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Glutamine metabolism links growth factor signaling to the regulation of autophagy

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Activation of the PI3K-AKT1-FOXO module by growth factors increases survival and stress resistance. We identified the gene encoding glutamine synthetase (GLUL, glutamate-ammonia ligase) as a novel transcriptional target of this signaling cascade. Growth factor removal increases glutamine synthetase expression and activity through activation of FOXO transcription factors. Surprisingly, increased levels of glutamine synthetase inhibit MTOR signaling by blocking its lysosomal translocation. Furthermore, FOXO activation induces autophagosome formation and autophagic flux in a glutamine synthetase-dependent manner. This may be important for maintaining cell survival during conditions of growth factor and nutrient deprivation since inhibition of autophagy induces cell death. These studies reveal that glutamine metabolism can play an important regulatory role in the regulation of autophagy by growth factor signaling. In addition, the induction of autophagy by FOXO-mediated glutamine synthetase expression might contribute to the tumor suppressive function of FOXOs.

Keywords: PI3K, FOXO, glutamine metabolism, MTOR, autophagy

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The PI3K-AKT1-FOXO signaling module plays a pivotal role in a wide variety of cellular processes and represents one of the most diverse yet significant signaling systems regulating intracellular communication. Activated by growth factors, cytokines and hormones, the lipid kinase PI3K regulates cell growth, proliferation, differentiation, motility, survival and intracellular trafficking. Many of these functions relate to the ability of PI3K to activate the serine/threonine kinase AKT1 (also known as PKB). AKT1 itself can regulate transcriptional programs through inhibitory phosphorylation of Forkhead Box O (FOXO) transcription factors: FOXO1, FOXO3 and FOXO4. During periods of growth factor withdrawal and nutrient depletion, inactivation of PI3K-AKT1 and subsequent activation of FOXOs can influence a wide range of biological processes including cell cycle regulation, stress resistance, development, reproduction and aging. Additionally, FOXOs have been shown to function as tumor suppressors by blocking cell cycle progression and actively inducing apoptosis. In the absence of growth factors, complex regulation of stress resistance, cell cycle progression and apoptosis ensures cellular survival while minimizing cellular damage. Perturbing the balance between these processes can result in carcinogenesis, and sequencing of diverse malignancies has revealed multiple genomic alterations of PI3K-AKT1 signaling pathway components, making the PI3K pathway one of the most commonly activated pathways in human cancer.

Screening for transcriptional targets involved in survival and stress resistance regulated by PI3K, AKT1 and FOXOs we identified the glutamine synthetase gene as a novel FOXO transcriptional target. Glutamine synthetase expression is inhibited by growth factor stimulation or selective activation of either PI3K or AKT1. In contrast, inhibition of PI3K

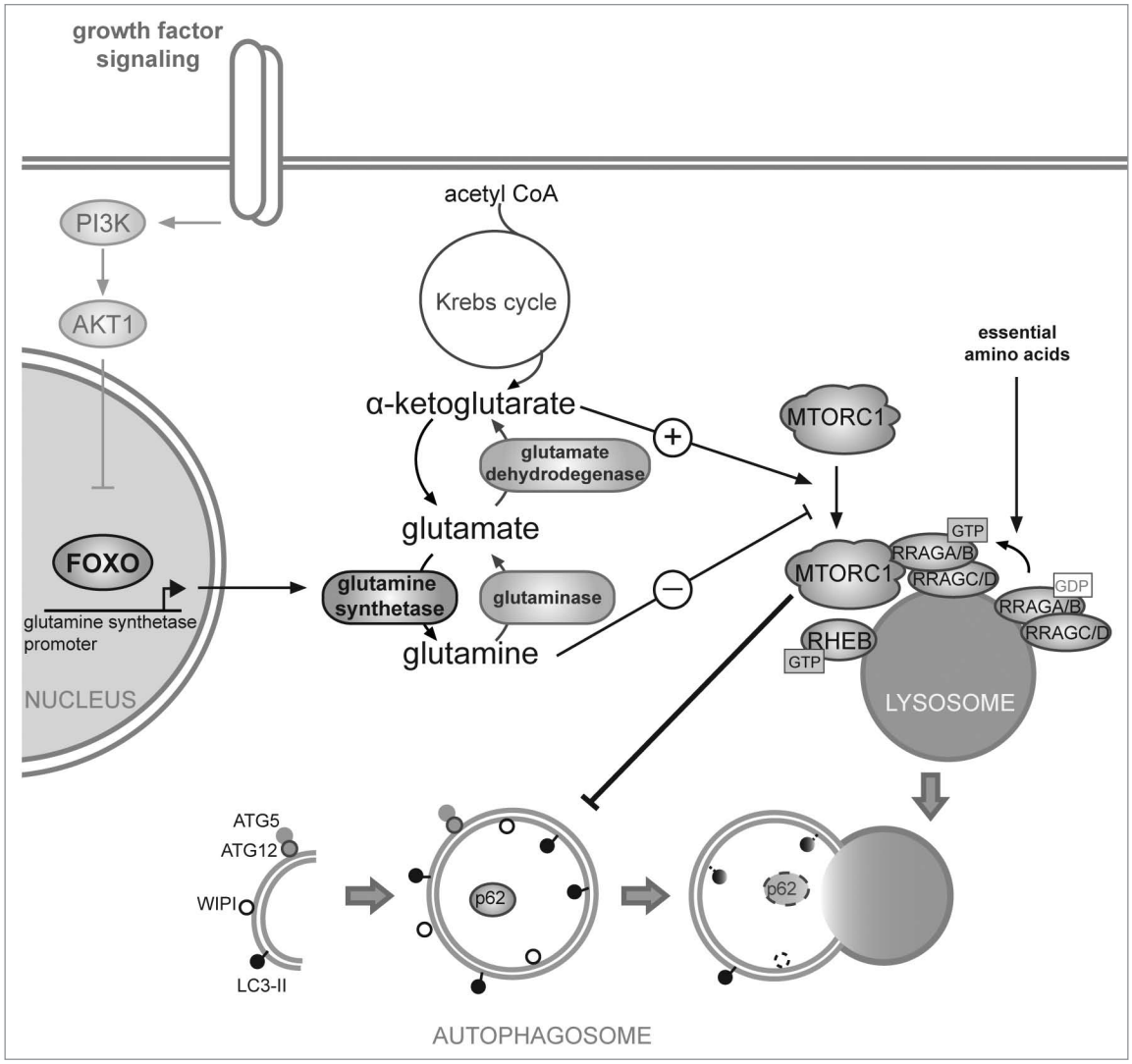

Figure 1. Schematic model showing the interplay between glutamine metabolism, MTOR activation and autophagy. Growth factor deprivation, caloric restriction or oxidative stress trigger the activation of FOXO resulting in upregulation of glutamine synthetase expression. The subsequent rise in glutamine levels inhibits MTOR by preventing lysosomal localization, through currently undefined mechanisms, thereby inducing the formation of autophagosomes. In contrast glutaminolysis, resulting in the generation of α-ketoglutarate can result in activation of MTOR and inhibition of autophagy. The removal of damaged proteins and organelles can decrease the level of reactive oxygen species (ROS), which in turn might prevent genomic instability and/or cellular senescence. These mechanisms have the potential to reduce the incidence of cancer and prolong life span (see text for full details).

activity induces binding of activated FOXO3 to the *GLUL* promoter and increases transcription. Elevated enzyme activity levels result in a specific increase in glutamine levels, while the levels of other amino acids remain unaffected by FOXO-activation. Surprisingly, we observed that FOXO3 activation results in inhibition of MTORC1 activity in a GS-dependent manner. This is in contrast to essential amino acids such as leucine, which activate the RRAG GTPases enabling their interaction with the MTORC1 complex facilitating its relocalization to the lysosomal surface. This is a crucial step in amino acid-mediated activation of MTORC1 since binding to the lysosomal membrane positions the MTORC1 complex in close proximity to its upstream activator RHEB. Our current findings demonstrate that activation of FOXO3 can abrogate the lysosomal localization of MTOR, which once again is dependent on glutamine synthetase activity (**Fig. 1**).

A recent study has further extended our understanding of MTOR regulation by glutamine metabolism. Similar to FOXO-mediated upregulation of glutamine synthetase, knockdown of glutaminase and glutamate dehydrogenase that together convert glutamine into α-ketoglutarate also inhibits MTOR signaling. Interestingly increasing the levels of α-ketoglutarate were reported to activate MTOR by increasing GTP loading of RRAGB and lysosomal translocation, resulting in MTORC1 activation. This suggests that MTOR activity can be controlled by glutamine metabolism at multiple levels: an increase in glutaminolysis and increased α-ketoglutarate production

activates MTOR, whereas decreased hydrolysis and increased glutamine synthesis block MTOR activation. It also suggests that one direct mechanism by which increased glutamine synthetase activity could inhibit MTOR is through glutamate consumption thereby reducing α-ketoglutarate levels.

MTOR is a well-characterized inhibitor of autophagy, and the FOXO3-mediated increase in glutamine synthetase activity, or addition of glutamine to nutrient-starved cells, results in increased autophagy as measured by multiple autophagic markers. FOXO3 activation increases WIPI puncta formation and induces ULK2 and LC3 colocalization as well as WIPI1 and ATG12. In addition, lipidated LC3 levels are elevated in FOXO3-activated cells and increase further upon treatment with the lysosomal inhibitor bafilomycin A_1. Importantly, the induction of FOXO3-mediated autophagy is dependent on glutamine production since l-methionine sulphoximine (MSO), a specific glutamine synthetase inhibitor, abrogates autophagosome formation. Taken together, this demonstrates that FOXO3 modulates multiple steps in the autophagic cascade through an increased activity of glutamine synthetase resulting in autophagosome formation as well as increased autophagic flux.

Regulation of glutamine synthetase activity by the PI3K-AKT1-FOXO signaling module appears to be conserved in the nematode worm *C. elegans*. In *C. elegans* activation of an ortholog of the insulin receptor, DAF-2 results in activation of a PI3K ortholog, AGE-1, which induces activation of an AKT1 ortholog (AKT). In the absence of nutrients, the nematode worm can enter a stress-resistant stage in which the worms can survive up to four times longer. The DAF-2 pathway negatively regulates this process and inactivation of the AGE-1-AKT pathway results in a long-lived phenotype, which is dependent on activation of the worm FOXO transcription factor DAF-16. We have shown that glutamine synthetase activity is dramatically increased in *daf-2* mutant worms compared with wild-type worms. Importantly, this is DAF-16 dependent and suggests that *GLUL* is an evolutionarily conserved FOXO transcriptional target. Autophagy may modulate animal aging by decreasing cellular damage, and in several *C. elegans* models for dietary restriction, longer life span is associated with increased autophagy. Whether DAF-16-mediated increases in glutamine levels also triggers autophagosome formation in *C. elegans*, and whether this is required for the life-span promoting properties of DAF-16, awaits further study.

Autophagy is often considered to be a double-edged sword in terms of its potential role in tumor development. It has been reported to inhibit tumorigenesis by degrading damaged proteins and organelles resulting in lower oxidative stress and thereby reducing DNA damage. However, an antitumorigenic role for autophagy is highlighted by the fact that many oncogenes inhibit autophagy, whereas tumor suppressors can increase autophagy, often through modulation of the mTORC1 complex. Our findings reveal a novel mechanism by which the PI3K-AKT1-FOXO pathway modulates autophagy, and provide further insight into the regulation of autophagy by amino acids. Importantly, blocking autophagosome formation after FOXO activation reduces cell viability, indicating that the induction of autophagy is required for cellular survival under stress conditions. The induction of autophagy may thus be an important downstream effect of FOXO activation that mediates the tumor suppressive properties of the FOXO transcription factors.