responses in animals in response to an abundance of nutrients (glucose). The pathway most famously transduces insulin signals in vertebrates and related growth factors in invertebrates. Akt generally facilitates actions that go forward when fuel supplies to cells are plentiful, while also inhibiting arrest or death signals (Brazil et al., 2004). Active Akt stimulates glucose uptake, glycogen synthesis [inhibiting glycogen synthase kinase 3 (GSK3)], lipogenesis and protein synthesis (by regulating TOR), while also inhibiting pro-apoptosis factors (e.g. BAD) and suppressing forkhead box class O transcription factors (FoxOs), which variously mediate cell cycle

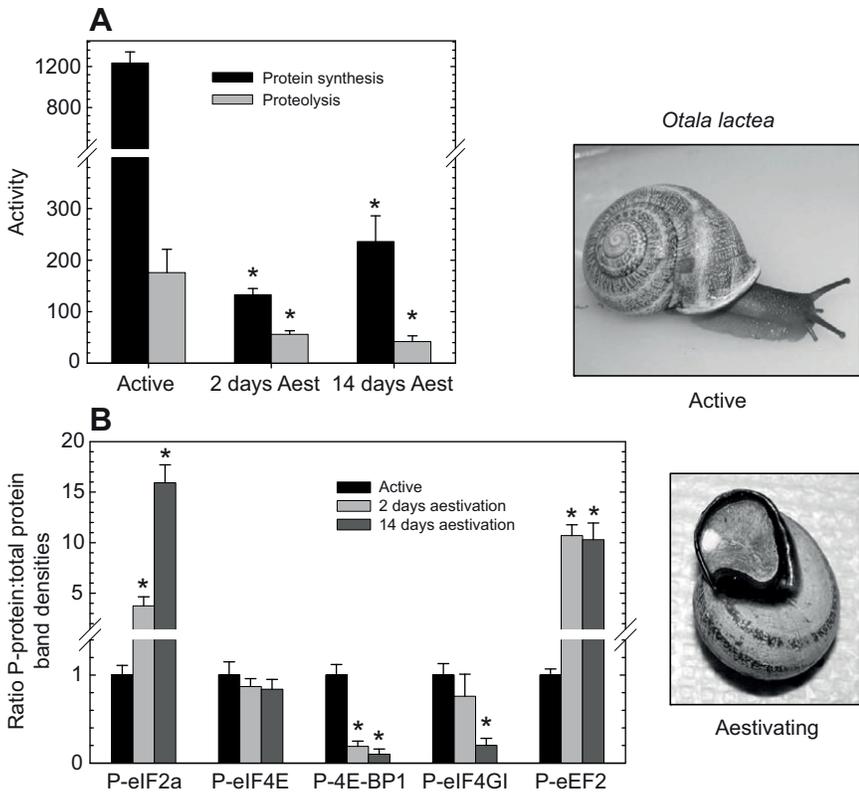


Fig. 1. Aestivation and the control of protein synthesis in the hepatopancreas of *Otala lactea*. (A) Rates of protein synthesis determined as [ $^3$ H]-leucine incorporation into acid-precipitable protein and activity of the 20S proteasome (measured with Z-LLE peptide substrate) in active (control) snails, and snails after 2 and 14 days of aestivation (Aest). (B) Responses of ribosomal proteins showing the ratio of band densities for phosphorylated inactive versus total for each protein. Phosphospecific antibodies detected eIF2 $\alpha$  (Ser51), eIF4E (Ser209), 4E-BP1 (Ser65), eIF4GI (Ser1108) and eEF2 (Thr56). Total protein band densities did not change significantly during aestivation except for eIF4E, where both total and P-eIF4E (Ser209) decreased by ~20% during aestivation. Data are means  $\pm$  s.e.m. and are shown relative to the active value ( $N=4-5$ ). Asterisks indicate a significant difference from the active value ( $P<0.05$ ). Compiled from Ramnanan et al. (Ramnanan et al., 2009). Photo credits: J.M.S.

arrest, apoptosis, quiescence, stress resistance and life extension (Brazil et al., 2004; Proud, 2006; Maiese et al., 2008; Sengupta et al., 2010). Given all of these functions influenced by Akt, regulation of this pathway would clearly be important for aestivation success. Indeed, multiple studies have generally shown reduced Akt action (i.e. growth-inhibiting, quiescence-promoting) in situations of hypometabolism ranging from mammalian hibernation to *C. elegans* dauer (Mukhopadhyay et al., 2006; Storey et al., 2010).

However, recent analysis of Akt and its downstream targets in aestivating snails suggest a modified picture of Akt action in aestivation. Several criteria indicated that Akt was activated in foot and hepatopancreas of aestivating *O. lactea*, including an approximately twofold rise in Akt activity, enhanced Akt substrate affinity and higher amounts of active phosphorylated Akt (Ser473). Furthermore, differential responses by Akt to incubation with protein kinases versus protein phosphatases indicated a higher phosphorylation state of Akt in aestivating snails (Ramnanan et al., 2007). However, when several well-known downstream targets of Akt were analyzed, some intriguing results were seen. Two expected consequences of activated Akt occurred. The first was a twofold increase in phospho-BAD (Ser136) that would inhibit BAD from interacting with the mitochondrial apoptosis machinery. The second was a twofold to fourfold increase in phospho-Foxo3a (Ser253); phosphorylation of this transcription factor leads to its expulsion from the nucleus and would thereby suppress the pro-apoptosis gene expression actions of this FoxO (Ramnanan et al., 2007; Maiese et al., 2008). Hence, both of these Akt-mediated phosphorylation events would help to suppress cell death signaling during aestivation, thereby contributing to long-term life extension.

However, other common targets of Akt responded anomalously during aestivation. The central regulator of protein translation, target of rapamycin (TOR), showed no change in phosphorylation state during aestivation, which suggests that: (1) it is not under Akt control

in aestivation, and (2) it is not the regulator of the observed changes in 4E-BP1 phosphorylation during aestivation (Fig. 1), although 4E-BP1 is a well-known target of mTOR in other systems. Furthermore, Akt control over GSK-3 was clearly lacking in aestivating snails, as the amount of phospho-GSK3 $\beta$  (Ser9) decreased by approximately half in both muscle and hepatopancreas. The activation of GSK3 that should result was also not evident because GS was inhibited. Note, however, that direct phosphorylation of GS by AMPK (discussed above) probably ensured inhibition of glycogen synthesis in the aestivating snails. Thus, it appears that the growth and proliferation actions of Akt that are normally mediated via phosphorylation of mTOR and GSK3 were uncoupled from Akt influence during aestivation. Overall, this suggests that influences from growth factor (nutrient) signaling are much less important to snail hypometabolism than signals from energy availability (AMPK). These data also support an emerging principle of hypometabolism. The traditional view of metabolic regulation is an either-or (ying-yang) relationship between catabolism and anabolism; e.g. in well-fed, high-energy states, anabolism is favored and catabolism is low, whereas in starved, stressed or low-energy states, catabolism is favored and anabolism is suppressed. Anabolism is clearly also suppressed in all forms of hypometabolism but so too is catabolism; global suppression of all metabolic activities is needed to achieve long-term life extension during aestivation or any other form of hypometabolism (Storey and Storey, 2004; Storey and Storey, 2010). Hence, what we would consider to be 'normal' signaling by Akt in response to fed or starved states breaks down during hypometabolism, as is clearly evidenced here by the differential responses of metabolic functions that are traditionally under Akt control.

#### Cell responses to dehydration: ERK signaling

Control over body hydration (and the related ionic strength and osmolality of body fluids) is an important issue for aestivators that

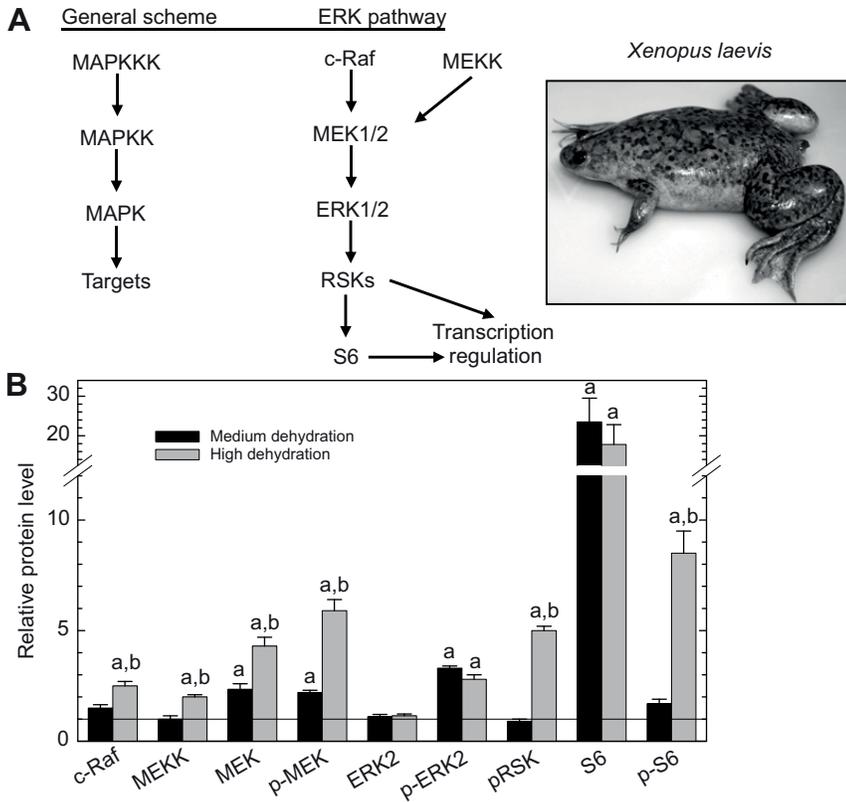


Fig. 2. Activation of the ERK signaling pathway in the lung of African clawed frogs, *Xenopus laevis*, during whole-body dehydration. (A) Schematic of ERK pathway members within the three-tier signaling cascade that typifies the MAPK family. (B) Relative changes in protein or phosphoprotein (p-) levels, compared with controls (mean control levels were set to 1 as illustrated by the horizontal line), in response to medium or high dehydration ( $16.6 \pm 1.6$  or  $28.0 \pm 1.6\%$  of total body water lost, respectively). Immunoblotting analyzed total protein levels of MEKK, MEK1/2 and ERK2 and phosphorylated active forms: p-cRaf<sup>ser338</sup>, p-MEK1/2<sup>ser217/221</sup> and p-ERK<sup>thr202/tyr204</sup>. Downstream targets of ERK2 were also assessed: p-p90 ribosomal S6 kinase (RSK)<sup>ser380</sup>, total S6 ribosomal protein and p-S6<sup>ser235/236</sup>. Data are means  $\pm$  s.e.m. from three to six independent trials. Different letters indicate a significant difference ( $P < 0.05$ ) from the control value (a) or the medium dehydration value (b). Compiled from Malik and Storey (Malik and Storey, 2009a). Photo credit: J.M.S.

may spend weeks, months or years of dormancy in an arid environment. The signaling pathways that are activated by dehydration are of major interest in aestivation. To explore this we have been examining the responses to dehydration by African clawed frogs, *Xenopus laevis*. Although almost exclusively aquatic, in their native environment in southern Africa this species is faced with seasonal drying of its ponds and responds in one of two ways: (1) by nocturnal overland migration to a new water source or (2) by digging into the cooler, damper subsoil of their evaporating pond and entering aestivation (Alexander & Bellerby, 1938). These frogs can endure substantial desiccation (losing as much as 32–35% of total body water) (Romspert, 1975; Malik and Storey, 2009a) and under dehydration stress they elevate the production of nitrogenous osmolytes including amino acids, ammonia (twofold to threefold increase) and urea (15- to 20-fold increase) to enhance water retention and/or uptake from damp soil (Balinsky et al., 1967).

To evaluate dehydration-responsive signal transduction in *X. laevis*, we looked first at the mitogen-activated protein kinase (MAPK) signaling cascades that are prominent responders to environmental stresses; for example, MAPKs are well-known to respond to anoxia, freezing or osmotic stresses in amphibians and reptiles (for a review, see Cowan and Storey, 2003). Responses to whole-body dehydration by the extracellular signal-regulated kinase (ERK) signaling cascade (one member of the MAPK superfamily) in *X. laevis* illustrate this idea. Fig. 2 shows the coordinated responses by ERK family members in lung of *X. laevis* challenged by medium and high levels of dehydration (Malik and Storey, 2009a). Dehydration resulted in elevated levels of phosphorylated active kinases in all three tiers of the MAPK cascade: the initiating MAPK kinase kinases (c-Raf and MEKK), the MAPK kinase (MEK1/2) and finally the MAPK (ERK1/2). Two downstream targets of ERK2 also showed robust increases in active phosphorylated protein content: the p90 ribosomal S6 kinase

(RSK<sup>ser380</sup>) and the transcription factor STAT3<sup>ser727</sup> (not shown). Finally, RSK activation led to strong phosphorylation of its best known target, the S6 ribosomal protein, although multiple other targets of RSK action have now been identified in protein synthesis, cell survival and others (Fenton and Gout, 2011).

Dehydration also enhanced ERK signaling in most other *X. laevis* tissues, implicating the ERK pathway as a key mediator of cellular responses to low water stress with consequences for both gene expression and reversible control of enzymes and other proteins (Malik and Storey, 2009a). Significantly, this role now appears to be universal because new work on both mammalian embryonic kidney cells and *C. elegans* shows a conserved activation of the ERK pathway in response to desiccation and inhibition of desiccation early response genes by an ERK inhibitor (Huang et al., 2010). Although multiple actions for ERK in cellular adjustments to desiccation are indicated, one intriguing action can be postulated for the lung. Recent studies showed that the expression of mucin genes in the mammalian respiratory tract is under ERK1/2 control (Choi et al., 2009) and this suggests that one consequence of ERK activation in lung of water-stressed *X. laevis* could be to mediate changes in the amount or composition of mucins in airway epithelia to contribute to limiting respiratory water loss.

Much remains to be learned about the interactions between dehydration and cell signaling in aestivation. For example, the role of TOR signaling in controlling protein synthesis certainly needs study. In mammals, cell hydration state influences the rates of protein synthesis versus proteolysis largely by regulating TOR (Schliess et al., 2006). Hyperosmotic dehydration inactivates mTOR signaling and attenuates activities including insulin-induced glucose uptake, glycogen synthesis and lipogenesis. A natural effect of dehydration in reducing protein synthesis could be one of the mechanisms exploited by aestivators.

### Cell responses to dehydration: antioxidant defenses

Multiple studies have shown that enhanced antioxidant defenses are an integral part of hypometabolism, including aestivation (Hermes-Lima and Zenteno-Savin, 2002; Storey and Storey, 2010; Page et al., 2010). Antioxidant enzymes are typically upregulated as an early response during hypometabolism, which was initially hard to explain because oxidative stress appeared to be most intense during the arousal period from the hypometabolic state. The hypothesis of preparation for oxidative stress was put forward – this posited that antioxidant genes or proteins were upregulated during the transition into hypometabolism (before full global suppression of transcription and translation) so that they were in place when dormancy was broken and levels of reactive oxygen species soared in concert with a rapid increase in oxygen uptake and consumption by the arousing animal (Hermes-Lima and Zenteno-Savin, 2002). There is good support for this; for example, plasma ascorbate levels are rapidly depleted during arousal from hibernation, indicating that oxidative stress accompanies the rapid rise in oxygen consumption demanded by thermogenesis (Drew et al., 2002). Equally, however, high antioxidant defenses could have a key role in cell preservation in the hypometabolic state. For example, 2 month aestivation by spadefoot toads (*Scaphiopus couchii*) resulted in decreased amounts of reduced glutathione and accumulation of lipid peroxidation products (conjugated dienes and lipid hydroperoxides) in tissues (Grundy and Storey, 1998). Over extended periods of dormancy, the capacity for degradation and resynthesis of oxidatively damaged macromolecules would be low because of global suppression of these energy-expensive processes. Thus, elevation of antioxidant defenses provides a means to limit oxidative damage to macromolecules over long-term dormancy in the same way as elevated chaperones limit the accumulation of misfolded proteins. These adaptations give organisms in hypometabolic states ways to defend themselves against external stresses (e.g. temperature change, UV radiation, chemical exposures, etc.) and preserve viability for as long as possible. Recent research reported that starvation was not the trigger that elevated antioxidant enzymes in aestivating lungfish (Page et al., 2010). Another component of aestivation is dehydration, and recent studies on *X. laevis* indicate that this stress is the more likely trigger; indeed, dehydration triggered both the Nrf2 and FoxO1 signal transduction pathways, leading to enhanced antioxidant capacities in *X. laevis* tissues (Malik and Storey, 2009b; Malik and Storey, 2011).

The Nrf2 transcription factors are major players in the inducible response to oxidative stress (Giudice et al., 2010). Nrf2 normally resides in the cytoplasm anchored by a repressor protein, Keap1. Oxidative stress modifies Keap1 and Nrf2 is freed to translocate to the nucleus, where it forms heterodimers with other proteins (e.g. small Mafs) and binds to the antioxidant response element in genes that code for antioxidant and detoxifying proteins. Studies with *X. laevis* found that dehydration to 28% of total body water lost triggered organ-specific changes consistent with Nrf2 activation: a twofold increase in *nrf2* mRNA in liver; twofold to fourfold increases in total Nrf2 protein in lung, heart, skin and liver; and a 4.3-fold increase in Nrf2 protein in nuclear fractions of muscle (Malik and Storey, 2009b). As a consequence, dehydrated frogs showed enhanced protein expression by members of two gene families under Nrf2 control: glutathione-S-transferase (GST) and aldo-keto reductase (AKR). Peripheral tissues (e.g. skeletal muscle and skin) were particularly affected; for example, GST isozymes kappa, mu and theta rose approximately twofold in muscle along with three AKR isozymes (AFAR1, AKR1B4 and AKR1A3). All

organs showed elevated amounts of at least one GST and one AKR isozyme during dehydration.

The forkhead box class O type 1 (FoxO1) transcription factor also responds to environmental stress and regulates two major antioxidant enzymes: catalase and manganese-dependent superoxide dismutase (MnSOD) (Van der Horst and Burgering, 2007). Transcript and protein levels of both enzymes increased by 1.5-fold to twofold in *X. laevis* liver under dehydration stress and catalase also doubled in skeletal muscle. Multiple lines of evidence indicated that enhanced FoxO1 transcriptional activity was responsible: a 1.8-fold increase in FoxO1 protein in liver nuclear fractions, reduced phospho-FoxO1 (Ser 245) levels (the phosphorylated form is excluded from the nucleus) and a 60% increase in FoxO1 binding to DNA (Malik and Storey, 2011). Notably, both enzymes are also enhanced in other forms of hypometabolism (diapause and dauer) and linked with FoxO signaling (Lee et al., 2003; Van der Horst and Burgering, 2007; Sim and Denlinger, 2011).

Overall, these studies of Nrf2 and FoxO1 provide the first demonstrations of transcriptional responses by antioxidant systems to dehydration in amphibians (Malik and Storey, 2009b; Malik and Storey, 2011). They also add to evidence from a wide number of sources that stress-responsive enhancement of antioxidant defenses is an integral part of all forms of hypometabolism, including aestivation, with two main functions: (1) protection of cells and/or tissues during long-term dormancy, when degradation and resynthesis of oxidatively damaged proteins is not energetically favorable, and (2) defense against the rapid generation of reactive oxygen species when animals transition back from hypometabolism to high rates of oxygen-based metabolism (Hermes-Lima and Zenteno-Savin, 2002; Storey and Storey, 2010).

### Perspectives and conclusions

Available data to date have only begun to unravel the molecular level regulation of aestivation and the complex web of signal transduction pathways, reversible controls on metabolic pathways and gene expression that allows organisms to turn down the fires of life and persist in stable hypometabolic states for lengthy times. We know that aestivation involves ancient signaling and regulatory systems that allow organisms to respond to water, nutrient and energy levels, and that these are used to suppress growth, proliferation and development when these key elements of life are limiting. Many of the underlying tenets of hypometabolism are being worked out with the *C. elegans* dauer model and are proving to be generally applicable across the animal kingdom (Lant and Storey, 2010). However, as the current Commentary shows, there are significant deviations, such as the anomalous regulation of Akt in aestivation.

Other aspects of regulatory control in aestivation remain to be investigated, a prominent and topical one being the modes of transcriptional suppression involved. These are currently coming to the forefront in mammalian hibernation research, but little is yet known about how well they are conserved in other systems of hypometabolism, although global suppression of gene expression does occur in all systems of hypometabolism that have been analyzed to date (Storey and Storey, 2004; Storey and Storey, 2007). Epigenetic mechanisms are key candidates for regulators of transcriptional suppression because they produce widespread gene silencing in many aspects of life (e.g. development, differentiation, aging, etc.) (Fraga et al., 2007). DNA methylation and post-transcriptional modification of histones are two main mechanisms. Histone modifications include acetylation and phosphorylation,

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which reduce chromatin packing to increase transcription factor accessibility to gene promoters, and methylation, which acts oppositely. In hibernating mammals, relative levels of histone acetylation and phosphorylation decrease during torpor and activities of histone deacetylases rise, all indicative of transcriptional silencing (Morin and Storey, 2009). Only one study to date has approached epigenetic regulation in aestivation. Analysis of the mRNA transcript abundance of seven genes whose proteins play roles in gene silencing found that two (transcriptional co-repressor SIN3A and DNA cytosine-5-methyltransferase 1) were upregulated in cruralis muscle of aestivating frogs (*C. alboguttata*) (Hudson et al., 2008). Exploration of the role of chromatin remodeling in long-term gene silencing in aestivation and other forms of hypometabolism will be one of the key challenges of the next few years.

Another potential mechanism of global gene silencing is inhibition of transcription factor action. Clearly individual regulatory mechanisms exist for each transcription factor but global methods are also called for. A reversible posttranslational modification called SUMOylation does just that. Ground squirrel organs showed strong increases in the levels of small ubiquitin-related modifier (SUMO)-conjugated proteins during hibernation and transcription factors are known to be primary targets of SUMOylation (causing mainly negative effects on gene expression) (Lee et al., 2007). Although little else is known about SUMOylation in natural hypometabolic states, the mechanism has obvious wide-ranging potential to provide a simple way to reversibly control transcriptional capacity without the cost of wholesale degradation and resynthesis of transcription factors.

Other potential controls are post-transcriptional but pre-translational – that is, they affect mRNA processing and availability. When cells are exposed to stress, mRNAs encoding most cellular proteins are reprogrammed and recruited to stress granules (Kedersha and Anderson, 2009) that contain mRNAs, small ribosomal subunits and selected translation initiation factors. Concomitant with this, most polysomes dissociate; indeed, this is well documented in both hibernation and anaerobiosis (Storey and Storey, 2004). The mRNAs in stress granules are preserved and subsequently made available for rapid translation when organisms arouse from hypometabolism. In mammalian hibernation, this seems to be a key way to ‘jump-start’ repair and/or renewal processes as animals rewarm. The role of stress granule formation is certainly another area that needs to be examined in aestivation.

Finally, a new principle of gene regulation that will undoubtedly prove to be universally important in hypometabolic systems is control by various kinds of non-coding RNA. In particular, the crucial role of microRNA (miRNA) has come to the forefront in recent years. These small non-coding transcripts (19–25 nt long) bind to mRNA transcripts and modulate their fate, targeting mRNAs into either stress granules for storage or processing bodies for degradation (Kedersha and Anderson, 2009). Hundreds of studies have now linked miRNAs to translational control in health and disease (Sayed and Abdellataif, 2011) and differential expression of selected miRNAs occurs in multiple forms of hypometabolism, including *C. elegans* dauer (Karp et al., 2011), hibernation and anaerobiosis (Biggar and Storey, 2011). Comparable studies of miRNA involvement in aestivation will be highly instructive. Indeed, a comprehensive analysis of all of these mechanisms of transcriptional and translational control will both greatly expand our knowledge about the regulation of aestivation and contribute to our broader understanding of the principles of metabolic arrest and life extension in all organisms.

## ACC

Acetyl-CoA carboxylase, a biotin-dependent enzyme that catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA, a main precursor for fatty acid biosynthesis. The enzyme is an important regulatory step for lipogenesis.

## AKR

Aldo-keto reductases are a large group of monomeric NADPH-dependent oxidoreductases. Among 14 families are aldehyde reductases (AKR1A group), aldose reductases (AKR1B group) and aflatoxin aldehyde reductases (AFARs), which catalyze the reduction of dicarbonyl-containing xenobiotic compounds.

## Akt

A serine/threonine protein kinase that is also known as protein kinase B. Akt is a positive effector of cell survival and growth and a key mediator of insulin signaling.

## AMPK

The heterotetramer 5-AMP-activated protein kinase plays a central role in cellular energy homeostasis, promoting catabolic reactions and inhibiting anabolic ones when energy levels are low.

## Chaperones

Proteins that assist in the folding and assembly of macromolecular structures, also preventing stress-induced (e.g. by heat) aggregation into nonfunctional structures.

## Dauer

A state of stasis in *C. elegans* nematodes, triggered by poor environmental conditions in larval stages L1 and L2 and leading to exit into the dauer instead of molt into L3. Dauer larvae are highly resistant to environmental stress.

## Diapause

A temporary pause in the growth and development of an organism in response to adverse environmental conditions, triggered and broken by specific stimuli (often hormonal) and an obligatory part of the life cycle for many species in seasonally harsh environments.

## ERK

Extracellular signal-regulated kinases are one of three families of MAPKs that are primarily known to be activated in response to growth factors with functions in regulating cell proliferation and differentiation.

## FoxO

Forkhead box (Fox) proteins are a huge family of transcription factors. Members of the O subfamily have in common regulation by insulin/PI3K/Akt signaling and are well known for roles in controlling apoptosis, cell-cycle arrest, DNA damage repair and oxidative stress resistance.

## GST

Glutathione *S*-transferases catalyze the conjugation of reduced glutathione to electrophilic centers on many kinds of substrates, including peroxidized lipids and a wide variety of xenobiotics and toxins to facilitate their export from the body.

## LKB1

Liver kinase B1 is an upstream protein kinase that regulates AMPK. Mutations that inactivate LKB1 cause a dominantly inherited human cancer called Peutz–Jeghers syndrome.

## MAPK

Mitogen-activated protein kinases are serine-/threonine-specific protein kinases that respond to numerous extracellular stimuli and regulate many cellular activities including gene expression, mitosis, differentiation, proliferation and cell survival/apoptosis. Each MAPK is activated *via* a cascade of upstream kinases (a MAPK kinase and MAPKK kinase) allowing massive amplification of signal and crosstalk between the ERK, JNK and p38 families of MAPKs.

## Nrf2

The Nuclear factor (erythroid-derived 2)-like 2 protein is a transcription factor with a primary role in the regulation of antioxidant genes. In unstressed situations, it is tethered in the cytoplasm by the Kelch-like ECH-associated protein 1 (Keap1).

## STAT

The Signal Transducer and Activator of Transcription (STAT) transcription factors regulate many aspects of growth, survival and differentiation in cells.

## TOR

Target of rapamycin is a serine/threonine protein kinase that has a major role in the positive regulation of mammalian protein synthesis in response to insulin, growth factors, nutrients and other signals.

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