Forkheads in neuroblastoma & human longevity: From the womb to the tomb

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Chapter 1

Introduction
Forkhead Box (FOX) Family Transcription Factors

The first member of what was to become the forkhead family (fork head, Fkh) was identified in Drosophila melanogaster by a genetic screen which yielded flies whose heads exhibited abnormal involution patterns early in development (Jurgens 1988). Fkh was subsequently cloned and described as being a nuclear protein with no identifiable sequence similarities to contemporary transcription factors of the time (Weigel 1989). The DNA binding domain now termed the forkhead domain was identified when Fkh was aligned with the independently discovered HNF-3A in rats (Lai 1990 & Weigel 1990). Throughout the last 25 years additional members of this superfamily which all contain the approximately 100 amino acid forkhead domain (the only criteria for inclusion) have been identified. So far phylogenetic analyses have resulted in the identification of 19 different subfamilies containing 50 members in humans and 44 in mice (Jackson 2010 & Fig. 1). The members of these subfamilies typically have sequence features outside of the forkhead domain common with each other but these features are not found within other FOX subfamilies. This familial tree structure was annotated using a nomenclature where FOX describes the membership in the superfamily, a letter of the alphabet describes membership in a subfamily and a number describes the particular subfamily member; e.g. FOXA1, FOXA2, FOXA3 and FOXD1, FOXD2, FOXD3 and FOXD4 (Hannenhalli 2009). Forkheads also exhibit extreme conservation with orthologues identifiable in yeast (fkh1 & 2, Zhu 2000). The following is an overview of select forkhead subfamilies that have exhibited powerful roles in shaping mammalian biology.
**Figure 1** Evolutionary tree of mouse forkhead box (Fox) genes (adapted from Hannenhalli 2009). A neighbor-joining tree is shown that is based on the protein sequence of the forkhead domain. The relationships shown in the tree are based on multiple alignment with each branch annotated with a bootstrap value that was based on 1000 permutations. The branch lengths are proportional to the mutation rate. In red boxes the forkheads which are the primary subjects of this Thesis are indicated.
**FOXA**

The FOXA subfamily was first identified from rat liver extracts as a protein that bound to the promoters of the transthyretin (Ttr), α1-antitrypsin (Serpina1) and albumin (Alb1) genes that contained a hepatocyte nuclear factor 3 (HNF-3) DNA binding element and were subsequently named HNF-3A, B & C (now FOXA1, 2 & 3 respectively) (Lai 1990). Since their discovery they have been shown to be involved in organ development and are thought to function as “pioneer” chromatin modification factors (for review see Friedman 2006). In addition to being fundamental for liver development they have also been shown to be pivotal for the maintenance of glucose homeostasis (for review see Le Lay 2010). Despite the expected and previously demonstrated redundancy of function, FOXA1 knockout mice exhibit severe hypoglycemia. This may be at least in part due to a deficiency of pancreatic glucagon production as well as an attenuated transcriptional gluconeogenic response to glucagon by the liver. Liver-specific deletion of FOXA2 was euglycemic while FOXA3 knockouts only exhibit mild hypoglycemia after prolonged fasting. Surprisingly, the FOXA1 orthologue in c. elegans, PHA-4, has been demonstrated to be robustly essential for dietary restriction-induced longevity as well as longevity in response to the loss of mTORC1 signaling (Panowski 2007 & Sheaffer 2008).

**FOXD**

The FOXD subfamily consists of four members (FOXD1-4) with 2 & 4 being poorly characterized. FOXD1 is pivotal in eye and kidney formation; playing an organogenic role like that of the FOXA subfamily (Herrera 2004 & Fetting 2014). FOXD3 is of particular interest due to its emerging roles as a maintenance factor in embryonic stem cells, an important player in neural crest development and a tumor suppressor. FOXD3 knockout mice were embryonic lethal with the epiblasts depleted of their progenitor cells (Hanna 2002). It was also impossible to derive teratomas or culture embryonic stem cells from FOXD3 null embryos; demonstrating that FOXD3 was essential for ES cell survival. Using a neural crest targeted Cre knockout model FOXD3 loss caused severe defects in NC development such as impaired formation of craniofacial features, abolishment of the enteric nervous system and reduction of the peripheral nervous system (Teng...
A follow-up study demonstrated that the neural crest stem cell features of multipotency, self-renewal capacity and ability to form neuronal lineages were maintained by FOXD3 with its loss causing a strong mesenchymal differentiation (Mundell 2011). In the neural crest derived tumor neuroblastoma FOXD3 was shown to have tumor suppressive functions (Li 2013). Overexpression of FOXD3 inhibited growth and invasion in cell lines and xenografts while knockdown enhanced these tumor driving properties. A similar role was discovered for FOXD3 in hepatocellular carcinoma and gastric cancer (Cheng 2013 & Liu 2014).

**FOXM**
FOXM1 is the only member of its subfamily and is an important factor for cell cycle progression at the G2 checkpoint as well as for faithful chromosomal segregation; although it is not completely essential (Laoukili 2005). Its expression pattern is tightly linked to the cell cycle with an onset in S phase and loss of expression by the end of mitosis (Korver 1997). Unsurprisingly, FOXM1 has also become a very well documented tumor driver in numerous systems. It has been found amplified in 5.6% of breast cancers, 42% of non-Hodgkin’s lymphomas and 58% of malignant peripheral nerve sheath tumors (Katoh 2013). Its potency has been further demonstrated in neuroblastoma and nearly every other tumor type (Wang 2011 & Wierstra 2013). FOXM1 has also been found to promote resistance to the chemotherapeutic cisplatin in breast cancer (Kwok 2010).

**FOXO**
The FOXO subfamily (FOXO1, 3, 4 & 6) is the best studied and perhaps most famous subfamily of forkheads to-date. Across mammalian tissues their expression patterns differ with FOXO3 being the most ubiquitous and FOXO1, FOXO4 and FOXO6 exhibiting more restrictive profiles (for review see Salih 2008). They directly interact with DNA by binding the “DBE” (DNA Binding Element, TTGTGTA) and the more degenerate thymidine-rich IRS (Insulin Responsive Sequence) elements (Furuyama 2000 & Brent 2008). Although they have specific roles they also have many redundant functions due to their sometimes overlapping expression and DNA binding patterns. This redundancy was especially relevant in regard to their tumor suppressive capabilities. In genetic mouse models of single
FOXO knockouts their roles as tumor suppressors were heavily masked by this redundancy and not revealed until a conditional triple knockout mouse for FOXO1/3/4 was constructed which then developed leukemia (Paik 2007). Beyond tumor suppression they have been implicated in many processes with some of the most important being apoptosis, cell cycle regulation, metabolism, longevity, response to oxidative stress, stem cell maintenance, cell-type specification, differentiation and immune response modulation.

FOXO1 (previously FKHR) was first discovered as an aveolar rhabdomyosarcoma fusion gene where the n-terminus of PAX3 and PAX7 (which contains the PAX DNA binding domains) are found fused to the c-terminus of FOXO1; this portion of FOXO1 contains the familial FOXO transactivation domain (Davis 1994 & Fredericks 1995). This fusion product is oncogenic as demonstrated by its ability to transform primary cells (Scheidler 1996). Physiologically, FOXO1 has been described as being of central importance in the liver where it acts to maintain whole-body glucose homeostasis. It is a potent activator of gluconeogenesis and VLDL cholesterol biosynthesis that in turn is tightly inhibited by the Insulin/Ins1-2/PI3K/Akt pathway (Nakae 2002, Dong 2008, Kamagate 2008 & Lu 2012). Recently, knockout of FOXO1/3/4 in the liver has demonstrated that FOXO3 and FOXO4 act redundantly with FOXO1 in both the activation of gluconeogenesis and suppression of lipogenesis (Haeusler 2014). Initially, the importance of FOXO1 in whole-body glucose homeostasis could only be assessed in heterozygous and conditional knockouts because the earlier FOXO1 whole-body knockouts were embryonic lethal owing to defects in vascular formation (Hosaka 2004). This defect in vasculature remodeling was later found to be caused by defects in the endothelial cells rather than defects in myocardium development when conditional knockouts were performed in both lineages (Sengupta 2012). Another important role recently emerged for FOXO1 as an essential factor in human embryonic stem cell (hESC) maintenance where it was found to directly drive the expression of Oct4 and Sox2 (Zhang 2011). Interestingly, this essential role was not conserved in mouse ESCs. In the immune system FOXO1 knockout specifically within T_{Reg} cells caused a debilitating inflammatory response similar to the scurvy
phenotype of FOXP3 knockout mice (Ouyang 2012). This was largely due to an over-production of IFN-γ which was transcriptionally suppressed by FOXO1.

Mammalian FOXO3A (previously FKHRL1) was discovered and cloned using probes derived from FOXO1 (Anderson 1998). By sequence homology FOXO3A is the FOXO most closely related to the c. elegans orthologue, Daf-16, although Daf-16 was first recognized as an orthologue of FOXO1 and FOXO4 (Ogg 1997).

FOXO3A knockout mice exhibit surprisingly mild phenotypes under normal conditions (Hosaka 2004). The only obvious defect was sterile females which was traced to uncontrolled follicular activation (John 2008). However, closer analysis has uncovered additional defects in these animals. FOXO3A has been implicated in the maintenance of lineage specific progenitor cells; although this function is not exclusive to FOXO3A with FOXO1 and FOXO4 also contributing. In the case of hematopoietic stem cells FOXOs are essential for the repression of reactive oxygen species and the establishment of quiescence via cell cycle repression (Tothova 2007). Following this finding a similar role was found for FOXOs in neural progenitor cells where they were essential for cell cycle and ROS repression; FOXO3A had the largest contribution in this lineage (Paik 2009 & Webb 2009). Recently, FOXO3A knockout alone in muscle satellite cells was sufficient to reverse quiescence and cause depletion of this progenitor pool as well (Gopinath 2014). This recurrent stem cell defect of hyperproliferation caused by FOXO3A loss supports the myriad of studies and observations demonstrating that FOXO3A has tumor suppressive roles in many systems.

FOXO3A has been shown to form oncogenic fusions with the MLL gene in leukemia creating products capable of cellular transformation (So 2003). Given the role of FOXO3A in suppressing the HSC cell cycle it is also plausible that the fusion event creates a haploinsufficiency of wild-type FOXO3A which may further contribute to tumorigenesis. Additional genetic evidence for FOXO3A acting as a tumor suppressor comes from reports of genomic deletions in natural killer (NK) cell neoplasms as well as in lung cancer (Mikse 2010 & Karube 2011). In both systems restoration of FOXO3A expression induced apoptosis. The ability of FOXO3A in
particular to induce apoptosis is thought to be central to its role as a tumor suppressor (Zhang 2011). The role of FOXO3A in oncogenesis was also probed by the creation of compound p53/FOXO3A knockout mice (Renault 2011). Here it was observed that the p53 tumor spectrum was expanded by the loss of FOXO3A. This demonstrated that the tumor suppressive pathways of FOXO3A and p53 are somewhat overlapping.

FOXO3A also has an important role in the immune system that involves the regulation of T cell and inflammation responses. In one seminal study it was discovered that FOXO3A acts to suppress T cell expansion in response to viral infection by suppressing the production of inflammatory cytokines by dendritic cells (Dejean 2009). This finding was shown to be of great importance in a subsequent study where the intronic FOXO3A SNP rs12212067 was found to be associated with the severity of the inflammatory Crohn’s disease and rheumatoid arthritis (Lee 2013). Minor allele carriers exhibited a decreased inflammatory response in these conditions and this was due to an increased FOXO3A mRNA expression from the minor allele in monocytes specifically following antigenic stimulation. This in turn suppressed inflammatory cytokine production via a TGFβ dependent mechanism.

FOXO4 (previously AFX) was discovered as a fusion partner of MLL in leukemia and of PAX3 in aveolar rhabdomyosarcoma (Borkhardt 1997 & Barr 2002). In addition to the canonical product splice variants have also been described for FOXO4; some of which may have dominant negative activity (Yang 2002 & Lee 2008). The FOXO4 knockout mouse has no detectable abnormalities (Hosaka 2004). Despite this it was recently reported that FOXO4 was necessary for the commitment of human embryonic stem cells to the neural lineage (Vilchez 2013). In light of the FOXO4 knockout mouse not having overt defects in neuronal lineages this seems to be a human-specific role for FOXO4 in embryonic stem cell neural commitment. This suggests that like FOXO1, FOXO4 may also have evolved new roles in ESC biology.

FOXO6 is the most recently described member of the FOXO family (Jacobs 2003). In some studies its expression pattern is described as being restricted to the brain
while in others it is described as more ubiquitously expressed. One reason for the discrepancy may be the existence of FOXO6 isoforms which may have confounded detection on the mRNA and protein levels (Chung 2013). Like FOXO1 is has been described as an activator of gluconeogenesis and VLDL cholesterol biosynthesis in the liver (Kim 2011 & Kim 2014). In another study whole-body FOXO6 knockout mice were found to have defects in memory consolidation following learning tasks (Salih 2012). This study supported the original claims about FOXO6 having a brain specific expression pattern and no metabolic phenotype was reported. More work is clearly needed to resolve the wide discrepancies between the existing studies.

**FOXP**

The FOXP subfamily contains four members (FOXP1-4) in humans who exhibit a very high degree of conservation with their corresponding mouse homologues. This has created a situation where mutations derived in mice and/or observed in humans have been predictive and explanatory for a large spectrum of disorders caused by those mutations in both mice and humans. The FOXP subfamily is also unique among forkheads in that they bind DNA by forming homo and heterodimers with each other via a unique leucine zipper motif (Li 2004). This subfamily exhibits exceptionally diverse functionality across a wide swath of critical biology from the development of speech to the regulation of autoimmunity.

FOXP1 is demonstrated to be involved in naïve T cell development, establishment of naïve T cell quiescence, the pro-B to pre-B cell transition, cardiomyocyte proliferation and differentiation, motor neuron connectivity as well as mental and verbal development (Hu 2006, Dasen 2008, Feng 2010, Zhang 2010, Feng 2011, Fevre 2013). The defects in motor neuron connectivity observed in mouse models may partly explain the haploinsufficient mutations observed in humans that result in severe speech impairment and slow motor responses. However, immunological defects were not observed in these haploinsufficient humans indicating that FOXP1 immunological functions may be sufficiently supported by one functional allele. In cancer FOXP1 has been shown to act as both an oncogene and a tumor
suppressor (for review see Koon 2007). Recently, FOXP1 has been shown to have tumor suppressive properties in neuroblastoma (Ackermann 2014).

In a role somewhat overlapping FOXP1 but non-redundant with it FOXP2 is intimately linked to the development of vocalization/speech and a driver behind the evolution of language. A mutation in the forkhead domain of FOXP2 was initially discovered in a familial pedigree exhibiting a severe speech and language disorder (Lai 2001). Subsequently, an evolutionary analysis demonstrated that FOXP2 only exhibited two amino acid changes in humans compared to mice and that these changes were not found in chimpanzees or other apes; indicating human specificity and hence segregation with the unique phenotype of language (Enard 2002). Molecular confirmation of the relevance of these human-specific FOXP2 mutations by generating mice with the humanized FOXP2 allele (Enard 2009). These mice had different vocalizations and exhibited increased synaptic plasticity and dendrite lengths in medium spiny neurons of the striatum; suggesting FOXP2 functions were enhanced. In another seminal experiment knockdown of FOXP2 by RNAi in the basal ganglia of songbirds greatly impaired the ability to imitate songs which may have been due to disruption of reinforcement learning mechanisms (Haesler 2007 & Murugan 2013).

FOXP3 was discovered in 1991 as the mutated gene responsible for the immunologically defective scurfy mice whose X-linked phenotypic hallmark is the hyperproliferation of CD4+CD8- T lymphocytes (Brunkow 2001). Shortly thereafter this finding was translated to humans when people with the autoimmune immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome were shown to all have FOXP3 mutations (Bennett 2001 & Wildin 2001). This implicated FOXP3 as being essential in the development and functionality of CD4+ T\textsubscript{Reg} suppressor cells. Subsequently, FOXP3 expression was shown to be specific for the CD25+ subset of CD4+ T\textsubscript{Reg} cells and that these cells could be created by the overexpression of FOXP3 in naïve T cells (Hori 2003). These CD4+CD25+FOXP3+ T\textsubscript{Reg} cells are now known to be absolutely essential in the modulation of T cell responses to host and foreign antigens. Without this regulation T cell hyperproliferation and autoimmune disease can occur.
The FOXR subfamily consists of FOXR1 and FOXR2 (formerly FOXN5 and FOXN6, respectively). There is very little known about these transcription factors from a fundamental perspective; although it is thought that their expression is highly restricted to the early stages of embryogenesis (Katoh 2004a, Katoh 2004b & Schuff 2006). The first functional work on FOXR was the characterization of FOXR1 fusion genes in neuroblastoma tumors (Santo 2010, this thesis). Here it was shown that FOXR1 expression is essentially undetectable in normal human tissues, most neuroblastomas and only in isolated cases in other tumors. However, a small subset of neuroblastomas exhibit fusions between FOXR1 and the promoter regions and first exons of MLL and PAFAH1B2 as a result of interstitial deletions at 11q23. These fusion events cause FOXR1 to be aberrantly expressed and it was demonstrated that this is an oncogenic event. This first report of FOXR1 as an oncogene has proven to be quite prescient as the same has been found for FOXR2.

FOXR2 was first discovered as an oncogene in a transposon mutagenesis screen for tumor drivers of malignant peripheral nerve sheath tumors (MPNSTs) (Rahrmann 2013). FOXR2 expression was found to be associated with MPNSTs and required for anchorage independent growth and tumorigenic capacity in vivo.

In another forward genetic screen FOXR2 was discovered as acting oncogenically in the pediatric tumor medulloblastoma (Koso 2014). FOXR2 was specifically found to be expressed within the sonic the hedgehog subtype of medulloblastoma and was implicated as having a direct role in SHH signaling. The findings to-date unequivocally indicate that the FOXR subfamily can act as powerful oncogenes when aberrantly expressed outside of their normal early embryonic context. Given the ability of the FOXR subfamily to block normal differentiation and drive the cell cycle it may be interesting to explore if these factors play a fundamental role in embryonic stem cell maintenance.

**Forkhead Interplay**

Given the broad spectrum of biology that forkheads govern and the complementary, overlapping and sometimes opposing functions these different subfamilies represent it will be of interest to define the points of contact that must
inevitably exist between them. Some work has already been done in this area. This has become increasingly interesting in the realm of cancer where FOXO3A and FOXM1 are described as opposing each other on many functional levels (Zhao 2012). In one study FOXO3A was found to directly repress FOXM1 mRNA transcription and in another FOXO3A was found to compete directly for binding to FOXM1 target genes; counteracting FOXM1 oncogenic functions (McGovern 2009, Karadedou 2012). Similarly, in the osteosarcoma cell line HOS it was found that FOXR1 is capable of suppressing FOXO target genes (Santo 2010). However, it remains to be established if this was direct competition with FOXO for promoter occupancy or a more indirect suppression of FOXO transcription factor activity. In another study involving multiple cancer cell lines FOXO3A was found to up-regulate FOXP1 expression. FOXP1 then bound to FOXO3A occupied enhancers to suppress FOXO3A target gene induction and consequently FOXO3A induced apoptosis (van Boxtel 2013). In the immune system FOXOs were found to be essential for the establishment of the T_{Reg} cell lineage because they were necessary for the expression of FOXP3 and other crucial genes (Ouyang 2010). Undoubtedly these stories are only the “tip of the iceberg” and this area will prove a fertile ground for further research.

The FOXO3A Post-Translational Regulatory Landscape
Over the last 15 years many post-translational modifications that impact the functionality of FOXO3A have come to light. The primary modifications identified for FOXO3A so far are phosphorylation, acetylation and methylation while some others with lesser understood consequences have also been described (for review see Eijkelenboom 2013). Befitting the highly-conserved status of FOXO3A many of the pathways that influence FOXO3A function are some of the most ancient and therefore fundamental ones known. Despite the identification of individual modifications and their effects very little is known about how they behave in concert to establish the ultimate functional output of FOXO3A in any given context. The following is a synopsis of the primary avenues for FOXO3A regulation identified to-date and the general context in which they occur.
Figure 2  Domain map and post-translational modifications of FOXO3A.  a. A domain and PTM map of human FOXO3A.  FOXO domain information was compiled from Biggs 1999, Nasrin 2000, Brownawell 2001, Rena 2001, Brunet 2002, So 2002 and Wang 2012.  Within the domain structure the forkhead domain is colored blue, NLSs are red, NESs are green and the transactivation domain is yellow.  Phosphorylation events are marked with green lines, acetylation with red lines and methylation with purple lines.  b. Protein secondary structure map of the FOXO3A forkhead domain (left) and amino acids within the forkhead domain that contact DNA (right) adapted from crystallographic study of Tsai 2007.  DNA contact residues highlighted with a red box are among the residues known to be post-translationally modified.

The PI3K/Akt pathway provided the first and perhaps most important insights into basic FOXO3A mechanics and functionality.  The first description of Akt phosphorylating and inhibiting FOXO came from the c. elegans FOXO orthologue Daf-16 (Paradis 1998).  This finding cemented the centrality of FOXO in the Insulin/PI3K/Akt pathway.  Shortly thereafter, the discoveries that mammalian FOXO1, FOXO3A and FOXO4 were phosphorylated and inhibited by Akt were reported nearly simultaneously demonstrating the extreme conservation of this pathway (Brunet 1999, Kops 1999, Nakae 1999).  These reports provided instant insight into the orientation of FOXOs in regards to cell survival and their pleiotropic roles in growth factor signaling.
The PI3K/Akt pathway is activated in response to a wide array of growth factors, cytokines, stressors and the critical regulator of metabolism insulin. When the PI3K/Akt axis is active Akt phosphorylates both cytosolic and nuclear FOXO3A at the sites Thr32, Ser253 and Ser315 (Fig. 2a). These phosphorylations terminate FOXO3A transcriptional activity by promoting the association of 14-3-3 proteins with FOXO3A and the pooling of FOXO3A in the cytoplasm via CREM1-mediated nuclear export (Brunet 2002). Mutation of these three Akt sites to alanine produced a constitutively active form of FOXO3A capable of directly inducing apoptosis (Brunet 1999). These Akt site-mutant constructs have since been widely used to elucidate the functions of FOXO in many different systems without the need for PI3K/Akt pathway inhibition.

Akt is not the only kinase downstream of PI3K known to phosphorylate FOXO3A. It was reported that SGK1 could do so as well in response to growth factor stimulation and that this occurred in parallel with Akt phosphorylation (Brunet 2001). This study suggested that Ser315 was primarily the target of SGK1 while Akt optimally phosphorylated Thr32 and Ser253. Interestingly, other AGC kinases were also tested to see if they were capable of phosphorylating FOXO3A. Mainly they were not capable of this; implying relative specificity for SGKs and Akts in regard to FOXO3A phosphorylation. Very recently, the paradigm that SGK1 is an inhibitor of FOXO3A function was challenged. In c. elegans it was shown that sgk-1 activity actually promoted stress resistance and longevity and did not influence Daf-16 subcellular localization (Chen 2013). However, the exact mechanism underlying this new found relationship has yet to be described.

IKKβ was another kinase found to be capable of phosphorylating and inhibiting FOXO3A (Hu 2004). In breast cancer cells IKKβ phosphorylation at Ser644 induced the nuclear exclusion of FOXO3A as well as its ubiquitination and proteasomal degradation. This was completely independent of Akt-mediated phosphorylation demonstrating that it is a true parallel pathway. This parallel pathway proved important in myeloid leukemia where IKKβ could maintain FOXO3A inhibition in the presence of PI3K inhibition (Chapuis 2010).
The phosphorylation of FOXO3A is not only inhibitory, it can be activating as well. AMPK is a multi-subunit kinase that is progressively activated as ATP/ADP levels fall and AMP levels rise (for review see Hardie 2012). Hence AMPK senses the energetic state of a cell by directly measuring available ATP. When energy stress occurs and AMPK is activated it is capable of phosphorylating FOXO3A on six sites: Thr179, Ser399, Ser413, Ser555, Ser588 and Ser626 (Greer 2007a). These phosphorylations mildly enhanced the transcriptional activity of FOXO3A under energy stress conditions and altered the target gene set of FOXO3A. Some of these phosphorylations were also found to be conserved on Daf-16 and AMPK/Daf-16 could be placed in a cascade mediating dietary-restriction induced longevity in c. elegans (Greer 2007b). However, the exact consequences of these phosphorylations were unclear in c. elegans.

Acetylation of FOXOs has been another running story in the field. The study of this modification in regards to FOXO function was initiated with the discovery that p300 interacted with Daf16 and FOXO1 to regulate the IGFBP-1 promoter (Nasrin 2000). Shortly following this p300/CBP (CREB Binding Protein) was found to acetylate FOXO1 and FOXO3A when erythroid progenitor cells were growth factor deprived (Mahmud 2002). Subsequently, PCAF (p300/CBP associated factor) was found as an additional acetyltransferase while deacetylation was found to be carried out by SIRT1 (Class III HDAC family) as well as other unspecified Class I & II HDACs (Brunet 2004). In this study oxidative stress was found to induce FOXO3A acetylation and concomitant cell death. This could be counteracted by SIRT1-mediated deacetylation causing a switch in the FOXO3A gene expression program towards stress resistance genes. A more detailed view of the acetylation came about with the identification of the conserved lysines K242 and K245 as the primary sites for this modification (Daitoku 2004). The location of these residues in the c-terminal region of the DNA binding domain led to experiments demonstrating that acetylation at these sites decreased the ability of FOXO1 to bind DNA (Matsuzaki 2005). Interestingly, it was also found that acetylation promoted the phosphorylation of DNA-bound FOXO1 on Ser256 (FOXO3A Ser253) specifically because the interaction between FOXO1 and the DNA was loosened by the acetylation. Mutants that mimicked deacetylation were not phosphorylated...
by Akt when FOXO1 was pre-bound to DNA. This raised the possibility of a model where acetylation could reduce the overall DNA binding affinity of FOXO and also promote its nuclear export in the presence of active Akt. Additional biochemical studies with accompanying crystal structures of FOXO1 and FOXO3A bound to DNA confirmed that acetylation was inhibitory of FOXO DNA binding (Fig. 2b, Tsai 2007 & Brent 2008). These studies also reinforced the point that FOXO1 and FOXO3A bind most optimally to DBE elements and more loosely to IRS elements. This observation may help explain how FOXO3A changed its set of target genes when deacetylated by SIRT1 (Brunet 2004). In theory, acetylation of nuclear FOXO3A may preferentially cause its detachment from promoters that contain IRS elements more readily; leaving DBE-containing promoters relatively more occupied (although bound with less affinity). In support of this concept analysis of promoter sequences from genes regulated by acetylated and deacetylated FOXO3A revealed that promoters targeted by deacetylated FOXO3A were enriched for IRS & IRS-like elements while acetylated FOXO3A regulated promoters that were enriched for DBE elements (Santo 2006).

Although a relatively new arrival on the scene, the methylation of FOXO3A may yet prove to be of relevance to its function. It was recently demonstrated that the arginine methyltransferase PRMT1 could methylate FOXO1 on Arg248 and Arg250 (Yamagata 2008). These methylations were capable of preventing FOXO1 phosphorylation by Akt on Ser256 (FOXO3A Ser253) which led to nuclear trapping of FOXO1 and apoptosis under reactive oxygen species stress. It was also found that PRMT1 could methylate all the FOXOs and that these particular arginines were conserved in all family members and Daf-16. FOXO3A has also been shown to be mono-methylated on lysine 271 by the methyltransferase Set9 (Calnan 2012). Set9 was not capable of methylating the other FOXOs. It caused an increase in FOXO3A transcriptional activity and a decrease in the stability of the protein that seemed to be due to proteasomal degradation. However, phenotypic consequences were not demonstrated and the condition under which this occurred was not defined.
Phosphoinositide-3-OH Kinase (PI3K) Signaling

PI3K signaling is centered upon the phosphorylation of the inositol ring of phosphatidylinositol lipid substrates at the 3-hydroxyl group (for review see Vanhaesebroeck 2010). There are three classes of PI3K enzymes responsible for these inositol ring phosphorylations; Class IA, II & III. Class IA consists of the four catalytic subunits p110α/β/δ/γ along with the two primary regulatory subunits p85α/β. Class IA converts PtdIns-4,5-biphosphate (PtdIns(4,5)P$_2$) to PtdIns-3,4,5-triphosphate (PtdIns(3,4,5)P$_3$) which is the primary output of the PI3K pathway responsible for much of its downstream signaling. Class II includes the three members PI3K-C2 α/β/γ which catalyze the conversion of PtdIns-4-phosphate (PtdIns4P) to PtdIns(3,4)P$_2$. Class III only has one member, Vps34 which converts PtdIns to PtdIns3P. The activity of Class IA PI3Ks can be counteracted by the phosphatase PTEN (PtdIns(3,4,5)P$_3$ → PtdIns(4,5)P$_2$) as well as the phosphatases INPP5E and SHIP2 (PtdIns(3,4,5)P$_3$ → PtdIns(3,4)P$_2$). These competing activities maintain tight control over the size of the potent PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$ pools which in turn limits the magnitude of the downstream signaling.

Class IA PI3K activity is primarily triggered by the activation of receptor tyrosine kinases, GPCRs and RAS. Some of the earliest identified examples of this were the PDGF and insulin receptors (Auger 1989 & Ruderman 1990). It is now known that the Class IA PI3K enzymes are recruited to target receptors by the binding of their p85 regulatory subunits to adaptor molecules such as Irs1/2 and Grb2 (Myers 1992 & Besset 2000). The PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$ products in turn bind to the pleckstrin homology (PH) domains of target kinases (i.e. Akt and Pdk1) as well as those of GEFs and GAPs (i.e. regulators of Rac/Rho signaling) which are the primary effectors and signaling branch points in the pathway (for review see Vanhaesebroeck 2012).

A particularly important subset of PI3K effectors fall under the category of AGC kinases (for review see Pearce 2010). These kinases are so named because their catalytic domains resemble that of protein kinases A, G & C (PKA, PKG & PKC). The subset most important to the proper functioning of the PI3K pathway are Akt1/2/3, SGK1/2/3, p70S6K and Phosphoinositide-dependent kinase 1 (PDK1). All
of the AGC kinases typically have to be phosphorylated two times to be fully activated, once in a domain referred to as the hydrophobic motif (sometimes dispensable) and again at an absolutely required site in a region referred to as the activation domain. In this regard, PDK1 is a “master” member of the AGC kinase family as it phosphorylates and activates at least 23 AGC members at their activation domains. Unlike most other AGC kinases PDK1 is catalytically constitutively active due to its ability to trans-autophosphorylate itself on Ser241. It is found throughout the cell and can be recruited to the plasma membrane upon binding to PtdIns(3,4,5)P$_3$. This recruitment can potentiate the phosphorylation of Akt, a particularly crucial and well-studied mediator of PI3K signaling. Akt binds to PDK1 both in the cytosol and at the membrane independently of PtdIns(3,4,5)P$_3$ (Calleja 2007). Upon the binding of PtdIns(3,4,5)P$_3$ to the PH domain of Akt, Akt undergoes a conformational change that separates the PH domain from the catalytic domain, exposing the activation loop. PDK1 can then phosphorylate Akt at Thr308 which is an absolute requirement for Akt catalytic activity (Alessi 1996). To achieve full kinase activity, Akt can be additionally phosphorylated at Ser473 within its hydrophobic motif by the mTORC2 complex (Sarbassov 2005). Like Akt, both PDK1 and mTORC2 phosphorylate the SGKs on their activation loops and hydrophobic motifs, respectively (Biondi 2001, Garcia-Martinez 2008). The phosphorylation of SGKs on their hydrophobic motifs by mTORC2 potentiates their interaction with PDK1 via the PDK1 PIF pocket. Subsequently, they are phosphorylated on their activation loops by PDK1. The SGKs parallel the Akts in many ways and even share many of the same substrates. However, SGKs do not have a PH domain with which to interact with phosphoinositides. p70S6Kinase is a critical regulator of 5’ cap-dependent mRNA translation which is also activated by PDK1 phosphorylation on its activation loop (Pullen 1998). p70S6Kinase is also an important substrate of the mTORC1 complex which phosphorylates it on its hydrophobic motif (Ali 2005). This phosphorylation on Thr389 potentiates activation loop phosphorylation by PDK1. These events place this critical mediator of translation and its effector substrate S6 downstream of both PI3K and mTORC1 signaling.
PI3K signaling is involved in many cellular and biological contexts as a promoter of cell growth, survival, protein synthesis and metabolism (for review see Engelman 2006). One of the most physiologically important roles of PI3K in mammals is the maintenance of whole-body glucose homeostasis. Indeed, discovery of PI3K as the major activity associated with insulin and IGF-1 receptor signaling provided crucial mechanistic insights into the regulation of glucose uptake, gluconeogenesis and glycogen storage. One of the first reports in this area characterized the repression of glycogen synthase kinase (GSKα/β) by Akt in rat adipocytes and skeletal muscle in response to insulin (Cross 1997). In mice compound heterozygous deletion of p110α/β, a heterozygous knockin of a catalytically dead p110α or muscle-specific deletion of p85α/β all exhibited impaired insulin-stimulated glucose clearance (Brachmann 2005, Foukas 2006 & Luo 2006). Similarly, whole-body knockout of Akt2 but not Akt1 or Akt3 caused fasting hyperglycemia, impaired insulin-stimulated glucose uptake and elevated circulating insulin levels (for review see Gonzalez 2009). Additionally, liver-specific Akt2 knockout mice exhibited decreased liver triglyceride levels of both normal and high fat diets; demonstrating a positive role for Akt in triglyceride synthesis (Leavens 2009). The greatly reduced glucose uptake caused by loss of Akt activity is now known to result from the impairment of Glut4 glucose transporter translocation to the membrane which is caused by the loss of Akt phosphorylation of AS160 (Tanti 1997 & Bruss 2005). The centrality of this pathway has been further highlighted in humans where a family with a severe form of type II diabetes carries a point mutation disabling Akt2 (George 2004). Not only is glucose uptake affected by loss of PI3K signaling, so is gluconeogenesis. In the liver, loss of Insulin/PI3K/Akt signaling caused high levels of gluconeogenesis due to elevated FOXO1 transcription factor activity (Dong 2008).

**Mechanistic Target Of Rapamycin (mTOR) Signaling**

TOR was originally discovered when a genetic screen was conducted to identify genes involved in the sensitization and resistance of yeast to G1 growth arrest in response to the immunosuppressive drug rapamycin (Heitman 1991). Rapamycin was found to inhibit yeast proliferation by forming a complex with the FKBP12 protein. This complex was later co-purified with its target proteins in rat cells; the
largest of which was named RAFT1 which was found to be homologous with the genetically identified yeast TOR1 and TOR2 genes (Sabatini 1994). Simultaneously, the human cDNA was cloned and named FRAP (Brown 1994). Both RAFT1 and FRAP are now simply referred to as MTOR. At the time MTOR was also recognized to contain a kinase domain that exhibited high homology with the PI3K class of enzymes and was termed a PI3K Class IV enzyme. Interestingly, MTOR was even identified as a kinase phosphorylating the 4th position on the phosphatidylinositol ring, making it a putative “PI4” kinase (Sabatini 1995). This activity was found to be independent of rapamycin. However, MTOR was soon discovered to be a serine/threonine protein kinase phosphorylating its now well-known substrates 4E-BP1 and p70S6K which strongly implicated MTOR in the regulation of protein translation (Burnett 1998). Gradually, it emerged that the protein kinase MTOR was a participant in large, multi-subunit complexes that regulated its subcellular localization, catalytic activity and substrate specificity. These complexes now referred to as mTORC1 and mTORC2 have since been the focus of intensive investigation (for review see Laplante 2012). These complexes have common and unique members. The common ones are MTOR, deptor, mLST8, tti1 and tel2. Raptor and pras40 are specific to mTORC1 while Rictor, mSin1 and protor1/2 are specific for mTORC2. A single molecule of MTOR can only participate in one complex at any given time making these complexes mutually exclusive. This exclusivity extends to the cell signaling and biological roles of these complexes.

mTORC1

With the discovery of raptor, mTORC1 was the first MTOR complex to be described which set the stage for it to be the best studied of the two complexes (Kim 2002). mTORC1 responds to diverse inputs but the two primary points of control are amino acid levels and growth factor signaling (for review see Huang 2014). Very recently the lysosome has been identified as a crucial site for the proper regulation of mTORC1 activity.

Upon amino acid stimulation (especially leucine) mTORC1 and its essential activating GTPase RHEB<sup>GTP</sup> are recruited to the outer lysosomal membrane by the Ragulator complex (composed of LAMTOR1-5). This recruitment is dependent
upon the formation of active heterodimers between the RagA/B and RagC/D GTPases. This means that RagA can dimerize with either C or D as can RagB. The GDP/GTP bound state of each Rag in the heterodimer determines whether or not mTORC1 will be activated. An active combination would be $\text{RagA/B}^{\text{GTP}}\text{-RagC/D}^{\text{GDP}}$ while an inactive combination would be $\text{RagA/B}^{\text{GDP}}\text{-RagC/D}^{\text{GTP}}$. Importantly, the Ragulator complex acts as a GEF for the RagA/B members (converting GDP $\rightarrow$ GTP) while the proteins FLCN and FNIP1/2 act as a GAP for the RagC/D members (converting GTP $\rightarrow$ GDP). The GEF activity of the Ragulator complex can be counteracted by the GAP activity of the GATOR1 complex which can strongly suppress mTORC1 activity in the absence of amino acids. GATOR1 GAP activity can in turn be repressed by the GATOR2 complex. Recently, a family of GDIs (guanine nucleotide disassociation inhibitors) known as the sestrins have been shown to play a central role in the suppression of mTORC1 signaling by prevention of RagA/B GDP/GTP exchange (Peng 2014). Strikingly, TSC2 was shown to be non-essential for sestrin-mediated mTORC1 suppression. These lysosome/Ragulator-based mechanisms demonstrate that amino acid stimulation and sensing is the dominant mechanism of mTORC1 activation. The “classical” but more subservient route to activation runs via the inactivation of TSC2 by Akt phosphorylation in response to growth factor stimulation. Akt phosphorylation of TSC2 has recently been found to delocalize the TSC1/TSC2 complex from the lysosome (Menon 2014). Thus growth factor stimulation can modulate mTORC1 activity but cannot activate mTORC1 in the absence of amino acids.

mTORC1 activates many anabolic processes for the synthesis of the basic building blocks of life. The synthesis of proteins, lipids and nucleotides have all been found to be powerfully and quite directly activated by mTORC1 (for review see Shimobayashi 2014). This occurs while mTORC1 suppresses catabolic processes such as autophagy. It does so by phosphorylating a number of substrates either directly or via other serine/threonine protein kinases under its control such as p70S6K. Specifically, mTORC1 can activate 5’ cap-dependent mRNA translation via its phosphorylation and inhibition of 4E-BP1 and its activation of p70S6K which in turn phosphorylates many other proteins involved in translation. This effect on translation seems to be limited to transcripts that contain a 5’ oligopyrimidine
tract (5’ TOP). p70S6K also promotes pyrimidine biosynthesis by phosphorylating and activating the multi-functional enzyme CAD. Additionally, p70S6K drives lipid synthesis by phosphorylating and activating SREBPs. Independently of p70S6K, mTORC1 directly phosphorylates and suppresses LIPI1 which further activates SREBPs.

Mice where mTORC1 components and regulators have been manipulated yielded a wealth of phenotypes and insight into the biological roles of mTORC1 in mammals, especially in respect to metabolic control. TSC1 knockout in the liver hyperactivated mTORC1 and this resulted in the suppression of Akt activity and lipogenesis on both normal and high fat diets (Yecies 2011). Conversely, raptor knockout in the liver increases lipogenesis (Sengupta 2010). Adipose-specific knockout of raptor revealed that mTORC1 is necessary for adipogenesis as well as fat formation (Polak 2008). Although not fully explained, these mice were also more tolerant of glucose with enhanced glucose clearance capacity. Interestingly, increased Akt activity was observed not in adipocytes but in the muscle which was thought to be the cause. Whole-body p70S6kinase knockout recapitulated the insulin sensitivity and greatly reduced adiposity observed in the adipose-specific raptor KO mice; demonstrating the importance of p70S6kinase as an mTORC1 target (Um 2004). Raptor knockout in skeletal muscle does not affect whole-body glucose homeostasis but it does cause muscle atrophy with progressive dystrophy along with increased glycogen storage and hyperactive Akt (Bentzinger 2008).

mTORC2
This complex was only recently defined and is not as well understood in terms of its regulation or functions. A probable complicating factor is the existence of at least five isoforms of mSin1 that can form at least three different mTORC2 complexes which may have different activities and/or mechanisms of activation (Frias 2006). Currently, mTORC2 is believed to be activated by growth factors but the mechanism underlying this is completely unknown. The best work so far on its activation demonstrates that active mTORC2 is physically bound to ribosomes and that this association is required for its activation by growth factors (Zinzalla 2011).
As previously mentioned, mTORC2 is best known for phosphorylating the hydrophobic motif of AGC kinases such as those of Akt, SGKs and PKCs. This gives mTORC2 the potential to regulate a plethora of functions by modulating the activity of the AGC family. One paper using conditional whole-body knockout of the essential mTORC2 component Rictor demonstrated marked glucose intolerance and insulin insensitivity which was mapped to hepatic mTORC2 activity by tissue-specific deletion (Lamming 2012). Specifically, hepatic insulin insensitivity led to heightened gluconeogenesis which suggests the Akt/FOXO1 axis might have been disrupted, although this was not shown to be causative. Another study did show the same increase in gluconeogenesis along with a defect in lipid synthesis caused by liver-specific Rictor KO (Hagiwara 2012). In this study overexpression of an Akt2 Ser474 phosphomimetic (same site as Ser473 on Akt1) construct was able to rescue the gluconeogenesis and lipogenesis defects. The defect in lipogenesis was SREBP1c-dependent, indicating a role for the mTORC2/Akt axis in SREBP1c regulation. This finding also demonstrated that normally active mTORC1 is not sufficient to sustain lipogenesis if Akt signaling is impaired.

Originally, mTORC2 was described as also being important for the maintenance of cytoskeletal organization and integrity (Sarbassov 2004). The mechanism for this was largely missing but the recent discovery that mTORC2 stabilizes and activates PKCζ by phosphorylating it at its hydrophobic motif (Thr560) is enlightening (Li 2014). This report even demonstrated that a PKCζ phosphomimetic mutant of Thr560 could greatly rescue the cytoskeletal defects of Rictor KO MEFs by normalizing Rac/Rho signaling (increasing Rac/Cdc42 activity and suppressing RhoA activity). Intriguingly, it was also noted that inhibition of PI3K with LY294002 had absolutely no effect on endogenous PKCζ Thr560 phosphorylation while catalytic mTOR inhibitors could abolish it. Additionally, insulin stimulation was unable to drive the phosphorylation of PKCζ Thr560. These data strongly challenge the “dependency” of mTORC2 activity on PI3K signaling and its activation by growth factor signaling. Could it be that the phosphorylation of Akts and SGKs by mTORC2 in response to growth factors has more to do with substrate availability under growth factor stimulating conditions rather than regulation of mTORC2
catalytic activity? This cytoskeletal role for mTORC2 has even been extended to memory formation where mTORC2 was found necessary for the formation of long-term memory in flies and mice where again mTORC2 increased Rac activity (Huang 2013).

PI3K/mTOR/FOXO Crosstalk & Feedback Control
Although composed of distinct components the PI3K/mTOR/FOXO pathways exhibit enormous levels of crosstalk and overlap (Fig. 3). This tight integration is necessary for the maintenance of cellular and organismal homeostasis under just about all physiologic conditions. One of the inaugural findings in this area was the discovery that Akt could directly activate mTORC1 by phosphorylating TSC2 thereby disrupting the TSC1/TSC2 complex (Inoki 2002). More detailed analysis revealed that Akt phosphorylation delocalizes TSC2 from RHEB, relieving RHEB of TSC2 GAP activity (Cai 2006). Another point of contact was found to be Akt phosphorylation of the mTORC1 component PRAS40 which relieves repression of mTORC1 activity (Sancak 2007). In order to maintain balanced mTORC1 activity mTORC1 suppresses PI3K/Akt signaling at the growth factor receptor level. It is now known to do so via two distinct mechanisms. The first discovered was the phosphorylation and destabilization of the Irs1 adaptor molecule by p70S6kinase (Um 2004). Recently, mTORC1 has been found to directly phosphorylate and stabilize Grb10 which interacts with the insulin and IGF-1 receptors to suppress receptor-mediated signaling (Hsu 2011 & Yu 2011). mTORC2 is also capable of suppressing PI3K/Akt signaling from the receptor level. mTORC2 can phosphorylate and stabilize a subunit of the E3 ubiquitin ligase complex CUL7 (Fbw8); CUL7 is then targeted to ubiquitinate and degrade cytosolic pools of Irs1 (Kim 2012). PI3K and mTORC1 are also capable of repressing mTORC2 activation. Akt and p70S6kinase can phosphorylate the mTORC2 component Sin1 causing its disassociation from the complex and mTORC2 inactivation (Liu 2013). Being a key downstream mediator of the PI3K/Akt pathway, FOXOs can also potently regulate growth factor receptor, PI3K and mTORC1/2 activity.
Figure 3  Diagram of the PI3K/mTOR pathway. Kinases are colored blue, phosphatases are red, GTPases are orange, transcription factors are purple and green circles are phosphorylation sites.
When FOXOs are transcriptionally active they can act on the growth factor receptor level by driving the expression of receptor tyrosine kinases such as the insulin, IGF-1 and HER3 receptors (Chandarlapaty 2011). FOXOs can also directly bind to the promoter of p110α (PIK3CA) and upregulate its expression (Hui 2008). Taken together, FOXOs can act as inducers of growth factor-stimulated PI3K activity when they themselves are transcriptionally active. Under physiologic conditions, FOXO activity could be achieved through reduction of PI3K/Akt signaling and/or the modulation of other regulatory pathways. FOXOs can also powerfully regulate the balance of mTOR activity. In a seminal study both sestrin3 and Rictor were identified as direct targets of FOXOs (Chen 2010). Upon FOXO activation sestrin3 and Rictor were simultaneously upregulated. Sestrin3 was capable of repressing mTORC1 activity while the increased amount of free Rictor drove mTORC2 complex assembly and activity. Using FOXO3A null MEFs, FOXO3A was activated using H₂O₂ (oxidative stress) which functions regardless of Akt status. Shortly after activation the wild-type MEFs demonstrated elevated Akt activity along with repressed mTORC1 activity while the null MEFs showed sustained mTORC1 activity and repressed Akt activity. The authors hypothesized that FOXO may act to suppress anabolic metabolism mediated by mTORC1 and promote energy production via enhanced mTORC2/Akt activity during periods of cellular stress.

PI3K/mTOR/FOXO In Neuroblastoma & Other Cancers

Clinical & Genetic Features Of Neuroblastoma
Neuroblastoma is a tumor arising from neural crest sympathoadrenal progenitor cells within the adrenal medulla (for review see Mueller 2009, Cheung 2013 & Huang 2013). Although rare, it is the most common extra-cranial solid pediatric tumor with an incidence of 10.2 per million children under 15 years of age. Tumors are histologically classified based primarily on the degree of differentiation and Schwannian cell stromal content. This yields the categories neuroblastoma (primarily undifferentiated neuroblasts), ganglieneuroma (primarily Schwann cells) and ganglioneuroblastomas (composite of neuroblasts and Schwann cells). The most widely used staging system for neuroblastoma is the International
Neuroblastoma Staging System (INSS). This system is primarily based on degree of metastasis and surgical resection. Stage 1 tumors are localized and fully resectable while Stage 2 is either completely or partially resectable with only minor lymph node involvement. Stage 3 includes unilateral or bilateral unresectable tumors that exhibit considerable lymph node involvement while Stage 4 is any primary tumor with sites of distal metastasis. Stage 4S is a special case of a primary tumor with a localization of Stage 1 or 2 in a child of less than 1 year old showing only one site of metastasis (either skin, liver or bone marrow) that consists of < 10% malignant cells. Stage 4S is unique in that these tumors may spontaneously regress without treatment. Risk classification is based on histology, INSS stage and age. For unknown reasons age is an independent risk factor in neuroblastoma with children ≤ 18 months of age having considerably better prognosis then children beyond this cutoff. This phenomena even applies to Stage 3 & 4 neuroblastomas. Patients ≤ 18 months of age generally have a good prognosis even with high stage disease while older patients with high stage disease typically have a poor prognosis. In high stage patients the most common sites of metastasis are bone marrow (70%), bone (55%), lymph nodes (30%), liver (30%) and skull/orbital (18%). In high risk patients treatment includes surgical resection, intensive chemotherapy, radiotherapy and myeloablative therapy followed by hematopoietic stem cell transplantation. Despite this intensive multimodal treatment high risk patients have an overall survival rate of less than 40%.

In contrast to other tumors, neuroblastoma is a cancer with a very limited recurrent mutational spectrum (Molenaar 2012). The most common single-gene mutations are MYCN amplification (22%) and ALK receptor activation (8 – 10%). Full genome sequencing also suggested that Rac/Rho signaling along with other genes involved in neuritogenesis may be perturbed in high stage neuroblastomas and form a subgroup distinct from MYCN amplified tumors; although no single player in these pathways was mutated at a high frequency. More commonly, large chromosome gains and losses are identified in high stage tumors. Heterozygous loss of 1p36 occurs in 20 – 35% of all neuroblastomas and in 70% of aggressive ones (for review see Domingo-Fernandez 2012). Heterozygous loss of 11q occurs in 40 – 45% of tumors and rarely co-occurs with MYCN amplification or 1p36 loss.
Although candidate tumor suppressor genes have been identified on both 1p36 and 11q it is likely that haploinsufficiency of many genes from both these regions contribute to neuroblastoma pathogenesis. Gain of 17q is a non-prognostic widespread event in neuroblastoma of all stages (70%). Although it is not prognostic its common occurrence may indicate that 17q candidate genes are more generally involved in transformation rather than aggressive tumor properties or therapy resistance.

**PI3K/mTOR/FOXO In Neuroblastoma**

Previous work has repeatedly highlighted the centrality of the PI3K/mTOR pathway in neuroblastoma pathogenesis. The first indication of the importance of PI3K signaling in neuroblastoma came from a study demonstrating the efficacy of PI3K inhibition in a transgenic MYCN model (Chesler 2006). Shortly thereafter, immunohistochemical analysis of tumors for phosphorylated Akt demonstrated the highly significant poor prognosis of increased Akt pThr308 and pSer473 (Opel 2007). Interestingly, phosphorylation of the MAPK kinase Erk was not prognostic. This indicated that PI3K/Akt signaling was specifically important and not general RTK signaling. Subsequent work with PI3K-targeting compounds such as PI-103 (dual PI3K/mTOR inhibitor) and MK-2206 (Akt inhibitor) demonstrated that PI3K/mTOR/Akt inhibition was capable of sensitizing neuroblastoma cells to chemotherapeutic agents as well as having significant single-agent affects (Bender 2011 & Li 2012). Recently, a compound mouse model was constructed combining overexpression of mutant ALK with MYCN (Berry 2012). This yielded very aggressive, high-penetrance tumors that exhibited enhanced Akt and mTOR signaling. Combining the ALK inhibitor Crizotinib with the mTOR inhibitor Torin2 caused full tumor regression and dramatically extended the lifespan of the mice. These results strongly indicate that the primary mechanism of mutant ALK oncogenicity in neuroblastoma is via the PI3K/mTOR pathway.

Although much has been shown generally about the importance of the PI3K/mTOR pathway in neuroblastoma, comparatively little has been reported about FOXO in this system. In an overexpression system it was demonstrated that FOXO3A could activate cell death in SHEP cells via upregulation of the pro-apoptotic factors Bim
and Noxa (Obexer 2007). Importantly, IGF-1 was shown to be capable of inactivating endogenous FOXOs via PI3K signaling in neuroblastoma cells (Schwab 2005). FOXOs were also implicated in the TPA-induced differentiation of the SY5Y cell line (Mei 2012). These findings demonstrated that FOXOs had the potential to act as tumor suppressors in neuroblastoma cells and hence counteract oncogenic PI3K signaling.

**PI3K/mTOR/FOXO In Other Cancers**

The PI3K/mTOR pathway is a notorious force across the entire spectrum of cancers and tumor predisposing syndromes. The most frequently mutated component of the pathway is the PtdIns(3,4,5)P$_3$ phosphatase PTEN (for review see Hollander 2011). Indeed, PTEN was discovered due to its location on 10q23 which was a region widely lost in many cancers. It was also identified as the cause of Cowden Syndrome where germline loss of PTEN causes a condition where sporadic tumors occur with strong predispositions for breast, endometrium and thyroid carcinomas. PTEN is also known to be silenced by promoter methylation and microRNAs. Also quite prevalent across cancer are activating mutations in the PIK3CA (p110α) catalytic subunit (for review see Hafsi 2012). The most common activating mutations are E542K, E545K and H1047R. E542K and E545K disrupt the interaction between p110α and the p85 regulatory subunits, allowing for enhanced membrane localization and signaling. H1047R is located within the catalytic domain and directly enhances p110α catalytic activity. PTEN and PIK3CA mutations confer on cancer cells increased resistance to apoptosis, enhanced metastatic capacity as well as an increased capacity for glycolysis and hence promotion of the Warburg effect. Akt is much less frequently mutated but an Akt1 E17K mutation within the PH domain was uncovered in breast, colon and ovarian cancer. This mutation causes Akt1 to localize to the membrane independently of PtdIns(3,4,5)P$_3$, leading to constitutive signaling. Strikingly, this Akt1 E17K mutation seems to the cause of Proteus Syndrome (Lindhurst 2011). This syndrome is characterized by overgrowth of many tissues leading to severe morphological deformations. Genetic defects in the mTOR pathway itself are so far much less common in cancer. However, Tuberous Sclerosis Complex (TSC) is a condition of sporadic tumors arising from inactivation of the mTOR suppressors
TSC1/2 (for review see Orlova 2010). Outside of PI3K/mTOR components themselves, hyperactivation of receptor tyrosine kinases (RTKs) by either mutation or overstimulation almost all commonly result in activation of PI3K/mTOR signaling. This simple fact puts PI3K/mTOR signaling high on the list of drug targets across all cancers.

Due to the relatively strict inverse correlation between PI3K pathway activity and FOXO activity FOXOs were largely expected and found to be tumor suppressive. As previously mentioned, knockout of all FOXOs in the hematopoietic lineage causes leukemia (Paik 2007). FOXOs act as tumor suppressors primarily by promoting cell cycle arrest, differentiation and apoptosis. In various systems FOXOs have been found to drive the expression of the cell cycle suppressors p16, p21 and p27 which contributed significantly to their tumor suppressive capability (Medema 2000, Stahl 2002, Seoane 2004 & de Keizer 2010). FOXOs have also been found capable of upregulating Bim and driving mitochondrial apoptosis (Dijkers 2000, Essafi 2005 & Krol 2007). FOXOs have also proven to be a fundamental check on the oncogenicity of Myc. An initial indication of this came from work showing that PI3K activity was essential for Myc-induced transformation and that constitutively active FOXO3a could prevent transformation (Bouchard 2004). One very direct mechanism by which FOXO could squelch Myc transcriptional activity came with the finding that FOXO can drive the expression of the Max inhibitory factor Mxi1-SRα (Delpuech 2007 & Gan 2010). FOXOs are also capable of driving p19Arf expression which can activate p53-dependent cell cycle arrest in response to oncogenic Myc activity (Bouchard 2007).

**PI3K/mTOR/FOXO In Longevity**

**C. elegans**

A genetic screen for mutations that extended lifespan in c. elegans uncovered some of the first mutations at a locus subsequently described as age-1 (Klass 1983 & Friedman 1988). Mutants carrying the most potent allele had a mean lifespan increase of 65% and a maximum lifespan increase of 110%. A few years later another breakthrough story emerged in longevity. The c. elegans dauer formation
mutants daf-2 and daf-16 were found to regulate adult lifespan with daf-2 mutation doubling maximal lifespan and daf-16 mutation completely suppressing this extension (Kenyon 1993). These two separate discoveries were united when age-1 was placed downstream of daf-2 and upstream of daf-16 (Dorman 1995). These exciting genes were rapidly identified as orthologues of the then emerging mammalian INSR (daf-2)/PI3K (age-1)/FOXO (daf-16) pathway (Morris 1996, Kimura 1997, Ogg 1997 & Lin 1997). Based on this parallel the c. elegans orthologues of PTEN (daf-18), Akt1/2 (akt-1/2) and Pdk1 (pdk-1) were also cloned and found to function as expected with regard to mammalian PI3K signaling (Ogg 1998, Paradis 1998 & Paradis 1999). Incredibly, even more dramatic lifespan extensions were discovered in 2nd generation age-1 mutants that saw 10-fold increases in median lifespan and large increases in tolerance for various stressors (Ayyadevara 2008). The lifespan extension was fully reversible by daf-16 mutation as was some of the stress resistance. Despite these strong effects, daf-2/age-1/daf-16 signaling is not required for caloric restriction induced longevity (Lakowski 1998 & Kaeberlein 2006). This highlights that caloric restriction operates via a distinct pathway. A main question in the longevity field has been how daf-16 mediates such a powerful and robust effect. From multiple mRNA profiling and ChIP studies daf-16 target genes have been identified. Major classes of daf-16 targets include reactive oxygen species (ROS) suppressive genes, heat shock proteins, genes involved in resistance to heavy metal toxicity, pathogens and even hypertonic stress (for review see Murphy 2006). Another major class of genes identified are involved in protein, carbohydrate and lipid metabolism. Despite these lists, no complete mechanisms have yet been identified that can recapitulate the entire effect of daf-16 on longevity. Studying daf-16 is further complicated by the existence of different daf-16 isoforms (daf-16a/b/d/f) that have their own tissue-specific expression distributions, respond somewhat differently to upstream pathways and may have different downstream targets (Kwon 2010). This highlights that tissue-specific studies may be necessary to further delineate the exact roles of daf-16 in longevity.

Like the PI3K/FOXO pathway, mTOR in c. elegans (a.k.a. CeTOR) and raptor (daf-15) were also shown to modulate both dauer formation and lifespan (Jia 2004).
Reduction of mTORC1 signaling mainly by heterozygous mutation of daf-15 extended mean lifespan 30% and maximum lifespan 19% and also caused fat accumulation. Together with daf-2 mutation, daf-15 heterozygous mutation extended lifespan an additional 12%. As with daf-2, daf-16 mutation could revert the daf-15 lifespan extension but not the fat accumulation. This suggested that daf-16 was downstream of mTORC1 in lifespan regulation and that mTORC1 was acting in parallel with the daf-2/age-1 pathway to modulate lifespan. Unlike the daf-2/age-1/daf-16 pathway, mTOR was found to be involved in the longevity induced by dietary restriction (Hansen 2007). Using RNAi directed against mTOR itself no additional lifespan extension occurred in DR animals, although mTOR RNAi could extend lifespan on its own. This clearly separated aspects of mTOR function from the PI3K/FOXO pathway in terms of longevity. This relationship was further clarified when mTORC1 and mTORC2 were individually tested. It was confirmed that daf-16 was required for lifespan extension downstream of mTORC1 inhibition, however mTORC2 inhibition resulted in lifespan extension that was daf-16 independent (Robida-Stubbs 2012). This suggests that reduction of mTORC2 and not mTORC1 activity is responsible for DR-based longevity, although this remains to be tested. It would also be informative to inhibit Raptor and Rictor in an age-1 mutant background to see if lifespan can be further extended. This would help clarify the degree of dependence of mTORC1 and mTORC2 signaling on PI3K signaling which is most likely not fully abrogated in daf-2 mutants.

**D. melanogaster**

Due to the reproducible role of Insulin/PI3K/mTOR/FOXO signaling in modulating c. elegans lifespan researchers set out to see if these findings were conserved. For this they turned to fruit flies which are a more complex yet genetically tractable organism with a reasonably short lifespan. From the beginning it was clear that these pathways had taken on more complex roles then those observed in c. elegans. Whereas it is possible to completely inactivate components of the Insulin/PI3K pathway in c. elegans this was much less possible in drosophila. Unlike c. elegans, inactivation of Dp110 (age-1/PIK3CA), InR (daf-2/INSR), dAkt1 (akt-1/2) and p60 (p85α/β) were either homozygous lethal, had an unaffected
lifespan or were short-lived (Clancy 2001). However, CHICO (orthologue of mammalian Irs1-4) homozygous mutant females had a median lifespan increase of 48% while homozygous males were slightly shorter-lived. Heterozygous mutation was sufficient to achieve a 36% median lifespan increase in females and 13% increase in males. Additionally, an increase in female mean lifespan of 85% was observed with a certain allele combination of InR mutations (Tatar 2001). This was not totally unexpected because previous work had shown that the Insulin/PI3K pathway was vital for organ and cell growth in drosophila (Leevers 1996, Bohni 1999 and Gao 2000). FOXO was first shown to extend drosophila lifespan when a constitutively active version of dFOXO (Akt phosphorylation sites mutated) was overexpressed in the pericerebral fat body (Hwangbo 2004). This led to reduced insulin signaling in the brain and peripheral fat body. Homozygous null dFOXO animals had shorter lives then controls; however, like c. elegans, homozygous dFOXO mutants completely ablated lifespan extension in CHICO heterozygous mutants and other PI3K pathway mutant backgrounds (Yamamoto 2011 & Slack 2011). Unlike c. elegans, dFOXO mutation was unable to completely rescue all phenotypes caused by reduced Insulin/PI3K signaling; such as reduced body size and increased stress resistance. This indicated that even as early in evolution as drosophila the Insulin/PI3K pathway had acquired other effectors. In agreement with c. elegans, dFOXO was not required for DR-induced lifespan extension (Min 2008 & Giannakou 2008). One potential mechanism underlying dFOXO lifespan extension downstream of Insulin/PI3K signaling may be direct repression of the TGFβ Activin-related ligand dawdle (Bai 2013). Repression of dawdle by dFOXO caused repression of TGFβ signaling which resulted in upregulation of the autophagy-inducing gene atg8a in muscle. Muscle-specific RNAi against atg8a or dawdle was sufficient to extend fly lifespan.

Like c. elegans CeTOR, homozygous inactivation of drosophila TOR (dTOR) resulted in severely impaired development with obvious defects in cell size and number and yielded early larval lethality (Oldham 2000). Overexpression of dTOR inhibitors dTSC1, dTSC2, dominant-negative p70S6kinase or the rapamycin binding domain of FKBP12 all extended mean lifespan 12 – 37% (Kapahi 2004). This study also demonstrated that dTOR inhibition in fat was sufficient for lifespan
extension and that dTOR inhibition could suppress lifespan reduction by over-feeding. Therefore like c. elegans CeTOR, dTOR is also involved in caloric/dietary restriction mediated longevity. Rapamycin treatment was also found to extend fly lifespan and this could be blocked by overexpression of constitutively active mutants of the mTORC1 downstream effectors p70S6Kinase and 4E-BP1 (Bjedov 2010). Interestingly, very little work has been done on the individual mTOR complexes in drosophila. Specific mTORC1 inhibition with raptor RNAi resulted in flies with bent wings which could be rescued by overexpression of p70S6kinase (Lee 2007). In the same study Rictor null flies had a reduced body size which corresponded to reduced Akt activity and increased dFOXO activity. Unfortunately, epistasis between Rictor and Insulin/PI3K mutants was not tested and lifespan results were not reported. However, epistasis between dTSC1/dTSC2 and Insulin/PI3K signaling has been evaluated. In one study co-overexpression of dTSC1 and dTSC2 could suppress lethality caused by InR overexpression (Potter 2001). Another study showed that dTSC1/dTSC2 were downstream of InR/PI3K/Akt signaling but also parallel to it (Gao 2001). On one hand heterozygous loss of dTSC1/dTSC2 could rescue InR mutant lethality, placing these genes directly downstream of Insulin/PI3K/Akt signaling. On the other, double mutation of dPTEN and dTSC1 gave a cell size increase much greater than either mutation alone, strongly arguing for parallelism. Hence mTOR signaling is activated by Insulin/PI3K/Akt signaling but is also independently activated. This could again explain how mTOR functions in DR-induced longevity that is Insulin/PI3K/FOXO-independent. Again, further work would be needed to breakdown mTORC1/2 function in drosophila in regard to DR-induced longevity and dFOXO function.

**Mice**

One of the first long-lived mouse models described was the Ames Dwarf (df/df) mouse. These mice are much smaller in size than normal laboratory mice and are deficient in growth hormone (GH) production; as a consequence they also have low circulating levels of IGF-1 (Slabaugh 1981, Chandrashekar 1993 & Brown-Borg 1996). The role of GH in mouse lifespan was additionally confirmed when the GH receptor was specifically ablated, recapitulating the Dwarf phenotype along with
reduction of IGF-1 levels (Coschigano 2003). The same year heterozygous IGF1 receptor (IGF1R) knockout mice were reported showing significantly extended life for females but not for males (Holzenberger 2003). Interestingly, the dwarfism phenotype did not occur suggesting additional roles for GH signaling in longevity beyond those of IGF1 signaling. Recently, IGF1 signaling was specifically restored in the GHRKO background and reversion of pro-longevity phenotypes was observed but the effect on lifespan is yet to be determined (Arum 2014). Strikingly, GHRKO can also completely block the pro-longevity effects of caloric restriction; meaning CR cannot extend the GHRKO phenotype any further (Bonkowski 2006). This effect extended to the enhanced insulin sensitivity that both CR and GHRKO provide with no further increase in the combination (Bonkowski 2009).

Due to the findings in Dwarf mice, c. elegans and drosophila other models that reduced IGF/Insulin/PI3K signaling were constructed. Adipose-specific insulin receptor knockout mice (FIRKO) exhibited decreased adiposity, increased insulin sensitivity at older ages and extended lifespan (Bluher 2002 & Bluher 2003). The increased insulin sensitivity is somewhat shocking but may be due to endocrine signaling from adipocytes to other insulin target organs such as muscle and liver. Lifespan was also extended by whole-body homozygous deletion of Irs1 (Selman 2008 & Selman 2011). Unlike the FIRKO mice, Irs1 nulls have life-long insulin insensitivity. However, they do have enhanced glucose clearance and preserved Beta cell mass (leading to higher insulin production) at older ages. Together with the FIRKO mouse data one interpretation may be that insulin sensitivity itself may not be a key parameter in longevity, but enhanced glucose clearance achieved by any means may be. Interestingly, whole-body heterozygous inactivation of one allele of PIK3CA resulted in lifespan extension as well as enhanced insulin sensitivity and glucose clearance (Foukas 2013). These same effects were observed in whole-body PTEN overexpressing mice (Ortega-Molina 2012). Both of these studies suggested that the enhanced insulin sensitivity may have been caused by reduction of mTORC1/p70S6kinase activity which led to decreased Irs1 phosphorylation and degradation. However, this hypothesis was untested. Another model tested was whole-body heterozygous deletion of Akt1 (Nojima
These mice lived longer but had normal insulin sensitivity and glucose clearance. They exhibited reduced mTORC1 activity but no change in FOXO activity.

In accordance with other model organisms, treatment of mice with rapamycin extended the lifespans of both males and females across heterogeneous genetic backgrounds (Harrison 2009). Interestingly, rapamycin is known to induce loss of insulin sensitivity and glucose intolerance in mice (Cunningham 2007). However, whole-body deletion of p70S6kinase was found to extend female but not male lifespan with only females having increased insulin sensitivity and glucose clearance (Selman 2009). Since rapamycin has been found to inhibit both mTORC1 and mTORC2 as well as induce glucose intolerance in vivo further genetic studies were required to clarify the role of mTOR in mammalian longevity and glucose homeostasis (Lamming 2012). Hepatic deletion of raptor (mTORC1) yielded mice with normal insulin sensitivity and glucose tolerance while hepatic deletion of Rictor (mTORC2) caused glucose intolerance and insulin insensitivity. No effect on lifespan was observed in these mice and mTORC2 inhibition was implicated as the cause of rapamycin-induced glucose intolerance and insulin insensitivity. These authors also constructed mice with whole-body compound heterozygous deletion of mTOR/raptor and mTOR/mlst8; one to create a maximal reduction of mTORC1 activity and the other to reduce the activity of both complexes. Only the mTOR/mlst8 model gave an increase in female but not male lifespan with no changes in insulin sensitivity or glucose tolerance; leaving the rapamycin effect unresolved.

**Primates & Humans**

Despite the existence of various FOXO knockout mouse models no role has yet been described for any of the FOXOs in mouse longevity. However, a connection to human longevity may have been uncovered with various genetic studies linking intronic polymorphisms of FOXO3A to centenarians (Willcox 2008, Flachsbart 2009, Anseimi 2009, Li 2009, Pawlikowska 2009 & Soerensen 2010). Another genetic association study discovered exonic polymorphisms in IGF1R that were associated with long-lived humans (Suh 2008). These polymorphisms reduced the
ability of IGF1R to activate Akt signaling. A study of individuals deficient in GHR signaling (GHRD) has added more support for IGF/insulin signaling in human healthspan (Guevera-Aguirre 2011). Compared to controls (including family members), GHRD individuals had low circulating IGF1 and IGF2 levels, no instances of cancer or diabetes and also exhibited very high insulin sensitivity. Unfortunately, lifespan studies on these individuals were inconclusive due to a relatively high number of deaths in the GHRD cohort attributable to alcoholism, accidents and convulsive disorders. Furthermore, in two different cohorts of controls and long-lived individuals increased insulin sensitivity was positively associated with longevity (Paolisso 2001 & Wijsman 2011). Exciting data has recently been reported in monkeys showing that 30% caloric restriction can extend their lifespan 3-fold; indicating that CR is also an operable mechanism for lifespan extension in primates and perhaps humans (Colman 2014). In a separate study CR increased insulin-stimulated PI3K pathway activity in muscle, insulin sensitivity and muscle-mediated glucose clearance (Wang 2009). Together, these findings suggest that the IGF/Insulin/PI3K/FOXO axis may be of relevance for primate and human longevity.
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