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“SLY AS A FOXO”: New Paths with Forkhead Signaling in the Brain

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Abstract

The Forkhead transcription factor FOXO3a has emerged as a versatile target for diseases that impact upon neuronal survival, vascular integrity, immune function, and cellular metabolism. Enthusiasm is high to fill a critical treatment void through FOXO3a signaling for several neurodegenerative disorders that include aging, neuromuscular disease, systemic lupus erythematosus, stroke, and diabetic complications. Here we discuss the influence of FOXO3a upon cell survival and longevity, the intricate signal transduction pathways of FOXO3a, insights into present disease models, and the potential clinical translation of FOXO3a signaling into novel therapeutic strategies.

Keywords

Amyotrophic lateral sclerosis; diabetes; apoptosis; FOXO3a; FKHRL1; Akt; erythropoietin; neuromuscular disease; oxidative stress; psychiatric; systemic lupus erythematosus; stroke; stem cells

INTRODUCTION

The mammalian forkhead transcription factor family regulates processes ranging from cell longevity to cell apoptosis and function as transcription factors by preferentially binding to the core consensus DNA sequence 5'-TTGTTTAG-3', the Forkhead response element (Chong *et al.*, 2005e, Chong *et al.*, 2004c, Wijchers *et al.*, 2006). The first member of this family was the *Drosophila melanogaster* gene *Fork head*. Since this time, greater than 100 forkhead genes and 19 human subgroups are known to exist that extend from FOXA to FOXS (Wijchers *et al.*, 2006). The forkhead box (FOX) family of genes is characterized by a conserved forkhead domain commonly noted as a “forkhead box” or a “winged helix” as a result of the butterfly-like appearance on X-ray crystallography (Clark *et al.*, 1993) and nuclear magnetic resonance (Jin *et al.*, 1998). All Fox proteins contain the 100-amino acid winged helix domain, but it should be noted that not all winged helix domains are Fox proteins (Larson *et al.*, 2007). The forkhead domain may have a number of variations from the usual three α -helices, three β -sheets, and two loops that are referred to as the wings. This domain defines the Fox class of transcription factors, but additional portions of the Fox proteins can be divergent. A unified nomenclature has been described that has now replaced prior terms such as forkhead in

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rhabdomyosarcoma (*FKHR*), the *Drosophila* gene fork head (*fkh*), and Forkhead Related Activator (FREAC)-1 and -2 (Kaestner *et al.*, 2000). Within the subclasses of the Fox proteins that are each designated by a letter, an Arabic number is provided such that the actual name of a Fox protein would follow the direction of “Fox, subclass or subgroup “Letter”, and member “Number”. In relation to human Fox proteins, all letters are capitalized, otherwise only the initial letter is listed as uppercase.

FOXO3a EXPRESSION

Of the Forkhead transcription factors, FOXO3a is one member that has emerged as a versatile target for a number of disorders. In tissues such as alveolar rhabdomyosarcoma tumor samples, the FOXO3a gene consists of at least three exons and leads to a protein containing 673 amino acids. The initial exon contains the N-terminal portion of the protein including the initiating ATG and 60 amino acids of the forkhead domain. This portion is GC rich and contains the methylation-sensitive restriction sites. The second exon contains the C-terminal 50 amino acids of the forkhead domain and the remaining translated region (Anderson *et al.*, 1998).

FOXO3a, also known as FKHL1 (forkhead in rhabdomyosarcoma like 1), is expressed in a wide variety of human cell types. For example, FOXO3a protein is present in human megakaryocytes (Tanaka *et al.*, 2001) and in the lamina propria and muscular of the gastro intestine of the pig (Zhou *et al.*, 2007). Furthermore, FOXO3a transcripts are expressed in multiple tissue that involve the ovary, prostate, skeletal muscle, brain, heart, lung, liver, pancreas, spleen, thymus, and testis. In murine models, mRNA expression is highest in muscle, adipose tissue, and embryonic liver (Furuyama *et al.*, 2000). With *in situ* hybridization experiments on adult and embryonic mouse brains, Foxo3 is expressed throughout the brain including all hippocampal areas, cortex and cerebellum (Hoekman *et al.*, 2006).

FOXO3a IN VIVO DELETION

Interestingly, Foxo1^{-/-} mouse embryos usually die upon embryonic day 10.5 as a consequence of incomplete vascular development. In contrast, Foxo3a^{-/-} as well as Foxo4^{-/-} mice can be viable and similar in appearance to controls, suggesting that at least for vascular development Foxo3a and Foxo4 are not critical (Hosaka *et al.*, 2004). Yet, the studies on Foxo3a^{-/-} null mice suggest that in the absence of Foxo3a, development of specific organs can be affected that may later have consequences for the nervous system. Foxo3a^{-/-} null mice become infertile and have been reported to experience follicular activation to the extent that ovarian follicles are depleted with the subsequent death of oocytes (Castrillon *et al.*, 2003). In addition, absence of Foxo3a leads to lymphoproliferation, organ inflammation of the salivary glands, lung, and kidney, and increased activity of helper T cells, indicating an important role for Foxo3a in immune regulation and T cell tolerance (Lin *et al.*, 2004). Foxo3a also regulates hematopoietic stem cell development (Miyamoto *et al.*, 2007) and neutrophil activity. For example, hematopoietic stem cell numbers are significantly decreased in aged Foxo3^{-/-} mice compared to the littermate controls, suggesting that Foxo3a plays a pivotal role in maintaining the these stem cell pool (Miyamoto *et al.*, 2007). Foxo3a null mice also are resistant to two models of neutrophilic inflammation that involve immune complex-mediated inflammatory arthritis and thioglycollate-induced peritonitis (Jonsson *et al.*, 2005). In addition, Foxo-deficient mice (Foxo1, Foxo3a, Foxo4) also exhibit an increase in reactive oxygen species (ROS) and changes in the expression of regulated-genes of ROS in hematopoietic stem cell populations. Thus, Foxo3a proteins may play an essential role in the response to physiologic oxidative stress (Tothova *et al.*, 2007).

CLINICAL IMPLICATIONS FOR FOXO3a IN THE NERVOUS SYSTEM

Ischemia and Oxidative Stress

FOXO3a may play a significant role during injuries that involve cerebral ischemia and oxidative stress (Chong *et al.*, 2005b, Chong *et al.*, 2005e). Hypoxic-ischemic conditions as well as oxidative stress represent significant pathways for the apoptotic destruction of cells (Chong *et al.*, 2005f, Li *et al.*, 2006a). Apoptosis consists of membrane phosphatidylserine (PS) exposure and DNA fragmentation (Maiese *et al.*, 2005a) and can contribute to a variety of disease states that especially involve the nervous system such as diabetes, stroke, vascular dementia, Alzheimer's disease, and trauma (Chong *et al.*, 2004b, Han and Suk, 2005, Li *et al.*, 2004a). As an early event in the dynamics of cellular apoptosis, membrane PS externalization can become a signal for the phagocytosis of cells (Chong *et al.*, 2005e, Hong *et al.*, 2004). Cells expressing externalized PS may be removed by microglia (Li *et al.*, 2004b, Lin and Maiese, 2001) as well as lead to the dysfunction of inflammatory cells (Chong *et al.*, 2007b, Li *et al.*, 2006b).

In clinical work, the effects of the genetic variance in FOXO3a on metabolic profile, age-related diseases, fertility, fecundity, and mortality were analyzed in 1245 aged persons. Increased risk of stroke was observed in two haplotypes of FOXO3a block-A suggesting an association with FOXO3a (Kuningas *et al.*, 2007). In animal studies, Foxo3a expression has been associated with c-Jun N-terminal kinase 3 activity and the potential to promote stroke (Pirianov *et al.*, 2007). Other studies of cerebral ischemia also have indicated that inhibitory phosphorylation of Foxo3a may be associated with the neuroprotective effects of estradiol during stroke (Won *et al.*, 2006). In neuronal cell culture models, inhibition or gene knockdown of Foxo3a activity can mediate the ischemic protective effects of metabotropic glutamate receptors (Chong *et al.*, 2006), enhance neuronal survival through NAD⁺ precursors (Chong *et al.*, 2004c, Li *et al.*, 2006a, Li *et al.*, 2004b, Maiese and Chong, 2003, Maiese *et al.*, 2007), and offer trophic factor protection in both neurons and vascular cells during oxidative stress such as with erythropoietin (Chong and Maiese, 2007a), insulin-like growth factor-1 (Zheng *et al.*, 2000), neurotrophins (Zheng *et al.*, 2002), and vascular endothelial growth factor (Abid *et al.*, 2004). In fact, agents that rely upon the modulation of FOXO3a, such as erythropoietin, commonly employ a number of pathways tied to FOXO3a signaling such as protein kinase B (Akt) (Chong *et al.*, 2003a, Chong *et al.*, 2003b, Chong *et al.*, 2002a, Chong *et al.*, 2005d, Chong *et al.*, 2003c, Chong and Maiese, 2007a).

Diabetes, Metabolism, and Cell Senescence

Diabetes mellitus is believed to affect more than 165 million individuals worldwide (Quinn, 2001) and by the year 2030 more than 360 million individuals will be afflicted with diabetes (Maiese *et al.*, 2007) and its debilitating conditions (Wild *et al.*, 2004). Type 2 diabetes represents at least 80 percent of all diabetics and is dramatically increasing in incidence as a result of changes in human behavior and increased body mass index (Laakso, 2001). Type 1 insulin-dependent diabetes mellitus accounts for only 5–10 percent of all diabetics. Yet, disease of the nervous system can become one of the most debilitating complications for diabetes and affect sensitive neuronal and vascular regions of the brain (Chong *et al.*, 2007c) resulting in significant functional impairment and dementia (Awad *et al.*, 2004, Gerozissis, 2003). Furthermore, both focal and generalized neuropathies, especially in conjunction with vascular disease, can result in severe disability (Perkins and Bril, 2002).

The forkhead transcription factor family may be involved in pathways responsible for cell metabolism, diabetes mellitus onset, and diabetic complications (Chong and Maiese, 2007b, Maiese *et al.*, 2005a, Maiese *et al.*, 2004, Maiese *et al.*, 2005b). In a prospective population based study of 1245 participants aged 85 years or more, haplotype analyses of FOXO1a

revealed that carriers of haplotype 3 'TCA' had higher HbA_{1c} levels to suggest evidence of at least disorders with glucose intolerance (Kuningas *et al.*, 2007). In particular for FOXO1, this transcription factor may be responsible for the prevention of beta cell injury during against oxidative stress. This process requires the complex formation with the pro-myelocytic leukemia protein Pml and the NAD-dependent deacetylase SIRT1 to activate expression of the insulin2 gene transcription factors NeuroD and MafA (Kitamura *et al.*, 2005). Yet, the role of Forkhead transcription factors can vary among different cells and tissues. For example, mice overexpressing FOXO1 in skeletal muscle suffered from reduced skeletal muscle mass and poor glycemic control (Kamei *et al.*, 2004). In regards to FOXO3a, a clinical exam of 734 individuals that sequenced all exons of the FOXO genes FOXO1a, FOXO3a, and FOXO4 found one promoter single nucleotide polymorphism in the 5' flanking region of FOXO3a that displayed a significant association with body mass index such that the highest body mass index was present in individuals homozygous for this allele (Kim *et al.*, 2006). In experimental models, administration of a high-fat diet induced hyperinsulinemic insulin-resistant obesity, increased expression of Foxo3a, and impaired cardiomyocyte function (Relling *et al.*, 2006). Other work has linked diabetic nephropathy to Foxo3a by demonstrating that phosphorylation of Foxo3a increased in rat and mouse renal cortical tissues two weeks after the induction of diabetes by streptozotocin (Kato *et al.*, 2006). Furthermore, enteric neurons can be protected from hyperglycemia by glial cell line-derived neurotrophic factor that can affect protein kinase B signaling and prevent Foxo3a activation and nuclear translocation (Anitha *et al.*, 2006). Yet, some studies in other experimental models suggest that Foxo3a may block oxidative stress during diabetes. For example, interferon γ -driven expression of tryptophan catabolism by cytotoxic T lymphocyte antigen 4 may activate Foxo3a to protect dendritic cells from oxidative stress in nonobese diabetic mice (Fallarino *et al.*, 2004).

Closely linked to the diabetic complications that can affect the nervous system is the regulation of cellular metabolism. One potential pathway to consider is the precursor for the coenzyme β -nicotinamide adenine dinucleotide (NAD⁺), namely nicotinamide (Li *et al.*, 2006a, Li *et al.*, 2004b, Maiese and Chong, 2003). Nicotinamide can be directly utilized by cells to synthesize NAD⁺ (Jackson *et al.*, 1995) and participates in energy metabolism through the tricarboxylic acid cycle by utilizing NAD⁺ in the mitochondrial respiratory electron transport chain for the production of ATP, DNA synthesis, and DNA repair (Hageman and Stierum, 2001, Magni *et al.*, 2004). Furthermore, nicotinamide can significantly increase NAD⁺ levels in vulnerable regions of the ischemic brain, suggesting that nicotinamide may offer cytoprotection of injured tissue through the maintenance of NAD⁺ levels (Sadanaga-Akiyoshi *et al.*, 2003). Administration of nicotinamide can significantly improved glucose utilization, prevent excessive lactate production and improve electrophysiologic capacity in ischemic animal models (Li *et al.*, 2006a).

Treatment with nicotinamide can approximate normal fasting blood glucose in animals with streptozotocin-induced diabetes (Hu *et al.*, 1996, Reddy *et al.*, 1995). Oral nicotinamide (1200mg/m²/day) protects β -cell function and prevents clinical disease in islet-cell antibody-positive first-degree relatives of type-1 diabetes (Olmos *et al.*, 2005). In addition, treatment with nicotinamide (25mg/kg) in patients with recent onset type-1 diabetes mellitus combined with intensive insulin therapy for up to two years after diagnosis can reduce HbA_{1c} levels (Crino *et al.*, 2005). Potentially relevant to diabetic patients with renal failure, nicotinamide also has been shown to reduce intestinal absorption of phosphate and prevent the development of hyperphosphatemia and progressive renal dysfunction (Eto *et al.*, 2005). Therefore, it should be of interest that nicotinamide may derive its protective capacity through two separate mechanisms of post-translational modification of Foxo3a. Nicotinamide can not only maintain inhibitory phosphorylation of Foxo3a, but also preserve the integrity of total Foxo3a and phosphorylated Foxo3a (Chong *et al.*, 2004c) to block Foxo3a proteolysis that can yield potentially apoptotic amino-terminal (Nt) fragments (Charvet *et al.*, 2003).

However, nicotinamide has many roles especially in relation to sirtuins. Nicotinamide offers protection in millimole concentrations against free radicals (Lin *et al.*, 2000), anoxia (Lin *et al.*, 2001), and oxygen glucose deprivation (Chong *et al.*, 2004c). Yet in relation to cell longevity, nicotinamide is an inhibitor of sirtuins in concentrations that range from 50–100 μ M (Porcu and Chiarugi, 2005). Increased longevity in yeast has been shown to be dependent upon silent information regulator 2 (Sir2) protein. The *Sir2* gene belongs to a family of genes which is a highly conserved group in the genomes of organisms ranging from archaeobacteria to eukaryotes (Frye, 2000, Vaziri *et al.*, 2001). Physiological concentrations of nicotinamide noncompetitively inhibit Sir2, suggesting that nicotinamide is a physiologically relevant regulator of Sir2 enzymes (Bitterman *et al.*, 2002). Other studies have shown that stimulation of the NAD⁺-dependent deacetylase SIRT1, a mammalian ortholog of Sir2, in mammalian cells during starvation is dependent upon Foxo3a expression (Nemoto *et al.*, 2004). This process may require a close feedback loop since SIRT1 can deacetylate Foxo3a during oxidative stress (Wang *et al.*, 2007) and inhibit its activity (Motta *et al.*, 2004).

Irrespective of the direct relationship to sirtuins, FOXO3a also has been linked to cell aging and senescence (Kyoung Kim *et al.*, 2005, Ramnanan *et al.*, 2007), suggesting a more broader impact for FOXO3a in studies that involve the effects of neurodegeneration associated with cognitive decline (Chong *et al.*, 2007a, Chong *et al.*, 2005c, Chong *et al.*, 2005f, Maiese *et al.*, 2005a). It has been shown that protein kinase B (Akt) activity can increase with cellular senescence in primary cultured human endothelial cells and inhibition of FOXO3a by Akt is essential for this growth arrest to occur (Miyachi *et al.*, 2004). Interestingly, FOXO3a inactivation in older animals may limit tissue antioxidant properties through decreased manganese-superoxide dismutase and lead to enhanced cell susceptibility to injury with aging (Li *et al.*, 2006c).

Inflammatory and Neuromuscular Disease

As previously noted, FOXO3a has a significant role in the immune system as evidenced by studies that illustrate that Foxo3a-deficient mice are resistant to neutrophilic inflammation and immune complex-mediated inflammatory arthritis (Jonsson *et al.*, 2005). For the nervous system, control of systemic lupus erythematosus (SLE) may require Foxo3a activation. Amelioration of SLE in animal models is achieved with administration of peptides based upon the complementary determining region-1 of an anti-DNA antibody. In these studies, transforming growth factor-beta (TGF- β) is increased with subsequent upregulation of Foxo3a that is believed to block NF-kappaB activation and interferon-gamma secretion (Sela *et al.*, 2006). In addition, models of multiple sclerosis with experimental autoimmune encephalomyelitis have shown that osteopontin, a protein expressed in multiple sclerosis lesions, was found to block Foxo3a activity that subsequently leads to activation of nuclear factor kappaB and promote the activity of myelin-reactive T cells resulting in disease progression (Hur *et al.*, 2007).

A number of disease entities are associated with skeletal muscle atrophy, such as cancer, diabetes, cardiac and renal failure, and sepsis. In muscles that undergo atrophy, the ubiquitin ligase atrogen-1 is increased that leads to muscle loss. In studies using cultured myotubes, atrophy was linked to the activation of Foxo3a (Sandri *et al.*, 2004). However, in clinical studies involving biopsies from twenty-two patients with amyotrophic lateral sclerosis, patients had a significant increase in atrogen-1 mRNA and protein content, but no detectable increase in FOXO3a mRNA and protein content (Leger *et al.*, 2006). This work may suggest that other factors in addition to FOXO3a are necessary to initiate neuromuscular disease.

FOXO3a SIGNALING IN THE NERVOUS SYSTEM

A number of regulatory pathways are essential to control cell survival, such as the phosphatidylinositol 3-kinase (PI 3-K) pathway through protein kinase B (Akt) (Chong *et al.*, 2005e, Chong and Maiese, 2007a). Regulation of Akt activity can affect a number of cellular functions that relate to growth, survival, injury, and metabolism. In particular, Akt can prevent apoptotic cell injury during late genomic DNA destruction and early apoptotic signaling with membrane PS exposure (Chong *et al.*, 2005f, Maiese and Chong, 2004). Enhanced Akt activity can increase cell survival during several injuries such as free radical exposure (Chong *et al.*, 2003b, Matsuzaki *et al.*, 1999), matrix detachment (Rytomaa *et al.*, 2000), neuronal axotomy (Namikawa *et al.*, 2000), DNA damage (Chong *et al.*, 2004a, Chong *et al.*, 2002a, Henry *et al.*, 2001, Kang *et al.*, 2003a), oxidative stress (Chong *et al.*, 2003b, Kang *et al.*, 2003a, Kang *et al.*, 2003b, Yamaguchi and Wang, 2001), hypoxic preconditioning (Wick *et al.*, 2002), β -amyloid exposure (Martin *et al.*, 2001), metabotropic receptor signaling (Chong *et al.*, 2005a, Chong *et al.*, 2006, Maiese *et al.*, 2005a), and cell metabolic pathways (Chong *et al.*, 2005g, Maiese and Chong, 2003). Activation of Akt also can prevent membrane PS exposure on injured cells (Chong *et al.*, 2004a, Li *et al.*, 2006b) and block the activation of microglia during oxidative stress (Chong *et al.*, 2005a, Kang *et al.*, 2003a, Kang *et al.*, 2003b).

Intimately linked to these survival pathways with Akt is FOXO3a (Fig. 1). Akt can phosphorylate FOXO3a and inhibit its activity to sequester FOXO3a in the cytoplasm by association with 14-3-3 proteins (Brunet *et al.*, 2002, Chong and Maiese, 2007a, Dong *et al.*, 2007, Kino *et al.*, 2005, Munoz-Fontela *et al.*, 2007). In the absence of inhibitory Akt1 phosphorylation, FOXO3a is activate, can translocate to the nucleus, and controls a variety of functions that involve cell cycle progression, cell longevity, and apoptosis (Lehtinen *et al.*, 2006, Li *et al.*, 2006a, Maiese *et al.*, 2007). Site-directed mutagenesis of the potential Akt phosphorylation has shown that FOXO3a is phosphorylated by Akt on Thr-32, Ser-253 and Ser-315. Each phosphorylation site for FOXO3a may function independently that determines subcellular localization. Akt may first phosphorylate FOXO3a at Ser-253, create a docking motif for 14-3-3 proteins, which then may participate in the nuclear export of FOXO3a (Brunet *et al.*, 1999, Cahill *et al.*, 2001). A 14-3-3 dimer requires stable binding to the phosphorylated N-terminal Akt site prior to the other half of the dimer binding to the phosphorylated Akt motif in the forkhead domain (Van Der Heide *et al.*, 2004).

Additional mechanisms exist independent from Akt that can phosphorylate and retain Foxo3a in the cytoplasm. As a close homolog to Akt, serum- and glucocorticoid-inducible protein kinase (Sgk) can increase cell survival through inhibition of Foxo3a (Leong *et al.*, 2003). However, Sgk and Akt have different efficacies with which they phosphorylate Foxo3a. Both kinases can phosphorylate Thr-32, but Sgk preferentially phosphorylates Ser-315 whereas Akt favors Ser-253. This work suggests that Sgk and Akt may control Foxo3a through different phosphorylation sites (Brunet *et al.*, 2001). The ability of Sgk to oversee Foxo3a activity also may involve p53. In studies that examine DNA damage, p53 led to the induction of Sgk that was necessary to maintain phosphorylation of Foxo3a (You *et al.*, 2004). Additional work also has demonstrated that IkappaB kinase (IKK) in the absence of activated Akt can phosphorylate Foxo3a and retain Foxo3a in the cytoplasm (Finnberg and El-Deiry, 2004, Hu *et al.*, 2004).

Downstream from the regulation of Foxo3a, a number of target genes for Foxo3a have been elucidated (Fig. 1). For example, FOXO3a activation in colonic cancer cells resulted in the expression of several members of Myc target genes that involve the Mad/Mxd family of transcriptional repressors and may account for the growth inhibitory effects of FOXO3a (Delpuech *et al.*, 2007). In relation to apoptotic death, Foxo3a has been shown to modulate a ligand activating a Fas-mediated death pathway (Barthelemy *et al.*, 2004) and to induce tumor-

necrosis-factor-related apoptosis-induced ligand (TRAIL) and the BH3-only proteins Noxa and Bim (Obexer *et al.*, 2007). FOXO3a also can modulate growth-arrest and DNA-damage-response protein 45 (Gadd45) and influence cell cycle regulation (Furukawa-Hibi *et al.*, 2002, Tran *et al.*, 2002). In regards to aging in a model of vascular smooth muscle cells, FOXO3a inactivation in older rats was found to reduce the transcription of manganese-superoxide dismutase, a major cellular anti-oxidant that may work to reduce cell disability with advancing age (Li *et al.*, 2006c). Furthermore, immune system regulation by FOXO3a is believed to be mediated, in part, through the regulation of nuclear factor-kappa B (NF-kappaB) (Lin *et al.*, 2004).

FUTURE PERSPECTIVES

There are several levels to target the activity of FOXO3a. FOXO3a may be regulated at three distinct levels. First, FOXO3a-dependent transcription depends upon the translocation of FOXO3a to the nucleus. As a result, kinases such as Akt exert control over FOXO3a through phosphorylation to sequester FOXO3a in the cytoplasm through association with 14-3-3 proteins (Brunet *et al.*, 2002, Chong and Maiese, 2007a, Dong *et al.*, 2007, Kino *et al.*, 2005, Munoz-Fontela *et al.*, 2007). Without Akt phosphorylation, FOXO3a is activate to translocate to the cell nucleus (Lehtinen *et al.*, 2006, Li *et al.*, 2006a, Maiese *et al.*, 2007). Second, the integrity of the FOXO3a protein represents another level of regulation. IKK can phosphorylate and lead to proteolysis of FOXO3a *via* the Ub-dependent proteasome pathway (Hu *et al.*, 2004). In addition, modulation of caspase 3 activity also appears to be closely associated with a unique regulatory mechanism that blocks the proteolytic degradation of phosphorylated FOXO3a. FOXO3a has been shown to be a substrate for caspase 3-like proteases at the consensus sequence DELD³⁰⁴A (Charvet *et al.*, 2003). As a result, blockade of caspase 3 - like activity can prevent the destruction of phosphorylated FOXO3a during conditions of oxidative stress (Chong *et al.*, 2004c, Chong and Maiese, 2007a). Agents that prevent FOXO3a degradation through caspase 3 inhibition (Chong *et al.*, 2002b, Chong *et al.*, 2004c, Chong and Maiese, 2007a) can be cytoprotective since proteolysis of FOXO3a has been shown to yield amino-terminal (Nt) fragments that may further precipitate cellular injury (Charvet *et al.*, 2003). As additional mechanisms, FOXO3a can become acetylated, such as during growth factor deprivation (Mahmud *et al.*, 2002), or deacetylated by the sirtuin Sir2 homolog SIRT1 (Brunet *et al.*, 2004, Motta *et al.*, 2004, Wang *et al.*, 2007), to either disrupt FOXO3a association with target DNA or to alter the ability of FOXO3a to lead to cell cycle arrest and cell death.

Future directives that focus upon FOXO3a as a therapeutic target should consider the multiple pathways that can govern FOXO3a activity. In relation to cell survival, trophic factors such as erythropoietin, insulin growth factor, and brain-derived growth factor can inhibit activity of FOXO3a and sequester it in the cytoplasm of cells (Chong *et al.*, 2006, Chong *et al.*, 2004c, Chong and Maiese, 2007a, Zheng *et al.*, 2000, Zhu *et al.*, 2004). Although several agents that include growth factors, metabotropic receptors, and NAD⁺ precursors (Chong *et al.*, 2006, Chong *et al.*, 2004c, Chong and Maiese, 2007a) can prevent cell injury through reductions in FOXO3a activity, future work that intends to prevent apoptotic injury should strive to modulate FOXO3a at multiple levels that involve inhibitory phosphorylation of FOXO3a, prevention of phosphorylated FOXO3a destruction, cytoplasmic retention of FOXO3a, and gene knockdown of FOXO3a. Yet, it is clear that such a recipe is overly simplistic and must proceed with caution, since FOXO3a activity is required as a significant regulator of cell growth, cell senescence, and in some cases for the prevention of invasive neoplasms (Belguise *et al.*, 2007, Real *et al.*, 2005, Rice *et al.*, 2007).

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Fig. 1. Potential cellular signaling pathways for FOXO3a

A representative illustration of possible “upstream” and “downstream” signal transduction pathways for the FOXO3a system that may be set in motion, such as during the presence of the growth factor erythropoietin (EPO).