Grasping Forkhead Box O functions by a bilateral approach

De functies van Forkhead Box O eiwitten doorgronden middels een bilaterale aanpak.

(met een samenvatting in het Nederlands)

Proefschrift

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Far away in the sunshine are my highest aspirations. I may not reach them, but I can look up and see their beauty, believe in them, and try to follow where they lead.

Louisa May Alcott
US juvenile novelist (1832 - 1888)

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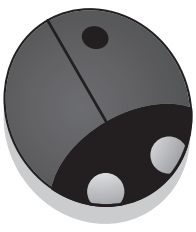


Introduction

Metabolic adaptation to environmental alterations is fundamental for long term survival of the organism. Therefore, signaling mechanisms that govern adaptation control lifespan.

At the molecular level, information about the environment is brought to the cells by extracellular signaling molecules, which engaged by cell-surface receptors activate intracellular signaling pathways. This allows transmission of information into the nucleus and results in alteration of the gene expression repertoire that is crucial for adaptation.

Two major mechanisms have evolved for the rapid and accurate transmission of signals from cell-surface receptors to the nucleus, both involving protein phosphorylation. One of them depends on the regulated translocation of activated kinases from the cytoplasm to the nucleus where they can phosphorylate transcription factors and alter their activity. The second mechanism relies on the sequestration of transcription factors in the cytoplasm. This keeps them in the latent state and upon phosphorylation they translocate to the nucleus where they can activate their target genes [1].

The Insulin/Insulin-like growth factor (INS/IGF) signaling (IIS) pathway is considered to be the central regulator of metabolism in metazoans (Box 1). Insulin action depends on the activity of a linear cascade of kinases including PI3K phosphoinositide 3- kinase (PI3K), 3-phosphoinositide dependent protein kinase 1 (PDK1) and protein kinase B (PKB/AKT). Upon activation, PKB phosphorylates numerous proteins in both the cytoplasm and the nucleus. which is crucial for the metabolic response [2]. Recent studies have signified the role of the c-Jun-N-Terminal kinase (JNK) pathway in regulation of IIS signaling [3]. Since JNK is activated by a broad range of cellular stresses, its ability to modulate insulin signaling provides an important mechanism that warrants metabolic adaptation to environmental challenges. Given that active PKB promotes growth and energy storage but at the expense of lifespan, JNK promotes lifespan extension by opposing INS signaling. Importantly, both the IIS pathway and JNK signaling converge on Forkhead Box O (FOXO) transcription factors and a vast

Box 1: Insulin signaling

Insulin and insulin-like growth factor (IGF) belong to the same group of evolutionary related proteins that possess a variety of hormonal activities [203]. Insulin is mainly involved in the regulation of carbohydrate and fat metabolism and thereby the storage of energy, whereas IGF plays a vital role in the regulation of somatic growth and cellular proliferation [2, 203]. Insulin signaling is mediated by a complex, highly integrated network of proteins, which transmit the signal downstream of insulin receptor (IR) tyrosine kinase [204]. IR has two splice isoforms that are usually co-expressed in cells that also express the highly related insulin-growth factor-1 receptor (IGF1R), which can also be activated by insulin. Similarly, IGF activates its own receptor but in addition can also activate IR [204]. The interaction of IGF with the IGF1R is tightly controlled by the family of insulin-like growth factor binding proteins (IGFBPs), which demonstrate high affinity binding to both IGF but no binding to insulin. Thus IGFBPs scavenge away IGF and thereby promote the binding of insulin to its receptor [205]. The binding of ligand to IR/IGF1R triggers the phosphorylation of at least six known substrate proteins including insulin receptor substrate (IRS) proteins and Shc proteins that are linked to the activation of two main signaling pathways: the phosphatidylinositol 3-kinase (PI3K)–AKT/protein kinase B (PKB) pathway, which is responsible for most of the metabolic actions of insulin, and the Ras–mitogen-activated protein kinase (MAPK) pathway, which regulates expression of particular genes and cooperates with the PI3K pathway to control cell growth and differentiation [204].

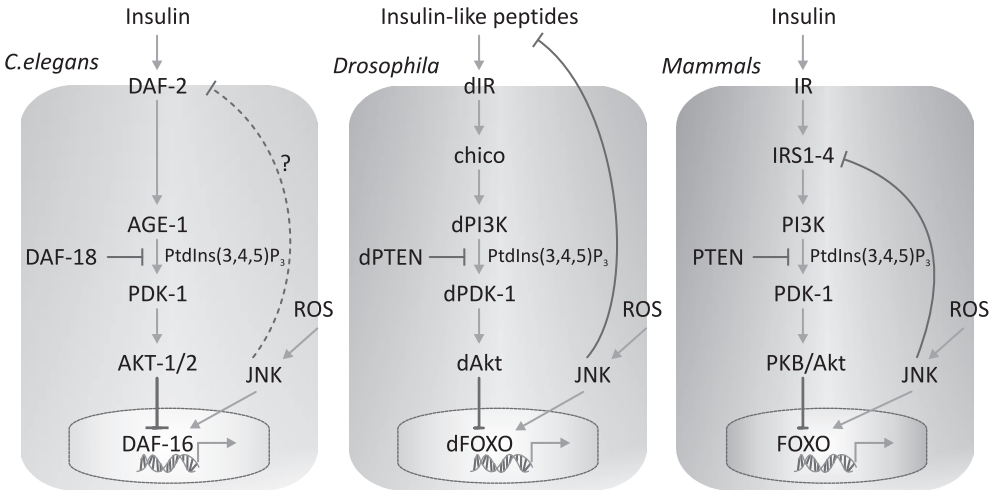


Figure 1. Conservation of insulin and stress signaling towards FOXO between *C. elegans*, *D. melanogaster* and mammals

Upon engagement of insulin, the insulin receptor (IR) phosphorylates insulin receptor substrate (IRS) proteins, which triggers activation of the phosphatidylinositol 3-kinase (PI3K)–AKT/protein kinase B (PKB) pathway. PI3K is a lipid kinase that phosphorylates inositol lipids in the plasma membrane. These phosphorylated lipids form docking sites for the 3-phosphoinositide-dependent protein kinase 1 (PDK1) and protein kinase B (PKB/Akt). This allows activation of PKB, which in turn directly phosphorylates FOXO proteins and thereby inhibits their transcriptional activity. This can be overruled by ROS signaling pathway, which via activation of c-Jun-N-Terminal kinase (JNK) promotes FOXO-dependent transactivation. Additionally, JNK can also directly inhibit insulin signaling. It has not been established whether this is also conserved in *C. elegans*. Nevertheless, PKB has an opposing effect on the lifespan of worms in comparison to JNK.

amount of studies indicate that IIS- and JNK-dependent regulation of lifespan requires FOXO-mediated transcription (Figure 1) [4].

FOXO proteins in the regulation of lifespan

The first indications for the biological functions of FOXO came from genetics studies on the *Caenorhabditis elegans* ortholog of FOXO, Abnormal dauer formation protein 16 (DAF-16) [5]. Normally, *C. elegans* has a relatively short lifespan of only a few weeks. However, under adverse conditions, such as changes in temperature, reduction in nutrients and overcrowding, juvenile animals can enter an alternative dormant larval stage referred to as "dauer". This diapause stage is DAF-16 dependent and can last for months allowing animals to wait for conditions to improve, before proceeding to adulthood [5, 6].

Additionally, a null mutation in DAF-2 (an ortholog of the mammalian INS/IGF receptor) or AGE-1 (an ortholog of the catalytic subunit of mammalian PI3K) arrests the development of *C. elegans* at dauer stage even in the presence of sufficient amounts of food [7, 8]. Interestingly, partial reduction of DAF-2 levels decelerates the rate of aging and greatly increases lifespan of *C. elegans* without substantially affecting its activity or fertility [5, 9, 10]. Both processes require the presence of functionally active DAF-16, which implies that under normal conditions DAF-2 negatively regu-

lates the activity of DAF-16 [11, 12]. Moreover, it appears that DAF-16 can also regulate lifespan non-autonomously and its activity in the intestine seems to be more relevant for promotion of lifespan than in other tissue. Furthermore, neuronal activity of DAF-16 is important for dauer formation and to a lesser extent for lifespan regulation [13, 14].

Similarly, in *Drosophila melanogaster*, attenuation of IIS due to mutations in the insulin-like receptor (InR) or receptor substrate Chico significantly increases lifespan of flies [15, 16]. In addition, ectopic expression of dFOXO (*Drosophila* FOXO) in the fat body of adult flies, which corresponds to mammalian white adipose tissue and liver, also extends lifespan [17, 18]. This indicates that signaling pathways that tune the lifespan of worms also operate in flies. Therefore, the question rises whether this stays also true for mammals. Whereas, both *C. elegans* and *D. melanogaster* express a single copy of INS/IGF receptor, mammals possess distinct receptors for insulin and IGF1, with the insulin receptor being generally associated with metabolic regulation and glucose homeostasis, and the IGF1 receptor being responsible for growth [19, 20]. In addition, in mammals the FOXO class of transcription factors consists of four members: FOXO1, FOXO3a, FOXO4 and FOXO6 (Box 2) [21]. Nevertheless, current findings indicate that a decrease in the IIS pathway prolongs lifespan in mice, which demonstrates that this pathway of lifespan determination has been conserved throughout evolution [22, 23]. Moreover, similarly to invertebrates, mammalian lifespan can also be regulated cell non-autonomously since impaired insulin signaling in both adipose tissue and brain are sufficient to increase lifespan of mice [24, 25].

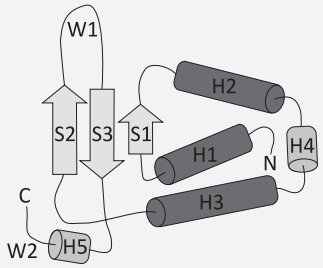
Whether these phenotypes are at least partially FOXO-dependent still needs to be determined. Interestingly, perturbation in insulin signaling has been associated with increased lifespan in humans [26, 27]. What is even more remarkable is that some polymorphisms of FOXO proteins have been linked to longevity in several cohorts located throughout the world [28]. This suggests that both IIS and FOXO are involved in determining aging and longevity in humans.

It is now gradually recognized that metabolic syndromes are often associated with precocious aging and increased mortality. Therefore, it has become apparent that maintenance of metabolic homeostasis, especially under stress conditions, is crucial for longevity. Notably, moderate activation of the stress-responsive JNK pathway results in extended lifespan in worm and flies [29]. In both species JNK-mediated prolongation of lifespan requires DAF-16/dFOXO [30, 31]. This implies that JNK is opposing the IIS pathway through activation of its downstream target DAF-16/FOXO. Activation of FOXO by JNK has also been reported in mammalian cells. In addition, in mammalian systems JNK also inhibits INS signaling upstream at the level of IRS proteins. Moreover, JNK has also been reported to regulate IIS via an endocrine mechanism. In *Drosophila*, activation of JNK results in the down-regulation of DILP2 expression, an insulin resembling peptide, in a dFOXO-dependent manner [31]. It has been also shown that JNK-1 influences IIS through the transcriptional activation of lipocalin signals [32].

In invertebrates, activation of the JNK pathway does not only promote lifespan but also increases resistance to environmental insults [29-31]. Similarly to longevity, JNK-mediated activation of stress defenses is dependent on FOXO-

Box 2: FOXO proteins within the family of Forkhead transcription factors

The forkhead superfamily of transcription factors is characterized by a specific type of monomeric DNA-binding domain known as the forkhead box (FOX) [206]. The crystal structure of the FOX domain revealed that it is made up out of three α helices and two characteristic large loops, or "wings" [207]. Therefore, forkheads are also called winged-helix transcription factors. The panel on the right depicts a ribbon representation of the solution structure of the FOXO4 forkhead domain sequence Ser-92 to Gly-181.



The *Drosophila melanogaster* fork head (fkh) gene was the first identified member of the forkhead/winged-helix class of transcription factors and it has been shown to be essential for the proper formation of terminal structures of the embryo. Since then over 100 forkheads have been recognized throughout the animal kingdom as well as in fungi and yeast [208]. Genetic studies revealed that forkhead transcription factors besides having functions in development, also play an essential role in the regulation of metabolism and cellular proliferation and survival.

Forkheads are divided in several subclasses based on structural similarities and conservation levels in the FOX domain. The nomenclature of chordate FOX transcription factors uses FOX (for "Forkhead box") as the root symbol. This is followed by a letter that indicates the subfamily. Within a subfamily, each gene is identified by a number (e.g., FOXO3a). Abbreviations for the chordate FOX proteins contain all uppercase letters for human (e.g., FOXO3a); only the first letter capitalized for mouse (e.g., Foxo3); and the first and subclass letters capitalized for all other chordates (e.g., FoxO3) [209].

In mammals, the class O of forkhead transcription factors (FOXO) consists of four members: FOXO1, FOXO3a, FOXO4 and FOXO6. FOXO2 is identical to FOXO3a and FOXO3b represents a pseudogene. FoxO5 is expressed only in the zebrafish *Danio rerio* [210]. FOXOs are classical transcription factors as they contain besides DNA binding domain an atypical nuclear localization signal (NLS) and a nuclear export signal (NES), which is surrounded by transactivation domain (TAD) (panel below, the numbers indicate the amino acids corresponding to FOXO4). The DNA binding domain of FOXOs, in particular

helix three, binds to the consensus motif 5'-TTGTTTAC-3' [211]. Therefore, theoretically, all FOXOs have similar activating or repressing functions. Nevertheless, they also exhibit more specific functions, which is most probably determined by structural differences among FOXO proteins in combination with specific expression patterns and isoform-specific regulation.

mediated transcription. In addition, FOXO proteins have been implicated in enhanced stress resistance of certain long-lived mice such as mice lacking the p66Shc protein [33].

Taken together, this provides an important mechanism that allows maintenance of energy resources under unfavorable conditions and simultaneously increases environmental stress resistance.

Further studies showed that the longevity of DAF-2 mutants also depends on the activity of AAK-2, the catalytic subunit of AMP activated kinase (AMPK) [34]. AMPK functions as a sensor of low energy levels and becomes active when the AMP/ATP ratio increases. Consequently, AMPK activates catabolic pathways and represses anabolic pathways in order to restore normal energy levels. Originally, it has been shown that AMPK acts downstream of DAF-2 but parallel to DAF-16 to regulate lifespan [34, 35]. However, AMPK can also extend lifespan in response to dietary restric-

tion and at least in some cases this appears to be DAF-16 dependent [36, 37]. Moreover, treatment of mice with the anti-diabetic drug metformin, which activates AMPK, can slow down the aging processes and extend lifespan [38].

Interestingly, AMPK was also found to be important for Sirtuin-mediated promotion of longevity [35]. The SIR2 protein of *Saccharomyces cerevisiae* is the founding member of a large family of $(NAD)^+$-dependent protein deacetylases called the Sirtuins. Overexpression of SIR2 has been reported to extend lifespan in yeast, worms and flies [39-41]. Notably, the SIR2-mediated extension of lifespan in worms has been shown to be DAF-16-dependent [41]. Whether dFOXO is required for the SIR2-promoted longevity of flies is not known. Similarly, it is not established whether sirtuins extend lifespan in mammals. However, it has been revealed that SIRT1, a mammalian ortholog of SIR2, can regulate the activity of FOXO proteins resulting in expression of genes involved in stress resistance. Since increased stress defenses are beneficial for longevity, it is likely that SIRT1 through FOXO might promote lifespan. Likewise, heat and oxidative stress in worms increases lifespan in both a SIR2- and DAF-16-dependent manner [42, 43].

Recently, it has been revealed that signals from the reproductive system influence lifespan. Removal of germ cells, but not somatic reproductive tissues, in worms and flies, dramatically lengthens their lifespan [44, 45]. In *C. elegans*, the loss of germline activates DAF-16 by a novel mechanism that involves the adaptor protein KRI-1. It is not clear whether a similar system exists in mammals, although the mammalian reproductive system can affect lifespan. If ovaries from young mice are transplanted into old recipients, the lifespans of the recipients is extended. Moreover, it has recently been reported that Krev interaction trapped protein 1 (KRIT1, also known as the CCM1), the mammalian ortholog of KRI-1, promotes expression and activity of FOXO and loss of KRIT1 significantly impairs intracellular defenses against oxidative stress [46].

It is apparent that diverse pathways involved in the regulation of lifespan modulate FOXO transcriptional activity. This raises the possibility that FOXO proteins functions as a node at which multiple pathways intersect to control aging. However, it also indicates that there are several ways to influence lifespan. Even stress, normally detrimental to lifespan, at low levels can have beneficial effects [28].

FOXO proteins as tumor suppressors

Aging of an organism is associated with increased onset of cancer. Recently, compelling data signify that FOXO transcription factors, besides their role in promoting longevity, act as tumor suppressors [47]. This might prove to be an important safety mechanism in which the same proteins would protect against development of cancer while at the same extending lifespan.

The first indication about FOXO proteins acting as a tumor suppressor was the observation that FOXO1, FOXO3a and FOXO4 are present at chromosomal breakpoints associated with several forms of human tumors: rhabdomyosarcomas for FOXO1 and acute myeloid leukaemias for FOXO3a and FOXO4 [48-52]. These chromosomal translocations produce chimeric proteins consisting of the C-terminal transactivation domain of FOXO factors fused to the N-terminal domain of other transcriptional regulators including PAX3 or PAX7 for FOXO1

and the mixed lineage leukemia (MLL) gene for FOXO3a and FOXO4. It is postulated that the presence of fusion proteins that exhibit MLL or PAX gain-of-function characteristics is a driving factor for tumors to arise. However, since mice bearing the PAX3-FOXO1 transgene fail to develop tumors it is possible that the increased tumorigenicity of these chimeric proteins results, at least partially, from loss of FOXOs functions [53]. This is supported by the observation that the MLL–FOXO4 fusion leads to transdominant repression of wild-type FOXO expressed from the remaining intact allele [54].

Another important indication supporting tumor suppressive functions of FOXOs came from research performed in *C. elegans*. In *C. elegans*, self-renewal of germline stem cells is controlled by defective in germ line development-1 (GLD-1). Mutations in GLD-1 cause reentry of germ cells, during the early stages of oogenesis, into the mitotic cell cycle and thus overproliferation [55]. Akin to transformed vertebrate cells, these cells proliferate in a growth factor–independent manner, and do not undergo apoptosis, ultimately leading to the death of the animal. Therefore, this type of germ cell hyperplasia can be considered as a germ-line tumor in *C. elegans*. Interestingly, a mutation in DAF-2 that impairs IIS reduces hyperplasia in GLD-1 mutants through inhibition of proliferation and increased cell death. This is mediated by enhanced activity of DAF-16, which operates along with a p53-dependent pathway, specifically in GLD-1 mutant cells, without affecting cell division in the normal germ line [56].

Hyperactivation of PKB/AKT is one of the most common characteristics of human cancer. Increased PKB/AKT activity is caused by aberrant expression or mutation of PKB itself, by overexpression of tyrosine kinase receptor, or by mutations in Ras, phosphatase and tensin homolog (PTEN) or PI3K [57]. Notably, increased activation of PKB results in the inhibition of FOXO functions. This implicates that activity of FOXO proteins might be inhibitory to the development of tumor and further supports their tumors suppressive functions. In addition, it has been shown that in breast cancer tissue sections the presence of cytoplasmic FOXO3a highly correlates with poor survival of breast cancer patients [58].

Compelling evidence that FOXO proteins are indeed true tumor suppressors came from studies employing genetically modified mice. Firstly, employing nude mice it has been shown that an active form of FOXO3a can suppress tumorigenicity induced by IκB kinase β (IKKβ) [58]. Similarly, a constitutively active version of FOXO4 reduces tumor onset as well as progression and size in nude mice transplanted with cells expressing the human epidermal growth factor receptor 2 (HER2) oncogene [59]. Finally, expression of active FOXO1 decreases tumorigenesis of PTEN null cells [60].

In addition, it has been reported that combined broad somatic deletion of three FOXO genes (FOXO1, FOXO3a and FOXO4), results in a lineage restricted tumor phenotype in mice characterized by thymic lymphomas and hemangiomas, which ultimately were capable of spreading to various tissues [61]. This demonstrates that FOXO proteins are indeed genuine tumor suppressors.

Importantly, complete loss of all three FOXO alleles was required for tumor formation since disruption of any two genes together shows less severe phenotypes. This underlies the functional redundancy among FOXO tran-

scription factors [61].

With respect to the ubiquitous expression of FOXO proteins, the narrow spectrum of tumors in triple FOXO-null mice, restricted to thymocytes and endothelial-derived cells, is rather unexpected. Remarkably, not all tissues containing endothelial cells developed hemangiomas. This strengthens the tissue specific functions of FOXO proteins, which is supported by gene expression profile studies indicating that FOXOs regulate the expression of different genes in diverse tissues [61].

In the aforementioned studies, the oncogenic background driving the development of FOXO-null tumors has not been defined. Therefore, it is of great interest to uncover how loss of FOXO protein cooperates with expression and activity of various oncogenes. Moreover, even though there is some degree of functional redundancy among FOXOs, it cannot be excluded that in combination with the deregulation of certain oncogenes, loss of a single FOXO is sufficient to increase tumorigenesis.

It has been shown that active FOXO3a can inhibit the induction of multiple target genes of the oncogenic protein Myc [62]. In addition, a dominant-negative form of FOXO (dnFOXO), which abrogates the function of all FOXO isoforms, enhances lymphomagenesis in the E-m-Myc mouse model [63]. One way to enhance Myc-induced tumorigenesis is by enhancing the apoptotic threshold and indeed it has been shown that dnFOXO attenuates Myc-induced apoptosis. At the same time, there is no effect on Myc-induced proliferation.

Another important mechanism that represses tumor formation is oncogene-induced senescence (OIS). Importantly, it has been revealed recently that FOXO4 plays a role in BRAF kinase-induced senescence [64]. BRAF is a potent oncogene that is commonly upregulated in different human tumors with the highest occurrence in melanomas. The constitutively active form of BRAF ($BRAF^{V600E}$) initially supports cell cycle progression, which ultimately is impeded due to BRAF-induced senescence [65]. Therefore, in order to obtain a tumorigenic phenotype, cells need to bypass senescence by acquiring additional mutations.

The increased activity of BRAF results in elevated levels of reactive oxygen species (ROS), which are crucial for the activation of FOXO4. Once activated, FOXO4 triggers expression of $p21^{cip1}$, which in turn is necessary for the induction of senescence. Moreover, ectopic expression of FOXO4 in melanoma-driven cells reinduces senescence [64]. This suggests that in order to bypass senescence cells need to inactivate FOXO4. This assumption is supported by the fact that loss of PTEN synergizes with $BRAF^{V600E}$ to induce melanomas [66]. Importantly, the lack of PTEN activity upregulates PKB, which on its turn, inhibits FOXO functions.

Taken together, the conclusion can be drawn that FOXO transcription factors are *bona fide* tumor suppressor genes. Given that they are inactivated in a high number of human tumors, it will be interesting to see whether reactivation of FOXOs could revert fully developed cancer.

Involvement of FOXO proteins in regulation of the cell cycle

The ability of FOXO to regulate cell-cycle progression constitutes one of the most relevant features for their tumor suppressive functions. In general, ectopic expression of FOXO proteins results in inhibition of cell proliferation even in cell lines transformed due to for instance

aberrant activation of Ras or deficiency of PTEN [60, 67]. Hence, FOXO proteins exert opposite effects on proliferation in comparison to PKB, which promotes cell cycle progression and growth (Figure 2) [68, 69].

In normal dividing cells, progression through the different cell-cycle phases is coordinated by the sequential and periodic activation of positive regulators, cyclins, and CDKs (cyclin-dependent kinases). Several cyclin-CDK complexes have been identified, each promoting progression through a distinct phase of the cell cycle. The activities of cyclin-CDK complexes are counterbalanced by two classes of inhibitors namely CKIs (cyclin-dependent kinase inhibitors) and proteins of the retinoblastoma (Rb) family [70].

FOXO transcription factors affect progression of the cell-cycle in two major ways, firstly, by inducing cell cycle arrest and secondly by promoting apoptosis [71]. Activation of FOXOs takes place under conditions that have a rather anti-proliferative character including growth factor withdrawal or oxidative stress. Therefore, once activated, FOXO proteins block cell-cycle progression at the G1 phase by promoting expression of the specific CKI, $p27^{kip1}$ [67, 72]. Besides transcriptional upregulation of $p27^{kip1}$, FOXOs have been shown to increase its stability [59, 73]. Moreover, activation of FOXOs promotes expression of another CKI, $p21^{cip1}$ [74], which similarly to $p27^{kip1}$, interacts with cyclin-D/CDK4/6 and cyclin-E/CDK2 complexes [75, 76].

Finally, FOXOs have been implicated in the negative regulation of cyclin-D protein levels; however, the mechanism of this mode of FOXO action is at present unknown [77].

It is important to underline that FOXO-dependent expression of $p27^{kip1}$ seems to be mainly induced by growth factors deprivation, whereas $p21^{cip1}$ appears to be upregulated specifically in response to oxidative stress [64].Additionally, it has also been reported that transforming growth factor β (TGFβ) stimulation triggers the formation of a complex between FOXO and similar to mothers against decapentaplegic (SMAD) proteins, thereby promoting expression of $p21^{cip1}$ [74]. However, it became clear that TGFβ alters the expression of many other genes in a FOXO-dependent manner [78, 79]. TGFβ normally functions as a negative regulator of cell growth [80]. Therefore, it is not surprising that TGFβ employs FOXO proteins to execute the desired gene responses since FOXO-mediated transactivation also promotes cytostasis.

Interestingly, FOXO transcription factors are also able to directly upregulate expression of retinoblastoma-like p130 protein [68]. Protein levels of p130 positively correlate with the induction of quiescence [81]. Therefore, following FOXO-mediated cell-cycle arrest, cells enter a quiescent state commonly referred to as G0. Even though the G0 phase is associated with an exit of the cell-cycle, this process is reversible and cells can continue to proliferate. Thus, activation of the PI3K-PKB pathway through inhibition FOXO activity, decreases the levels of $p27^{kip1}$ and p130, thereby promoting the cell-cycle reentry of the quiescent cell [68].

In addition to inhibition of cell-cycle progression at the G1 phase, FOXO factors have been indicated to play a rather opposite role in the G2 phase of the cell-cycle.

Overexpression of a transcriptionally inactive mutant of FOXO3a induces accumulation of cells in G2/M and delayed progression from the M to G1 phase [82].

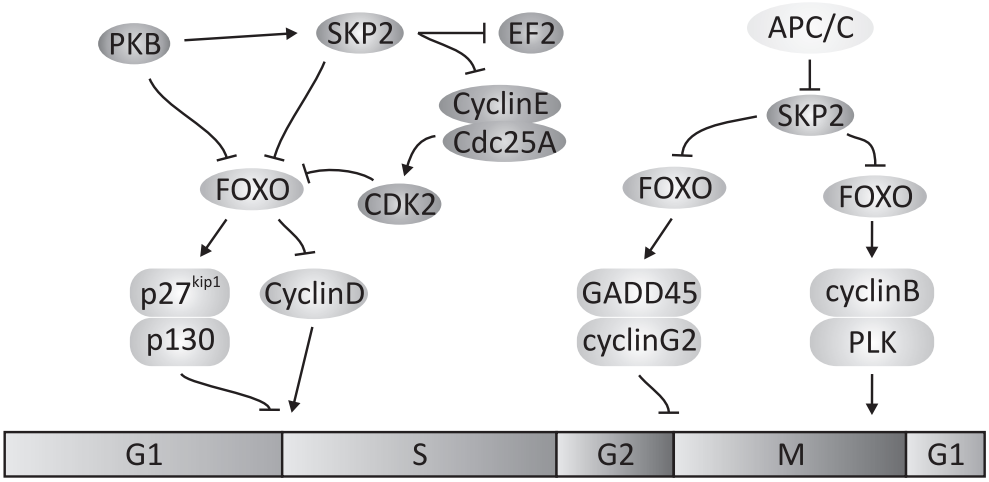


Figure 2. The involvement of FOXO proteins in regulation of cell cycle progression
In the absence of growth factor signaling FOXO transcription factors become active. This triggers transactivation of several negative regulators of the G1/S transition of the cell cycle and repression of positive regulators, which all together stops the cell cycle at the G1/S phase. In addition, activation of FOXO has been associated with induction of G2/M cell cycle arrest. Thus, progression through the cell cycle requires inactivation of FOXO proteins, which is accomplished through PKB-mediated exclusion of FOXO from the nucleus followed by degradation. In addition, CDK2 inhibits activity of FOXO in order to allow G1 to S transition. The inhibition of SKP2 during the later phase of mitosis allows accumulation of FOXOs and progression through cell cycle.

Furthermore, it has been reported that FOXOs promote expression of mitotic genes such as cyclin B and Polo-like kinase (PLK) [82]. Thus, it is suggested that FOXO-mediated transcription is crucial for cell-cycle progression. Interestingly, it points to a model in which activation of PKB and therefore inactivation of FOXOs is crucial for the initiation of cell-cycle progression (see Figure 2). However, efficient execution of the mitotic program requires decreased PKB activity followed by activation of FOXO-mediated transcription.

FOXO has also been indicated to play a role in the G2 checkpoint in response to oxidative stress. Ectopic expression of the active form of FOXO4 induces a G2 arrest of the cell cycle [83]. This seems to be independent of $p27^{kip1}$ as overexpression of $p27^{kip1}$ itself does not induce G2 arrest. In contrast, FOXO4-mediated induction of G2 arrest positively correlates with expression of GADD45, which has previously been shown to induce G2 arrest itself [83]. GADD45 is involved in DNA damage repair and protein levels of GADD45 are increased in response to oxidative stress. Moreover, GADD45 has been suggested to be a direct target of FOXO3a and FOXO4 [83]. Altogether, it has been proposed that in response to oxidative stress FOXOs enhance expression of GADD45 and thereby induce G2 arrest. This would promote resistance to oxidative stress through regulation of DNA repair.

Finally, activation of FOXO transcription factors induces apoptosis in hematopoietic cells in response to cytokine withdrawal [84, 85]. Similarly, oxidative stress-mediated activation of FOXOs in neuronal cells results in cell death [86]. It is unclear why in these cells apoptosis

and not the cell cycle arrest, is the main outcome of FOXO-mediated transcription. It is possible that these cells are somehow more sensitive to apoptosis and therefore this is the main response that is activated if the conditions are not optimal or damaging. Importantly, several proapoptotic genes been shown to be directly regulated by FOXO transcription factors [87]. This indicates that similar to the cell-cycle arrest FOXOs can promote cell death in multiple ways.

FOXO functions in oxidative stress resistance

FOXO-dependent protection against tumorigenesis might be associated with their ability to regulate cellular antioxidant capacity.

First of all, the DAF-16-dependent dauer state in *C. elegans* is characterized by increased resistance to oxidative stress. Most importantly, this elevated stress resistance is DAF-16 dependent, which suggests that DAF-16 supports expression of genes involved in stress defensive mechanisms [10, 88].

The activity of mammalian FOXO proteins can be directly modified by stress responsive kinases including JNK, Mammalian Ste20-like kinase (MST1) and p38 mitogen-activated protein kinase (p38 MAPK) [89-91]. This results in FOXO-dependent upregulation of antioxidant genes, which is crucial for resolving oxidative stress. Importantly, MST1-FOXO signaling is also operating in *C. elegans* [91]. Similarly, both JNK and p38MAPK have been shown to induce nuclear localization of DAF-16 in response to oxidative stress or heat respectively [30, 92], suggesting that these kinases promote DAF-16 activity. This assumption is further supported by the fact that JNK- and p38MAPK-mediated regulation of DAF-16 is coupled to synergistic lifespan extension [30, 92]. In addition, the JNK-FOXO signaling pathway is crucial for tolerance against oxidative stress insults in *D. melanogaster* [29, 31]. It has also been recently shown that dFOXO upregulates the expression of Jafrac1, an ortholog of human peroxiredoxin II, which plays crucial role in ROS scavenging [93]. This might extend the list of antioxidant that are upregulated by FOXO proteins, which include manganese superoxide dismutase (MnSOD), catalases and sestrins (Figure 3) [94, 95].

Cellular ROS are considered to function as signaling molecules. However, it is vital for cells to sense any changes in ROS levels, since once they are elevated this might result in oxidative stress and eventually become detrimental to the cells. Thus, there are various redox-sensing proteins within the cell that are modulated by reversible oxidation of their cysteine residues. This in turn alters activities of numerous signaling pathways and enables the cell to respond to ROS signals or to activate stress responses.

Recently, it has been shown that the activity of FOXO4 can be modulated by oxidation of its cysteine residues, which raises the possibility that FOXO proteins themselves can act as direct sensors of the redox status of cells [96]. The oxidation of cysteine-thiols of FOXO4 and most probably FOXO3a (unpublished data), is critical for the interaction of FOXOs with E1A binding protein p300/CREB-binding protein (p300/CBP), which is mediated through formation of disulphide bonds. p300 and CBP are essential regulators of FOXO-mediated transcription and they induce a shift in the FOXO-dependent transcriptional program from cell cycle arrest to apoptosis [96].

Since FOXO proteins themselves can also induce expression of antioxi-

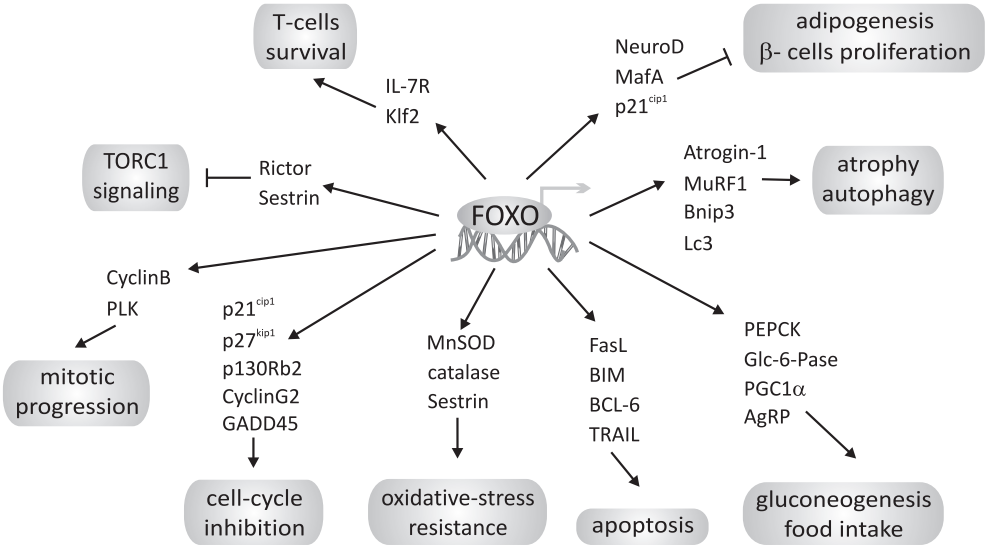


Figure 3. FOXO targets genes and the cellular outcome of their transactivation
FOXO transcription factors are implicated in the transcriptional regulation of a myriad of proteins. This in turn, involves FOXO in the regulation of diverse cellular processes most notably cell cycle progression, cell death, protection from cellular (oxidative) stress and cellular metabolism. Note that this figure does not include all FOXO target genes.

dant genes, it is possible that when the level of oxidative stress is relative low, they will primarily support cell cycle arrest and activation of defensive mechanisms. This provides both the time and the tools to reduce cellular ROS levels. Importantly, FOXOs have also been shown to induce expression of proteins involved in DNA damage repair [97], thus their activation is not only resolving oxidative stress but also allowing repair of the damage [98, 99].

Nevertheless, in response to severely damaging ROS levels, FOXOs can switch to induction of apoptosis. This is more beneficial for the whole organism, because it will prevent potentially dangerous mutations to be passed on to daughter cells. Therefore, it is possible that in response to different ROS levels, cysteine residues of FOXO proteins are differentially modified, which would allow fine tuning of cellular responses to various levels of ROS.

This is consistent with the tumor suppressive functions of FOXO proteins and the promotion of longevity. The elimination of cells damaged beyond repair has important implications in preventing tumor development that would otherwise limit lifespan. On the other hand, activation of repair mechanisms is important for tissue maintenance, as too extensive elimination of cells could result in depletion of sources of tissue self-renewal, which would also shorten lifespan [94].

The role of FoxO in the regulation of metabolism

Several *in vivo* studies employing various mouse models revealed that FOXOs are also involved in the regulation of metabolism, food intake, atrophy and angiogenesis. This implies that even though at the cellular level FOXOs are

generally recognized as being involved in the regulation of proliferation and resistance to stress, they also display various tissue specific functions. Importantly, both aspects of FOXO functions appear to be equally important with respect to environmental adaptation and therefore longevity.

Involvement of FOXOs in the regulation of metabolism is in accordance with them being the main targets of INS signaling, which is the master regulator of systemic energy management.

In order to modulate metabolism, FOXO proteins appear to function in a number of regulatory tissues including liver, pancreas, the hypothalamus-pituitary axis, and adipose tissue. This results in orchestrated upregulation of several genes promoting an increase in the circulating glucose levels. Therefore, ablation of FOXO1 in hepatic cells reduces glucose levels in newborn and adult mice [100]. Importantly, this suggests that FOXO1 might be the primary FOXO involved in regulation of glucose metabolism. However, other FOXOs may possibly contribute to the regulation of glucose homeostasis since liver-specific triple knockdown of FOXO1, FOXO3a and FOXO4 results in a much more pronounced phenotype [101].

The major FOXO1 transcriptional targets that are involved in gluconeogenesis include Glucose-6-phosphatase (G6Pase), Phosphoenolpyruvate carboxykinase (PEPCK) and PPARγ coactivator 1α (PGC1α). Interestingly, PGC1α promotes FOXO1 activity thereby providing positive feedback regulation of FOXO1-mediated transactivation. Moreover, in the neonatal liver FOXO1 interacts with another transcription factor CCAAT enhancer binding protein α (C/EBPα), which results in the cooperative induction of PEPCK. Binding of FOXO1 to the promoter of PEPCK occurs in a C/EBPα-dependent manner. At present it is not known whether FOXO1-mediated expression of gluconeogenic genes requires its assembly with other transcription factors in adults as well [102]. Besides being involved in glucose metabolism, FOXO1 is also implicated to regulate lipid metabolism. However, the role of FOXO1 in the regulation of lipid metabolism is rather inconclusive as FOXO1 has been reported to both reduce the expression of genes important for lipid/sterol synthesis and promote lipogenesis [103].

Nevertheless, FOXO1 can also promote expression of the apolipoprotein ApoC-III and thereby inhibit the activity of lipoprotein lipase, which is crucial for hydrolysis of triglycerides [104]. Therefore, FOXO1 through ApoC-III increases the levels of triglycerides in plasma.

Several studies reported that FOXO1 is involved in the regulation of pancreatic β-cells. Increased activation of FOXO1 appears to inhibit β-cell proliferation thereby regulating β-cell mass. This is most probably related to the ability of FOXO1 to negatively regulate the expression and activity of pancreatic and duodenal homeobox 1 (PDX1), which plays a pivotal role in the maintenance of β-cell function [105, 106].

However, in response to elevated ROS levels FOXO1 provides protection against β-cell failure [107]. Interestingly, this depends on FOXO1 acetylation, which triggers translocation of FOXO1 to PML bodies. However, it appears that in order to induce transactivation of target genes FOXO1 needs to be deacetylated by SIRT1. This triggers expression of the Ins2 gene transcription factors Neurogenic differentiation (NeuroD) and Transcription factor MafA (MafA) in a FOXO1-dependent manner [107]. How

this promotes stress resistance has not been elucidated but it might be related to the induction of premature senescence. Importantly, since uncontrolled activation of FOXO1 can be detrimental to β-cells, deacetylated FOXO1 seems to be more prone to degradation [107]. Thus, acetylation of FOXO1 might provide protection against degradation at the expense of decreased activity.

Recently, osteoblasts have been found to play a role in regulating glucose metabolism through secretion of osteocalcin [108]. In addition, mice lacking FOXO1 specifically in osteoblasts, exhibit enhanced proliferation of pancreatic β-cells, insulin secretion and insulin sensitivity. This suggests that FOXO1 acts in osteoblasts to regulate overall glucose homeostasis. Subsequently, it has been revealed that the ability of osteoblast-specific FOXO1 to modulate glucose metabolism depends on a FOXO1-induced decrease in osteocalcin expression and an increase in the expression of Osteotesticular protein tyrosine phosphatase (OST-PTP) [109]. In this way, FOXO1 not only reduces osteocalcin protein levels but also inhibits its activity as it has been shown that OST-PTP, which encodes a protein tyrosine phosphatase, inactivates osteocalcin by enhancing its γ-carboxylation [108, 109].

Importantly, FOXO1 is also implicated in the suppression of adipogenesis, which depends on FOXO1-mediated upregulation of $p21^{cip1}$ and the ability of FOXO1 to inhibit the expression and activity of Peroxisome proliferator-activated receptor γ (PPARγ) [110, 111]. Since adipose tissue plays a fundamental role in energy homeostasis, the capacity of FOXO1 to regulate the differentiation of adypocytes constitutes a potent way to modulate overall metabolism.

Many aspects of energy homeostasis, including food uptake, are regulated by the hypothalamus. Importantly, FOXO1 has been proven to function in the arcuate nucleus of the hypothalamus to promote food intake and an increase in bodyweight by inducing the expression of orexigenic neuropeptides Agouti-related protein (AgRP) and/or neuropeptide Y (NPY) [112, 113]. Simultaneously, FOXO1 suppresses expression of anorexigenic neuropeptides such as Pro-opiomelanocortin (POMC) via inhibition of the Signal transducer and activator of transcription 3 (STAT3)-dependent transactivation. Activation of the insulin and leptin signaling pathways decreases expression of FOXO1 in the hypothalamus [112], which is consistent with the inhibitory effect of these hormones on food intake.

Taken together, FOXO1 operates in several regulatory tissues to systematically modulate metabolism. This leads to a metabolical change from glucose utilization to glucose preservation and is crucial for surviving under unfavorable conditions such as like nutrient deprivation. However, when nutrients are available, FOXO1 induces food uptake, which allows restoration of the metabolic reserves.

Skeletal muscle is the largest organ in the human body and it acts as its major protein reservoir. During starvation or deprivation of nutrients, skeletal muscle exhibits increased protein degradation driven by two processes, namely ubiquitin-proteasomal atrophy and lysosomal autophagy. In the short term, this is highly beneficial as it provides energy but also free amino acids for the synthesis of acute-phase proteins in the liver, and for the maintenance of protein synthesis in obligatory working organs. Interestingly, conditions that promote muscle wasting augment expression of

FOXO proteins. Therefore, it has been revealed recently that FOXOs mediate both atrophy and autophagy in muscle in response to fasting, but also denervation, glucocorticoids, and hind limb suspension [114-117]. During fasting FOXO1 and FOXO3a induce transcription of two muscle-specific E3 ubiquitin ligases, Atrogin-1Muscle atrophy F-box protein (Atrogin-1/MAFbx) and Muscle-specific RING finger protein 1 (MuRF1). This promotes proteolysis via an ubiquitin-dependent proteasome system without induction of apoptosis [115, 116]. In addition, constitutively active FOXO4 has been shown to enhance expression of Zinc finger protein 216 (ZNF216), a novel ubiquitin-binding protein, which is necessary for denervation-induced atrophy [114]. Importantly, in human muscle the protein levels of FOXO1, atrogin-1 and MuRF1 positively correlate with muscle atrophy following the de-training period [118].

In parallel, FOXO3a has been demonstrated to promote autophagy in response to fasting or denervation by directly controlling the transcription of several autophagy-related genes including Microtubule-associated protein 1 light chain 3 (LC3), BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (Bnip3), Gamma-aminobutyric acid receptor-associated protein-like 1 (Gabarapl1), and Ubiquitin-like protein ATG12 (Atg12l) [119, 120]. This FOXO3a-induced autophagy is mainly mediated by Bnip3 [119]. Interestingly, also in *D. melanogaster* activation of dFOXO in response to starvation promotes autophagy in the fat body [121]. Furthermore, BECLIN-1, a *C. elegans* ortholog of the yeast and mammalian autophagy gene Vacuolar protein sorting-associated protein 30 (APG6/VPS30), is necessary for life extension of DAF-2 mutants [122]. At present, it is not known whether this is also DAF-16-dependent. Nevertheless, it might be possible that FOXO-mediated regulation of autophagy is evolutionary conserved.

FOXO in the maintenance of stem cell pools

Longevity of higher-level organisms requires preservation of tissue homeostasis, which in many tissues types strongly depends on their regenerative capacities. The regeneration of tissues relies on the presence and maintenance of tissue-specific stem cells. Importantly, several studies have proven that FOXO transcription factors are crucial for the long-term regenerative potential of stem cells [123]. The simultaneous ablation of FOXO1, FOXO3a and FOXO4 specifically in hematopoietic system results in a significant decrease in the population of long-term hematopoietic stem cells LT-HSCs. The reduction in LT-HSCs correlated with a higher rate of proliferation in FOXO-deficient HSCs and expansion of both the myeloid and lymphoid lineages. This indicates that FOXO proteins are crucial for the maintenance of HSCs in the quiescent state, thereby preserving their replicative and self-renewal capacity. This is in accordance with the ability of FOXOs to promote expression of several cell cycle inhibitors.

In addition, FOXO-deficient HSCs exhibit higher levels of apoptosis. This however, was also observed in the more mature populations [123].

The molecular mechanisms underlying the ability of FOXOs to control proliferation and apoptosis in HSCs depend on the regulation of ROS levels in those cells (Figure 4). This is supported by the fact that treatment of mutant mice with antioxidants rescues the proliferative and survival defects of FOXO-null

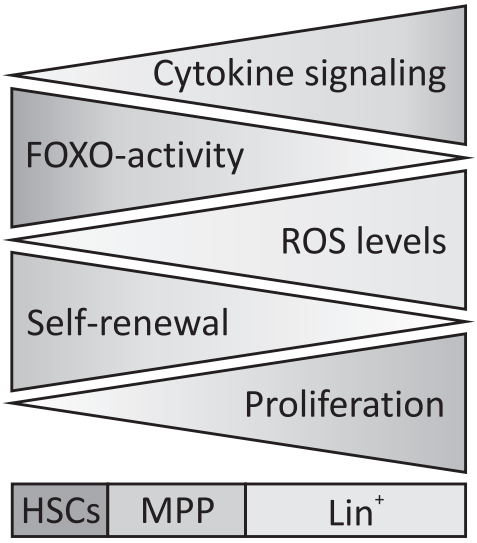


Figure 4. FOXO proteins are essential for the maintenance of the HSC pool
The preservation of HSC functions depends on their ability to maintain cellular ROS at low levels. This strongly depends on FOXO activity as loss of FOXO significantly increases ROS levels within HSCs and subsequently decreases the lifespan of these cells. Thus, the progression of HSCs towards more mature state likely requires cytokine-dependent inactivation of FOXO activity. This increases ROS levels and drives HSCs to proliferate and allows their differentiation of lineage-restricted cells.

HSCs [123].

Thus, depletion of FOXO proteins results in elevated ROS levels, thereby promoting cell cycling of HSCs and consequent loss of quiescence. This is paralleled by reduced expression of a subset of genes involved in the metabolism of ROS including several previously identified targets of FOXOs. The ROS-dependent regulation of quiescent state of HSCs has been reported previously and it seems to depend on the activity of p38 MAPK [124].

Interestingly, recent studies employing PKB-knockout mice revealed that PKB-deficient HSCs persist in the G0 phase of the cell cycle and exhibit lower levels of ROS in comparison to wild type HSCs [125]. This could be a result of the enhanced activity of FOXOs; however, reduced ROS levels in the PKB-null HSCs do not correlate with increased expression of known FOXO targets. Nevertheless, since loss of PKB is associated with increased FOXO activity, it cannot be excluded that this accounts, at least partially, for the observed phenotype.

Additionally, this nicely correlates with other studies showing that specific ablation of PTEN in the hematopoietic system results in the increased proliferation of HSCs and progressive reduction in the HSC compartment [126]. This is in accordance with FOXO knockout studies where reduced activity of FOXOs also resulted in increased proliferation of HSC.

Altogether, this illustrates that cellular ROS levels in HSCs need to be precisely balanced in order to maintain their functions.

The FOXO-dependent regulation of stem cell maintenance is not restricted to HSCs. Recently, two independent studies have shown that FOXO proteins are crucial regulators of neuronal stem cells (NSCs). Similarly to HSCs, ablation of FOXOs in NSCs initially promotes proliferation, which eventually results in depletion of the NSC pool and a decline in neurogenesis [127]. In addition, involvement of several new FOXO-specific target genes has been implicated in FOXO-dependent regulation of NSCs. Moreover, FOXO-deficient NSCs exhibit enhanced Wnt pathway activity, which is crucial for an acute short-term increase in their proliferation [127]. Previously, FOXO proteins have been reported to interact with β-catenin in response to oxidative stress [128, 129]. This enhances FOXO-dependent transactivation; how-

ever it diverts β-catenin from T-cell factor (TCF) and thereby attenuates Wnt/TCF-mediated transcription [129]. Thus, enhanced Wnt signaling in FOXO-null NSCs is in accordance with the idea that lack of FOXOs would promote binding of β-catenin to TCF and thereby its activation.

Additionally, FOXO3a appears to be necessary for the expression of hypoxia-dependent genes in NSCs, several of which are known to be targets of the hypoxia-inducible factor 1 (HIF1) [130]. Interestingly, FOXOs have previously been shown to inhibit HIF1 activity [131], which suggests that depending on cell type FOXOs may exhibit different functions. This is further supported by the observation that the subset of genes that is regulated by FOXOs in HSCs shows rather little overlap with the program of genes controlled by FOXO factors in NSCs.

Strikingly, deletion of only FOXO3a in both HSCs and NSCs was sufficient to mimic the phenotype of a complete lack of all FOXOs [130, 132]. This implies that FOXO3a might be the primary FOXO factor involved in the regulation of stem cells. This is further supported by the fact that FOXO3a$^{-/-}$ female mice display a distinctive ovarian phenotype, with global follicular activation leading to oocyte death and premature depletion of functional ovarian follicles [133, 134].

It is evident now that FOXO transcription factors have many cell- and tissue-specific functions. This is not unexpected as different cells respond in their own specific way to external stimuli and this requires modulation of diverse sets of genes. The ability of FOXOs to coordinate multiple processes in various cell types provides an efficient way to retain homeostasis under changing environmental conditions. This in turn is absolutely essential for promoting longevity.

Regulation of FOXO activity

The involvement of FOXO transcription factors in tumor development and longevity is enabled by the ability of FOXOs to promote various cellular processes including cell cycle arrest, DNA damage repair, ROS detoxification, autophagy and apoptosis. In addition, FOXO proteins are also involved in the regulation of cell differentiation and therefore development and finally, they are also engaged in the modulation of metabolism. Because of the diverse characters of the cellular functions of FOXO, their activity needs to be tightly controlled.

A vast number of external stimuli regulate FOXO proteins, including insulin, IGF1, other growth factors, nutrients, neurotrophins, cytokines and oxidative stress (Figure 5). Importantly, the modulation of FOXO activity is involving many different posttranslational modifications PTMs, which promote or inhibit functions of FOXOs by changing their cytoplasmic localization, creating new protein binding sites or abrogating protein interactions (Figure 6). In addition, PTMs may alter the stability of FOXO proteins or through allosteric effects modify their ability to bind to DNA.

FOXO regulation by phosphorylation

Growth factors

Since FOXO proteins predominantly operate as effective activators of transcription, they need to localize within the nucleus in order to perform their functions. Therefore, one way to regulate FOXO transcriptional activity is by altering their subcellular localization. This is executed via multiple types of PTMs,

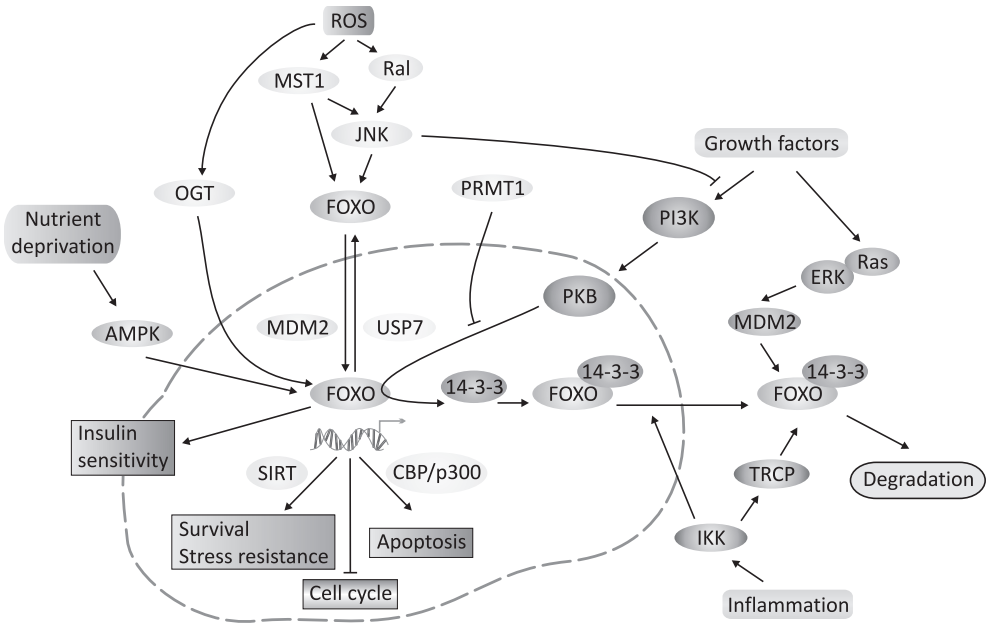


Figure 5. The regulation of FOXO proteins in response to different biological conditions
FOXO functions are predominantly regulated by two modes of cellular signaling. The first one, involves growth factor signaling, which via activation of the PI3K/PKB pathway and the Ras/ERK pathway negatively regulates the activity of FOXO proteins. Secondly, cellular stress signaling, especially oxidative stress, promotes FOXO functions. It appears that oxidative stress signals towards FOXO proteins in multiple ways consisting of several feed-forward and feedback loops. In addition, the activity of FOXO proteins is regulated by a signaling pathway activated in response to inflammation or nutrient deprivation.

among which phosphorylation seems to be the major species.

In response to growth factors, such as insulin and IGF the PI3K/PKB signaling pathway becomes active, which results in PKB-mediated phosphorylation of all FOXO proteins as well as DAF-16. This subsequently leads to their exclusion from the nucleus. PKB phosphorylates FOXO1, FOXO3a and FOXO4 on three conserved residues, which provide a recognition site for the chaperone protein 14-3-3 [135]. The binding of 14-3-3 to FOXOs not only allows active transport of FOXO factors out of the nucleus and possibly impairs their ability to bind DNA, but in addition it sequesters them in the cytoplasm [136]. Interestingly, FOXO6 is constitutively localized in the nucleus and even in the presence of active IIS, it does not translocate to the cytoplasm [137]. Notably, FOXO6 contains only two PKB motifs, which emphasizes that phosphorylation of all three sites is necessary for efficient nuclear exclusion of FOXO proteins. Nevertheless, FOXO6 functions are still modulated upon activation of the IIS pathway; however, it is not clear by what mechanism.

Serum and glucocorticoid-induced kinase (SGK) is highly homologous to PKB and, like PKB, SGK is activated by PI3K and translocates to the nucleus upon growth factor stimulation [138]. Since SGK shares the same consensus phosphorylation sequence with PKB, it can phosphorylate FOXO proteins at the same sites (i.e. Thr-32, Ser-253 and Ser-

315 in FOXO3a). However, SGK and PKB exhibit differential specificity towards these three regulatory sites on FOXO3a. While both kinases can phosphorylate Thr-32, SGK displays a marked preference for Ser-315, whereas PKB favors Ser-253 [139]. Nevertheless, SGK also promotes nuclear exit of FOXO3a and it has been postulated that cooperative phosphorylation of FOXO3a by PKB and SGK is critical for growth factors to inhibit FOXO3a-mediated transcription [139].

Phosphorylation of the second regulatory site on FOXOs is not only important for recruitment of 14-3-3 but it also creates a docking site for another kinase Casein kinase 1 (CK1). It has been shown for FOXO1 that phosphorylation of Ser-319 functions as a priming site for CK1 to phosphorylate Ser-322 and Ser-325 [140]. Finally, FOXO1 has been shown to be phosphorylated on another highly conserved residue, Ser-329, by Dual-specificity tyrosine-phosphorylated and regulated kinase 1A (DYRK1A) [141]. Even though phosphorylation of this site is not regulated by growth factors and appears to be constitutive, it negatively regulates FOXO1-dependent transactivation and impairs its nuclear localization in unstimulated cells [141].

Altogether, combined phosphorylation by PKB, CK1 and DYRK1A creates a cluster of phosphorylated residues and it has been postulated that this negatively charged stretch enhances nuclear export of FOXO proteins by directly increasing their interaction with the export machinery [142].

Recently, the TGFβ-activated kinase 1/Nemo-like kinase (TAK1/NLK) pathway has been identified to induce phosphorylation of multiple residues in FOXO1 [143]. One of the sites has been identified as Ser-329, which is also phosphorylated by DYRK1A. Thus, similar to DYRK1A, NLK-mediated phosphorylation induces exit of FOXO1 from the nucleus and thereby attenuates FOXO1-dependent transcription. However, it has not been elucidated, whether any of the stimuli known to activate NLK are also promoting phosphorylation of Ser-329. In addition, it cannot be excluded that instead of TAK1 another upstream kinase activates NLK to promote phosphorylation of FOXO1.

In addition to 14-3-3-mediated cytoplasmic sequestration of FOXO proteins, it has been suggested that another mechanism might support cytoplasmic localization of FOXOs. Studies performed in *D. melanogaster* revealed an interaction between dFOXO and the pleckstrin homology domain-containing protein Melted [144]. Melted recruits dFOXO to the plasma membrane and activity of dFOXO is increased in Melted-null mutants. This implies that Melted might function as an adaptor protein and colocalizes dFOXO in close proximity to PKB, thereby facilitating its phosphorylation. In addition, recruitment of dFOXO to the plasma membrane might enhance its cytoplasmic sequestration. Importantly, the Melted protein is highly conserved between flies and mammals; however, it has not been revealed yet whether the ability of Melted to regulate mammalian FOXO proteins is also conserved.

IκB kinase (IKK)

Immunohistochemical analysis of certain tumors unexpectedly revealed that even though these tumors did not exhibit any form of active PKB, FOXO3a was still excluded from the nucleus [58]. This made it plausible that in these tumors other, PKB-independent mechanisms are responsible for the retention

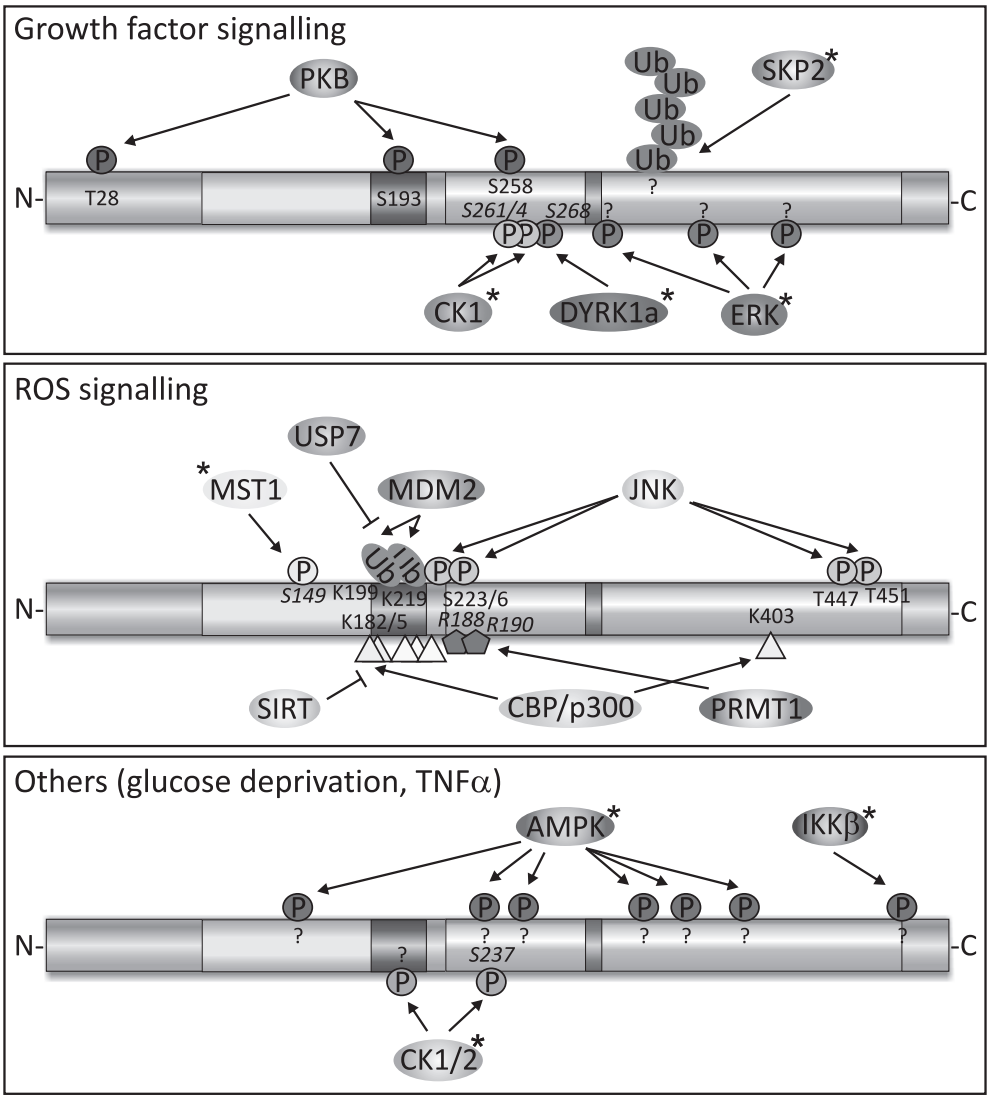


Figure 6. Overview of posttranslational modifications of FOXO4
A great number of enzymes including kinases, acetyltransferases and methyltransferases modify FOXO proteins. This mostly alters FOXO subcellular localization, but also affects FOXO degradation, DNA-binding ability, transcriptional specificity, or protein–protein interactions. Sites that are conserved in FOXO members but that have not yet been confirmed to be modified in FOXO4 are italicized. Enzymes that are marked with an asterisk have been reported to regulate the activity of other FOXO proteins but are not validated for FOXO4.

of FOXO3a in the cytoplasm. Consequently, IKK has been identified to phosphorylate FOXO3a on Ser-644 and thereby induces its exit from the nucleus [58]. In addition, it has been shown that the IKK-mediated phosphorylation of FOXO3a is induced in response to TNFα stimulation. Thus, IKK in a similar man-

ner to PKB represses FOXO3a-mediated transcription. However, the phosphorylation of Ser-644 seems to be specific for human and mouse FOXO3a since this residue is not conserved in other FOXOs. In addition, the molecular mechanism that mediates IKK-dependent modulation of nucleo-cytoplasmic shuttling of FOXO3a still needs to be elucidated.

Importantly, the IKK signaling pathway via activation of Nuclear factor κB (NFκB), promotes cell proliferation and anti-apoptotic responses [145]. Therefore, inhibition of FOXO3a is consistent with pro-survival features of IKK signaling.

Cyclin-dependent kinases (CDKs)

Sequential activation of various CDK complexes is the driving force for cell cycle progression. Recently, it has been shown that CDK2 directly phosphorylates FOXO1 on Ser-349. Phosphorylation of this residue resulted in the cytoplasmic localization of FOXO1 and consequent inhibition of its transcriptional activity [146]. The activity of CDK2 is essential for the G1 to S transition. On the other hand, inhibition of CDK2 plays a central role in DNA damage–induced cell cycle arrest and DNA repair [147]. Thus, this raises the possibility that CDK2-mediated phosphorylation of FOXO1 is essential for cell cycle progression. However, in the case of DNA damage this phosphorylation is abolished, and this restores the activity of FOXO1 resulting in induction of cell cycle arrest and DNA damage repair or apoptosis. Importantly, the CDK2-dependent regulation of FOXO appears to be FOXO1 specific [146]. Furthermore, it has been revealed that CDK1 can also phosphorylate FOXO1 on the same residue [148]. CDK1 in complex with cyclin B plays a central role in the G2 to M transition as well as in mitotic progression. Therefore, CDK1-mediated phosphorylation of FOXO1 keeps it in an inactive state and this is necessary for successful completion of the cell cycle. However, it is also apparent that FOXO1 transcriptional activity can be restored upon DNA damage in both the G1/S and G2/M phases of the cell cycle.

In contrast to the above, another group reported that CDK1-dependent phosphorylation on Ser-349 results in activation of FOXO1 [149]. Using post-mitotic neurons they showed that this phosphorylation disturbs interaction of FOXO1 with 14-3-3 and thereby promotes its nuclear localization. This in turn, stimulates FOXO1-dependent transcription and results in induction of apoptosis. It is important to note that at least *in vivo,* differentiated neurons are mostly not dividing and activation of cell cycle-related proteins leads to aberrant reentry of the cell cycle and subsequent apoptosis. Therefore, in post-mitotic neurons activation of CDK1 has been associated with induction of cell death. Thus, it is probable that FOXO1 also displays distinct functions in non-dividing cells in comparison to cells that are actively cycling. However, and arguing against, the same authors pointed out the role of CDK1-FOXO1 pathway in proliferating cells [149]. Also in this system they observed that CDK1-mediated phosphorylation of FOXO1 promotes its transcriptional activity, which is crucial for expression of Polo-like kinase (PLK) and therefore for proper progression of cells through mitosis.

Therefore, it is unclear at the moment how these opposing data regarding CDK1-dependent regulation of FOXO1 can be reconciled.

Oxidative stress signaling induced phosphorylations

Besides the inhibitory role of phosphorylation on the FOXO transcriptional

activity, there is substantial evidence indicating that phosphorylation promotes FOXO-mediated transcription.

The observation that under stress conditions FOXOs translocate to the nucleus even in the presence of growth factors [90, 146, 150] suggests that FOXO proteins are positively regulated by signaling pathways involved in stress responses. Interestingly, it became apparent that several kinases transmit signals from stress stimuli to FOXOs, via direct phosphorylation of FOXO proteins on multiple residues.

One of the kinases that are involved in the activation of FOXO transcription factors under stress conditions is JNK kinase. In response to oxidative stress JNK phosphorylates FOXO4 on at least four different residues. This induces translocation of FOXO4 to the nucleus and promotes its transcriptional activation [90]. JNK-mediated phosphorylation depends on upstream signaling, which involves Mixed lineage kinase 3 (MLK3) and the small GTPase Ral [under submission M.C. van der Berg et al.]. The JNK consensus sites in FOXO4 are not conserved in other FOXOs, which might indicate different susceptibility to JNK regulation among FOXO family members.

Interestingly, JNK has also been reported to phosphorylate 14-3-3, thereby releasing it from its binding partners [151]. In this manner JNK antagonizes PKB-mediated survival signals as JNK-mediated phosphorylation of 14-3-3 releases several proapoptotic proteins, thereby promoting their activity. A similar mechanism has been proposed for FOXO3a [152]. Because 14-3-3 is involved in the regulation of all FOXO transcription factors and since JNK promotes nuclear localization of other FOXOs including DAF-16, this might point to a more general mechanism for JNK to modulate the functions of FOXO proteins.

Although, it has been shown that JNK-mediated phosphorylation of 14-3-3 is necessary to hinder its binding to other proteins, it is not known whether phosphorylation of 14-3-3 binding partners is also involved in this process. Because JNK phosphorylates both 14-3-3 and FOXOs it will be interesting to see whether JNK-mediated phosphorylation of FOXOs themselves is necessary for release of 14-3-3. This has been partially addressed for FOXO4. Mutation of two JNK-phosphorylation sites in FOXO4 seemingly has no effect on binding between FOXO4 and 14-3-3 [90]. However, since JNK can also phosphorylate FOXO4 on additional sites, and because the stress factor was missing in those experiments, it is difficult to draw any final conclusions. Therefore, additional studies are necessary to elucidate whether JNK-dependent phosphorylation of FOXO proteins can obstruct their binding to 14-3-3 or whether it alters FOXO activity in different way.

MST1, another stress-activated protein kinase, has been shown to promote FOXO-mediated transactivation. In response to oxidative stress, MST1 phosphorylates FOXO3a and FOXO1 at a conserved residue within the DNA binding domain corresponding to Ser-207 of FOXO3a [91, 153]. Interestingly, MST1-induced phosphorylation of this site does not affect binding of FOXO3a to DNA but it induces dissociation of 14-3-3 and thereby triggers the relocalization of FOXO3a to the nucleus. This results in activation of apoptosis in both primary mammalian neurons and several cell lines [91]. In addition, the MST1-FOXO pathway has been implicated in the protection of naïve T lymphocytes against cellular oxidative stress and by

doing so it promotes their survival [154]. This indicates that depending on the cell type, MST1 can upregulate a different set of FOXO target genes.

Interestingly, structural analysis revealed that phosphorylation of Ser-212 (corresponding to Ser-207 in FOXO3a) hinders the ability of FOXO1 to bind to DNA [155]. This suggests that upon MST1-induced phosphorylation and translocation into the nucleus, FOXOs would have to be dephosphorylated to be able to bind to DNA and trigger the transcription of their target genes.

Since the MST1 targeted residue in FOXOs is highly conserved across different species, the MST1-FOXO pathway has also been revealed to operate in *C. elegans*. It has been shown that the worm ortholog of MST1, CST1 is essential for DAF-16-mediated lifespan extension [91].

Importantly, several studies revealed the crosstalk between MST1 and JNK signaling and it has been shown that MST1 promotes JNK activity [156]. In addition, recent studies revealed that JNK phosphorylates MST1 on Ser-82. This phosphorylation correlates with enhanced activity of MST1 and is crucial for its ability to regulate FOXO3a [157]. This might constitute an interesting feedback signaling loop where MST1 can be activated by its own substrate in order to activate FOXO-mediated transcription. However, since JNK can itself promote the activity of FOXO, additional studies are necessary to further elucidate the relationship between MST1 and JNK in FOXO regulation.

Interestingly, MST1 itself is also a substrate of the PKB kinase, which negatively regulates its activity and thereby inhibits MST1-mediated activation of FOXO3a [158]. Therefore, PKB can suppress FOXOs in two ways: firstly, by inhibiting FOXO proteins directly and secondly by indirectly repressing their upstream activators.

This signifies a dynamic interplay among various kinases that are involved in the modulation of FOXO activity. Importantly, they can counteract or promote each other's signaling at multiple levels by differential regulation of FOXO factors but also through direct inhibition or activation of each other's activity.

AMPK- and nutrient-related regulation of FOXOs

All kinases discussed above modulate transcriptional activity of FOXO transcription factors by altering FOXO localization within the cell. In contrast AMPK activates FOXO proteins without the ability to influence their cellular localization. In response to glucose deprivation or more general nutrient deprivation, AMPK phosphorylates FOXO3a on multiple sites and by doing so activates its downstream transcriptional program [159]. Since AMPK cannot promote nuclear translocation of FOXO3a, it suggests that FOXO3a localizes in the nucleus prior to being phosphorylated by AMPK. Because nutrient deprivation would inhibit PKB signaling, it is possible that this also triggers nuclear accumulation of FOXOs. Moreover, since glucose deprivation has been shown to elevate cellular ROS levels [60], it is likely that following nutrient deprivation activation of JNK and MST1 would also contribute to nuclear localization of FOXO proteins.

Genome-wide microarray analysis revealed that AMPK phosphorylation of FOXO3a alters the expression of particular target genes involved in the regulation of energy metabolism and stress resistance [159]. Therefore, it can be assumed that AMPK modifies the specificity of FOXO3a toward its targets

genes and thereby promotes expression of a particular subset of proteins, which are crucial for the adaptation to changes in energy levels. This highlights the important issue that targeted activation of transcription factors most likely does not result in upregulation of all putative target genes, but rather alters expression of only those genes that are most relevant to the environmental conditions.

AMPK has also been shown to regulate the activity of FOXO1; however, these results are rather inconsistent with the ones obtained for FOXO3a. In response to shear stress human endothelial cells activate AMPK, which promotes exit of FOXO1 from the nucleus followed by its degradation [160]. In this way, AMPK negatively regulates FOXO1-dependent transactivation. In addition, it has been reported that glucose deprivation or artificial activation of AMPK decrease protein levels of ectopically expressed FOXO1 in hepatoma cell lines [161].

At the moment, it is unclear what might account for the observed discrepancy. It is possible that different FOXOs are differentially regulated by AMPK. However, it is also important to underline that different cell lines and stress stimuli have been used in those studies making it difficult to compare. Nevertheless, this suggests that AMPK might affect the activity of FOXO proteins in a cell type- and stimulus-dependent manner.

However, studies performed in *C. elegans* identified DAF-16 as a target for AMPK-mediated phosphorylation. AMPK increases stress resistance and extends longevity in a DAF-16-dependent manner [36]. This suggests that AMPK-dependent positive regulation of FOXO transcriptional activity is conserved among different species.

The role of phosphatases in opposing growth factor regulation of FOXO proteins

Even though it has been suggested that the growth factor-stimulated phosphorylation and inhibition of FOXO proteins can be overruled by stress-induced phosphorylation, it is not clear whether growth factor-induced phosphorylations need to be removed in order to allow those induced by stress to promote FOXO-dependent transactivation. In addition, it is not unlikely that precise regulation of the activity of FOXOs requires the involvement of phosphatases, as it seems to be the fastest and easiest way to erase regulatory marks.

Protein phosphatase 2A (PP2A) was revealed to dephosphorylate FOXOs directly; however, there are some discrepancies between various studies with respect to the specificity of PP2A towards different FOXO family members. Firstly, it has been shown that knockdown of PP2A but not Protein phosphatase 1 (PP1) increases phosphorylation of FOXO1 on the PKB sites and delays its nuclear translocation [162]. Furthermore, inhibition of PP2A reduces FOXO1-mediated cell death due to decreased expression of the proapoptotic protein BIM [162]. This indicates that PP2A positively influences FOXO1 functions.

Subsequently, another group showed similar results for FOXO3a yet they additionally verified the reactivity of PP2A towards other FOXOs. In contrast to what had been shown previously, they reported that PP2A cannot dephosphorylate FOXO1 and is hardly able to dephosphorylate FOXO4 [163].

Importantly, PP2A can also dephosphorylate PKB, thereby abolishing its activity. This implies that the effect of PP2A on FOXO phosphorylation could also be indirect. Nevertheless, both

studies support rather direct, PKB-independent regulation of FOXO activity by PP2A.

Intriguingly, it has been suggested that binding of 14-3-3 protects FOXO3a from being dephosphorylated by PP2A [163]. Withdrawal of growth factors and consequent attenuation of PI3K/PKB activity seems to induce dissociation of 14-3-3 from FOXO3a, yet this process is PP2A dependent. This signifies a dynamic interplay between FOXO3a, PP2A and PKB signaling and further studies are required to elucidate the exact mechanism.

In addition, a calcium-dependent cytosolic protease, termed calpain, has been shown to regulate FOXO3a functions in a PP2A-dependent manner [164]. Calpain attenuates the activity of PP2A through proteolysis of its regulatory subunits, which are believed to be important in determination of the substrate specificity. Thus, cells lacking calpain exhibit a lower degree of phosphorylation of the PKB sites on FOXO3a and thereby increased FOXO3a activity. However, because phosphorylation of PKB itself is also reduced, activation of FOXO3a has been linked to a decrease in PKB activation [164]. Nevertheless, it cannot be excluded that the lack of calpain increases the activity of PP2A towards FOXO3a and not only towards PKB.

Interestingly, sustained activation of FOXO1 and FOXO3a in cardiomyocytes has been reported to increase basal phosphorylation of PKB and thereby its activity. This is most probably due to FOXO-mediated inhibition of PP2A and calcineurin (PP2B), thereby providing a regulatory feedback loop [165].

Additional data supporting the involvement of phosphatases in the regulation of FOXO functions has been obtained in worms. The *C. elegans* ortholog of mammalian SMEK1, which encodes the regulatory subunit of Protein phosphatase 4 (PP4) modulates transcriptional activity of DAF-16 and is important for some forms of DAF-16-dependent stress resistance and extension of lifespan [88].

Other PTMs in regulation of FOXOs

Methylation

Phosphorylation constitutes one way to modulate the transcriptional activity of FOXO. Interestingly, several other PTMs, including acetylation, ubiquitination and methylation, have been revealed to regulate FOXO transcription factors.

Two arginine residues in mouse Foxo1, Arg-248 and Arg-250, are subjected to methylation mediated by protein arginine methyltransferase 1 (PRMT1) [166]. Since these arginines are localized within the PKB consensus sequences, once modified by PRMT1, they preclude PKB-mediated phosphorylation of FOXO1 on Ser-253. Hence, next to dephosphorylation, methylation constitutes another way to limit phosphorylation of FOXO1 by PKB.

Importantly, elevated cellular ROS levels promote recruitment of PRMT1 to FOXO1 and facilitate its methylation. Thus, PRMT1 in response to oxidative stress augments FOXO1-dependent transactivation and thereby enhances oxidative stress-induced apoptosis. Moreover, PRMT1 can also methylate other FOXO family members including FOXO3a, FOXO4, and FOXO6. However, at present it is not known how methylation influences the activities of these proteins. Interestingly, FOXO3a has been implicated to positively regulate expression of BTG1, which in turn

enhances PRMT1 activity. In this way FOXO3a would potentiate its own activity [167].

Oxidative stress-stimulated increase in methylation of FOXO1 and the consequential decrease in PKB-dependent phosphorylation comprise a coordinated way of the regulation of two different PTMs. In addition, involvement of methylation in the modulation of phosphorylation illustrates the dynamic crosstalk between different PTMs. Because methylation promotes the apoptotic response, whereas PKB phosphorylation promotes survival, the combination of these two together demonstrate a way to fine-tune FOXO functions.

Acetylation

Apart from ROS induced phosphorylation and methylation of FOXOs, a large amount of data indicate that FOXO proteins are also acetylated upon treatment of cells with ROS [168-171]. This acetylation is mediated by the acetyl transferases CBP and p300. Importantly, acetylation has a dual effect on FOXO activity. With respect to FOXOs themselves acetylation hampers FOXO-DNA interactions, thereby negatively regulating FOXO-dependent transactivation [169]. However, binding of CBP and p300 to FOXOs might facilitate acetylation of histones thereby opening up the chromatin and promoting transcription [172]. Therefore, the data from studies regarding acetylation of FOXOs are often contradictory.

The level of FOXO acetylation is modulated by the opposing action of the SIR2/SIRT family of protein deacetylases [150, 171-174]. Similarly to CBP and p300, SIRT binds to FOXO in response to elevated ROS levels. In *C. elegans* SIR2 promotes DAF-16-dependent lifespan extension [42], which indicates that acetylation would negatively regulate FOXO functions.

Nevertheless, several studies signify that CBP and p300 are necessary components for FOXO-mediated transcriptional activity [172, 175, 176]. Therefore, it is believed now that CBP and p300 function as FOXO cofactors that shift the balance from target genes associated with cell cycle and stress resistance towards genes important for apoptotic responses [170]. This is consistent with results showing that ectopic expression of SIRT1 promotes FOXO3a-induced cell cycle arrest and inhibition of SIRT1 enhances FOXO3a-driven expression of the pro-apoptotic gene Bcl2-interacting mediator of cell death (BIM) [150].

Interestingly, it has been recently revealed that the binding of FOXO to CBP and p300, and subsequent acetylation of FOXO4 is entirely dependent on the presence of redox sensitive cysteines in FOXO4. In addition, an intermolecular disulfide bond was found to be formed between FOXO4 Cys-477 and p300 [96]. At present, it is not clear whether this is a general mechanism of the regulation of FOXO proteins by p300. Moreover, additional studies are required to further elucidate the exact molecular mechanism of disulphide bond formation between p300 and FOXO4.

Acetylation of FOXO proteins has also been indicated to modulate their cellular localization. For example in pancreatic β-cells, acetylation of FOXO1 induces its interaction with PML and the relocalization of FOXO1 to the PML nuclear bodies [107]. In addition, acetylation seems to promote the nuclear exclusion of FOXOs. It has been demonstrated that acetylation enhances the sensitivity of FOXOs towards PKB-mediated phosphorylation, thereby decreasing their nuclear accumulation [169, 173, 177, 178]. In contrast, it has also been shown that

FOXO3a and FOXO1a are acetylated during growth factor deprivation and their acetylation state reversibly correlates with phosphorylation on the PKB sites [179]. Nevertheless, this implies that there might be a correlation between phosphorylation of FOXOs and their acetylation state. Recently, another acetyltransferase PCAF was shown to inhibit FOXO1 in a PKB-dependent manner, as interaction between FOXO1 and PCAF requires phosphorylation of the PKB site Ser-253. However, PCAF attenuates FOXO1-mediated transactivation independently of its acetyltransferase activity [180]. Thus, further studies are necessary to elucidate the relationship between acetylation of FOXO proteins and PKB-induced phosphorylation.

It is important to emphasize that CBP and p300 as well as SIRT1 localize in the nucleus and they both interact and modify FOXOs in response to elevated ROS levels. In addition, SIRT1 directly interacts with p300 and p300 itself is deacetylated by SIRT1 [181]. Finally, CBP and p300 counteract the SIRT1-dependent effect on FOXO functions. Therefore, it is likely that proper regulation of FOXOs by these proteins requires yet an additional level of control.

Ubiquitination: poly- versus mono-

The modulation of the stability of FOXO proteins provides another way to regulate FOXO-dependent transcription.

Even though FOXOs are relatively stable proteins, several studies revealed that they are degraded in a proteasome-dependent manner. Importantly, regulation of FOXO stability is closely related to their phosphorylation state.

In response to insulin or growth factors, FOXO1 is degraded and this is triggered by PKB-mediated phosphorylation. This process depends on the activity of the F-box protein S-phase kinase-associated protein 2 (Skp2), which is responsible for polyubiquitination of FOXO1 [182, 183]. Thus, PKB signaling suppresses FOXO functions via both cytoplasmic sequestration and downregulation of protein levels. Interestingly, Skp2 has been identified as a putative E3 ligase that targets several other PKB substrates including $p27^{kip1}$, tuberin and merlin [184-186]. Importantly, many of these proteins have a negative effect on cell cycle progression and are considered to function as tumor suppressors. Thus, Skp2-mediated degradation might constitute a general mechanism for PKB to promote cell proliferation and thereby tumorigenesis.

In addition, PKB can directly phosphorylate Skp2 [187]; however, in this case the PKB-mediated phosphorylation most probably provides protection against degradation and thereby promotes the activity of Skp2.

It has been shown that Skp2-mediated degradation is specific for FOXO1 since no interaction between Skp2 and FOXO3a or FOXO4 has been detected. However, the stability of FOXO3a is also affected by PKB signaling and therefore it cannot be excluded that Skp2 also targets FOXO3a [186].

Recently, another pathway activated by growth factors has been identified as a putative regulator of FOXO stability. Growth factors are well defined activators of the Ras–MEK–ERK pathway. It has been revealed that activation of this pathway induces ERK-dependent phosphorylation of FOXO3a at three previously unidentified serine residues (Ser-344, Ser-294 and Ser-425). This in turn promotes interaction of FOXO3a with the E3 ubiquitin ligase Murine double minute 2 (Mdm2), which subsequently drives polyubiquitination of FOXO3a

followed by its proteasomal degradation [188, 189]. Importantly, ERK has previously been shown to promote Mdm2 transcription and translation [190, 191]. Thus, similarly to PKB and FOXO1, ERK enhances the degradation of FOXO3a not only by modifying the substrate itself but also by enhancing the activity of the ubiquitin E3 ligase.

Importantly, Mdm2 triggers degradation of another well defined tumor suppressor p53 [192]. Moreover, Mdm2 as well as Ras signaling are often aberrantly activated in human cancers. Therefore, it is possible that inhibition of FOXO3a functions is necessary for Ras- and Mdm2-induced tumor development. This is supported by the fact that breast cancer cells expressing a mutant form of FOXO3a that cannot be phosphorylated by ERK, displayed a reduction in tumor growth *in vivo*.

Previously, it has been reported that IKK phosphorylation of FOXO3a not only sequesters FOXO3a in the cytoplasm but also induces its degradation [58]. However, the E3 ligase involved in this process was not identified at that moment. Interestingly, it has recently been revealed that the oncogenic ubiquitin E3 ligase βTRCP1 induces degradation of FOXO3a in an IKKβ phosphorylation dependent manner [193]. In agreement with this, silencing of βTRCP1 enhances FOXO3a protein levels and promotes apoptosis in cancer cells. In addition, in a mouse model overexpressing βTRCP1, FOXO3a protein levels are downregulated, which promotes tumorigenesis and tumor growth [193]. Taken together, this further elucidates the mechanism of IKK-dependent regulation of FOXO3a functions.

Besides polyubiquitination, FOXO proteins are also subjected to monoubiquitination. In response to elevated cellular ROS levels FOXO4 becomes monoubiquitinated [194, 195]. The monoubiquitination of FOXO4 promotes its nuclear accumulation thereby increasing FOXO4-mediated transcription. However, it is not known via which mechanism monoubiquitination induces nuclear localization of FOXO4 [195]. Surprisingly, Mdm2 has been identified as an ubiquitin E3 ligase that mediates monoubiquitination of FOXO4 [194]. Importantly, it is suggested that monoubiquitination of FOXO4 is depending on the activity of MKK3/Ral/JNK pathway; however, at the moment the exact mechanism is not known [under submission M.C. van der Berg et al.]. This might explain the apparent inconsistency with the data indicating that Mdm2 targets FOXO3a for degradation.

Stress signaling also induces interaction of FOXO4 with the deubiquitinating enzyme USP7, which can remove ubiquitin moieties from FOXO4 and thereby inhibit its activity [195]. Moreover, the activity of ubiquitin-specific peptidase 7 (USP7) towards FOXO4 is enhanced by peptidyl-prolyl isomerase 1 (PIN1) [196]. The binding of PIN1 to FOXO4 is also stimulated by elevated cellular ROS levels and depends on the phosphorylation state of FOXO4. Phosphorylations of serine or threonine residues followed by a proline (S/T-P) are docking sites for PIN1. Thus, oxidative stress-induced phosphorylation of FOXO4 on one hand induces monoubiquitination of FOXO4 and on the other hand induces the recruitment of PIN1 [196]. This in turn might stimulate deubiquitination of FOXO4 in a USP7-dependent manner. Since monoubiquitination of FOXO4 requires active JNK signaling [under submission M.C. van der Berg et al.], this raises the possibility that JNK-mediated phosphorylation of FOXO4 itself is essential

for ubiquitination or deubiquitination of FOXO4.

Interestingly, FOXO1 and FOXO3a have been shown to be polyubiquitinated by muscle specific E3 ligase atrogin-1 in response to cardiac hypertrophy [197]. However, this polyubiquitination, which involves lysine 63–linked ubiquitin chains, does not mediate the degradation of FOXOs but promotes their transcriptional activity [197]. Moreover, since atrogin-1 is a transcriptional target of FOXO1 and FOXO3a [115, 116], this might provide a positive feedback loop supporting activities of both the E3 ligase and its substrates. Next to FOXO4 monoubiquitination, this is an example of ubiquitination being employed to stimulate FOXO-dependent transactivation.

O-GlcNAcylation

Several studies revealed that FOXO proteins are also modified by O-glycosylation. O-GlcNAcylation is a common PTM that occurs on serine and threonine residues. Two highly conserved proteins, OGT and OGA are responsible for the addition and removal of O-GlcNAc, respectively [198].

It has been reported that in response to oxidative stress FOXO4 binds to OGT and undergoes O-GlcNAcylation and this in turn promotes its activity [199]. Since O-GlcNAcylation is known to be required for cell survival under stress conditions [200], the activation of FOXO4 might account, at least partially, for the protective functions of O-GlcNAcylation.

Additionally, FOXO1 has been shown to be modified by O-GlcNAcylation in response to high glucose levels [201]. Since O-GlcNAcylation increases the activity of FOXO1, it has been suggested to promote FOXO-driven expression of gluconeogenic genes [201, 202]. This is rather paradoxical as it would result in a further increase in glucose levels. Thus, the physiological relevance of this PTM in respect to FOXO functions requires further investigation. Interestingly, O-GlcNAcylation might compete with phosphorylation and therefore it is possible that these PTMs might be regulated in a coordinated manner [198].

In summary, it is evident that the activities of FOXO transcription factors are modified through multiple layers of reversible PTMs. This in turn is executed by a complex and highly dynamic network of many enzymes that are activated in response to various external stimuli. Moreover, one modification can trigger or inhibit another, which suggests dynamic crosstalk between different PTMs. This is however still highly unexplored and therefore it will be highly interesting to unravel how exactly different PTMs of FOXOs can affect each other.

Moreover, as it is often observed that enzymes having opposite effects on FOXO functions are activated in response to the same stimuli, it is intriguing how these interactions are regulated in time and space.

Outline of this thesis

The focus of thesis is the *in vitro* and *in vivo* regulation of FOXO functions. In the first two chapters we provide new mechanistic insights into the ROS-dependent regulation of the activity of FOXOs within cells. Specifically, in Chapter 2 we describe the involvement of Nemo-like kinase (NLK) as a novel kinase that modulates FOXO-dependent transcription. One of the important findings presented in this chapter is the crosstalk between two different classes of PTMs, where NLK-dependent phosphorylation of FOXO4 inhibits its monoubiquitination and by doing so represses the transcriptional activity of FOXO4.

In Chapter 3 we provide further evidence for a dual role of acetylation in the regulation of FOXO functions. Importantly, we show that acetylation of FOXO4 is not altered only due to changes in the activity of HATs and HDACs but can be influenced by other proteins interacting with FOXO4. In particular, we identified Myb binding protein 1a (MYBBP1a) as a novel interactor of FOXO4. Moreover, we showed that MYBBP1a attenuates the acetylation of FOXO4 and most probably histones, and thereby can both promote and suppress the activity of FOXO4.

It is not always possible to address a question about specific physiological functions of distinct FOXO proteins in *in vitro* cellular studies. Therefore, we generated GFP-FOXO4 knock-in mice and analyzed the spatiotemporal expression pattern of FOXO4, which is described in Chapter 4. To our knowledge this is the first attempt to analyze the expression levels of FOXO protein in the context of a whole organism.

The main findings that emerge from the studies presented in this thesis and their relevance for the current understanding of the regulation of FOXO functions are discussed in the final Chapter 5.

Finally, in Chapter 6 we discuss the ROS-dependent regulation of insulin signaling.

References

1. Karin, M. and T. Hunter, Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. Curr Biol, 1995. 5(7): p. 747-57.
2. Avruch, J., Insulin signal transduction through protein kinase cascades. Mol Cell Biochem, 1998. 182(1-2): p. 31-48.
3. Karpac, J. and H. Jasper, Insulin and JNK: optimizing metabolic homeostasis and lifespan. Trends Endocrinol Metab, 2009. 20(3): p. 100-6.
4. van den Berg, M.C. and B. Burgering, Integrating opposing signals towards FOXO. Antioxid Redox Signal.
5. Kenyon, C., The first long-lived mutants: discovery of the insulin/IGF-1 pathway for ageing. Philos Trans R Soc Lond B Biol Sci. 366(1561): p. 9-16.
6. Lin, K., et al., daf-16: An HNF-3/forkhead family member that can function to double the lifespan of Caenorhabditis elegans. Science, 1997. 278(5341): p. 1319-22.
7. Johnson, T.E., Increased lifespan of age-1 mutants in Caenorhabditis elegans and lower Gompertz rate of aging. Science, 1990. 249(4971): p. 908-12.
8. Morris, J.Z., H.A. Tissenbaum, and G. Ruvkun, A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in Caenorhabditis elegans. Nature, 1996. 382(6591): p. 536-9.
9. Kenyon, C., et al., A C. elegans mutant that lives twice as long as wild type. Nature, 1993. 366(6454): p. 461-4.
10. Honda, Y. and S. Honda, The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans. FASEB J, 1999. 13(11): p. 1385-93.
11. Gottlieb, S. and G. Ruvkun, daf-2, daf-16 and daf-23: genetically interacting genes controlling Dauer formation in Caenorhabditis elegans. Genetics, 1994. 137(1): p. 107-20.
12. Ogg, S., et al., The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature, 1997. 389(6654): p. 994-9.
13. Libina, N., J.R. Berman, and C. Kenyon, Tissue-specific activities of C. elegans DAF-16 in the regulation of lifespan. Cell, 2003. 115(4): p. 489-502.
14. Wolkow, C.A., et al., Regulation of C. elegans lifespan by insulinlike signaling in the nervous system. Science, 2000. 290(5489): p. 147-50.
15. Clancy, D.J., et al., Extension of lifespan by loss of CHICO, a *Drosophila* insulin receptor substrate protein. Science, 2001. 292(5514): p. 104-6.
16. Tatar, M., et al., A mutant *Drosophila* insulin receptor homolog that extends lifespan and impairs neuroendocrine function. Science, 2001. 292(5514): p. 107-10.
17. Giannakou, M.E., et al., Long-lived *Drosophila* with overexpressed dFOXO in adult fat body. Science, 2004. 305(5682): p. 361.
18. Hwangbo, D.S., et al., *Drosophila* dFOXO controls lifespan and regulates insulin signalling in brain and fat body. Nature, 2004. 429(6991): p. 562-6.
19. Barbieri, M., et al., Insulin/IGF-I-signaling pathway: an evolutionarily conserved mechanism of longevity from yeast to humans. Am J Physiol Endocrinol Metab, 2003. 285(5): p. E1064-71.
20. Nakae, J., Y. Kido, and D. Accili, Distinct and overlapping functions of insulin and IGF-I receptors. Endocr Rev, 2001. 22(6): p. 818-35.
21. Calnan, D.R. and A. Brunet, The FoxO code. Oncogene, 2008. 27(16): p. 2276-88.
22. Holzenberger, M., et al., IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature, 2003. 421(6919): p. 182-7.
23. Liang, H., et al., Genetic mouse models of extended lifespan. Exp Gerontol, 2003. 38(11-12): p. 1353-64.
24. Bluher, M., B.B. Kahn, and C.R. Kahn, Extended longevity in mice lacking the insulin receptor in adipose tissue. Science, 2003. 299(5606): p. 572-4.
25. Taguchi, A., L.M. Wartschow, and M.F. White, Brain IRS2 signaling coordinates lifespan and nutrient homeostasis. Science, 2007. 317(5836): p. 369-72.
26. Suh, Y., et al., Functionally significant insulin-like growth factor I receptor mutations in centenarians. Proc Natl Acad Sci U S A, 2008. 105(9): p. 3438-42.
27. Kojima, T., et al., Association analysis between longevity in the Japanese population and polymorphic variants of genes involved in insulin and insulin-like growth factor 1 signaling pathways. Exp Gerontol, 2004. 39(11-12): p. 1595-8.
28. Kenyon, C.J., The genetics of ageing. Nature. 464(7288): p. 504-12.
29. Wang, M.C., D. Bohmann, and H. Jasper, JNK signaling confers tolerance to oxidative stress and extends lifespan in *Drosophila*. Dev Cell, 2003. 5(5): p. 811-6.
30. Oh, S.W., et al., JNK regulates lifespan in Caenorhabditis elegans by modulating nuclear translocation of forkhead transcription factor/DAF-16. Proc Natl Acad Sci U S A, 2005. 102(12): p. 4494-9.
31. Wang, M.C., D. Bohmann, and H. Jasper, JNK extends lifespan and limits growth by antagonizing cellular and organism-wide responses to insulin signaling. Cell, 2005. 121(1): p. 115-25.

32. Hull-Thompson, J., et al., Control of metabolic homeostasis by stress signaling is mediated by the lipocalin NLaz. PLoS Genet, 2009. 5(4): p. e1000460.
33. Nemoto, S. and T. Finkel, Redox regulation of forkhead proteins through a p66shc-dependent signaling pathway. Science, 2002. 295(5564): p. 2450-2.
34. Apfeld, J., et al., The AMP-activated protein kinase AAK-2 links energy levels and insulin-like signals to lifespan in C. elegans. Genes Dev, 2004. 18(24): p. 3004-9.
35. Curtis, R., G. O'Connor, and P.S. DiStefano, Aging networks in Caenorhabditis elegans: AMP-activated protein kinase (aak-2) links multiple aging and metabolism pathways. Aging Cell, 2006. 5(2): p. 119-26.
36. Greer, E.L., et al., An AMPK-FOXO pathway mediates longevity induced by a novel method of dietary restriction in C. elegans. Curr Biol, 2007. 17(19): p. 1646-56.
37. Williams, D.S., et al., Oxaloacetate supplementation increases lifespan in Caenorhabditis elegans through an AMPK/FOXO-dependent pathway. Aging Cell, 2009. 8(6): p. 765-8.
38. Anisimov, V.N., et al., Metformin slows down aging and extends lifespan of female SHR mice. Cell Cycle, 2008. 7(17): p. 2769-73.
39. Guarente, L. and F. Picard, Calorie restriction--the SIR2 connection. Cell, 2005. 120(4): p. 473-82.
40. Rogina, B. and S.L. Helfand, Sir2 mediates longevity in the fly through a pathway related to calorie restriction. Proc Natl Acad Sci U S A, 2004. 101(45): p. 15998-6003.
41. Tissenbaum, H.A. and L. Guarente, Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. Nature, 2001. 410(6825): p. 227-30.
42. Berdichevsky, A., et al., C. elegans SIR-2.1 interacts with 14-3-3 proteins to activate DAF-16 and extend lifespan. Cell, 2006. 125(6): p. 1165-77.
43. Heidler, T., et al., Caenorhabditis elegans lifespan extension caused by treatment with an orally active ROS-generator is dependent on DAF-16 and SIR-2.1. Biogerontology. 11(2): p. 183-95.
44. Flatt, T., et al., *Drosophila* germ-line modulation of insulin signaling and lifespan. Proc Natl Acad Sci U S A, 2008. 105(17): p. 6368-73.
45. Hsin, H. and C. Kenyon, Signals from the reproductive system regulate the lifespan of C. elegans. Nature, 1999. 399(6734): p. 362-6.
46. Goitre, L., et al., KRIT1 regulates the homeostasis of intracellular reactive oxygen species. PLoS One. 5(7): p. e11786.
47. Dansen, T.B. and B.M. Burgering, Unravelling the tumor-suppressive functions of FOXO proteins. Trends Cell Biol, 2008. 18(9): p. 421-9.
48. Galili, N., et al., Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma. Nat Genet, 1993. 5(3): p. 230-5.
49. Borkhardt, A., et al., Cloning and characterization of AFX, the gene that fuses to MLL in acute leukemias with a t(X;11)(q13;q23). Oncogene, 1997. 14(2): p. 195-202.
50. Corral, J., et al., Acute leukemias of different lineages have similar MLL gene fusions encoding related chimeric proteins resulting from chromosomal translocation. Proc Natl Acad Sci U S A, 1993. 90(18): p. 8538-42.
51. Hillion, J., et al., AF6q21, a novel partner of the MLL gene in t(6;11)(q21;q23), defines a forkhead transcriptional factor subfamily. Blood, 1997. 90(9): p. 3714-9.
52. Parry, P., Y. Wei, and G. Evans, Cloning and characterization of the t(X;11) breakpoint from a leukemic cell line identify a new member of the forkhead gene family. Genes Chromosomes Cancer, 1994. 11(2): p. 79-84.
53. Lagutina, I., et al., Pax3-FKHR knock-in mice show developmental aberrations but do not develop tumors. Mol Cell Biol, 2002. 22(20): p. 7204-16.
54. So, C.W. and M.L. Cleary, MLL-AFX requires the transcriptional effector domains of AFX to transform myeloid progenitors and transdominantly interfere with forkhead protein function. Mol Cell Biol, 2002. 22(18): p. 6542-52.
55. Francis, R., et al., gld-1, a tumor suppressor gene required for oocyte development in Caenorhabditis elegans. Genetics, 1995. 139(2): p. 579-606.
56. Pinkston, J.M., et al., Mutations that increase the lifespan of C. elegans inhibit tumor growth. Science, 2006. 313(5789): p. 971-5.
57. Tokunaga, E., et al., Deregulation of the Akt pathway in human cancer. Curr Cancer Drug Targets, 2008. 8(1): p. 27-36.
58. Hu, M.C., et al., IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. Cell, 2004. 117(2): p. 225-37.
59. Yang, H., et al., Constitutively active FOXO4 inhibits Akt activity, regulates p27 Kip1 stability, and suppresses HER2-mediated tumorigenicity. Oncogene, 2005. 24(11): p. 1924-35.
60. Simons, A.L., et al., Glucose deprivation-induced metabolic oxidative stress and cancer therapy. J Cancer Res Ther, 2009. 5 Suppl 1: p. S2-6.
61. Paik, J.H., et al., FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell, 2007. 128(2): p. 309-23.
62. Bouchard, C., et al., Myc-induced prolifera-

tion and transformation require Akt-mediated phosphorylation of FoxO proteins. EMBO J, 2004. 23(14): p. 2830-40.
63. Bouchard, C., et al., FoxO transcription factors suppress Myc-driven lymphomagenesis via direct activation of Arf. Genes Dev, 2007. 21(21): p. 2775-87.
64. de Keizer, P.L., et al., Activation of forkhead box O transcription factors by oncogenic BRAF promotes p21cip1-dependent senescence. Cancer Res. 70(21): p. 8526-36.
65. Dhomen, N., et al., Oncogenic Braf induces melanocyte senescence and melanoma in mice. Cancer Cell, 2009. 15(4): p. 294-303.
66. Dankort, D., et al., Braf(V600E) cooperates with Pten loss to induce metastatic melanoma. Nat Genet, 2009. 41(5): p. 544-52.
67. Medema, R.H., et al., AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature, 2000. 404(6779): p. 782-7.
68. Kops, G.J., et al., Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. Mol Cell Biol, 2002. 22(7): p. 2025-36.
69. Brunet, A., et al., Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell, 1999. 96(6): p. 857-68.
70. Li, A. and J.J. Blow, The origin of CDK regulation. Nat Cell Biol, 2001. 3(8): p. E182-4.
71. Burgering, B.M. and G.J. Kops, Cell cycle and death control: long live Forkheads. Trends Biochem Sci, 2002. 27(7): p. 352-60.
72. Dijkers, P.F., et al., Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). Mol Cell Biol, 2000. 20(24): p. 9138-48.
73. Nakamura, N., et al., Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN. Mol Cell Biol, 2000. 20(23): p. 8969-82.
74. Seoane, J., et al., Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell, 2004. 117(2): p. 211-23.
75. Besson, A., S.F. Dowdy, and J.M. Roberts, CDK inhibitors: cell cycle regulators and beyond. Dev Cell, 2008. 14(2): p. 159-69.
76. Blain, S.W., Switching cyclin D-Cdk4 kinase activity on and off. Cell Cycle, 2008. 7(7): p. 892-8.
77. Schmidt, M., et al., Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. Mol Cell Biol, 2002. 22(22): p. 7842-52.
78. Gomis, R.R., et al., A FoxO-Smad synexpression group in human keratinocytes. Proc Natl Acad Sci U S A, 2006. 103(34): p. 12747-52.
79. Gomis, R.R., et al., C/EBPbeta at the core of the TGFbeta cytostatic response and its evasion in metastatic breast cancer cells. Cancer Cell, 2006. 10(3): p. 203-14.
80. Bachman, K.E. and B.H. Park, Duel nature of TGF-beta signaling: tumor suppressor vs. tumor promoter. Curr Opin Oncol, 2005. 17(1): p. 49-54.
81. Grana, X., J. Garriga, and X. Mayol, Role of the retinoblastoma protein family, pRB, p107 and p130 in the negative control of cell growth. Oncogene, 1998. 17(25): p. 3365-83.
82. Alvarez, B., et al., Forkhead transcription factors contribute to execution of the mitotic programme in mammals. Nature, 2001. 413(6857): p. 744-7.
83. Furukawa-Hibi, Y., et al., FOXO forkhead transcription factors induce G(2)-M checkpoint in response to oxidative stress. J Biol Chem, 2002. 277(30): p. 26729-32.
84. Dijkers, P.F., et al., FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. J Cell Biol, 2002. 156(3): p. 531-42.
85. Peng, S.L., Foxo in the immune system. Oncogene, 2008. 27(16): p. 2337-44.
86. Davila, D. and I. Torres-Aleman, Neuronal death by oxidative stress involves activation of FOXO3a through a two-arm pathway that activates stress kinases and attenuates insulin-like growth factor I signaling. Mol Biol Cell, 2008. 19(5): p. 2014-25.
87. Fu, Z. and D.J. Tindall, FOXOs, cancer and regulation of apoptosis. Oncogene, 2008. 27(16): p. 2312-9.
88. Wolff, S., et al., SMK-1, an essential regulator of DAF-16-mediated longevity. Cell, 2006. 124(5): p. 1039-53.
89. Asada, S., et al., Mitogen-activated protein kinases, Erk and p38, phosphorylate and regulate Foxo1. Cell Signal, 2007. 19(3): p. 519-27.
90. Essers, M.A., et al., FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. EMBO J, 2004. 23(24): p. 4802-12.
91. Lehtinen, M.K., et al., A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends lifespan. Cell, 2006. 125(5): p. 987-1001.
92. Kondo, M., et al., The p38 signal transduction pathway participates in the oxidative stress-mediated translocation of DAF-16 to Caenorhabditis elegans nuclei. Mech Ageing Dev, 2005. 126(6-7):

p. 642-7.
93. Lee, K.S., et al., JNK/FOXO-mediated neuronal expression of fly homologue of peroxiredoxin II reduces oxidative stress and extends lifespan. J Biol Chem, 2009. 284(43): p. 29454-61.
94. de Keizer, P., B. Burgering, and T.B. Dansen, FOXO as a sensor, mediator and regulator of redox signaling. Antioxid Redox Signal.
95. Chen, C.C., et al., FoxOs inhibit mTORC1 and activate Akt by inducing the expression of Sestrin3 and Rictor. Dev Cell. 18(4): p. 592-604.
96. Dansen, T.B., et al., Redox-sensitive cysteines bridge p300/CBP-mediated acetylation and FoxO4 activity. Nat Chem Biol, 2009. 5(9): p. 664-72.
97. Tran, H., et al., DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. Science, 2002. 296(5567): p. 530-4.
98. Tsai, W.B., et al., Functional interaction between FOXO3a and ATM regulates DNA damage response. Nat Cell Biol, 2008. 10(4): p. 460-7.
99. Yalcin, S., et al., Foxo3 is essential for the regulation of ataxia telangiectasia mutated and oxidative stress-mediated homeostasis of hematopoietic stem cells. J Biol Chem, 2008. 283(37): p. 25692-705.
100. Matsumoto, M., et al., Impaired regulation of hepatic glucose production in mice lacking the forkhead transcription factor Foxo1 in liver. Cell Metab, 2007. 6(3): p. 208-16.
101. Haeusler, R.A., K.H. Kaestner, and D. Accili, FoxOs function synergistically to promote glucose production. J Biol Chem. 285(46): p. 35245-8.
102. Sekine, K., et al., Foxo1 links insulin signaling to C/EBPalpha and regulates gluconeogenesis during liver development. EMBO J, 2007. 26(15): p. 3607-15.
103. Gross, D.N., A.P. van den Heuvel, and M.J. Birnbaum, The role of FoxO in the regulation of metabolism. Oncogene, 2008. 27(16): p. 2320-36.
104. Altomonte, J., et al., Foxo1 mediates insulin action on apoC-III and triglyceride metabolism. J Clin Invest, 2004. 114(10): p. 1493-503.
105. Kawamori, D., et al., The forkhead transcription factor Foxo1 bridges the JNK pathway and the transcription factor PDX-1 through its intracellular translocation. J Biol Chem, 2006. 281(2): p. 1091-8.
106. Kitamura, T., et al., The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. J Clin Invest, 2002. 110(12): p. 1839-47.
107. Kitamura, Y.I., et al., FoxO1 protects against pancreatic beta cell failure through NeuroD and MafA induction. Cell Metab, 2005. 2(3): p. 153-63.
108. Lee, N.K., et al., Endocrine regulation of energy metabolism by the skeleton. Cell, 2007. 130(3): p. 456-69.
109. Rached, M.T., et al., FoxO1 expression in osteoblasts regulates glucose homeostasis through regulation of osteocalcin in mice. J Clin Invest. 120(1): p. 357-68.
110. Nakae, J., et al., The forkhead transcription factor Foxo1 regulates adipocyte differentiation. Dev Cell, 2003. 4(1): p. 119-29.
111. Dowell, P., et al., Convergence of peroxisome proliferator-activated receptor gamma and Foxo1 signaling pathways. J Biol Chem, 2003. 278(46): p. 45485-91.
112. Kim, M.S., et al., Role of hypothalamic Foxo1 in the regulation of food intake and energy homeostasis. Nat Neurosci, 2006. 9(7): p. 901-6.
113. Kitamura, T., et al., Forkhead protein FoxO1 mediates Agrp-dependent effects of leptin on food intake. Nat Med, 2006. 12(5): p. 534-40.
114. Hishiya, A., et al., A novel ubiquitin-binding protein ZNF216 functioning in muscle atrophy. EMBO J, 2006. 25(3): p. 554-64.
115. Sandri, M., et al., Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell, 2004. 117(3): p. 399-412.
116. Stitt, T.N., et al., The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. Mol Cell, 2004. 14(3): p. 395-403.
117. Sandri, M., et al., PGC-1alpha protects skeletal muscle from atrophy by suppressing FoxO3 action and atrophy-specific gene transcription. Proc Natl Acad Sci U S A, 2006. 103(44): p. 16260-5.
118. Leger, B., et al., Akt signalling through GSK-3beta, mTOR and Foxo1 is involved in human skeletal muscle hypertrophy and atrophy. J Physiol, 2006. 576(Pt 3): p. 923-33.
119. Mammucari, C., et al., FoxO3 controls autophagy in skeletal muscle in vivo. Cell Metab, 2007. 6(6): p. 458-71.
120. Zhao, J., et al., FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells. Cell Metab, 2007. 6(6): p. 472-83.
121. Juhasz, G., et al., Gene expression profiling identifies FKBP39 as an inhibitor of autophagy in larval *Drosophila* fat body. Cell Death Differ, 2007. 14(6): p. 1181-90.
122. Melendez, A., et al., Autophagy genes are essential for dauer development and lifespan extension in C. elegans. Science, 2003. 301(5638): p. 1387-91.
123. Tothova, Z., et al., FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell, 2007. 128(2): p. 325-39.

124. Ito, K., et al., Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. Nat Med, 2006. 12(4): p. 446-51.
125. Juntilla, M.M., et al., AKT1 and AKT2 maintain hematopoietic stem cell function by regulating reactive oxygen species. Blood. 115(20): p. 4030-8.
126. Zhang, J., et al., PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. Nature, 2006. 441(7092): p. 518-22.
127. Paik, J.H., et al., FoxOs cooperatively regulate diverse pathways governing neural stem cell homeostasis. Cell Stem Cell, 2009. 5(5): p. 540-53.
128. Essers, M.A., et al., Functional interaction between beta-catenin and FOXO in oxidative stress signaling. Science, 2005. 308(5725): p. 1181-4.
129. Hoogeboom, D., et al., Interaction of FOXO with beta-catenin inhibits beta-catenin/T cell factor activity. J Biol Chem, 2008. 283(14): p. 9224-30.
130. Renault, V.M., et al., FoxO3 regulates neural stem cell homeostasis. Cell Stem Cell, 2009. 5(5): p. 527-39.
131. Bakker, W.J., I.S. Harris, and T.W. Mak, FOXO3a is activated in response to hypoxic stress and inhibits HIF1-induced apoptosis via regulation of CITED2. Mol Cell, 2007. 28(6): p. 941-53.
132. Miyamoto, K., et al., Foxo3a is essential for maintenance of the hematopoietic stem cell pool. Cell Stem Cell, 2007. 1(1): p. 101-12.
133. John, G.B., et al., Foxo3 is a PI3K-dependent molecular switch controlling the initiation of oocyte growth. Dev Biol, 2008. 321(1): p. 197-204.
134. Hosaka, T., et al., Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. Proc Natl Acad Sci U S A, 2004. 101(9): p. 2975-80.
135. Rena, G., et al., Roles of the forkhead in rhabdomyosarcoma (FKHR) phosphorylation sites in regulating 14-3-3 binding, transactivation and nuclear targetting. Biochem J, 2001. 354(Pt 3): p. 605-12.
136. Van Der Heide, L.P., M.F. Hoekman, and M.P. Smidt, The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. Biochem J, 2004. 380(Pt 2): p. 297-309.
137. Jacobs, F.M., et al., FoxO6, a novel member of the FoxO class of transcription factors with distinct shuttling dynamics. J Biol Chem, 2003. 278(38): p. 35959-67.
138. Vanhaesebroeck, B. and D.R. Alessi, The PI3K-PDK1 connection: more than just a road to PKB. Biochem J, 2000. 346 Pt 3: p. 561-76.
139. Brunet, A., et al., Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). Mol Cell Biol, 2001. 21(3): p. 952-65.
140. Rena, G., et al., Two novel phosphorylation sites on FKHR that are critical for its nuclear exclusion. EMBO J, 2002. 21(9): p. 2263-71.
141. Woods, Y.L., et al., The kinase DYRK1A phosphorylates the transcription factor FKHR at Ser329 in vitro, a novel in vivo phosphorylation site. Biochem J, 2001. 355(Pt 3): p. 597-607.
142. Zhao, X., et al., Multiple elements regulate nuclear/cytoplasmic shuttling of FOXO1: characterization of phosphorylation- and 14-3-3-dependent and -independent mechanisms. Biochem J, 2004. 378(Pt 3): p. 839-49.
143. Kim, S., et al., Regulation of FOXO1 by TAK1-Nemo-like kinase pathway. J Biol Chem. 285(11): p. 8122-9.
144. Teleman, A.A., Y.W. Chen, and S.M. Cohen, *Drosophila* Melted modulates FOXO and TOR activity. Dev Cell, 2005. 9(2): p. 271-81.
145. Luo, J.L., H. Kamata, and M. Karin, The anti-death machinery in IKK/NF-kappaB signaling. J Clin Immunol, 2005. 25(6): p. 541-50.
146. Huang, H., et al., CDK2-dependent phosphorylation of FOXO1 as an apoptotic response to DNA damage. Science, 2006. 314(5797): p. 294-7.
147. Satyanarayana, A. and P. Kaldis, A dual role of Cdk2 in DNA damage response. Cell Div, 2009. 4: p. 9.
148. Liu, P., T.P. Kao, and H. Huang, CDK1 promotes cell proliferation and survival via phosphorylation and inhibition of FOXO1 transcription factor. Oncogene, 2008. 27(34): p. 4733-44.
149. Yuan, Z., et al., Activation of FOXO1 by Cdk1 in cycling cells and postmitotic neurons. Science, 2008. 319(5870): p. 1665-8.
150. Brunet, A., et al., Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science, 2004. 303(5666): p. 2011-5.
151. Tsuruta, F., et al., JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3 proteins. EMBO J, 2004. 23(8): p. 1889-99.
152. Sunayama, J., et al., JNK antagonizes Akt-mediated survival signals by phosphorylating 14-3-3. J Cell Biol, 2005. 170(2): p. 295-304.
153. Yuan, Z., et al., Regulation of neuronal cell death by MST1-FOXO1 signaling. J Biol Chem, 2009. 284(17): p. 11285-92.
154. Choi, J., et al., Mst1-FoxO signaling protects Naive T lymphocytes from cellular oxidative stress in mice. PLoS One, 2009. 4(11): p. e8011.
155. Brent, M.M., R. Anand, and R. Marmorstein, Structural basis for DNA recognition by FoxO1 and its regulation by posttranslational modifica-

tion. Structure, 2008. 16(9): p. 1407-16.
156. Graves, J.D., et al., Caspase-mediated activation and induction of apoptosis by the mammalian Ste20-like kinase Mst1. EMBO J, 1998. 17(8): p. 2224-34.
157. Bi, W., et al., c-Jun N-terminal kinase enhances MST1-mediated pro-apoptotic signaling through phosphorylation at serine 82. J Biol Chem. 285(9): p. 6259-64.
158. Jang, S.W., et al., Akt phosphorylates MstI and prevents its proteolytic activation, blocking FOXO3a phosphorylation and nuclear translocation. J Biol Chem, 2007. 282(42): p. 30836-44.
159. Greer, E.L., et al., The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3a transcription factor. J Biol Chem, 2007. 282(41): p. 30107-19.
160. Dixit, M., et al., Shear stress-induced activation of the AMP-activated protein kinase regulates FoxO1a and angiopoietin-2 in endothelial cells. Cardiovasc Res, 2008. 77(1): p. 160-8.
161. Barthel, A., et al., Regulation of the forkhead transcription factor FKHR (FOXO1a) by glucose starvation and AICAR, an activator of AMP-activated protein kinase. Endocrinology, 2002. 143(8): p. 3183-6.
162. Yan, L., et al., PP2A regulates the pro-apoptotic activity of FOXO1. J Biol Chem, 2008. 283(12): p. 7411-20.
163. Singh, A., et al., Protein phosphatase 2A reactivates FOXO3a through a dynamic interplay with 14-3-3 and AKT. Mol Biol Cell. 21(6): p. 1140-52.
164. Bertoli, C., et al., Calpain small-1 modulates Akt/FoxO3A signaling and apoptosis through PP2A. Oncogene, 2009. 28(5): p. 721-33.
165. Ni, Y.G., et al., FoxO transcription factors activate Akt and attenuate insulin signaling in heart by inhibiting protein phosphatases. Proc Natl Acad Sci U S A, 2007. 104(51): p. 20517-22.
166. Yamagata, K., et al., Arginine methylation of FOXO transcription factors inhibits their phosphorylation by Akt. Mol Cell, 2008. 32(2): p. 221-31.
167. Bakker, W.J., et al., FoxO3a regulates erythroid differentiation and induces BTG1, an activator of protein arginine methyl transferase 1. J Cell Biol, 2004. 164(2): p. 175-84.
168. Fukuoka, M., et al., Negative regulation of forkhead transcription factor AFX (Foxo4) by CBP-induced acetylation. Int J Mol Med, 2003. 12(4): p. 503-8.
169. Matsuzaki, H., et al., Acetylation of Foxo1 alters its DNA-binding ability and sensitivity to phosphorylation. Proc Natl Acad Sci U S A, 2005. 102(32): p. 11278-83.
170. van der Heide, L.P. and M.P. Smidt, Regulation of FoxO activity by CBP/p300-mediated acetylation. Trends Biochem Sci, 2005. 30(2): p. 81-6.
171. van der Horst, A., et al., FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2(SIRT1). J Biol Chem, 2004. 279(28): p. 28873-9.
172. Daitoku, H., et al., Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. Proc Natl Acad Sci U S A, 2004. 101(27): p. 10042-7.
173. Jing, E., S. Gesta, and C.R. Kahn, SIRT2 regulates adipocyte differentiation through FoxO1 acetylation/deacetylation. Cell Metab, 2007. 6(2): p. 105-14.
174. Kobayashi, Y., et al., SIRT1 is critical regulator of FOXO-mediated transcription in response to oxidative stress. Int J Mol Med, 2005. 16(2): p. 237-43.
175. Kwon, H.S., et al., Protein kinase B-alpha inhibits human pyruvate dehydrogenase kinase-4 gene induction by dexamethasone through inactivation of FOXO transcription factors. Diabetes, 2004. 53(4): p. 899-910.
176. Nasrin, N., et al., DAF-16 recruits the CREB-binding protein coactivator complex to the insulin-like growth factor binding protein 1 promoter in HepG2 cells. Proc Natl Acad Sci U S A, 2000. 97(19): p. 10412-7.
177. Qiang, L., A.S. Banks, and D. Accili, Uncoupling of acetylation from phosphorylation regulates FoxO1 function independent of its subcellular localization. J Biol Chem. 285(35): p. 27396-401.
178. Frescas, D., L. Valenti, and D. Accili, Nuclear trapping of the forkhead transcription factor FoxO1 via Sirt-dependent deacetylation promotes expression of glucogenetic genes. J Biol Chem, 2005. 280(21): p. 20589-95.
179. Mahmud, D.L., et al., Phosphorylation of forkhead transcription factors by erythropoietin and stem cell factor prevents acetylation and their interaction with coactivator p300 in erythroid progenitor cells. Oncogene, 2002. 21(10): p. 1556-62.
180. Yoshimochi, K., H. Daitoku, and A. Fukamizu, PCAF represses transactivation function of FOXO1 in an acetyltransferase-independent manner. J Recept Signal Transduct Res. 30(1): p. 43-9.
181. Motta, M.C., et al., Mammalian SIRT1 represses forkhead transcription factors. Cell, 2004. 116(4): p. 551-63.
182. Huang, H., et al., Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation. Proc Natl Acad Sci U S A, 2005. 102(5): p. 1649-54.
183. Matsuzaki, H., et al., Insulin-induced phos-

phorylation of FKHR (Foxo1) targets to proteasomal degradation. Proc Natl Acad Sci U S A, 2003. 100(20): p. 11285-90.
184. Nakayama, K., et al., Skp2-mediated degradation of p27 regulates progression into mitosis. Dev Cell, 2004. 6(5): p. 661-72.
185. Tang, X., et al., Akt phosphorylation regulates the tumour-suppressor merlin through ubiquitination and degradation. Nat Cell Biol, 2007. 9(10): p. 1199-207.
186. Plas, D.R. and C.B. Thompson, Akt activation promotes degradation of tuberin and FOXO3a via the proteasome. J Biol Chem, 2003. 278(14): p. 12361-6.
187. Lin, H.K., et al., Phosphorylation-dependent regulation of cytosolic localization and oncogenic function of Skp2 by Akt/PKB. Nat Cell Biol, 2009. 11(4): p. 420-32.
188. Yang, J.Y., et al., ERK promotes tumorigenesis by inhibiting FOXO3a via MDM2-mediated degradation. Nat Cell Biol, 2008. 10(2): p. 138-48.
189. Yang, W., N.G. Dolloff, and W.S. El-Deiry, ERK and MDM2 prey on FOXO3a. Nat Cell Biol, 2008. 10(2): p. 125-6.
190. Phelps, M., et al., MEK-ERK signaling controls Hdm2 oncoprotein expression by regulating hdm2 mRNA export to the cytoplasm. J Biol Chem, 2005. 280(17): p. 16651-8.
191. Phillips, A., C.J. Jones, and J.P. Blaydes, The mechanisms of regulation of Hdm2 protein level by serum growth factors. FEBS Lett, 2006. 580(1): p. 300-4.
192. Manfredi, J.J., The Mdm2-p53 relationship evolves: Mdm2 swings both ways as an oncogene and a tumor suppressor. Genes Dev. 24(15): p. 1580-9.
193. Tsai, W.B., et al., Inhibition of FOXO3a tumor suppressor function by betaTrCP1 through ubiquitin-mediated degradation in a tumor mouse model. PLoS One. 5(7): p. e11171.
194. Brenkman, A.B., et al., Mdm2 induces mono-ubiquitination of FOXO4. PLoS One, 2008. 3(7): p. e2819.
195. van der Horst, A., et al., FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP. Nat Cell Biol, 2006. 8(10): p. 1064-73.
196. Brenkman, A.B., et al., The peptidyl-isomerase Pin1 regulates p27kip1 expression through inhibition of Forkhead box O tumor suppressors. Cancer Res, 2008. 68(18): p. 7597-605.
197. Li, H.H., et al., Atrogin-1 inhibits Akt-dependent cardiac hypertrophy in mice via ubiquitin-dependent coactivation of Forkhead proteins. J Clin Invest, 2007. 117(11): p. 3211-23.
198. Hu, P., S. Shimoji, and G.W. Hart, Site-specific interplay between O-GlcNAcylation and phosphorylation in cellular regulation. FEBS Lett. 584(12): p. 2526-38.
199. Ho, S.R., et al., O-GlcNAcylation enhances FOXO4 transcriptional regulation in response to stress. FEBS Lett. 584(1): p. 49-54.
200. Zachara, N.E., et al., The dynamic stress-induced "O-GlcNAc-ome" highlights functions for O-GlcNAc in regulating DNA damage/repair and other cellular pathways. Amino Acids.
201. Kuo, M., et al., O-glycosylation of FoxO1 increases its transcriptional activity towards the glucose 6-phosphatase gene. FEBS Lett, 2008. 582(5): p. 829-34.
202. Kuo, M., et al., O-GlcNAc modification of FoxO1 increases its transcriptional activity: a role in the glucotoxicity phenomenon? Biochimie, 2008. 90(5): p. 679-85.
203. Yang, J., M. Anzo, and P. Cohen, Control of aging and longevity by IGF-I signaling. Exp Gerontol, 2005. 40(11): p. 867-72.
204. Taniguchi, C.M., B. Emanuelli, and C.R. Kahn, Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol, 2006. 7(2): p. 85-96.
205. Blakesley, V.A., et al., Signaling via the insulin-like growth factor-I receptor: does it differ from insulin receptor signaling? Cytokine Growth Factor Rev, 1996. 7(2): p. 153-9.
206. Weigel, D. and H. Jackle, The fork head domain: a novel DNA binding motif of eukaryotic transcription factors? Cell, 1990. 63(3): p. 455-6.
207. Clark, K.L., et al., Co-crystal structure of the HNF-3/fork head DNA-recognition motif resembles histone H5. Nature, 1993. 364(6436): p. 412-20.
208. Baldauf, S.L., A Search for the Origins of Animals and Fungi: Comparing and Combining Molecular Data. Am Nat, 1999. 154(S4): p. S178-S188.
209. Kaestner, K.H., W. Knochel, and D.E. Martinez, Unified nomenclature for the winged helix/forkhead transcription factors. Genes Dev, 2000. 14(2): p. 142-6.
210. Carter, M.E. and A. Brunet, FOXO transcription factors. Curr Biol, 2007. 17(4): p. R113-4.
211. Furuyama, T., et al., Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. Biochem J, 2000. 349(Pt 2): p. 629-34.

Chapter 2

Oxidative stress dependent regulation of Forkhead Box O 4 activity by Nemo-like kinase

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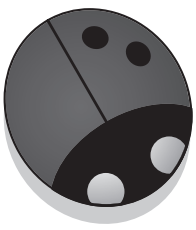

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Oxidative stress dependent regulation of Forkhead Box O4 activity by Nemo-like kinase

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Abstract

Forkhead Box O (FOXO) transcription factors are involved in various cellular processes including cell proliferation, stress resistance, metabolism and longevity. Regulation of FOXO transcriptional activity occurs mainly through a variety of posttranslational modifications (PTMs) including phosphorylation, acetylation and ubiquitination. Here we describe Nemo-like kinase (NLK) as a novel regulator of FOXOs. NLK binds to, and phosphorylates FOXO1, FOXO3a and FOXO4 on multiple residues. NLK acts as a negative regulator of FOXO transcriptional activity. For FOXO4 we show that NLK-mediated loss of FOXO activity co-occurs with inhibition of FOXO4 monoubiquitination. Previously, we have shown that oxidative stress-induced monoubiquitination of FOXO4 stimulates its transactivation, which leads to activation of an anti-oxidant defensive program. Conversely, NLK-dependent inhibition of FOXO4 activity can provide a means to downregulate this defensive program, when oxidative stress reaches a level beyond which repair is no longer feasible and cells need to undergo apoptosis.

Introduction

The family of Forkhead Box O (FOXO) transcription factors consists of 4 members denoted FOXO1, FOXO3a, FOXO4 and FOXO6 [1]. Of these, FOXO6 expression appears restricted to the brain whereas all others are ubiquitously expressed albeit at varying levels [2]. Regulation of FOXO activity occurs predominantly through two signaling pathways [3, 4]. First, FOXO activity is controlled by phosphoinositide 3-kinase PI3K/protein kinase B (PKB, also known as Akt) signaling and direct PKB mediated phosphorylation inhibits FOXO activity by inducing a relocalization of FOXO from the nucleus to the cytosol [5-7]. Second, an increased level of cellular oxidative stress results in FOXO activation and this is primarily mediated by direct phosphorylation by c-Jun N-terminal kinase (JNK). However, the pathway(s) leading to JNK activation with respect to FOXO activation is largely unknown and for FOXO4 we have obtained evidence that this at least requires the small GTPase Ral [8, 9]. Importantly, both regulation through PKB and JNK are evolutionary conserved. In *Caenorhabditis elegans*, the FOXO ortholog DAF-16 is required for lifespan determination through both the PI3K/PKB and the JNK pathway. Whereas PI3K/PKB activity shortens lifespan, JNK activity lengthens lifespan and both require DAF-16 [10, 11]. Despite the general picture, many details of FOXO regulation are still lacking and especially regulation by oxidative stress appears highly complex and involves,

besides JNK-mediated phosphorylation, many other PTMs including mono- and polyubiquitination, acetylation and methylation [12, 13]. Increased oxidative stress also induces binding of accessory proteins and we have described an evolutionary conserved role for binding of β-catenin to FOXO/DAF-16 in oxidative stress control [14]. The oxidative stress-induced binding of β-catenin to FOXO4 induces its transcriptional activity. In agreement with this, loss of BAR-1, the *C. elegans* ortholog of β-catenin reduces the activity of DAF-16 in dauer formation and lifespan. On the other hand, under normal physiological conditions binding between β-catenin and FOXO4 is reduced [15]. This allows β-catenin to form a complex with members of the Lymphoid enhancer factor/T-cell factor LEF-1/TCF transcription factor family, which promotes their activity leading to the expression of its targets genes. The binding of β-catenin to TCF is additionally regulated by Wnt signaling, which is involved in regulation of various developmental processes [16, 17]. Finally, elevated levels of β-catenin leading to the constitutive activation of TCF-mediated transcription are associated with carcinogenesis [18]. Therefore, it is important to tightly regulate the activity of the β-catenin/TCF complex. It has been shown that activation of a mitogen activated protein kinase (MAP) kinase pathway involving transforming growth factor β-activated kinase 1 (TAK1) and NLK leads to suppression of Wnt signaling [19]. TAK1 in complex with TAK 1-binding protein 1 (TAB1) activates NLK, which subsequently binds to and phosphorylates TCF. NLK-mediated phosphorylation of TCF impairs its ability to bind DNA and thereby decreases its transcriptional activity [20]. Additionally, NLK has been reported to stimulate ubiquitination of TCF by recruiting the E3 ligase NLK-associated ring finger protein (NARF), which targets TCF for degradation [21]. Moreover, it has been postulated that the TAK1-NLK pathway is activated directly by Wnt signaling and functions as a negative feedback mechanism [22, 23].

Besides, modulating TCF function, NLK is emerging as a regulator of many different transcription factors including Peroxisome proliferator-activated receptor-γ (PPAR-γ), Myb proteins and Notch1 [22, 24-26]. Surprisingly, the mechanism by which NLK controls the activity of this diverse set of transcription factors is highly versatile. For example, even though NLK has been reported to phosphorylate all three members of the mammalian Myb protein family, which results in suppression of their transcriptional activity, the molecular mechanism of this repression seems to be dissimilar for each Myb protein. NLK-directed phosphorylation of c-Myb in response to Wnt signaling leads to proteasomal degradation of c-Myb [22]. In contrast, phosphorylation of a-Myb does not affect its stability but prevents association of a-Myb with its coactivator CREB-binding protein (CBP). Additionally, ectopic expression of NLK has been associated with elevated levels of histone methylation, which might indirectly suppress activity of a-Myb [26]. Similarly, it has been reported that NLK inhibits transactivation of PPAR-γ by promoting histone-inactivating modifications. However, in this case NLK does not directly act on PPAR-γ itself but after being activated in response to Wnt-5a signaling NLK phosphorylates a histone methyltransferase, SET domain bifurcated 1 (SETDB1). This is driving the recruitment of the co-repressor complex to the PPAR-γ specific promoter regions

leading to an increase in histone H3-K9 methylation and blocking expression of PPAR-γ target genes [24].

Because of the observations described above we set out to investigate whether NLK could regulate FOXO function. Here we provide evidence that NLK inhibits FOXO transcriptional activity. NLK binds to FOXO and this binding is increased by elevated cellular oxidative stress and consequently FOXO is phosphorylated by NLK on a large number of sites both *in vitro* and *in vivo*. Finally, we show that NLK mediates FOXO4 repression by inhibiting the monoubiquitination of FOXO4. Taken together, our data reveal NLK as a novel regulator of FOXO function.

Materials and Methods

Cell culture and transfections

All cell lines except DLD1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin and 2 mM L-glutamine. DLD1 were maintained in RPMI medium 1640 supplemented as described above. $Pin1^{-/-}$ MEFs were a kind gift from Dr. P. van der Sluijs. $Mdm2^{-/-}p53^{-/-}$ MEFs were a kind gift from dr. A.G. Jochemsen. In all experiments, cells were cultured in the presence of 10% FBS. HEK293T and DLD1 cells were transiently transfected using FuGENE6 reagent according to the suggestions of manufacturer (Roche Applied Science). $Mdm2^{-/-}p53^{-/-}$ MEFs and $Pin1^{-/-}$ MEFs were transiently transfected using Effectene according to the protocol of the manufacturer (Qiagen). Total amounts of DNA were equalized using pcDNA4-TO. siRNA oligomers were transfected using HiPerFect according to the suggestions of the manufacturer (Qiagen). The following compounds were purchased: Cycloheximide and MG132 (Sigma and Biomol, respectively).

Plasmids, oligomers and recombinant proteins

pMT2-HA-FOXO4, GLOFlag3-Flag-FOXO4, HA-FOXO1, HA-FOXO3a, pBabe-puro, His-Ubiquitin, GloMyc-ubiquitin-specific peptidase 7 (USP7) [27], Flag-murine double minute 2 (Mdm2), Flag-peptidyl-prolyl isomerase 1 (Pin1) CMV-HA-$p27^{kip1}$ [28], pcDNA3-Flag-β-catenin [14] have been described previously. 6xDBE and $p27^{kip1}$ Luciferase have been describe before [9]. pRL-Tk (Tk *Renilla* luciferase) was purchased from Promega. Flag-NLK and Flag-NLK^{K155M} were a kind gift from dr. L. Smit [23]. myc-TAK1 and myc-$TAK1^{D175A}$ were kindly provided by dr. P. Cohen. pcDNA4-TO-StrepTag-NLK was generated by inserting phosphorylated oligonucleotides containing the BamHI-StrepTag-EcoRV sequence into BamHI/EcoRV digested pcDNA4-TO vector (Invitrogen) generating pcDNA4-TO-StrepTag. Subsequently, the NLK coding sequence was amplified using forward EcoRV primer 5'-CAGTCCAGTGAGATATCGCCGCGGCTTACAATGGC-3' and reverse XhoI primer 5'-GTGACTATCACTCGAGTCATCACTCCCACACCAGAG-3' and was cloned into EcoRV/XhoI digested pcDNA4-TO-StrepTag generating pcDNA4-TO-StrepTag-NLK. GLOFlag3-Flag-FOXO4Δ8 was generated by site directed mutagenesis according to the QuikChange Site-Directed Mutagenesis Kit protocol (Stratagene). Non-targeting siRNA (siCTR), siRNA against NLK (NLK #2, sense sequence 5'-GAAGTTGTTACTCAGTATTAT-dTdT-3'; NLK #3, sense sequence 5'-CTCCAACCTCCACACATTGAC-dTdT-3') and siRNA

against USP7 (described previously [27] were purchased from Dharmacon RNA. Recombinant proteins were purchased: His_6-NLK (Millipore), E1 (UBE1), E2 (UbcH5b), His_6-Mdm2, His_6-Ubiquitin (BostonBiochem, Cambridge, MA). Glutathione S-transferase (GST) or GST–FOXO4 fusion protein were expressed in Bl21DE3 Rosetta cells and purified by binding to glutathione-agarose beads.

Antibodies

Monoclonal antibodies: 12CA5 anti-HA and 9E10 anti-myc were produced in-house using a hybridoma cell line. The antibody against FOXO4 (834) has been described previously [29]. The following antibodies were purchased: NLK-H100, NLK-B5, GST-B14 and Mdm2-SMP14 (Santa Cruz Biotechnology); $p27^{kip1}$ (BD Biosciences); Acetylated-Lysine Antibody #9441 (Cell Signaling); USP7-BL851 (Bethyl Laboratories); tubulin and Flag-M2 (Sigma).

Cell lysates and western blot analysis

When preparing the total lysate samples, cells were washed with ice-cold phosphate buffered saline (PBS) and harvested in Laemmli sample buffer. Protein samples were resolved by SDS-PAGE and transferred to Polyvinylidene fluoride (PVDF) membrane (Perkin-Elmer). Western blot analysis was performed under standard conditions, using the indicated antibodies.

FOXO4 activity assays

To determine the expression of endogenous $p27^{kip1}$, HEK293T cells were transfected with empty vector or HA-FOXO4 together with pBabe-puro. Each of the constructs encoding Flag-NLK, Myc-TAK1, Flag-p38 was cotransfected and cells were left for twenty-four hours. To select for transfected cells, puromycin was added to the culture medium to a final concentration of 2 μg/ml for 48 hours. When indicated, the cells were additionally treated with 5 μM MG132 for a twelve hours period prior to harvesting. Cells were lysed in Laemmli sample buffer. The cell debris was pelleted by centrifugation and the supernatant was used for western blot analysis.

For luciferase assays DLD1 cells were transfected with either reporter construct bearing six canonical FOXO binding sites (6xDBE-luciferase) or human $p27^{kip1}$-promoter linked to luciferase (p27GL-1609) and the additional constructs were cotransfected as indicated. Luciferase counts were normalized using TK-*Renilla* luciferase. Luciferase levels were measured 48 hours after transfection employing a luminometer in combination with a dual luciferase assay kit according to the instructions of the manufacturer (Promega). All experiments were performed in triplicate.

Co-immunoprecipitations

Prior to the co-immunoprecipitations cells were treated with the optimized concentration of 200 μM of H_2O_2 (data not shown) in the presence of serum for the indicated duration. Cells were lysed in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 1% NP-40, 0.5% sodium deoxycholate, 10 mM EDTA, 150 mM NaCl, phosphatase and protease inhibitors and centrifuged at 20,800 x g for 10 minutes at 4°C. The cleared lysates were incubated with 5 μl solid anti-Flag M2 affinity beads for 2 hours at 4°C. Beads were washed four times with lysis buffer and proteins bound to the beads were eluted in Laemmli sample buffer, analyzed by SDS-PAGE and immunoblotting as described above using the indicated antibodies.

For the endogenous co-immunoprecipitations protein complexes within the cells were *in vivo* cross-linked by adding dithiobis(succinimidyl) propionate (DSP, Pierce) to the medium to a final concentration of 2.5 mM for 20 minutes at 37°C. The cross-linking reaction was quenched by adding Tris-HCl pH 7.5 to final concentration of 20 mM for 15 minutes at room temperature. Endogenous co-immunoprecipitations were performed using FOXO4 (834) antibodies.

In vitro kinase assay

GST or GST-FOXO4 were incubated with recombinant His-NLK in ATP buffer containing 30 mM Tris-HCl (pH 7.4), 15 mM $MgCl_2$, 1 mM EGTA, 100 μg/ml BSA, 200 μM DTT, 1 mM ATP and 5 μCi of [γ-^{32}P]ATP, at 30°C for 15 min. The reaction was terminated by adding 5x concentrated Laemmli sample buffer and heating the samples for 5 minutes at 95°C.

For *in vitro* kinase assays using exogenously expressed proteins, HEK293T cells were transfected with Flag-FOXO4 or Flag-NLK, respectively. The lysates were prepared and immunoprecipitated as described for the co-immunoprecipitation assay. The immunoprecipitations were subsequently washed three times with lysis buffer and then two times with kinase buffer containing 30 mM Tris-HCl (pH 7.4), 15 mM $MgCl_2$, 1 mM EGTA, and 100 μg/ml BSA**.** Flag-FOXO4 proteins coupled to the beads were eluted with 200 μg/ml 3x Flag-peptide (Sigma). Next, the FOXO4-containing eluates were incubated with Flag-NLK that was coupled to the beads, in ATP buffer for 15 min at 30°C. Phosphorylated proteins were analyzed by SDS-PAGE followed by autoradiography.

In vivo ubiquitination assay

The monoubiquitination assay was performed as described previously [27]. Shortly cells were transfected with the indicated constructs. When indicated, 5 μM MG132 was added to the cultures for twelve hours before harvesting the cells. Forty-eight hours after transfection, cells were treated as indicated and lysed in urea lysis buffer containing 8 M urea, 10 mM of Tris-HCl (pH 8.0), 100 mM of Na_2HPO_4/NaH_2PO_4 (pH8.0), 0.2% Triton X-100, 5 mM of NEM, and protease inhibitors. Ubiquitinated proteins were precipitated using 10 ul solid nickel-nitrilotriacetic acid (Ni-NTA) agarose (Sigma) and analyzed by SDS-PAGE followed by immunoblotting.

In vitro ubiquitination assay

Purified GST-FOXO4 (1.2 μg) was mixed with 275 ng UBE1, 85 ng UbcH5b, 400 ng His_6-Mdm2, and 10 ng His_6-Ubiquitin in a final volume of 40 μl reaction buffer containing 25 mM Tris-HCl pH 7.5, 60 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP. The reaction was carried out for 120 minutes at 30°C. When indicated 50 ng of His_6-NLK was added to the reaction. Ubiquitinated proteins were precipitated using 10 μl solid Ni-NTA agarose (Sigma) and analyzed by SDS-PAGE followed by immunoblotting. Additionally, when indicated GST-FOXO4 or His-Mdm2 were prephosphorylated using His-NLK for 30 minutes at 30°C. In parallel, control reactions containing GST-FOXO4 or His-Mdm2 but lacking His-NLK were treated identically. Subsequently, E1, E2 and His-ubiquitin were added and the reaction was carried out as described above.

FOXO4 half-life assay

HEK293T cells were transfected with

pBabe-puro plasmid and the indicated constructs. To select for transfected cells, puromycin was added to the culture medium to a final concentration of 2 μg/ml for 48 hours. Subsequently, cells were treated with cycloheximide for indicated time points. Protein samples were analyzed by SDS-PAGE followed by immunoblotting.. Relative FOXO4 protein expression levels were calculated using the Odyssey application software (LI-COR).

Results

Hydrogen peroxide increases interaction between NLK and FOXOs

To determine possible regulation of FOXO by NLK, we first tested whether FOXO4 can interact with NLK. To this end Flag-NLK and HA-FOXO4 were ectopically overexpressed in HEK293T cells. Following immunoprecipitation of Flag-NLK, we observed co-immunoprecipitation of FOXO4 and also in the reverse experiment we detected co-immunoprecipitation of NLK following immunoprecipitation of Flag-FOXO4, suggesting binding between NLK and FOXO4 (Figure 1A, B and Supplementary Figure S1A; Supplementary Data are available online at www.libertonline.com/ars). Similarly to FOXO4, we observed in co-immunoprecipitation assays that HA-FOXO1 and HA-FOXO3a interacted with Flag-NLK (Figure 1C, D and Supplementary Figure S1B, C). Moreover, hydrogen peroxide treatment of cells to increase the cellular level of reactive oxygen species (ROS) enhanced formation of this complex and this could be partially reversed upon pre-incubation of cells with the ROS scavenger N-Acetyl Cysteine (NAC) (Figure 1A, B and Supplementary Figure S1A).

However, the interaction between FOXO4 and NLK did not depend on NLK kinase activity since we observed also binding between kinase-dead NLK ($NLKK^{155M}$) and FOXO4.

Moreover, we were able to observe binding between FOXO4 and NLK under endogenous conditions (Figure 1E).

Thus, NLK interacts with FOXOs independent of its kinase activity and this interaction is enhanced by peroxide treatment of cells.

NLK phosphorylates FOXO4

Simultaneous expression of FOXO4 and wild type but not kinase-dead NLK resulted in the appearance of several forms of FOXO4 displaying reduced mobility on SDS-PAGE. This shift in the gel mobility of FOXO4 was abolished after treatment of immunocomplexes with λ phosphatase, which indicated that NLK expression results in increased FOXO4 phosphorylation *in vivo* (Figure 2A). To confirm direct phosphorylation of FOXO4 by NLK, we performed an *in vitro* kinase assay using Flag-FOXO4 and Flag-NLK that were both immunopurified from HEK293T cells and this further confirmed that NLK can directly phosphorylate FOXO4 (Figure 2B). Essentially the same result was obtained when using commercially obtained recombinant His-NLK and bacterially expressed GST-FOXO4, but not GST alone, as a substrate in an *in vitro* kinase assay (Figure 2C).

Taken together, these data indicate that NLK phosphorylates FOXO4 directly both *in vivo* and *in vitro*.

Because co-expression of NLK already results in an almost complete mobility shift of FOXO4 in the absence of peroxide treatment we wished to address whether peroxide treatment affected NLK kinase activity. To this end

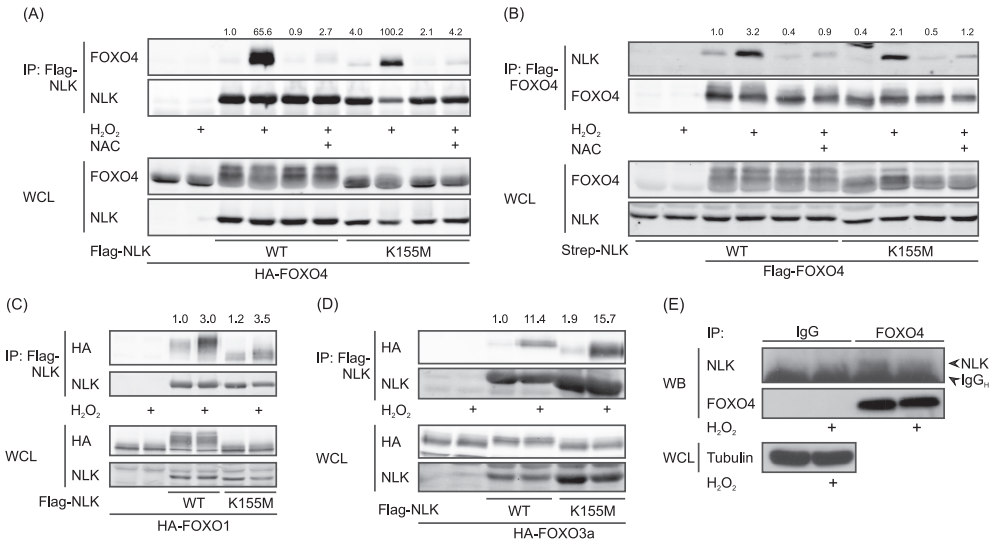


Figure 1. H_2O_2 induces interaction between NLK and FOXOs
(A, B) NLK binds to FOXO4. HEK293T cells were transfected with the indicated constructs. Cells were treated as indicated with 4 mM NAC for 24 hours followed by 200 μm H_2O_2 for 15 min. Cell lysates were subjected to immunoprecipitation using Flag-M2 affinity beads. Immunoprecipitated complexes were analyzed by SDS-PAGE and immunoblotting using the indicated antibodies. **(C, D)** NLK binds to FOXO1 and FOXO3a. Experiments were performed essentially as in Figure 1A, B using the indicated constructs. **(E)** NLK binds to FOXO4 under endogenous conditions. Cells were treated as indicated with 200 μm H_2O_2 for 30 min, followed by *in vivo* cross-linking using DSP. Cell lysates were subjected to immunoprecipitation using FOXO4 antibodies. Immunoprecipitated complexes were analyzed by SDS-PAGE and immunoblotting using the indicated antibodies. **(A, C, D)** Relative amounts of FOXOs bound to NLK for individual experiments are indicated. The protein levels were quantified using Odyssey application software (LI-COR). The levels of coimmunoprecipitated FOXOs were normalized to their total expression levels and the ratios between FOXOs and NLK were calculated. The amounts of FOXOs bound to NLK in the absence of H_2O_2 treatment were set to 1. **(B)** Relative amounts of NLK bound to FOXO4 are indicated and quantified as described above.

we performed an in vitro kinase assay on NLK precipitated from cells after various times of peroxide treatment (Figure 2D). We did not observe any change of NLK kinase activity measured in vitro suggesting that increased ROS does not affect NLK kinase activity. Thus we conclude that increased ROS stabilizes binding between NLK and FOXO4 by altering the on-off rate of the interaction. Apparently the on-off rate of the interaction is as such that after 48 hrs of transient expression NLK-mediated FOXO4 phosphorylation is complete. In agreement with this and because NLK activity is not regulated by peroxide, NAC treatment also does not affect NLK-mediated phosphorylation but only the apparent binding observed in co-immunoprecipitation.

NLK negatively regulates FOXO4 transcriptional activity

To analyze whether NLK can influence FOXO4 transcriptional activity, we first performed a luciferase reporter assay using the FOXO responsive 6xDBE-luciferase (data not shown) and p27-luciferase reporters [30, 31]. In both cases ectopic expression of NLK decreased

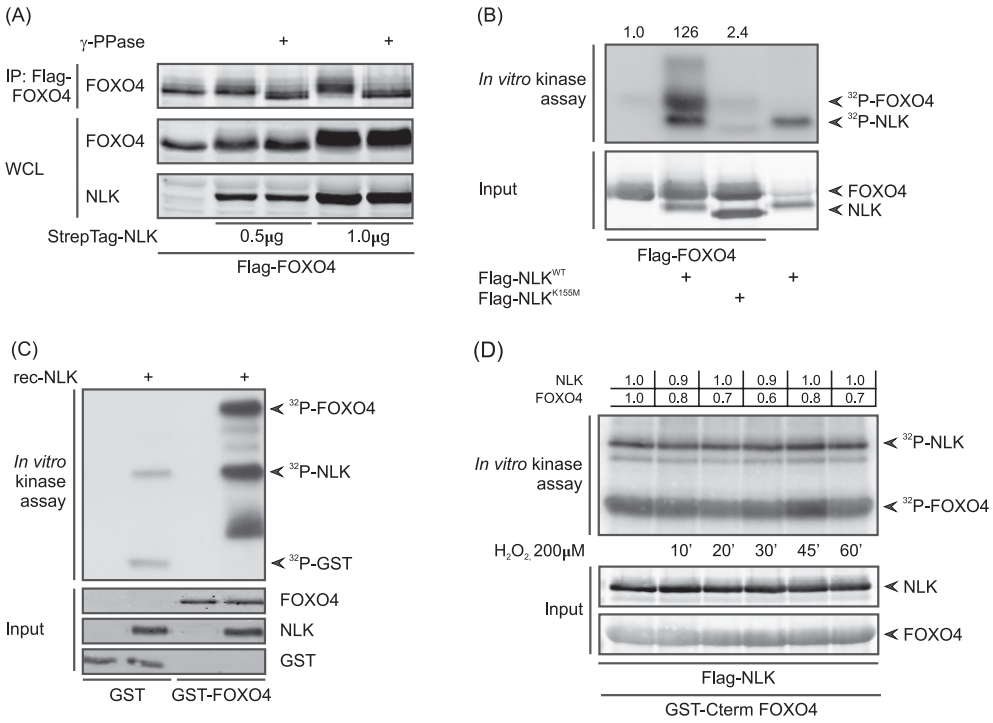


Figure 2. NLK phosphorylates FOXO4 *in vitro* and *in vivo*
(A) NLK induces phosphorylation of FOXO4 *in vivo*. Flag-FOXO4 was immunopurified from HEK293T cells and treated with λ-phosphatase (λ-PPase) for 30 min. as indicated. **(B)** NLK induces phosphorylation of FOXO4 *in vitro*. Flag-tagged FOXO4 and NLK were expressed in HEK293T cells and immunopurified. Aliquots of purified FOXO4 and NLK were subjected to an *in vitro* kinase assay in the presence of [γ-^{32}P]ATP. Phosphorylation was measured after 15 min of incubation by autoradiography. The levels of purified protein were verified by immunoblotting using the indicated antibodies. **(C)** GST or GST-FOXO4 was incubated with His_6-NLK in the presence of [γ-^{32}P]ATP. Phosphorylation was measured as in **(A)**. The levels of recombinant proteins were verified by immunoblotting using the indicated antibodies. **(D)** Hydrogen peroxide treatment has no effect on NLK activity. To assess the activity of NLK upon H_2O_2 treatment Flag-NLK was immunoprecipitated from HEK293T exposed to 200 μM of H_2O_2 for the indicated durations. Purified NLK was incubated with [γ-^{32}P]ATP and bacterially purified C-terminal fragment of FOXO4. The phosphorylation of proteins was measured as in **(A)**. Additionally, in **(B, D)** levels of phosphorylated FOXO4 and/or NLK for individual experiments are indicated. Intensities of individual bands were quantified as in Figure 1 and the ratios between the total protein levels and the levels of phosphorylated proteins were calculated. The basal phosphorylation of FOXO4 and NLK was set to 1.

FOXO4-induced expression of luciferase in a dose-dependent manner, whereas much weaker or no inhibition was observed using the kinase-dead mutant of NLK (Figure 3A).

To further explore the effect of NLK on FOXO activity, we analyzed the endogenous expression of $p27^{kip1}$, a well established FOXO target gene. Protein levels of $p27^{kip1}$ were strongly induced by exogenous expression of FOXO4 and FOXO1. More importantly, in both cases the FOXO-dependent induction of $p27^{kip1}$ was blocked by coexpression of NLK. Again, this inhibition was dependent on kinase activity since the

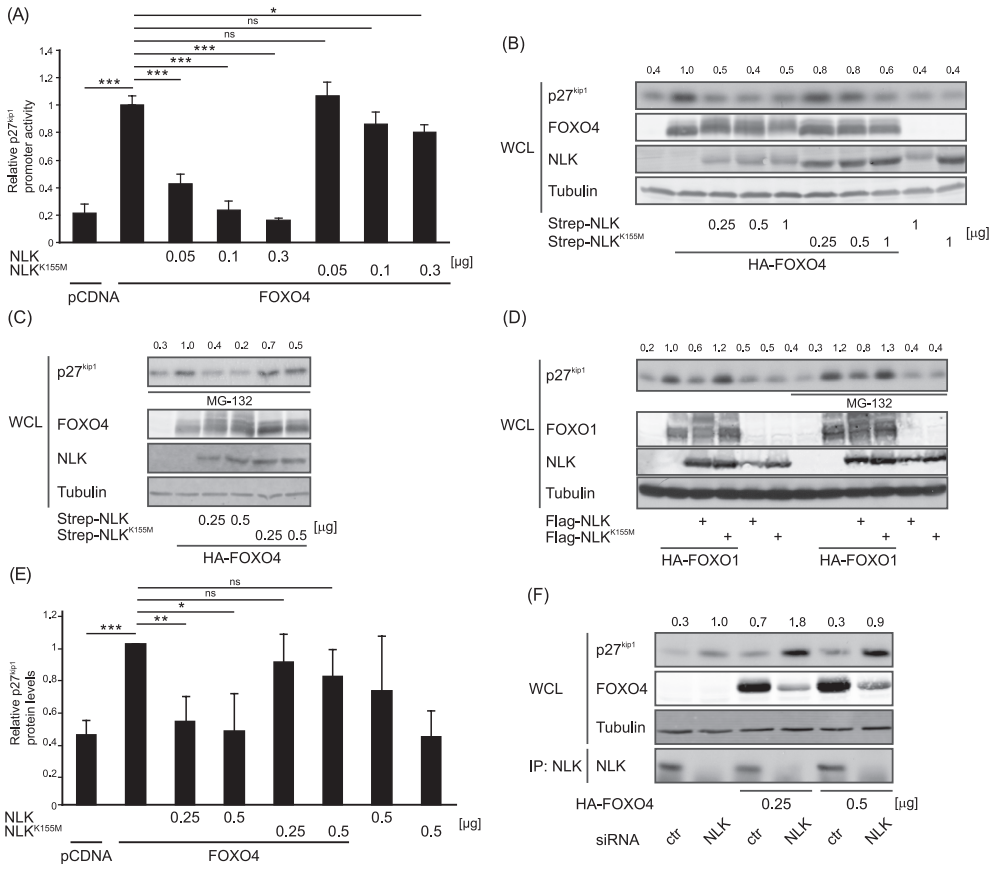


Figure 3. NLK negatively regulates FOXO transcriptional activity

(A) NLK inhibits the transcriptional activity of FOXO4. Luciferase assay in DLD1 cells transfected with FOXO-responsive luciferase-reporters, Tk-*Renilla*, HA-FOXO4 and WT NLK or NLK^{K155M}. Data presented are normalized to FOXO4 activity in the absence of NLK set at 1. Error bars represent s.d.; p values were calculated by t-tests between indicated data points (experiments performed three times with three replicates in each experiment). *p < 0.05; ***p < 0.0001; 'ns' – not significant. Protein levels were confirmed by immunoblotting using the indicated antibodies. **(B, C, D)** Exogenous expression of NLK reduces $p27^{kip1}$ protein levels. Western blot analysis of $p27^{kip1}$ expression of puromycin-selected HEK293T cells expressing the indicated proteins. Cells were treated with 5 µM MG132 for 12 hours when indicated. **(E)** $p27^{kip1}$ expression was quantified using Odyssey application software (LI-COR) and normalized to tubulin levels. The $p27^{kip1}$ level in the presence of FOXO4 was set to 1. Error bars represent s.d.; p values were calculated by t-tests between indicated data points (experiment performed three times); *p < 0.05; **p < 0.01; ***p < 0.0001; 'ns' – not significant. **(F)** Knockdown of NLK increases $p27^{kip1}$ protein levels. Western blot analysis of $p27^{kip1}$ expression of puromycin-selected HEK293T transfected with HA-FOXO4 and non-targeting siRNA or two combined siRNAs specific for NLK. To verify the efficiency of knockdown, endogenous NLK was immunoprecipitated. Additionally, in **(B–D, F)** relative $p27^{kip1}$ levels for individual experiments are indicated and quantified as in Figure 3E. The $p27^{kip1}$ levels in the presence of FOXO4 or FOXO1 were set to 1.

kinase-dead form of NLK could not inhibit FOXO-dependent induction of $p27^{kip1}$ protein expression (Figure 3B, C, D and quantification in Figure 3E). In accordance with these observations, targeted depletion of endogenous NLK in HEK293T cells using siRNA resulted in significant increase in $p27^{kip1}$ protein levels (Figure 3F and Supplementary Figure S2). Further, the NLK driven reduction in $p27^{kip1}$ protein could not be rescued by treating the cells with proteasomal inhibitor MG-132, which indicates that NLK does not influence stability of $p27^{kip1}$ but rather regulates its expression at the transcriptional level (Figure 3C, D).Taken together, these data show that NLK expression suppresses the ability of FOXOs to transactivate transcription.

NLK does not affect binding of FOXO4 to CBP or β-catenin

Next, we started to delineate the mechanism by which NLK could inhibit FOXO4 activity. As mentioned within the introduction, NLK regulates TCF activity by enhancing the dissociation of β-catenin/TCF complexes from the DNA, which leads to downregulation of TCF transcriptional activity. As we have shown that direct binding of β-catenin to FOXO4 following increased ROS positively regulates FOXO4 activity, we therefore first addressed whether NLK would affect the formation of the β-catenin/FOXO4 complex and by this would suppress its transactivation. However, upon co-expression of NLK, binding between FOXO4 and β-catenin did not significantly change suggesting NLK to act independent of β-catenin (Figure 4A).

NLK has been reported to reduce the activity of a Myb by inhibiting the association of a-Myb with its coactivator CBP. Again, we and others have shown

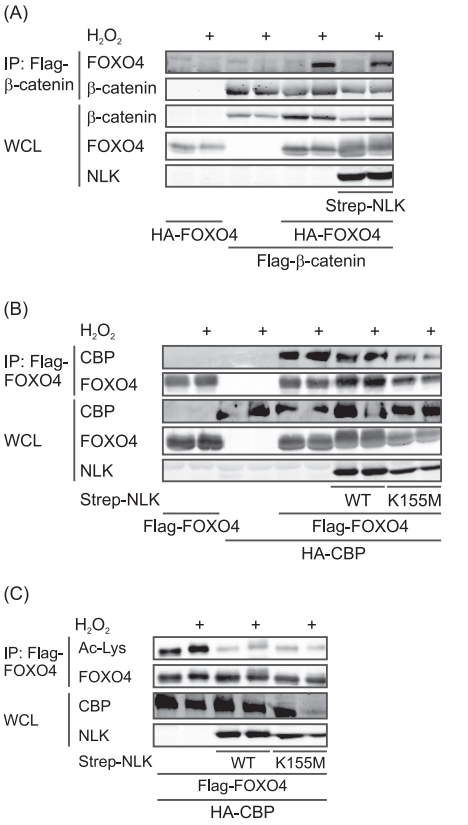


Figure 4. NLK does not regulate FOXO4 binding to β-catenin or CBP

(A) FOXO4 binds to β-catenin in the presence of NLK. HEK293T cells were transfected with the indicated constructs and treated with 200 μM H_2O_2 for 30 min as indicated. Flag-β-catenin was immunoprecipitated and binding of FOXO4 to β-catenin was analyzed by SDS-PAGE and immunoblotting. **(B)** FOXO4 binds to CBP in the presence of NLK. HEK293T cells were transfected with the indicated constructs and treated with 200 μM H_2O_2 for 30 min as indicated. Flag-FOXO4 was immunoprecipitated and binding of CBP to FOXO4 was analyzed by SDS-PAGE and immunoblotting. **(C)** Both WT-NLK and NLK^{K155M} reduce acetylation of FOXO4. To assess the acetylation of FOXO4, Flag-FOXO4 was immunopurified from HEK293T cells transfected with indicated constructs followed by SDS-PAGE and immunoblotting using *α*-Acetyl-Lysine antibody.

that CBP acetylates FOXO4 thereby reducing its activity [32]. NLK-mediated loss of CBP-binding to FOXO would therefore unlikely represent a mechanism for NLK-mediated FOXO inhibition. However, CBP binding to FOXO4 also results in recruitment of CBP to histones and CBP-mediated acetylation of histones is strongly associated with transcriptional activation [33]. Thus, in this way inhibition of the CBP-FOXO4 interaction would indirectly affect the ability of FOXO to stimulate transcription. Again, as shown in Figure 4B, co-expression of NLK did not affect binding of CBP to FOXO. Conversely, acetylation of FOXO4 was reduced by the presence of NLK but more importantly to the same extent by its kinase-dead mutant. Interestingly, we observed that both wild type NLK and NLK^{K155M} were acetylated by CBP (data not shown). This may explain the decrease in CBP-mediated acetylation of FOXO4 as this could result from competition between substrates. By taking into consideration that acetylation inhibits FOXO4 activity and that NLK-mediated inhibition of FOXO4 transactivation strongly depends on the kinase activity of NLK, it is unlikely that the decrease in acetylation of FOXO4 will account for NLK-mediated repression. Taken together, NLK does not interfere with binding of FOXO4 to β-catenin and CBP. Moreover, the NLK-induced decrease in CBP-directed acetylation of FOXO4 does not require its kinase activity and thereby most likely does not account for NLK-mediated inhibition of FOXO-induced $p27^{kip1}$ levels.

NLK regulates FOXO4 monoubiquitination.

In response to oxidative stress FOXO4 is rapidly monoubiquitinated and this results in upregulation of its transcriptional activity [27]. Therefore, we decided to next investigate whether the NLK-induced suppression of FOXO4 activity was linked with changes in the ubiquitination status of FOXO4. To address this possibility, we performed an *in vivo* ubiquitination assay of FOXO4 as described previously [27] in the presence or absence of NLK. Expression of wild type NLK but not its inactive mutant resulted in complete elimination of stress-induced FOXO4 monoubiquitination (Figure 5A and Supplementary Figure S3A). Moreover, NLK inhibited ubiquitination of FOXO4 in a concentration dependent manner (Figure 5B). This was not accompanied by a shift from mono- to enhanced poly-ubiquitination of FOXO4. In agreement with this, the protein half-life of FOXO4 was not affected by NLK co-expression (Figure 5C). In addition, NLK-dependent inhibition of monoubiquitination was specific for FOXO4 since we did not observe any effect on ubiquitination of $p27^{kip1}$ and USP7 (Figure 5D, Supplementary Figure S3B and data not shown). Thus, we conclude that NLK specifically inhibits monoubiquitination of FOXO4 following cellular oxidative stress and that this, in agreement with our previous studies, results in inhibition of FOXO4 transcriptional activity.

Phosphorylation of FOXO4 on S/T-P motifs is not required for NLK-mediated inhibition of FOXO4 monoubiquitination

Since we observed that NLK can phosphorylate FOXO4 *in vitro* and *in vivo*, we addressed the question whether NLK-dependent phosphorylation of FOXO4 is necessary for NLK-mediated inhibition of FOXO4 monoubiquitination and subsequent decrease in its transcriptional activity. Therefore, we first

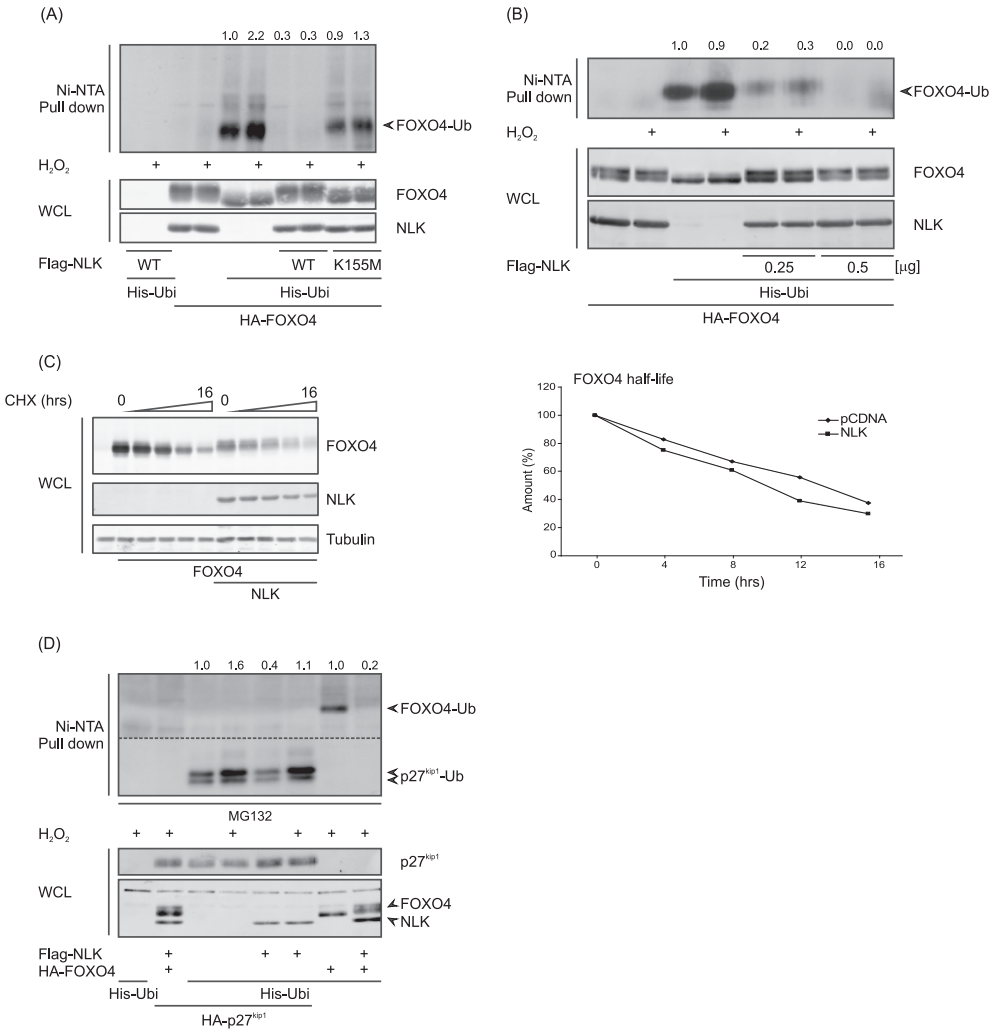


Figure 5. NLK inhibits monoubiquitination of FOXO4
(A, B) NLK negatively regulates ubiquitination of FOXO4. HEK293T cells were transfected with the indicated constructs and treated with 50 μM H_2O_2 for 15 min as indicated. Cell lysates were subjected to Ni-NTA pull-down to bind ubiquitinated proteins. Ubiquitinated FOXO4 was detected by SDS-PAGE and immunoblotting using α-FOXO4 antibody. **(C)** NLK does not affect protein stability of FOXO4. Analysis of the half-life of FOXO4. HEK293T cells were transfected with HA-FOXO4 alone or in combination with Flag-NLK. Transfected cells were treated with cycloheximide (CHX) for the indicated times. Using the Odyssey Infra-red imaging system, relative expression levels were calculated and displayed in a graph. **(D)** Monoubiquitination of $p27^{kip1}$ is not influenced by NLK. HEK293T cells were transfected with the indicated constructs and treated with 50 μM H_2O_2 for 15 min as indicated. Cell lysates were subjected to Ni-NTA pull down to bind ubiquitinated proteins. To detect ubiquitinated $p27^{kip1}$ and FOXO4, SDS-PAGE and immunoblotting was performed using α-$p27^{kip1}$ and α-FOXO4 antibodies. Additionally, in **(A, B, D)** relative levels of ubiquitinated FOXO4 and/or $p27^{kip1}$ for individual experiments are indicated. The levels of ubiquitinated proteins were quantified as in Figure 1 and normalized to their total expression levels. The levels of FOXO4 and $p27^{kip1}$ ubiquitinated in the absence of NLK and H_2O_2 treatment were set to 1.

sought to determine which residues in FOXO4 are specifically phosphorylated by NLK. For that reason we performed an *in vitro* kinase assay using bacterially expressed GST-FOXO4 and recombinant His-NLK. Subsequently, phosphorylated GST-FOXO4 was analyzed using liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) and we were able to identify twenty-eight different phosphorylation sites on FOXO4 (Figure 6A). Surprisingly, the identified phosphorylation sites did not adhere to a specific consensus sequence as suggested for NLK. NLK has been classified as a proline-directed MAP kinase, phosphorylating serine or threonine residues followed by proline (S/T-P motifs) [34]. Yet, although we observed six out of the eight possible S/T-P sites of FOXO4 to be phosphorylated we also observed S-Q (ATM consensus) and other types of phosphorylation sites. The reason for this apparent anomaly is at present unclear. LC-MS/MS analysis of Flag-FOXO4 extracted from cells after co-expression with NLK revealed phosphorylation of several SP/TP sites including Ser237 and 268 and Thr447 and 451. However, protein coverage in these experiments was not 100%, so additional phosphorylation sites may have been missed. Nevertheless, considering that NLK was originally classified as a MAP kinase, we therefore decided to narrow our *in vivo* analysis on investigating the role of the six S/T-P phosphorylated sites in the NLK-dependent shift in gel mobility of FOXO4. To this end, we substituted individual serine or threonine residues within S/T-P motifs of FOXO4 for alanine and we observed that substitution of Ser^{268} with Ala^{268} in FOXO4 resulted in significant loss of reduced mobility (data not shown). This indicated that *in vivo* at least Ser^{268} is phosphorylated by NLK. However, NLK expression could still inhibit ubiquitination of the Ser^{268} to Ala^{268} mutant of FOXO4 to the same extent as wild-type FOXO4 (data not shown). Additionally, simultaneous mutation of all eight serine and threonine residues to alanine also had no effect on the efficiency of phosphorylation of FOXO4 by NLK in an *in vitro* kinase assay even though this mutant of FOXO4 (FOXO4Δ8) did not exhibit any shift in gel mobility (Fig 6B, C). Consistent with the notion that NLK phosphorylated FOXO4Δ8 as efficiently as the wild-type FOXO4, mutation of these residues had no effect on NLK-mediated inhibition of ubiquitination of FOXO4 (Fig 6D and Supplementary Figure S4A). Moreover FOXO4Δ8 was still able to induce $p27^{kip1}$ and this induction was still reduced by ectopic overexpression of NLK (Fig 6E and Supplementary Figure S4B). Taken together, these data indicate that NLK-mediated phosphorylation of S/T-P in FOXO4 is unlikely to be required for inhibition of its monoubiquitination by NLK.

NLK-mediated inhibition of monoubiquitination occurs independently of Mdm2, USP7 or Pin1

To further understand at which level NLK regulates ubiquitination of FOXO4, we investigated whether NLK expression would affect the interaction between FOXO4 and its E3 ligase Mdm2 [35]. Ectopic expression of NLK did not change binding between Mdm2 and FOXO4 (Figure 7A). Moreover, NLK could still inhibit ubiquitination of FOXO4 in cells lacking Mdm2, suggesting that NLK does not block ubiquitination of FOXO4 through inhibition of Mdm2 activity (Figure 7B and Supplementary Figure S5A). To additionally support that NLK has no in-

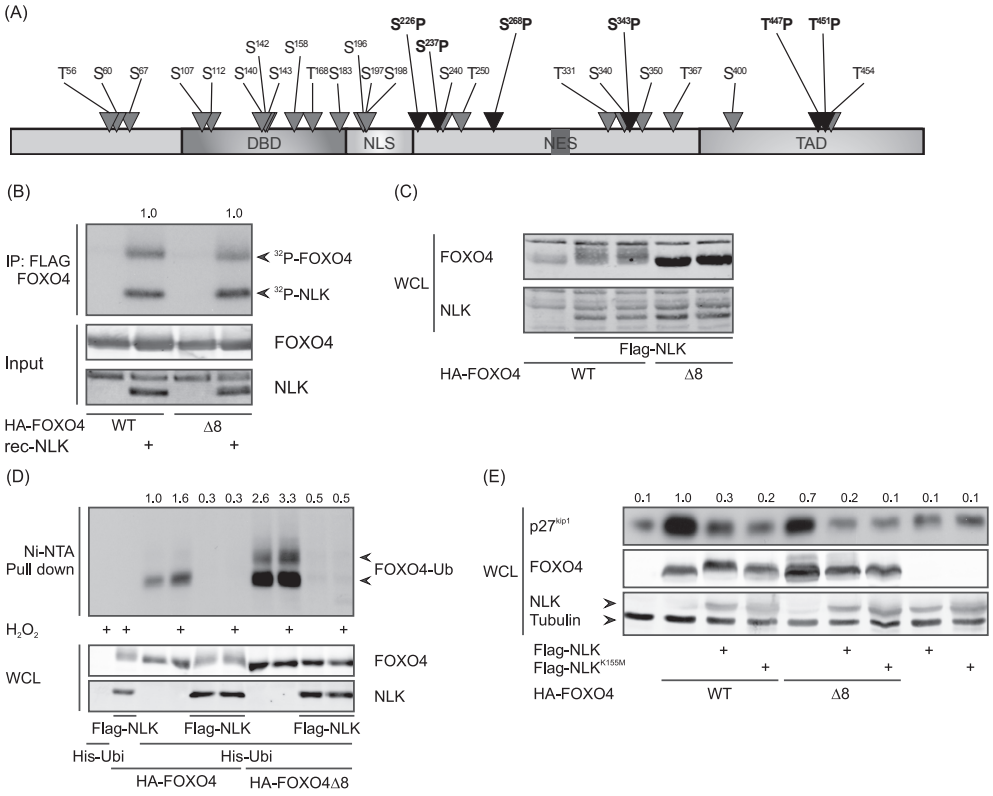


Figure 6. NLK-directed phosphorylation of FOXO4 on S/T-P motifs is not required for inhibition of its monoubiquitination

(A) NLK phosphorylates FOXO4 on multiple S and T residues in vitro. Identification of NLK-specific phosphosites in FOXO4. GST-FOXO4 was incubated with His_6-NLK in the presence of ATP and analyzed using LC-MS/MS. The identified S/T-P sites are indicated by black triangles. **(B)** Effect of S/T-P motifs mutations on NLK-mediated phosphorylation *in vitro*. Flag-FOXO4 WT or 8A mutant (Δ8) was expressed in HEK293T cells and immunoprecipitated. Aliquots of purified FOXO4 were incubated with or without NLK in presence of γ-P^{32}ATP. Phosphorylation was measured after 15 min. of incubation. Proteins levels were confirmed by SDS-PAGE and immunoblotting using the indicated antibodies. Additionally, relative levels of phosphorylated FOXO4 are indicated. Intensities of the individual bands were quantified as in Figure 2 and they were normalized to phosphorylation of wt-FOXO4 in presence of NLK set to 1. **(C)** Effect of S/T-P motifs mutations on the NLK-mediated shift in gel mobility of FOXO4. Total lysate of HEK293T cells expressing as indicated HA-FOXO4 WT and Δ8 and Flag-NLK were analyzed by SDS-PAGE and immunoblotting using the indicated antibodies. **(D)** NLK inhibits monoubiquitination of FOXO4Δ8. HEK293T cells were transfected with the indicated constructs and treated with 50 μM H_2O_2 for 15 min as indicated. Cell lysates were subjected to Ni-NTA pull down to bind ubiquitinated proteins. To detect ubiquitinated FOXO4 SDS-PAGE and immunoblotting was performed using α-FOXO4 antibody. Relative levels of ubiquitinated FOXO4 are indicated and quantified as in Figure 5. The level of ubiquitinated WT-FOXO4 in the absence of NLK and H_2O_2 treatment was set to 1. **(E)** NLK inhibits FOXO4Δ8-induced $p27^{kip1}$ expression. Total lysate of puromycin-selected HEK293T cells transfected with the indicated constructs were analyzed by SDS-PAGE and immunoblotting using the indicated antibodies. Relative levels of $p27^{kip1}$ normalized to tubulin levels are indicated. The $p27^{kip1}$ level in the presence of wt-FOXO4 was set to 1. DBD, DNA binding domain; NLS, nuclear localization signal; NES, nuclear export signal; TAD, transactivation domain.

fluence on Mdm2 activity, we also performed an *in vitro* ubiquitination assay for FOXO4. In this assay recombinant His-Mdm2 was able to ubiquitinate bacterially expressed GST-FOXO4 in the presence of recombinant E1 (UBE1), E2 (UbcH5b) and His-ubiquitin. However, as shown in Figure 7C, addition of recombinant NLK did not alter the ubiquitination pattern for FOXO4 under these conditions (lane 4, 5). Moreover, in order to enhance efficiency of NLK-directed phosphorylation and to enrich for the phosphorylated form of Mdm2 and FOXO4 (Mdm2-P, FOXO4-P) we pre-treated both His-Mdm2 and GST-FOXO4 with His-NLK before performing the ubiquitination assay. Nevertheless, we did not observe any significant changes in FOXO4-ubiquitination in the situation where both Mdm2 and FOXO4 were pre-phosphorylated (lane 9-12).

USP7 is a deubiquitinating enzyme (DUB) for FOXO4 [27] and therefore we considered the possibility that NLK enhanced deubiquitination of FOXO4 by promoting USP7 activity towards FOXO4. Using a co-immunoprecipitation assay we did not observe any effect of NLK on the interaction between USP7 and FOXO4 (Figure 7D). Consequently, NLK was still able to inhibit ubiquitination of FOXO4 after depletion of USP7 by siRNA, suggesting that NLK changes the ubiquitination status of FOXO4 regardless of USP7 (Figure 7E and Supplementary Figure S5B).

Finally, previously we have shown Pin1 to also act as a negative regulator of monoubiquitination of FOXO4 [28], suggesting the possibility that Pin1 and NLK act together in this process. Nevertheless, as shown in Figure 7F and Supplementary Figure S5C, NLK was still able to completely abolish ubiquitination of FOXO4 in cells lacking Pin1 indicating that Pin1 is not necessary for NLK-mediated inhibition.

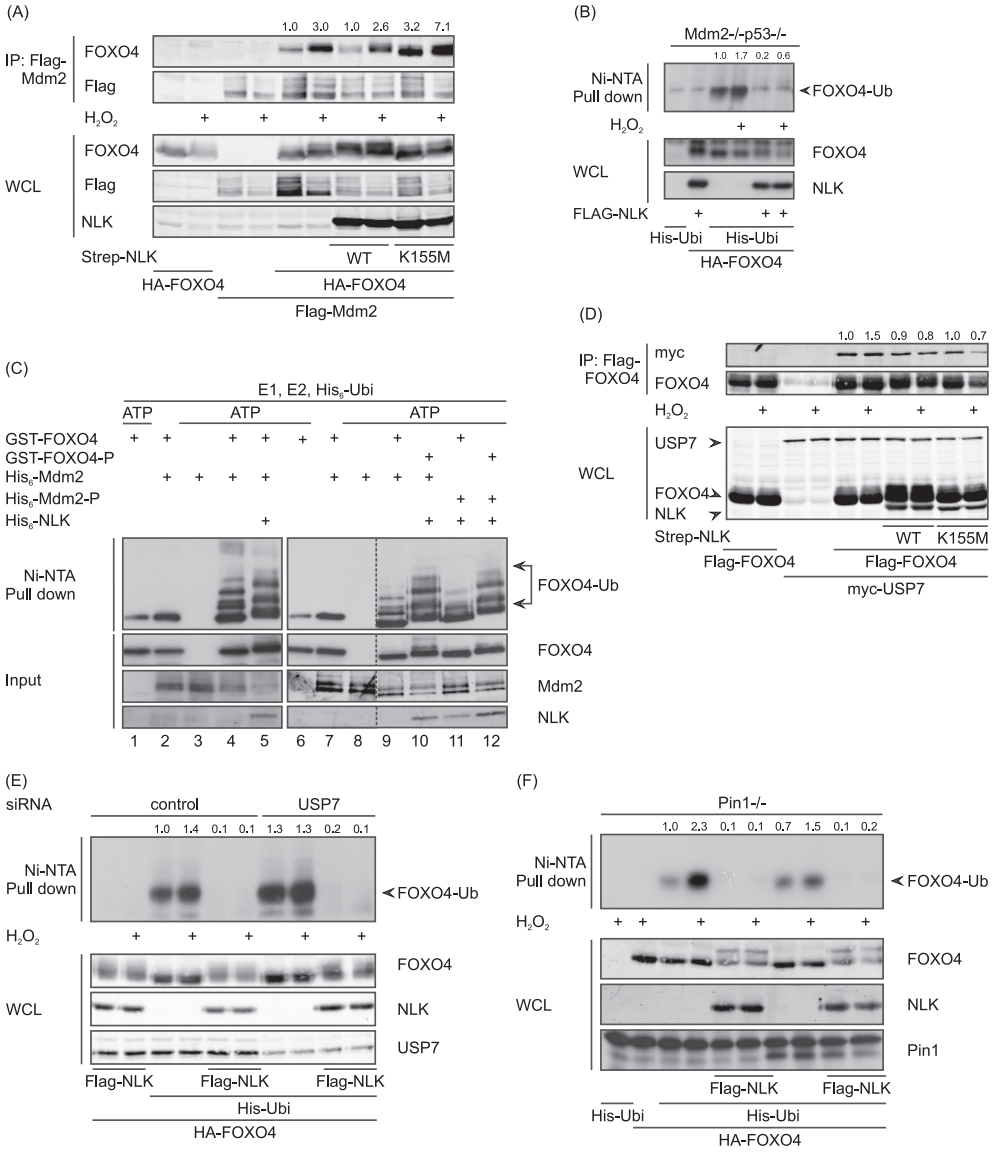
Thus, we conclude that negative regulation of monoubiquitination of FOXO4 by NLK occurs independently of Mdm2, USP7 and Pin1 activity.

Both NLK and p38 exhibit similar effects on monoubiquitination of FOXO4 as well as its transcriptional activity

It has been previously demonstrated that MAP3K TAK1 can activate NLK in response to Wnt signaling [19, 20, 22, 23, 26]. Therefore, we addressed the question whether TAK1 could also act as an upstream activator of NLK in case of FOXO4 repression. We performed an *in vivo* ubiquitination assay of FOXO4 and we observed that ectopic expression of TAK1 but not its kinase dead mutant ($TAK1^{D175A}$) led to the increase in ubiquitination of FOXO4 (Figure 8A and Supplementary Figure S6A). Hence, TAK1 is unlikely to be an upstream kinase of NLK in regard to the inhibition of FOXO4.

Given that it has been published recently that MAP kinase p38 serves as an activator of NLK we investigated the effect of p38 on ubiquitination of FOXO4 [36]. Similarly to NLK, p38 also exhibited a negative effect on ubiquitination of FOXO4 (Figure 8B and Supplementary Figure S6B). Moreover, ectopic expression of p38 suppressed the FOXO4 induced $p27^{kip}$ protein levels (Figure 8C andSupplementary Figure S6C). Taken together, these findings point towards p38 as a likely upstream activator of NLK that could potentiate its negative activity against FOXO4.

However, siRNA-mediated knockdown of NLK did not significantly change the ability of p38 expression to reduce FOXO4 mono-ubiquitination (data not shown). In addition we



observed direct phosphorylation of FOXO4 by p38 at least *in vitro* (BMTB unpublished observations) and thus p38 may regulate FOXO4 through NLK-dependent and –independent pathways (see summarizing Figure 8D).

Discussion

Here we provide evidence for NLK as a novel regulator of FOXO4 that negatively influences its transcriptional activity. In response to increased cellular oxidative stress complex formation between NLK and FOXO4 is strongly enhanced and this results *in vivo* in phosphorylation of FOXO4 on at least one of the eight S/T-P motifs i.e. Ser[268]. Although the interaction between NLK and

Figure 7. NLK-dependent inhibition of FOXO4 monoubiquitination does not require activity of Mdm2, USP7 and Pin1

(A) FOXO4 binds to Mdm2 in the presence of NLK. HEK293T cells were transfected with the indicated constructs and treated with 200 μM H_2O_2 for 30 min as indicated. Flag-Mdm2 was immunoprecipitated and binding of FOXO4 to Mdm2 was analyzed by SDS-PAGE and immunoblotting using α-FOXO4 antibody. **(B)** FOXO4 ubiquitination assay in Mdm2$^{-/-}$p53$^{-/-}$ MEFs. MEFs were transfected with the indicated constructs and treated with 50 μM H_2O_2 for 15 min as indicated. Cell lysates were subjected to Ni-NTA pull down to bind ubiquitinated proteins. Ubiquitinated FOXO4 was detected by SDS-PAGE and immunoblotting using α-FOXO4 antibody. **(C)** *In vitro* FOXO4 ubiquitination assay. Recombinant GST-FOXO4, His_6-Mdm2, E1, E2 and His_6-ubiquitin were incubated for 2 hours after adding ATP. To detect ubiquitinated GST-FOXO4, samples were subjected to Ni-NTA pull down followed by SDS-PAGE and immunoblotting using α-FOXO4 antibody. When indicated recombinant His_6-NLK was added to the reaction or GST-FOXO4 or His_6-Mdm2 were treated with His_6-NLK for 30 min prior to the addition of E1, E2 and ubiquitin. The levels of recombinant proteins were verified by SDS-PAGE and immunoblotting using the indicated antibodies. Separate lanes of SDS-PAGE are numbered 1-12 for reference purposes (see text results section). **(D)** FOXO4 binds to USP7 in the presence of NLK. Experiments were performed essentially as in Figure 7A using the indicated constructs. **(E)** Effect of USP7 knockdown on NLK-mediated inhibition of ubiquitination of FOXO4. HEK293T cells were transfected with the indicated constructs and treated with 50 μM H_2O_2 for 15 min as indicated. Cell lysates were subjected to Ni-NTA pull down to bind ubiquitinated proteins. Ubiquitinated FOXO4 was detected by SDS-PAGE and immunoblotting using α-FOXO4 antibody. **(F)** FOXO4 ubiquitination assay in Pin1$^{-/-}$ MEFs. Experiments were performed essentially as in Figure 7B using the indicated constructs but now using Pin1$^{-/-}$ MEFS.

Additionally, in **(A, D)** relative amounts of USP7 or Mdm2 bound to FOXO4 for individual experiments are indicated and quantified as in Figure 1. The amounts of USP7 or Mdm2 bound to FOXO4 in the absence of NLK and H_2O_2 treatment were set to 1. In **(B, E, F)** relative levels of ubiquitinated FOXO4 for individual experiments are indicated and quantified as in Figure 5. The levels of ubiquitinated FOXO4 in the absence of NLK and H_2O_2 treatment were set to 1.

FOXO4 is enhanced following increased ROS, we were unable to show that an increment in the ROS level directly increases the activity of NLK as measured by an *in vitro* kinase assay. In addition to the above, we were unable to show significant changes in the *in vitro* kinase activity of NLK after treatment of NLK-expressing HEK293T cells with Wnt5a or co-expression of TAK1. In agreement, whereas NLK inhibits FOXO4 monoubiquitination we observe TAK1 expression to enhance FOXO4 monoubiquitination in concordance with TAK1 signaling towards JNK [19] and JNK regulating FOXO4 monoubiquitination (manuscript in preparation). Taken together, this suggests the possibility that under the experimental conditions employed here NLK is actually a constitutively active kinase and its potential to specifically regulate FOXO4 activity therefore depends on the ability of NLK to co-localize with its substrate, which is in this case FOXO4. A similar mechanism has been described for some other kinases. For example, the activation of PKB by constitutively active PDK1 is regulated through the growth factor-induced Phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P3) production resulting in simultaneous recruitment of PKB and PDK1 to the plasma membrane. This induced co-localization of PKB and PDK1 then enforces a PtdIns(3,4,5)P_3-dependent conformational change of PKB to allow PDK1 to phosphorylate the critical threonine 308 within the T-loop of PKB [37].

Taking into consideration that NLK is mainly localized in the nucleus [34], it is likely that in this case FOXO4 translocates upon peroxide treatment in order to co-localize with NLK. This is consis-

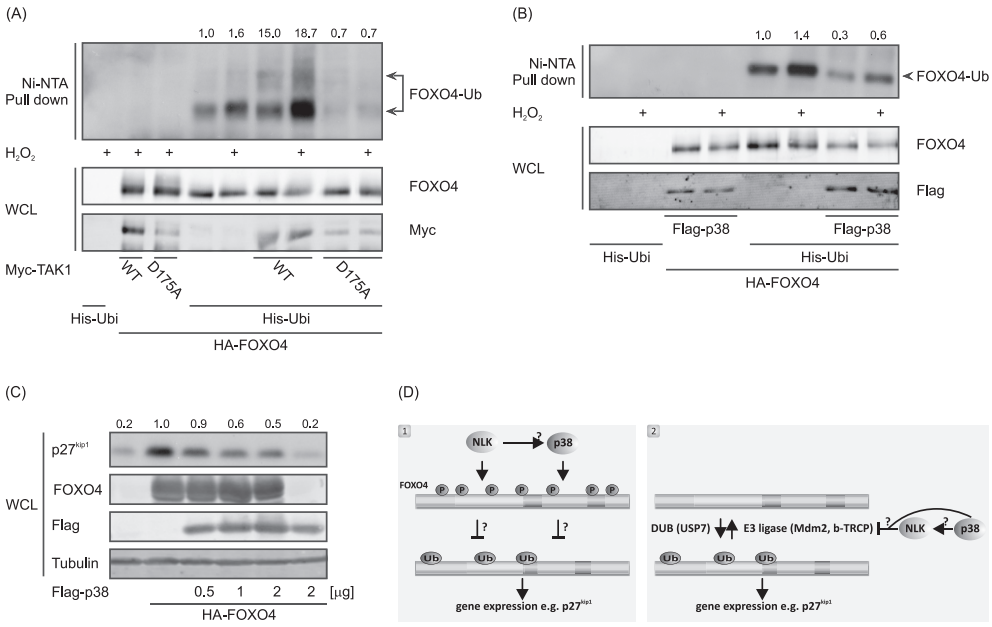


Figure 8. TAK1 enhances ubiquitination of FOXO4 whereas p38MAPK decreases ubiquitination of FOXO4 and FOXO4-induced $p27^{kip1}$ protein levels

(A) TAK1 enhances ubiquitination of FOXO4. HEK293T cells were transfected with HA-FOXO4 and wild type TAK1 or kinase dead TAK1 ($TAK1^{D175A}$). Cells were treated with 50 μM H_2O_2 for 15 min as indicated. Cell lysates were subjected to Ni-NTA pull down to bind ubiquitinated proteins. Ubiquitinated FOXO4 was detected by SDS-PAGE and immunoblotting using α-FOXO4 antibody. **(B)** p38MAPK decreases ubiquitination of FOXO4. Experiments were performed essentially as in Figure 8A using the indicated constructs. In **(A, B)** relative amounts of ubiquitinated FOXO4 for individual experiments are indicated and quantified as in Figure 5. The levels of ubiquitinated FOXO4 in the absence of TAK1 or p38MAPK and H_2O_2 treatment were set to 1. **(C)** Exogenous expression of p38MAPK reduces $p27^{kip1}$ protein levels. Western blot analysis of $p27^{kip1}$ expression in puromycin-selected HEK293T cells expressing HA-FOXO4 and Flag-p38 as indicated. Relative levels of $p27^{kip1}$ are indicated and quantified as in Figure 3. The $p27^{kip1}$ level in the presence of WT-FOXO4 was set to 1. **(D)** Here we show that NLK could interact with FOXO4 in a ROS-dependent manner. In addition, NLK can phosphorylate FOXO4 on multiple residues. NLK inhibits transcriptional activity and monoubiquitination of FOXO4; however, this appears not to be linked to its ability to directly phosphorylate FOXO4 (panel 1). Nevertheless, since NLK-mediated inhibition of FOXO4 ubiquitination and its activity depends on NLK kinase activity, we hypothesize that NLK could act on the ubiquitination machinery (e.g., E3 ligase) (panel 2). This is indicated by our result that NLK inhibits residual monoubiquitination in $Mdm2^{-/-}$ cells and Mdm2-reconstituted cells. The stress kinase p38, a potential upstream regulator of NLK, also inhibits FOXO4 monoubiquitination and directly phosphorylates FOXO4 in vitro (not shown). p38MAPK may therefore regulate monoubiquitination in an NLK-dependent and NLK-independent manner (panels 1 and 2).

tent with previous reports that elevated ROS leads to nuclear translocation of FOXO4 [9]. Moreover, the observation that in an *in vitro* kinase assay the presence of FOXO4 actually appears to increase NLK activity (Figure 2C) suggests the possibility that the actual increase in NLK activity is prompted by its direct binding to the substrate.

Oxidative stress regulates the tran-

scriptional activity of FOXOs in a positive manner [38]. This leads to expression of genes, which contribute to detoxification of cellular ROS and repair of DNA damage [39-42]. Furthermore, transactivation of FOXOs induces cell cycle arrest that provides time for scavenging of ROS and repairing of DNA damage to take place [29, 43-45]. In apparent contradiction, elevation in cellular ROS level simultaneously leads to inhibition of FOXO4 by NLK. However, it is important to note that the cellular response to ROS is tightly linked to the cellular concentration of ROS. Thus, whereas ROS is often studied in the context of a damaging signal eventually leading to cell death, ROS also performs a role in normal non-pathological signaling. Hence, it is conceivable that when the level of oxidative stress within the cell exceeds a certain threshold it becomes important to downregulate in this case the FOXO4 dependent anti-oxidant defense program in order to allow apoptosis. It has been reported previously that NLK induces apoptosis in DLD1 cells [46]. In addition, we observed that ectopic expression of NLK in U2OS cells suppressed the growth of these cells and induced apoptosis in a kinase-dependent manner (data not shown). Hence, the NLK-mediated transrepression of FOXO4 activity in response to elevated ROS might be important for overcoming the FOXO4-induced cell cycle arrest in order to trigger apoptosis. In agreement with this hypothesis, ectopic expression of NLK causes downregulation of FOXO4-induced $p27^{kip1}$ levels. This inhibition partially depends on kinase activity as much weaker inhibition is observed after overexpression of kinase-dead form of NLK.

In contrast, downregulation of NLK protein levels increases $p27^{kip1}$ expression. Our previous study has shown that monoubiquitination of FOXO4 increases its nuclear localization and consequently enhances its transcriptional activity [27]. Here we observe that ubiquitination of FOXO4 is abolished by NLK, which might provide a mechanism for the inhibition of FOXO4 by NLK. Considering that inhibition of both FOXO4 monoubiquitination and FOXO4-triggered expression of $p27^{kip1}$ requires kinase activity of NLK it became interesting to determine the involvement of NLK-dependent phosphorylation of FOXO4 in the inhibition of its activity. NLK belongs to the MAP kinase family and has been reported previously to phosphorylate a broad spectrum of transcription factors on multiple Ser or Thr residues lying within the S/T-P motif. For example, NLK-mediated repression of Notch1 requires phosphorylation of at least 7 residues in Notch1. Similarly, in case of c-Myb, NLK has been reported to phosphorylate 15 different residues and substitutions of all of them with Ala residues were necessary to reverse NLK-mediated degradation of c-Myb.

Despite the fact that FOXO4 contains only eight S/T-P motifs, we were unable to unequivocally define which residue(s) is targeted by NLK phosphorylation. Even mutation of all S/T-P sites failed to abrogate NLK-dependent repression of FOXO4 transcriptional activity, which may indicate that phosphorylation of FOXO4 is not essential for its inhibition. However, using an *in vitro* kinase assay followed by LC-MS/MS analysis we identified twenty-eight residues in FOXO4 being phosphorylated by NLK. This, together with previous studies, suggests that the extent of NLK-mediated phosphorylation rather than site-specific phosphorylation might be crucial for NLK to exert its negative ef-

fects on substrate activity or stability.

It has been shown for other transcription factors, including Ets-1 that decrease in its activity strongly correlates with the number of sites phosphorylated within its serine-rich region [47]. Therefore, it is possible that in order to efficiently repress FOXO4 activity NLK needs to target other residues in addition to the S/T-P motifs to achieve the required degree of phosphorylation. However, additional studies are needed to determine the relevance of this possibility with respect to FOXO4 regulation. Also it can be questioned how relevant the analysis of a mutant of any protein harboring such a number of mutations is in comparison to the function of wild-type protein.

To further clarify the molecular mechanism leading to NLK-mediated inhibition of monoubiquitination of FOXO4, we hypothesized that NLK could influence the activity of enzymes being directly involved in ubiquitination/deubiquitination of FOXO4. Previously, we have been able to provide evidence that Mdm2 acts as an E3 ligase for FOXO4 [35] which is counteracted by USP7-mediated deubiquitination [27]. However, based on our results obtained in $Mdm2^{-/-}p53^{-/-}$ cells as well as in an *in vitro* ubiquitination assay it seems unlikely that NLK decreases FOXO4 ubiquitination by repression of Mdm2 activity. In addition, also USP7 appears not required for NLK-mediated regulation of ubiquitination of FOXO4. Finally, even though the peptidyl isomerase Pin1 attenuates ubiquitination of FOXO4, loss of Pin1 does not eliminate the inhibitory effect of NLK.

Despite these essentially negative results with respect to how NLK may regulate ubiquitination of FOXO4, we cannot exclude the involvement of other E3 ligases or deubiquitinating enzymes (DUBs) necessary for NLK to negatively modulate ubiquitination of FOXO4. For instance, it has been shown previously that like FOXO4, p53 is also a substrate for USP7, yet other DUBs e.g. USP10 have been identified to also deubiquitinate p53 and contribute to its activation [48]. Therefore, it would be interesting to address the question whether USP10 can also target ubiquitinated FOXO4.

In mammals the FOXO family consists of four members, FOXO1, FOXO3a, FOXO4 and FOXO6 [1, 3]. We show here that similarly to FOXO4, NLK can also interact with FOXO1 and negatively modulate its transcriptional activity, suggesting that NLK acts as general regulator of all FOXOs. Moreover, while this manuscript was under preparation, it was reported that the TAK1-NLK pathway inhibits activity of FOXO1 via triggering FOXO1 translocation out of the nucleus [49]. Although the mechanism for this was not determined it is clear that these observations are complementary to what would be expected for inhibition of monoubiquitination. However, in contrast to FOXO4, mutation of eight S/T-P sites present in FOXO1 alleviated the effect of NLK. A rationale for this different behavior of mutant FOXO4 versus mutant FOXO1 is unclear at present. Nevertheless, it is apparent that NLK antagonizes FOXOs transcriptional activity. Therefore, it would be interesting to determine whether NLK-mediated regulation of FOXOs activity is conserved throughout evolution in a manner similar to the regulation by PKB and JNK [10, 11].

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References

1. Katoh, M., Human FOX gene family (Review). Int J Oncol, 2004. 25(5): p. 1495-500.
2. Jacobs, F.M., et al., FoxO6, a novel member of the FoxO class of transcription factors with distinct shuttling dynamics. J Biol Chem, 2003. 278(38): p. 35959-67.
3. Burgering, B.M., A brief introduction to FOXOlogy. Oncogene, 2008. 27(16): p. 2258-62.
4. Greer, E.L. and A. Brunet, FOXO transcription factors at the interface between longevity and tumor suppression. Oncogene, 2005. 24(50): p. 7410-25.
5. Brownawell, A.M., et al., Inhibition of nuclear import by protein kinase B (Akt) regulates the subcellular distribution and activity of the forkhead transcription factor AFX. Mol Cell Biol, 2001. 21(10): p. 3534-46.
6. Brunet, A., et al., Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell, 1999. 96(6): p. 857-68.
7. Kops, G.J., et al., Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature, 1999. 398(6728): p. 630-4.
8. De Ruiter, N.D., B.M. Burgering, and J.L. Bos, Regulation of the Forkhead transcription factor AFX by Ral-dependent phosphorylation of threonines 447 and 451. Mol Cell Biol, 2001. 21(23): p. 8225-35.
9. Essers, M.A., et al., FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. EMBO J, 2004. 23(24): p. 4802-12.
10. Oh, S.W., et al., JNK regulates lifespan in Caenorhabditis elegans by modulating nuclear translocation of forkhead transcription factor/DAF-16. Proc Natl Acad Sci U S A, 2005. 102(12): p. 4494-9.
11. Kenyon, C., et al., A C. elegans mutant that lives twice as long as wild type. Nature, 1993. 366(6454): p. 461-4.
12. Calnan, D.R. and A. Brunet, The FoxO code. Oncogene, 2008. 27(16): p. 2276-88.
13. van der Horst, A. and B.M. Burgering, Stressing the role of FoxO proteins in lifespan and disease. Nat Rev Mol Cell Biol, 2007. 8(6): p. 440-50.
14. Essers, M.A., et al., Functional interaction between beta-catenin and FOXO in oxidative stress signaling. Science, 2005. 308(5725): p. 1181-4.
15. Hoogeboom, D., et al., Interaction of FOXO with beta-catenin inhibits beta-catenin/T cell factor activity. J Biol Chem, 2008. 283(14): p. 9224-30.
16. Behrens, J., et al., Functional interaction of beta-catenin with the transcription factor LEF-1. Nature, 1996. 382(6592): p. 638-42.
17. Eastman, Q. and R. Grosschedl, Regulation of LEF-1/TCF transcription factors by Wnt and other signals. Curr Opin Cell Biol, 1999. 11(2): p. 233-40.
18. Roose, J. and H. Clevers, TCF transcription factors: molecular switches in carcinogenesis. Biochim Biophys Acta, 1999. 1424(2-3): p. M23-37.
19. Ishitani, T., et al., The TAK1-NLK-MAPK-related pathway antagonizes signaling between beta-catenin and transcription factor TCF. Nature, 1999. 399(6738): p. 798-802.
20. Ishitani, T., J. Ninomiya-Tsuji, and K. Matsumoto, Regulation of lymphoid enhancer factor 1/T-cell factor by mitogen-activated protein kinase-related Nemo-like kinase-dependent phosphorylation in Wnt/beta-catenin signaling. Mol Cell Biol, 2003. 23(4): p. 1379-89.
21. Yamada, M., et al., NARF, an nemo-like kinase (NLK)-associated ring finger protein regulates the ubiquitylation and degradation of T cell factor/lymphoid enhancer factor (TCF/LEF). J Biol Chem, 2006. 281(30): p. 20749-60.
22. Kanei-Ishii, C., et al., Wnt-1 signal induces phosphorylation and degradation of c-Myb protein via TAK1, HIPK2, and NLK. Genes Dev, 2004. 18(7): p. 816-29.
23. Smit, L., et al., Wnt activates the Tak1/Nemo-like kinase pathway. J Biol Chem, 2004. 279(17): p. 17232-40.
24. Takada, I., et al., A histone lysine methyltransferase activated by non-canonical Wnt signaling suppresses PPAR-gamma transactivation. Nat Cell Biol, 2007. 9(11): p. 1273-85.
25. Ishitani, T., et al., Nemo-like kinase suppresses Notch signaling by interfering with formation of the Notch active transcriptional complex. Nat Cell Biol. 12(3): p. 278-85.
26. Kurahashi, T., et al., The Wnt-NLK signaling pathway inhibits A-Myb activity by inhibiting the association with coactivator CBP and methylating histone H3. Mol Biol Cell, 2005. 16(10): p. 4705-13.
27. van der Horst, A., et al., FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP. Nat Cell Biol, 2006. 8(10): p. 1064-73.
28. Brenkman, A.B., et al., The peptidyl-isomerase Pin1 regulates p27kip1 expression through inhibition of Forkhead box O tumor suppressors. Cancer Res, 2008. 68(18): p. 7597-605.
29. Dijkers, P.F., et al., Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). Mol Cell Biol, 2000. 20(24): p. 9138-48.
30. Kwon, T.K., et al., Characterization of the murine cyclin-dependent kinase inhibitor gene p27Kip1. Gene, 1996. 180(1-2): p. 113-20.
31. Furuyama, T., et al., Identification of the dif-

ferential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. Biochem J, 2000. 349(Pt 2): p. 629-34.
32. van der Horst, A., et al., FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2(SIRT1). J Biol Chem, 2004. 279(28): p. 28873-9.
33. van der Heide, L.P. and M.P. Smidt, Regulation of FoxO activity by CBP/p300-mediated acetylation. Trends Biochem Sci, 2005. 30(2): p. 81-6.
34. Brott, B.K., B.A. Pinsky, and R.L. Erikson, Nlk is a murine protein kinase related to Erk/MAP kinases and localized in the nucleus. Proc Natl Acad Sci U S A, 1998. 95(3): p. 963-8.
35. Brenkman, A.B., et al., Mdm2 induces mono-ubiquitination of FOXO4. PLoS One, 2008. 3(7): p. e2819.
36. Ohnishi, E., et al., Nemo-like kinase, an essential effector of anterior formation, functions downstream of p38 mitogen-activated protein kinase. Mol Cell Biol. 30(3): p. 675-83.
37. Pearce, L.R., D. Komander, and D.R. Alessi, The nuts and bolts of AGC protein kinases. Nat Rev Mol Cell Biol. 11(1): p. 9-22.
38. Furukawa-Hibi, Y., et al., FOXO transcription factors in cell-cycle regulation and the response to oxidative stress. Antioxid Redox Signal, 2005. 7(5-6): p. 752-60.
39. Kops, G.J., et al., Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature, 2002. 419(6904): p. 316-21.
40. Tothova, Z., et al., FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell, 2007. 128(2): p. 325-39.
41. Tran, H., et al., DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. Science, 2002. 296(5567): p. 530-4.
42. Honda, Y. and S. Honda, The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans. FASEB J, 1999. 13(11): p. 1385-93.
43. Fernandez de Mattos, S., et al., FoxO3a and BCR-ABL regulate cyclin D2 transcription through a STAT5/BCL6-dependent mechanism. Mol Cell Biol, 2004. 24(22): p. 10058-71.
44. Medema, R.H., et al., AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature, 2000. 404(6779): p. 782-7.
45. Schmidt, M., et al., Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. Mol Cell Biol, 2002. 22(22): p. 7842-52.
46. Yasuda, J., et al., Nemo-like kinase induces apoptosis in DLD-1 human colon cancer cells. Biochem Biophys Res Commun, 2003. 308(2): p. 227-33.
47. Pufall, M.A., et al., Variable control of Ets-1 DNA binding by multiple phosphates in an unstructured region. Science, 2005. 309(5731): p. 142-5.
48. Yuan, J., et al., USP10 regulates p53 localization and stability by deubiquitinating p53. Cell. 140(3): p. 384-96.
49. Kim, S., et al., Regulation of FOXO1 by TAK1-Nemo-like kinase pathway. J Biol Chem. 285(11): p. 8122-9.

Chapter 3

MYBBP1a is a novel modulator of FOXO4-dependent transcription

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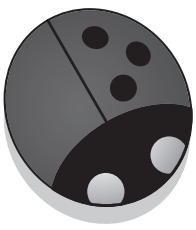

MYBBP1a is a novel modulator of FOXO4-dependent transcription

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Abstract

Acetylation of lysine residues within histones strongly correlates with regions of active transcription. Therefore, proteins possessing intrinsic histone acetyltransferase (HAT) and deacetylase (HDAC) activities are often functioning as regulators of transcription. Intriguingly, recent studies revealed that certain HATs, in addition to histones, acetylate specific transcription factors. As this might alter the activities of those factors in both a positive and negative manner, it is evident now that HATs not always act to enhance transcription.

The Forkhead Box O (FOXO) transcription factors have been described previously to be regulated by reversible acetylation, which is triggered by calcium response element-binding (CREB)-binding protein (CBP) and/or p300. However, the outcome of this regulation is rather complex, as it has been shown to result in the activation, modulation and repression of FOXO activity. Thus, CBP/p300 can act both as co-activators and co-repressors of FOXO functions. Here, we identify Myb binding protein 1a (MYBBP1a) as a novel interaction partner of FOXO4. Furthermore, we reveal that MYBBP1a can suppress acetylation of FOXO4 and thereby, similarly to CBP/p300, promote or impede the activity of FOXO4. Interestingly, MYBBP1a also appears to increase monoubiquitination of FOXO4, which previously has been associated with enhanced activation of FOXO4. Taken together, this suggests that MYBBP1a modulates FOXO4 functions by simultaneous alteration of multiple posttranslational modifications (PTMs) of FOXO4.

Introduction

Regulation of transcription by transcription factors primarily occurs by transcription factors acting not only as a sequence specific recruitment factor for RNA polymerase but also for chromatin modifiers. These modifiers have diverse enzymatic function and include HATs that are able to transfer an acetyl group onto the lysine residue within the N-terminal tail of histone proteins [1]. Several proteins that possess an intrinsic histone acetyltransferase activity have been identified, including p300 and CBP. Acetyl moieties can also be removed by deacetylases and reversible acetylation of histones is predominantly associated with gene activation, as it weakens the nucleosomal structure and thereby increases the accessibility of DNA by transcription factors [2]. Thus, HATs are recognized as coactivators of transcription and they are recruited to sites of transcription by DNA-bound transcription factors.

Interestingly, it has been demonstrated that HATs can also acetylate several nonhistone proteins including transcription factors themselves and thereby modulate their functional properties. Such a relationship has been revealed for FOXOs and CBP, and its homologue p300, which have been demonstrated to reversibly acetylate FOXO proteins [3-6].

The mammalian family of FOXO transcription factors consists of four members FOXO1, FOXO3a, FOXO4 and the recently identified, more distantly related FOXO6. FOXO proteins play an important role in a wide range of organismal functions including longevity, tumor suppression, metabolism and differentiation. In addition, at the cellular level FOXOs are involved in promotion of stress resistance, cell cycle arrest and apoptosis.

The activity of FOXO1, FOXO3a and FOXO4 is regulated by a complex combination of various PTMs, including phosphorylation, acetylation and ubiquitination. Interestingly, CBP/p300-dependent acetylation of FOXO transcription factors appears to modulate their activity in multiple ways [7-9].

First, several lysine residues within FOXOs have been identified as acetylated by various methods ([4-6, 10, 11] and unpublished data A.B. Brenkman). Importantly, this reveals that acetylation occurs within the DNA binding domain ([12-14] and unpublished data A.B. Brenkman). Therefore, acetylation impairs the ability of FOXOs to interact with DNA [15]. Consequently, one function of FOXO acetylation is to negatively regulate FOXO-dependent transactivation of their target genes by reducing DNA binding. However, other lysine residues outside the DNA binding domain are also acetylated. These include lysine residues within the NLS sequence, as well as within the transactivation domain. The effect of acetylation within the NLS is unknown, but since its function depends on lysine residues, it is likely that acetylation of NLS impairs nuclear entry of FOXO4. Acetylation within transactivation domain on the other hand opens the possibility to determine the FOXO transcriptional program. Indeed, it has also been shown that p300 enhances FOXO4-induced apoptosis, whereas deacetylation of FOXO3a promotes its ability to induce cell cycle arrest and resistance to oxidative stress [7, 16]. Thus, it is plausible that acetylation/deacetylation of FOXO proteins determines their specificity towards a certain subset of target genes. Therefore, one way to regulate transactivational properties of FOXO proteins is by altering their acetylation state and thereby shifting them from one set of target genes to another or alternatively, by prolonging or shortening the time during which FOXOs actively induce transcription. It is clear that detailed knowledge on when and how the various lysine residues of FOXO are modified is necessary to understand this differential regulation.

Several studies revealed that FOXOs physically interact with CBP/p300 and that this interaction is increased in response to elevated cellular levels of reactive oxygen species (ROS). Moreover, the binding between FOXO4 and p300 depends on the formation of ROS-induced intermolecular disulphide bonds between these proteins. Therefore, acetylation of a FOXO4 mutant that lacks all cysteine residues and thus is unable to form disulphide bonds is greatly reduced. Gene profiling by comparing the activity of wild-type FOXO4 versus FOXO4ΔCys indeed revealed an apparent partial shift in gene targeting [16].

However, FOXO1 has been reported

to recruit CBP/p300 to the promoter of several genes including the insulin-like growth factor binding protein 1 (IGFBP-1) gene and Agouti-related protein (AgRP) gene, and by doing so FOXO1 might promote acetylation of histones and thereby facilitate transcription. Therefore, it is suggested now that CBP/p300 has a dual role in the regulation of the activity of FOXO transcription factors. This is supported by several studies demonstrating that CBP/p300 is actually essential for FOXO-dependent transcription. This raises the possibility that the primary outcome of the interaction between FOXO and CBP/p300 is acetylation of histones and therefore stimulation of FOXO-dependent transactivation whereas acetylation of FOXOs themselves takes place at a later time point and would account for termination of transcription.

To complicate our understanding even further, CBP/p300-dependent acetylation of proteins and histones is also indirectly regulated by other PTMs, modulation of auto-acetylation of CBP/p300 and possible steric interference by other proteins interacting with FOXO. Recently, it has been shown that Myb-binding protein 1a (MYBBP1a) inhibits the activity of the Nuclear factor κB (NF-κB) transcription factor by competing p300 from binding to NF-κB. MYBBP1a, also referred to as $p160^{MYB}$, was originally identified as an interacting protein of the c-Myb transcription factor, which is critical for hematopoietic cell proliferation and differentiation. Subsequently, it has been shown that a shorter version of MYBBP1a, $p67^{MYB}$ inhibits c-Myb-dependents transcription.

MYBBP1a is predominantly localized in the nucleolus; however its precise function there remains unclear. Furthermore, MYBBP1a has been shown to be proteolytically processed in response to ribosomal stress, which results in removal of the C-terminal part of MYBBP1a and generation of two shorter versions, $p140^{MYB}$ and $p67^{MYB}$. Interestingly, the C-terminus of MYBBP1a contains seven short basic amino acid repeat sequences, which are responsible for both nuclear and nucleolar localization of MYBBP1a. Thus, MYBBP1a also translocates from the nucleolus to the nucleoplasm under conditions that inhibit ribosome biogenesis. In addition, endogenous $p67^{MYB}$ is found in myeloid cells; however, it is not known what triggers the proteolytically cleavage of MYBBP1a in these cells.

Besides being involved in regulation of NF-κB, MYBBP1a has also been shown to interact and inhibit several other transcription factors. For example, MYBBP1a is a negative regulator of PPARγ co-activator 1α (PGC-1α) functions, which are crucial for the regulation of metabolic processes such as mitochondrial biogenesis and respiration in muscle and gluconeogenesis in liver [17]. It has been demonstrated that both MYBBP1a and $p67^{MYB}$ bind to PGC-1α, and repress its transcriptional activity. The interaction of MYBBP1a and PGC-1α is sensitive to p38 mitogen-activated protein kinase (p38MAPK) as phosphorylation of PGC-1α induces dissociation of MYBBP1a from the complex [17].

Similarly, direct binding of MYBBP1a and $p67^{MYB}$ to another transcription factor, Homeobox protein Prep1, induces dissociation of its coactivator Pre-B-cell leukemia transcription factor 1 (Pbx1). Thus, MYBBP1a suppresses Prep1-dependent transactivation of its target genes, which play a key role in development and organogenesis. In addition, treatment of cells with actinomycin D thereby inducting ribosomal stress, promotes binding of MYBBP1a to Prep1.

MYBBP1a has been also implicated in the regulation of the expression of proteins involved in the regulation of circadian rhythms, as it has been demonstrated that MYBBP1a repressed expression of the Period 2 gene possibly via binding to Cryptochrome-1 (CRY1). Finally, MYBBP1a has recently been found to be a part of the Proto-oncogene tyrosine-protein kinase receptor Ret-Nuclear receptor corepressor 1 (Ret-CoR) co-repressor complex that facilitates the trans-repressive function of the photoreceptor cell-specific nuclear receptor (PNR) and thereby it regulates neuronal differentiation in the developing retina.

Taken together, MYBBP1a can regulate the activity of a broad range of transcription factors that are involved in diverse cellular processes. Thus, it is possible that MYBBP1a acts as a general repressor of transcription, although MYBBP1a can also bind to the aromatic hydrocarbon receptor (AhR) and promote its transcriptional activity.

Even though the molecular mechanism of the suppressive functions of MYBBP1a is not fully understood, it is likely that it depends, at least partially, on the counteracting the activity of HATs, since inhibition of protein deacetylation diminishes MYBBP1a-mediated repression [17].

Here, we identify a novel, MYBBP1a-dependent pathway that regulates FOXO4 signaling. In response to elevated cellular ROS levels, MYBBP1a interacts with FOXO4. This results in impaired acetylation of FOXO4 and consequently, an alteration of its activity.

Materials and methods

Cell culture and transfections

HEK293T and U2OS cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin and 2 mM L-glutamine. In all experiments, cells were cultured in the presence of 10% FBS. HEK293T cells were transiently transfected using FuGENE6 reagent according to the suggestions of manufacturer (Roche Applied Science). U2OS cells were transfected via the calcium-phosphate method.

Plasmids and oligomers

pMT2-HA-FOXO4, GLOFlag3-Flag-FOXO4, pBabe-puro, His-Ubiquitin, have been described previously [18]. 6xDBE Luciferase has been described before [19]. pRL-Tk (Tk *Renilla* luciferase) was purchased from Promega. Construct used for expression of HA-p300 and HA-CBP were described before [4]. GLOFlag3-Flag-FOXO4ΔCys has been characterized previously [16] and this construct was used to amplified cDNA encoding FOXO4ΔCys, which subsequently was cloned N-terminally to an HA tag in a PMT2 vector, using the Gateway system (Invitrogen). To generate the V5-MYBBP1a expression vector, MYBBP1a cDNA was cloned N-terminally to the V5 tag in a pcDNA3.1 vector using the Gateway system (Invitrogen). Similarly, cDNA encoding the N-terminal or C-terminal part of MYBBP1a was cloned to the same vector to generate V5-p67^{MYB} and V5-MYBBP1a$^{580\text{-}end}$, respectively.

cDNA of MYBBP1a was amplified using a myc-MYBBP1a construct purchased from Addgene as a template and forward primer: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCGGAGATGAAGAGCCCCACG-3' and reverse primer: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAAGGTGTCTGCACTCTCCTG-3'. cDNA encoding the N-terminal part of MYBBP1a (p67^{MYB}) was amplified using the same forward

primer as for full length MYBBP1a and reverse primer: 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTATTCCTTCAGAGTACTCATCATCTG-3′. cDNA encoding the C-terminal part of MYBBP1a (MYBBP1a$^{580\text{-end}}$) was amplified using forward primer: 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAATTAGAGGCCCGCTCCTCTGAG-3′ and the same reverse primer as for full length.

Antibodies

Monoclonal antibody: 12CA5 anti-HA was produced in-house using a hybridoma cell line. The antibody against FOXO4 (834) has been described previously [20]. The following antibodies were purchased: MYBBP1a-54160 (Abcam); p27^{kip1} (BD Biosciences); Acetylated-Lysine Antibody #9441 (Cell Signaling); monoclonal V5 (Invitrogen); tubulin and Flag-M2 (Sigma).

Cell lysates and western blot analysis

When preparing the total lysate samples, cells were washed with ice-cold phosphate buffered saline (PBS) and harvested in Laemmli sample buffer. Protein samples were resolved by SDS-PAGE and transferred to PVDF membrane (Perkin-Elmer). Western blot analysis was performed under standard conditions, using the indicated antibodies.

Co-immunoprecipitations

Prior to the co-immunoprecipitations cells were treated with the optimized concentration of 200 μM of H_2O_2 (data not shown) in the presence of serum for the indicated duration. For the semi-endogenous co-immunoprecipitations cells were lysed in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 1% NP-40, 0.5% sodium deoxycholate 10 mM EDTA, 150 mM NaCl, phosphatase and protease inhibitors and the lysate was centrifuged at 20,800 x g for 10 minutes at 4˚C. The cleared lysates were incubated with 7.5 μl solid anti-Flag M2 affinity beads for 2 hours at 4˚C. Beads were washed four times with lysis buffer and proteins bound to the beads were eluted in Laemmli sample buffer, analyzed by SDS-PAGE and immunoblotting as described above using the indicated antibodies.

For the co-immunoprecipitations of Flag-FOXO4 and HA-CBP or HA-p300 cells were lysed in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 1% Triton X-100, 5 mM EDTA, 100 mM NaCl, 1.5mM $MgCl_2$, phosphatase and protease inhibitors.

For the co-immunoprecipitations of Flag-FOXO4 and V5-MYBBP1a Flag-FOXO4 bound to the beads was eluted with 200 μg/ml 3x Flag-peptide (Sigma) for 45 min at 4˚C after the last washing of the anti-Flag M2 affinity beads,. Subsequently, samples were analyzed as described above.

FOXO4 activity assays

To determine the expression of endogenous p27^{kip1}, HEK293T cells were transfected with empty vector, HA-FOXO4 or HA-FOXO4ΔCys together with pBabe-puro. Each of the constructs encoding V5-MYBBP1a, V5-p67^{MYB} or V5-MYBBP1a$^{580\text{-end}}$ was cotransfected and cells were left for twenty-four hours. To select for transfected cells, puromycin was added to the culture medium to a final concentration of 2 μg/ml for 48 hours. Cells were lysed in Laemmli sample buffer. The cell debris was pelleted by centrifugation and the supernatant was used for western blot analysis.

For luciferase assays U2OS cells were transfected with the reporter construct

bearing six canonical FOXO binding sites (6xDBE-luciferase) and the additional constructs were cotransfected as indicated. Luciferase counts were normalized using TK-*Renilla* luciferase. Luciferase levels were measured 48 hours after transfection employing a luminometer and a dual luciferase assay kit according to the instructions of the manufacturer (Promega). All experiments were performed in triplicate.

In vivo ubiquitination assay

The monoubiquitination assay was performed as described previously [18]. Shortly, cells were transfected with the indicated constructs. Forty-eight hours after transfection, cells were treated as indicated and lysed in urea lysis buffer containing 8 M urea, 10 mM of Tris-HCl (pH 8.0), 100 mM of Na_2HPO_4/NaH_2PO_4 (pH 8.0), 0.2% Triton X-100, 5 mM of NEM, and protease inhibitors. Ubiquitinated proteins were analyzed by SDS-PAGE followed by immunoblotting.

Propidium Iodide Exclusion Assay

Cell viability was measured using propidiumiodide exclusion staining and flow cytometry. Spectrin-GFP cotransfection was used as a marker for transfected cells. Dead transfected cells were identified as both GFP and propidiumiodide positive.

Results

Binding between FOXO4 and MYBBP1a requires both N- and C-terminal domains of MYBBP1a and is promoted by elevated ROS levels

To gain a better insight into the functions of FOXO4 we searched for proteins that interact specifically with wild-type FOXO4 but not with the cysteine-free mutant of FOXO4, in which all cysteine residues are mutated to serine residues (hereafter referred to as FOXO4ΔCys).

We ectopically expressed Flag-tagged FOXO4 protein, wild-type or ΔCys versions, in HEK293T cells and purified FOXO4-containing protein complexes by immunoprecipitation using anti-Flag M2 affinity gel. Additionally, HEK293T cells were treated with hydrogen peroxide to enrich samples for the acetylated form of FOXO4 and allow for the formation of cysteine bridges between FOXO4 and its potential interactors. Subsequently, FOXO4-associated proteins were identified by liquid chromatography mass spectrometry/mass spectrometry (LC–MS/MS).

Several proteins were exclusively purified in the presence of wild-type FOXO4 but not FOXO4ΔCys. In addition, we were able to recover a number of proteins interacting with both wild-type and ΔCys FOXO4, indicating that binding of these proteins to FOXO4 does not depend on disulphide bond formation. Interestingly, MYBBP1a was reproducibly identified as a one of the most abundant FOXO4 interactors. Thus, even though binding of MYBBP1a was not restricted to wild-type FOXO4 we decided to further analyze its relationship with FOXO4. The main reason for this was that since MYBBP1a was previously shown to regulate binding between NF-κB and p300, suggesting that MYBBP1a could be involved in the regulation of acetylation of FOXO4 as well.

To confirm the physical interaction between MYBBP1a and FOXO4 we again overexpressed Flag-FOXO4 in HEK293T cells and performed a coimmunoprecipitation assay using anti-Flag M2 affinity gel. Indeed, we were able to detect endogenous MYBBP1a bound to Flag-FOXO4 by Western blot analysis

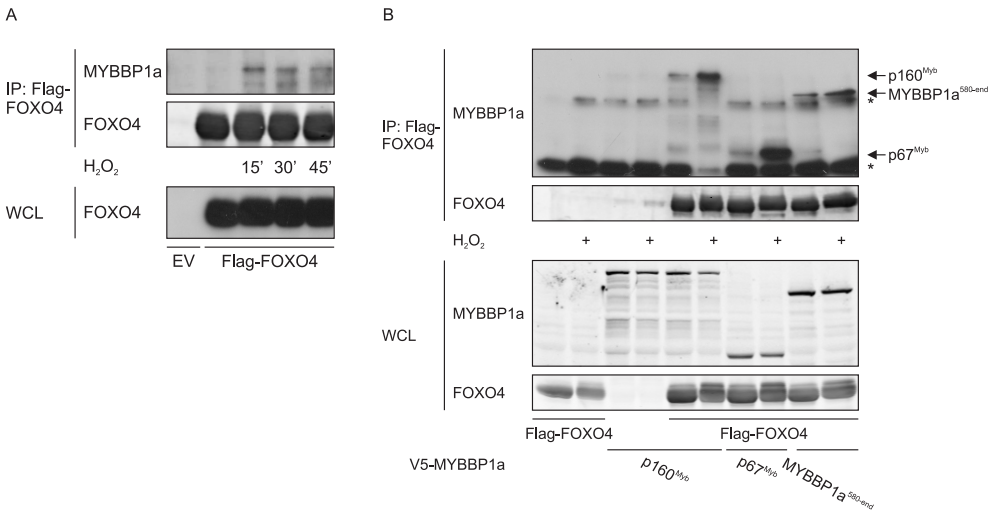


Figure 1. Hydrogen peroxide induces interaction between MYBBP1a and FOXO4
(A) Endogenous MYBBP1a binds to ectopically expressed FOXO4. HEK293T cells were transfected with the indicated constructs. Cells were treated as indicated with 200 μM H_2O_2 for indicated amount of time. Cell lysates were subjected to immunoprecipitation using Flag-M2 affinity beads. Immunoprecipitated complexes were analyzed by SDS-PAGE and immunoblotting using the indicated antibodies. **(B)** FOXO4 interacts with both N- and C-terminal parts of MYBBP1a in ROS-dependent manner. HEK293T cells were transfected with the indicated constructs and treated as indicated with H_2O_2 for 30 min. Immunoprecipitated complexes were eluted using 3x Flag peptide and analyzed by SDS-PAGE and immunoblotting using the indicated antibodies. Asterisks indicate nonspecific background bands.

and this binding was increased after treatment of cells with hydrogen peroxide (Figure 1A).

Subsequently, we decided to determine which part of MYBBP1a interacts with FOXO4. Therefore, we exogenously expressed Flag-FOXO4 together with full length V5-MYBBP1a or with two deletion mutants of MYBBP1a, one corresponding to the shorter version of MYBBP1a - $p67^{MYB}$ and a second mutant thatx encompassed the remaining C-terminal part of MYBBP1a, referred to as $MYBBP1a^{580\text{-end}}$. As shown in Figure 1B we observed that FOXO4 interacted with full length MYBBP1a in a ROS-dependent manner. Interestingly, we also observed that both mutants of MYBBP1a were able to interact with FOXO4 and in both cases the interaction was increased due to peroxide treatment of the cells. However, the binding of FOXO4 to $MYBBP1a^{580\text{-end}}$ was only weakly induced and binding to $p67^{MYB}$ was much more strongly induced. This might suggest that basal binding may proceed through the C-terminal part of MYBBP1a and the induced binding through its N-terminal part.

Thus, FOXO4 forms a complex with MYBBP1a and this involves both C- and N-terminal halves of MYBBP1a. In addition, hydrogen peroxide promotes formation of the complex between FOXO4 and MYBBP1a.

Ectopic expression of MYBBP1a affects the ability of FOXO4 to activate transcription.

Subsequently, we examined the effect of MYBBP1a on the activity of FOXO4. To this end, we performed luciferase reporter assays using FOXO-responsive

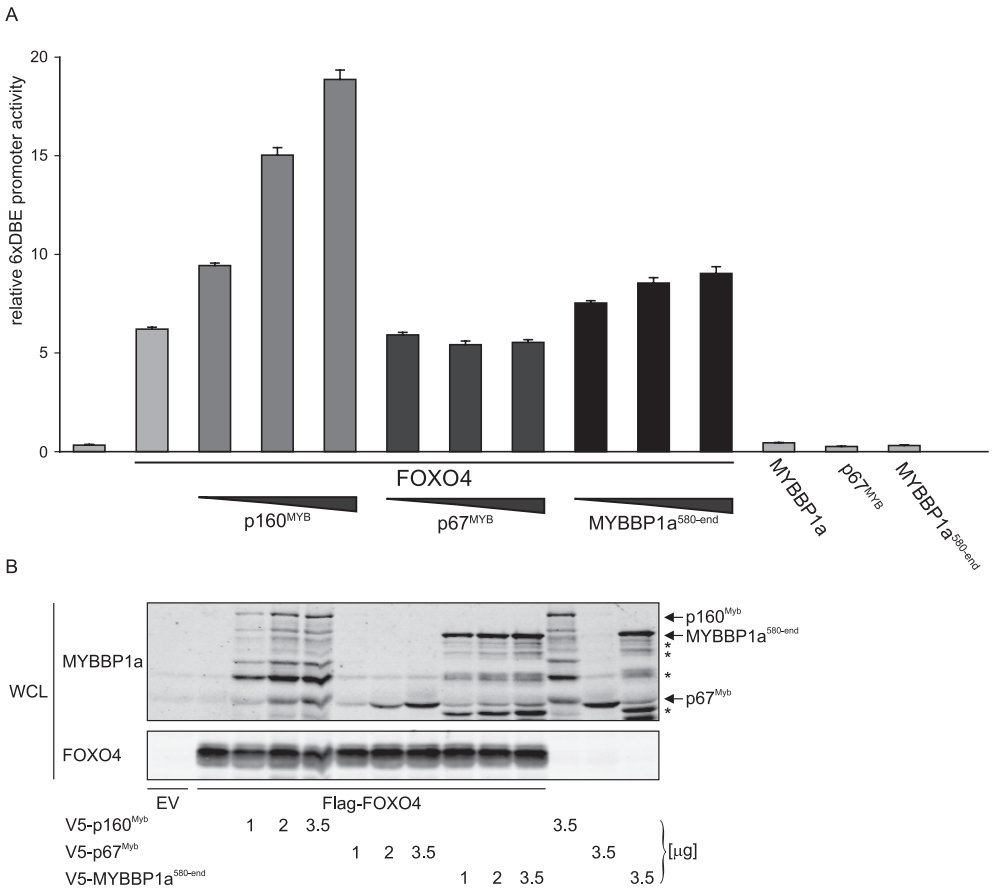


Figure 2. MYBBP1a negatively regulates FOXO transcriptional activity
(A) MYBBP1a inhibits the transcriptional activity of FOXO4. Luciferase assay in U2OS cells transfected with FOXO-responsive 6xDBE-luciferase constructs, Tk-*Renilla* as internal control, HA-FOXO4 and full length MYBBP1a or its N-terminal part, p67^{MYB} or C-terminal part, MYBBP1a$^{580\text{-}end}$. Data represent the average of three independent experiments. Error bars represent s.d. of triplicates. **(B)** Protein levels were confirmed by immunoblotting using the indicated antibodies. Asterisks indicate nonspecific background bands.

6xDBE-luciferase expressed in U2OS cells. We observed that ectopic expression of V5-MYBBP1a increased the activity of Flag-FOXO4 in this assay in a dose dependent manner. However, neither V5-p67^{MYB} nor V5-MYBBP1a$^{580\text{-}end}$ was able to alter the activity of Flag-FOXO4 (Figure 2). Taken together, MYBBP1a modulates the activity of FOXO4. This depends on both C- and N-terminal halves of MYBBP1a as V5-p67^{MYB} and V5-MYBBP1a$^{580\text{-}end}$ mutants of MYBBP1a are not able to alter the activity of FOXO4.

MYBBP1a curtails p300/CBP-induced acetylation of FOXO4, without altering its binding to p300/CBP

Previously, it has been shown that MYBBP1a can modulate the acetylation state of NF-κB and presumably other proteins. Thus, we first examined

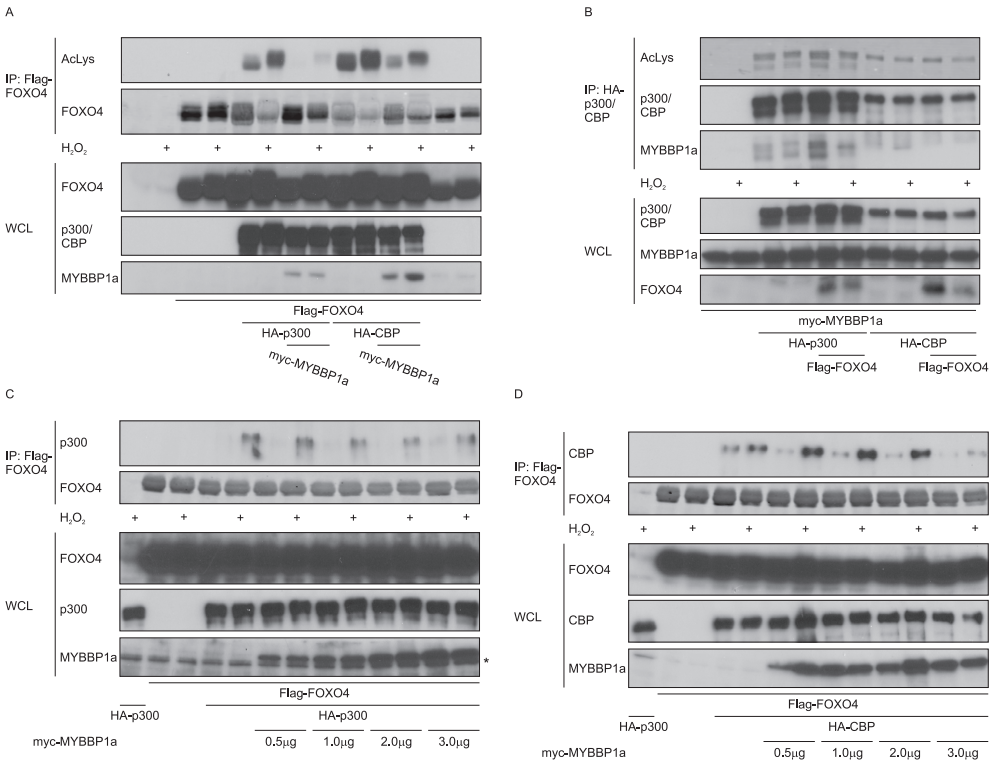


Figure 3. MYBBP1a attenuates the acetylation of FOXO4 without altering its binding to HATs
(A) MYBBP1a reduces CBP/p300-induced acetylation of FOXO4. To assess the acetylation of FOXO4, Flag-FOXO4 was immunopurified from HEK293T cells transfected with the indicated constructs followed by SDS-PAGE and immunoblotting using an α-Acetyl-Lysine antibody. **(B)** MYBBP1a does not inhibit the intrinsic acetyltransferase activity of p300 and CBP. The interaction between MYBBP1a and p300 is visible. The autoacetylation of p300 and CBP was examined as a measurement of their activity. Therefore, HA-p300 or HA-CBP was immunopurified from HEK293T cells transfected with the indicated constructs followed by SDS-PAGE and immunoblotting using an α-Acetyl-Lysine antibody. **(C, D)** MYBBP1a does not regulate FOXO4 binding to p300 or CBP. HEK293T cells were transfected with the indicated constructs and treated with 200 μM H_2O_2 for 30 min as indicated. Flag-FOXO4 was immunoprecipitated and binding of FOXO4 to p300 and CBP was analyzed by SDS-PAGE and immunoblotting. In **(C)** the asterisk indicates nonspecific background bands.

whether MYBBP1a influences the acetylation state of FOXO4.

To this end, we ectopically expressed Flag-FOXO4 together with HA-p300 or HA-CBP and observed that acetylation of FOXO4 was induced due to overexpression of HATs. In addition, hydrogen peroxide further promoted acetylation of FOXO4. However, co-expression of V5-MYBBP1 decreased CBP/p300-induced acetylation of FOXO4 in a dose dependent manner (Figure 3A). Thus, ectopic expression of MYBBP1a negatively regulates the acetylation of FOXO4.

Next, we analyzed whether the MYBBP1a-dependent decrease in acetylation of FOXO4 is due to impaired binding between FOXO4 and p300 or CBP. Therefore, we ectopically ex-

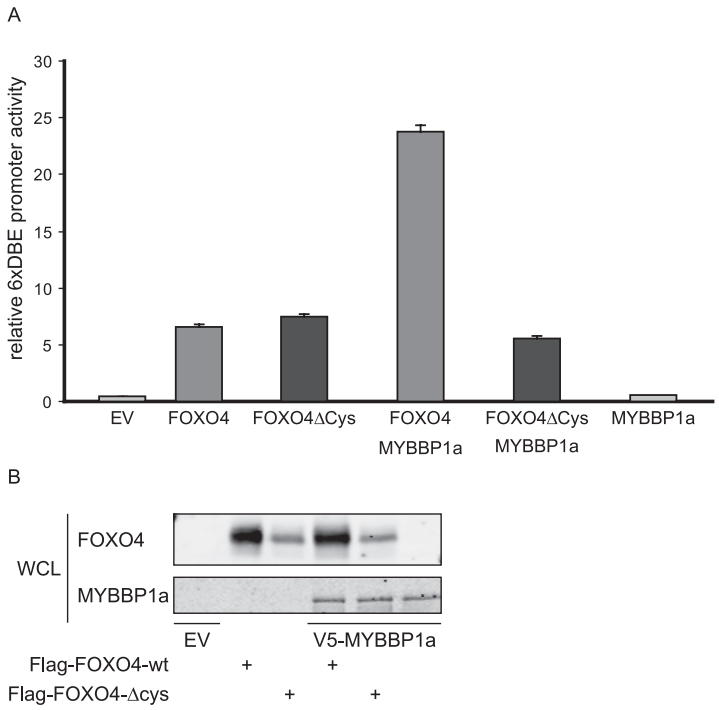


Figure 4. MYBBP1a does not regulate FOXO4ΔCys transcriptional activity
(A) Luciferase assay in U2OS cells transfected with FOXO-responsive 6xDBE-luciferase constructs, Tk-*Renilla* as internal control, and HA-FOXO4. Each experiment was performed in triplicate. Error bars represent s.d. of triplicates. **(B)** Protein levels were confirmed by immunoblotting using the indicated antibodies.

pressed Flag-FOXO4 together with HA-p300 or HA-CBP in HEK293T cells and we performed an immunoprecipitation assay using Flag M2 affinity gel. We observed that both p300 and CBP coimmunoprecipitated with Flag-FOXO4 and the binding of these proteins to FOXO4 was induced by hydrogen peroxide. However, simultaneous overexpression of V5-MYBBP1 did not alter the amount of CBP/p300 that was bound to FOXO4 (Figure 3C and 3D).

Since we were also able to detect binding between MYBBP1a and p300, it is possible that MYBBP1a can inhibit the activity of CBP/p300 and thereby decrease the acetylation of FOXO4. Therefore, we examined the auto-acetylation of p300 and CBP, as a measure of their activity, in the presence or absence of ectopically expressed MYBBP1a. However, as shown in Figure 3B we did not observe any differences when comparing the acetylation state of p300/CBP due to auto-acetylation in the absence or presence of MY BBP1a.

Therefore, we conclude that MYBBP1a suppresses the acetylation of FOXO4 and this is likely due to steric interference, which impedes the accessibility of lysine residues in FOXO4 by CBP/p300.

MYBBP1a-dependent activation of FOXO4 in a reporter assay involves acetylation of FOXO4

To establish whether the observed re-

duction in p300/CBP-mediated acetylation of FOXO4 has a role in the observed increase in transcriptional activity as measured by reporter assays, we compared the effect of MYBBP1a on wild-type FOXO4 versus FOXO4ΔCys. Due to the defective binding of p300/CBP to FOXO4ΔCys this mutant lacks regulation by p300/CBP. Therefore, we performed FOXO4 reporter assays and we observed that overexpression of MYBBP1a promoted the transcriptional activity of wild-type FOXO4, but importantly not of FOXO4ΔCys (Figure 4A). This suggests that the MYBBP1a-dependent decrease in acetylation of FOXO4 is indeed responsible for observed augmentation of FOXO4-mediated transcription of a luciferase reporter gene.

MYBBP1a enhances monoubiquitination of FOXO4

FOXO4 has previously been shown to be monoubiquitinated on multiple lysine residues and this promotes the activity of FOXO4. Since ubiquitination and acetylation might occur at the same lysine residues, we were interested whether MYBBP1a can increase monoubiquitination of FOXO4. Therefore we analyzed ubiquitination of FOXO4 with and without ectopic expression of MYBBP1a. Consistent with the negative effect of MYBBP1a on the acetylation of FOXO4, its presence clearly elevated monoubiquitination of FOXO4 (Figure 5).

Hence, this suggests that MYBBP1a hinders acetylation of FOXO4 and thereby facilitates its monoubiquitination.

MYBBP1a represses FOXO-induced p27^{kip} expression

As discussed above p300/CBP may have multiple functions in the regulation of

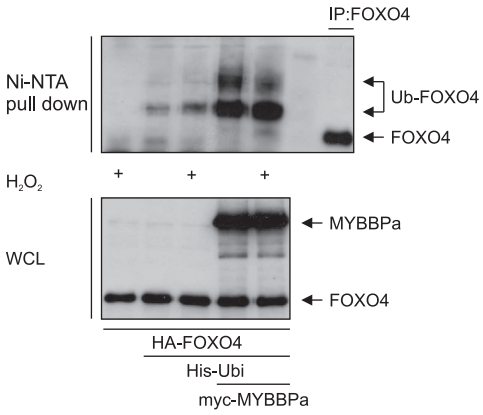


Figure 5. MYBBP1a enhances monoubiquitination of FOXO4
HEK293T cells were transfected with the indicated constructs and treated with 50 μM H_2O_2 for 15 min as indicated. Cell lysates were subjected to Ni-NTA pull-down to bind ubiquitinated proteins. Ubiquitinated FOXO4 was detected by SDS-PAGE and immunoblotting using α-FOXO4 antibody.

FOXO activity. Therefore, we analyzed whether MYBBP1a influences the expression of endogenous target genes of FOXO4.

Exogenous expression of FOXO4 in HEK293T cells strongly elevated the levels of p27^{kip1}, a well established FOXO target gene. However, surprisingly and in contrast to the reporter assays, simultaneous overexpression of V5-MYBBP1a inhibited the FOXO4-dependent induction of p27^{kip1} protein levels (Figure 6A). Similarly to the luciferase reporter assay, mutants of MYBBP1a were not able to decrease FOXO4-induced p27^{kip1} protein levels (Figure 6B and 6C). In addition, ectopically expressed MYBBP1a was still capable of inhibiting FOXO4ΔCys-induced p27^{kip1} protein levels to the same extent as wild-type FOXO4 (Figure 6D). Thus, we conclude that p300/CBP indeed exert control over FOXO function at multiple levels. Furthermore, transient reporter assays apparently predominantly reflect a role for di-

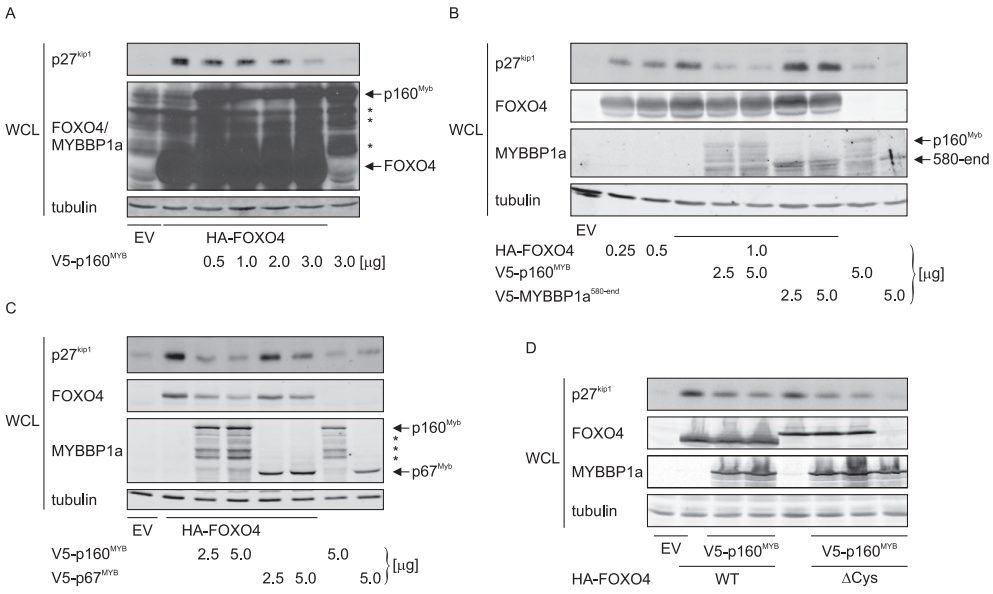


Figure 6. MYBBP1 decreases FOXO4-induced p27^{kip1} protein levels
(A) Exogenous expression of full length MYBBP1a reduces p27^{kip1} protein levels. Western blot analysis of p27^{kip1} expression of puromycin-selected HEK293T cells expressing the indicated proteins. **(B, C)** MYBBP1a-mediated reduction of p27^{kip1} protein levels requires both N- and C-terminal halves of MYBBP1a. Cells were treated as described in (A). **(D)** Ectopic MYBBP1a reduces p27^{kip1} protein levels induced by wild type FOXO4 and FOXO4Δcys. Cells were treated as described in (A). Additionally, in **(A, C)** asterisks indicate nonspecific background bands.

rect acetylation of FOXO, whereas in endogenous gene regulation the relevance of other p300/CBP effects, like histone acetylation become dominant, at least in this experimental set-up.

Taken together, MYBBP1a interferes with FOXO function at multiple levels one of which is FOXO acetylation. How, MYBBP1a interferes with endogenous gene regulation remains to be established. Furthermore, these results are consistent with previous reports showing that acetylation can have multiple effects on the activity of FOXO4.

MYBBP1a has no effect on FOXO4-induced cell death

It has been suggested that acetylation determines a shift of FOXO function from inducing cell cycle arrest towards apoptosis [7, 21]. In addition p300 enhances FOXO4-induced cell death and this is dependent on the acetylation state of FOXO4 as p300 has no effect on cell death induced by FOXO4ΔCys. For that reason, we examined whether MYBBP1a can also modulate this function of FOXO4.

In agreement with previous studies, exogenous expression of Flag-FOXO4 induced apoptosis in U2OS cells; however simultaneous overexpression of V5-MYBBP1a had mostly no effect on FOXO4-induced cell death and only sometimes we were also able to observe a minor increase in FOXO4-induced apoptosis due to overexpression of MYBBP1a (Figure 7). The lack of a genuine effect of MYBBP1a in this assay might result from a different ratio

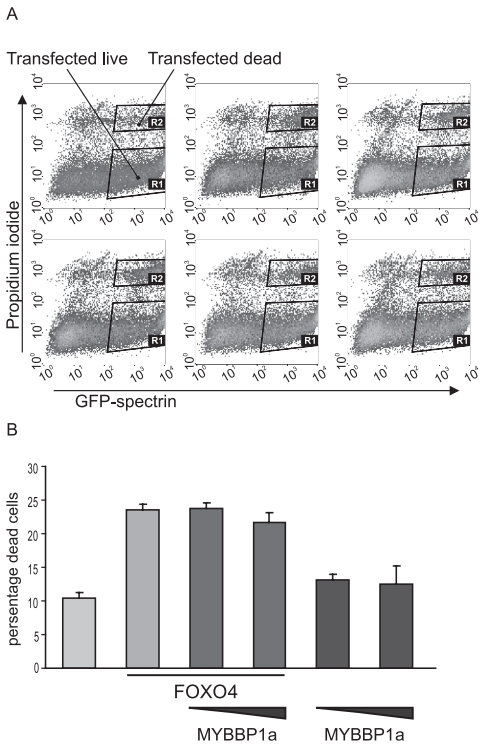


Figure 7. MYBBP1a does not reduce FOXO4-induced cell death
(A) Representative flow cytometry plots showing U2OS cell viability. Propidium iodide is only excluded from live cells. The indicated constructs were transfected together with limiting amounts of spectrin-GFP. Cells positive for spectrin-GFP and negative for propidium iodide are considered live transfected cells, whereas cells positive for both dyes are considered dead transfected cells. **(B)** The quantification of the experiment is represented in the bar graph. Error bars represent s.d. of triplicates.

of FOXO4 to MYBBP1a that has been used for the overexpression of these proteins. Importantly, in other experiments MYBBP1a had the greatest effect when relatively high amounts of plasmid were used for overexpression of MYBBP1a. This was not possible in the PI exclusion assay since high amounts of plasmids, which also required the use of high amounts of transfection reagent, somehow sensitized U2OS cells to apoptosis and thereby the FOXO4-dependent effect was not pronounced anymore.

Therefore, due to technical difficulties we cannot exclude that MYBBP1a can suppress apoptotic functions of FOXO4 and it is necessary to address this question using other approaches.

Discussion

Here we provide evidence that MYBBP1a alters the acetylation and ubiquitination state of FOXO4 and thereby modulates its activity. To our knowledge this is the first example of a FOXO transcription factor to be regulated by MYBBP1a. Interestingly, we observed that MYBBP1a can interact with FOXO4 in response to oxidative stress, which might provide further understanding of the stress-related functions of FOXO proteins.

MYBBP1a is a nuclear protein mainly localized to the nucleolus, which constitutes a specialized nuclear domain of ribosome biogenesis. MYBBP1a shares some homology with a yeast protein called POL5, which has been indicated to be involved in ribosomal RNA synthesis in *Saccharomyces cerevisiae*. Regardless of the homology with POL5, the role of MYBBP1a in the nucleolus and/or nucleus has not been revealed yet. However, MYBBP1a has been reported to be proteolytically processed in response to ribosomal stress and to translocate from the nucleolus to the nucleus, suggesting that MYBBP1a plays a role in the ribosomal stress response [22].

Therefore, MYBBP1a-dependent regulation of FOXO4 might provide a link between FOXO4 functions and ribosomal stress, being in accordance with the stress-protective functions of FOXO4.

This is further supported by the fact that the interaction between FOXO4 and MYBBP1a is enhanced due to hydrogen

peroxide treatment of cells. Importantly, elevated cellular ROS levels induce formation of intermolecular complexes between ribosomal proteins and RNA [23] as well as cause oxidative damage to RNA [24]. Thus, it is plausible that these processes might lead to induction of ribosomal stress and that this would trigger the translocation of MYBBP1a and its binding to FOXO4. However, further studies, employing agents specifically associated with induction of ribosomal stress, are required to elucidate this possibility.

Previously, MYBBP1a has been demonstrated to compete with p300 for the binding to NF-κB. This leads to the inhibition of NF-κB transcriptional activity, since p300 functions as a co-activator of NF-κB, facilitating its activity by acetylating histones and NF-κB itself [25].

In our study, we observed that MYBBP1a can negatively regulate the acetylation of FOXO4; however, this was not found to be associated with impaired binding of CBP or p300 to FOXO4. Moreover, ectopic expression of MYBBP1a increases the activity of FOXO4 in a reporter assay, but is unable to do so with FOXO4ΔCys. This indicates that decreased acetylation of FOXO4 is responsible for this effect, since acetylation of FOXO4ΔCys is already dramatically reduced and therefore MYBBP1a cannot improve its activity. In addition, MYBBP1a not only represses acetylation of FOXO4 but also promotes its monoubiquitination, which is also associated with increased activity of FOXO4. Thus, a combination of both reduced acetylation and increased monoubiquitination of FOXO4 might account for the MYBBP1a-dependent increase in FOXO4-mediated transcription of a luciferase reporter gene.

Despite the positive effect on FOXO4 activity in a reporter assay, we observed that overexpression of MYBBP1a negatively regulates FOXO4-dependent induction of its target gene $p27^{kip1}$.

Although this may appear inconsistent at first, it is in agreement with the rather complex effect that acetylation has on the activity of FOXO, as it has been shown to activate, modulate and inhibit FOXO-dependent transcription. This is due to the fact that CBP/p300-dependent acetylation of FOXOs themselves is hampering their ability to bind to DNA, which suppresses FOXO-induced transactivation. However, acetylation of histones by CBP/p300 is generally stimulating transcription, therefore it is required by FOXOs to induce expression of their target genes. Similarly, it has been shown for NF-κB that acetylation of certain residues enhances its DNA-binding ability and association into a transcriptionally active complex, whereas acetylation of distinct residues has been reported to lower its binding affinity for target sequences [26].

Thus, it is possible that in a luciferase reporter assay the most prominent effect is associated with acetylation of FOXO4 itself as expression of reporters might not be regulated by acetylation of histones. On the other hand, expression of endogenous genes is strongly dependent on histone modifications, therefore the FOXO4 effect might be less pronounced. However, we have at present no evidence that MYBBP1a has any influence on the acetylation of histones and further studies are required to elucidate this issue. In addition, it is also possible that MYBBP1a can enhance specificity of FOXO4 towards a subset of genes, as it has been proposed that acetylation mainly promotes expression of FOXO-target genes that are involved in apoptosis. It would be expected,

however, that MYBBP1a-dependent inhibition of FOXO4 acetylation should increase the expression of $p27^{kip1}$. However, since $p27^{kip1}$ is mainly upregulated by FOXO4 in response to growth factor deprivation, it is plausible that other targets of FOXO4, more specific for oxidative stress responses, are induced by MYBBP1a. Finally, it is not known whether MYBBP1a specifically inhibits the acetylation of certain lysine residues leaving the others unaffected and thereby determines the specificity of FOXO4.

Importantly, MYBBP1a has been also suggested to possess an intrinsic transcriptional repressive activity. When fused to the DNA binding domain of Gal4, it can suppress expression of a luciferase gene controlled by the TK promoter. Moreover, this repression was blocked upon treatment of cells with the histone deacetylase inhibitor trichostatin A (TSA), indicating that MYBBP1a can directly affect deacetylation of other proteins.

The MYBBP1a-mediated regulation of FOXO4-induced transcription might depend on both C- and N-terminal halves of MYBBP1a. This is supported by the observation that only full length MYBBP1a was able to modulate the activity of FOXO4 but not its shorter version $p67^{MYB}$. Interestingly, $p67^{MYB}$, when ectopically expressed is mislocalized to the cytoplasm and thereby excluded from the nucleus. Since the binding between FOXO4 and $p67^{MYB}$ is not impaired, this suggests that in order to regulate the FOXO4 transcriptional activity, MYBBP1a needs to localize within the nucleus. On the other hand, the C-terminal part of MYBBP1a, $MYBBP1a^{580\text{-}end}$, is localized in the nucleolus and like full length MYBBP1a translocates to the nucleus in response to actinomycin D treatment (data not shown). However, similar to $p67^{MYB}$, it has no effect on the activity of FOXO4. This indicates that MYBBP1a-mediated regulation of FOXO4 requires the N-terminal part of MYBBP1a, whereas the C-terminal part of MYBBP1a is necessary for its proper localization. Taken together, this implies that even though binding between FOXO4 and MYBBP1a can occur in the cytoplasm, the regulation of FOXO4 is restricted to the nucleus. Therefore, it can be hypothesized that it might require colocalization of both FOXO4 and MYBBP1a on the DNA.

Since $p67^{MYB}$ has been shown to be generated in response to ribosomal stress [22], this again suggests that regulation of FOXO4 by MYBBP1a might take place due to inefficient biogenesis of ribosome subunits. Nevertheless, it is necessary to examine whether MYBBP1a is also proteolytically modified in response to oxidative stress.

Previously, it has been shown that binding between PGC1α and MYBBP1a is inhibited due to p38MAPK-mediated phosphorylation of PGC1α [17]. Since FOXO4 has also been implicated to be a substrate for p38MAPK as well as other stress responsive kinases, it will be interesting to investigate whether the phosphorylation state of FOXO4 has any influence on its binding to MYBBP1a. Moreover, MYBBP1a has also been reported to interact with thioredoxin binding protein-2 (TBP-2) and TBP-2, similarly to p38, hampers the interaction between PGC1α and MYBBP1a [27]. Since binding of FOXO4 to p300 is negatively regulated by thioredoxin, whose activity is inhibited by TBP-2, it is tempting to speculate that regulation of FOXO4 by MYBBP1a might be affected by TBP-2.

Therefore, additional studies are required to expand the relationship between FOXO4 and MYBBP1a.

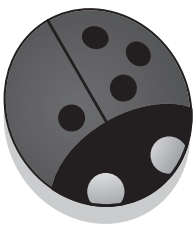
References

1. Nasrin, N., et al., DAF-16 recruits the CREB-binding protein coactivator complex to the insulin-like growth factor binding protein 1 promoter in HepG2 cells. Proc Natl Acad Sci U S A, 2000. 97(19): p. 10412-7.
2. Lee, D.Y., et al., A positive role for histone acetylation in transcription factor access to nucleosomal DNA. Cell, 1993. 72(1): p. 73-84.
3. Mahmud, D.L., et al., Phosphorylation of forkhead transcription factors by erythropoietin and stem cell factor prevents acetylation and their interaction with coactivator p300 in erythroid progenitor cells. Oncogene, 2002. 21(10): p. 1556-62.
4. van der Horst, A., et al., FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2(SIRT1). J Biol Chem, 2004. 279(28): p. 28873-9.
5. Fukuoka, M., et al., Negative regulation of forkhead transcription factor AFX (Foxo4) by CBP-induced acetylation. Int J Mol Med, 2003. 12(4): p. 503-8.
6. Daitoku, H., et al., Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. Proc Natl Acad Sci U S A, 2004. 101(27): p. 10042-7.
7. Brunet, A., et al., Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science, 2004. 303(5666): p. 2011-5.
8. van der Heide, L.P. and M.P. Smidt, Regulation of FoxO activity by CBP/p300-mediated acetylation. Trends Biochem Sci, 2005. 30(2): p. 81-6.
9. Motta, M.C., et al., Mammalian SIRT1 represses forkhead transcription factors. Cell, 2004. 116(4): p. 551-63.
10. Calnan, D.R. and A. Brunet, The FoxO code. Oncogene, 2008. 27(16): p. 2276-88.
11. van der Horst, A. and B.M. Burgering, Stressing the role of FoxO proteins in lifespan and disease. Nat Rev Mol Cell Biol, 2007. 8(6): p. 440-50.
12. Brent, M.M., R. Anand, and R. Marmorstein, Structural basis for DNA recognition by FoxO1 and its regulation by posttranslational modification. Structure, 2008. 16(9): p. 1407-16.
13. Tsai, K.L., et al., Crystal structure of the human FOXO3a-DBD/DNA complex suggests the effects of post-translational modification. Nucleic Acids Res, 2007. 35(20): p. 6984-94.
14. Matsuzaki, H., et al., Acetylation of Foxo1 alters its DNA-binding ability and sensitivity to phosphorylation. Proc Natl Acad Sci U S A, 2005. 102(32): p. 11278-83.
15. Hatta, M., F. Liu, and L.A. Cirillo, Acetylation curtails nucleosome binding, not stable nucleosome remodeling, by FoxO1. Biochem Biophys Res Commun, 2009. 379(4): p. 1005-8.
16. Dansen, T.B., et al., Redox-sensitive cysteines bridge p300/CBP-mediated acetylation and FoxO4 activity. Nat Chem Biol, 2009. 5(9): p. 664-72.
17. Fan, M., et al., Suppression of mitochondrial respiration through recruitment of p160 myb binding protein to PGC-1alpha: modulation by p38 MAPK. Genes Dev, 2004. 18(3): p. 278-89.
18. van der Horst, A., et al., FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP. Nat Cell Biol, 2006. 8(10): p. 1064-73.
19. Essers, M.A., et al., FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. EMBO J, 2004. 23(24): p. 4802-12.
20. Medema, R.H., et al., AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature, 2000. 404(6779): p. 782-7.
21. Accili, D. and K.C. Arden, FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. Cell, 2004. 117(4): p. 421-6.
22. Yamauchi, T., et al., Ribosomal stress induces processing of Mybbp1a and its translocation from the nucleolus to the nucleoplasm. Genes Cells, 2008. 13(1): p. 27-39.
23. Mirzaei, H. and F. Regnier, Protein-RNA cross-linking in the ribosomes of yeast under oxidative stress. J Proteome Res, 2006. 5(12): p. 3249-59.
24. Kong, Q. and C.L. Lin, Oxidative damage to RNA: mechanisms, consequences, and diseases. Cell Mol Life Sci. 67(11): p. 1817-29.
25. Furia, B., et al., Enhancement of nuclear factor-kappa B acetylation by coactivator p300 and HIV-1 Tat proteins. J Biol Chem, 2002. 277(7): p. 4973-80.
26. Calao, M., et al., A pervasive role of histone acetyltransferases and deacetylases in an NF-kappaB-signaling code. Trends Biochem Sci, 2008. 33(7): p. 339-49.
27. Yoshihara, E., et al., Disruption of TBP-2 ameliorates insulin sensitivity and secretion without affecting obesity. Nat Commun. 1(8): p. 127.

Chapter 4

Generation and analysis of GFP-Foxo4-expressing knock-in mice

Anna A. Szypowska, Catherine Robin, Toon A.B. van Veen, Niels Galjart, Boudewijn M.T. Burgering



Generation and analysis of GFP-Foxo4-expressing knock-in mice

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Abstract

A large number of cellular studies revealed that the Forkhead Box O (FOXO) subfamily of Forkhead transcription factors are involved in the regulation of a broad range of cellular processes, including cell cycle regulation, apoptosis and stress responses. It is also apparent that FOXO proteins have many redundant functions at the cellular level as well as in the context of the whole organism. Nevertheless, besides a functional redundancy, different FOXOs can transactivate a diverse set of target genes. This indicates that FOXO proteins also have non-redundant functions in the regulation of biological processes. This is especially appreciated in mouse model systems, which are instrumental in unravelling how diverse physiological processes are regulated by different members of the FOXO subfamily. Therefore, to advance our understanding of the functions of FOXO4 *in vivo* we decided to evaluate its expression pattern by generating GFP-Foxo4 expressing knock-in mice. Using these mice, we were able to demonstrate the expression of Foxo4 within the HSC compartment. At present, we are further characterizing these mice for the Foxo4 expression pattern. This will include the analysis of altered Foxo4 expression patterns after genetic challenges or other challenges such as nutrient deprivation.

Introduction

In mammals the FOXO subfamily of forkhead transcription factors consists of four members, i.e. FOXO1, FOXO3a, FOXO4 and FOXO6. FOXO proteins are homologous to the *Caenorhabditis elegans* transcription factor DAF-16, which regulates longevity downstream of the insulin-like signaling pathway [1]. Biochemical analysis in mammalian cells revealed that the activity of FOXOs is predominantly controlled by two modes of cellular signaling [2]. First, the growth factor signaling pathway regulates FOXO activity, which involves direct phosphorylation by protein kinase B (PKB) followed by the inactivation of FOXO-dependent transcription. The PKB-mediated inhibition of FOXOs is a consequence of a combination between cytoplasmic sequestration, reduced ability to bind DNA and decreased stability of FOXO factors. Secondly, the stress signaling pathway modulates FOXO activity through complex posttranslational modifications (PTMs), encompassing various degrees of phosphorylation, acetylation and ubiquitination [3]. This involves a dynamic network of several modifying enzymes, which generally

promote FOXO functions. The diverse gene programs that are activated by FOXOs enable these transcription factors to regulate a broad range of cellular processes including stress resistance, cell cycle arrest, apoptosis, metabolism, and differentiation [4]. In the context of the whole organism, FOXO cellular functions have been proven to be significant for the coordination of glucose homeostasis, angiogenesis and stem cell maintenance as well as immune, muscular, and neuronal functions [4].

FOXO transcription factors are considered to have many redundant functions. This seems to be particularly important for the tumor suppressive functions of FOXOs, as it has been shown that simultaneous loss of all three FOXO genes (FOXO1, FOXO3a and FOXO4) is a prerequisite for spontaneous tumor formation [5]. However, recent *in vivo* studies revealed that the roles of different members of the FOXO subfamily are not completely overlapping. This is supported by the observation that only deficiency of Foxo1, but not of any other Foxos, induces early embryonic death [6]. Since Foxo1-null embryos die due to incomplete vascular development [6], this indicates a fundamental role of this transcription factor in vascular formation. On the other hand liver-specific ablation of Foxo1 results in decreased glucose levels in new born mice, and in adult mice in response to fasting. This is associated with a diminution in fasting-induced glycogenolysis and gluconeogenesis [7]. In addition, Foxo3a$^{-/-}$ female mice exhibit a specific ovarian phenotype of global follicular activation leading to oocyte death and premature depletion of functional ovarian follicles followed by infertility [8].

Interestingly, growing evidence indicates the involvement of Foxo proteins in the regulation of immune system responses. Deficiency of Foxo3a leads to spontaneous, autoreactive activation of helper T cells followed by production of Th cytokines and a mild lymphoproliferative autoimmune syndrome [9, 10]. At the molecular level Foxo3a inhibits Nucelar factor κB (NFκB) and therefore loss of Foxo3a results in unrestrained activation of NFκB, whose overactivity is responsible for T cells hyperactivation. Similarly, Foxo1 has been shown to influence T cells in a distinctive way. Conditional deletion of Foxo1 specifically in these cells results in lower expression of the homing molecules L-selectin and C-C chemokine receptor type 7 (CCR7) thereby affecting T cell trafficking and survival [10]. The diminution of L-selectin levels is most probably an indirect effect of Foxo1-deficiency and originates from the Foxo1-dependent regulation of Kruppel-like factor 2 (KLF2) expression [11]. In addition, Foxo1 has also been shown to directly promote the expression of interleukin 7 receptor alpha chain (IL7R) by naïve T cells, which is crucial for survival of these cells [10].

Recently, Foxo transcription factors have been shown to regulate hematopoietic stem cell (HSC) number and functions. Loss of all Foxo proteins in the adult hematopoietic system increases the amount of lineage-negative, Sca/Kit-positive (LSK) cells in active phases of the cell cycle (S/G2/M) followed by depletion of HSC and multipotent progenitor pools [12]. This means that Foxos normally maintain LSK cells in the quiescent state of the cell cycle, which is crucial for maintenance of their self-renewal capacity. In addition, the depletion of Foxo proteins promotes apoptosis in both HSC and myeloid progenitor compartments and also elevates ROS levels in these cells [12]. Thus, a combi-

nation of aberrant cell cycle regulation, increased apoptosis and induction of oxidative stress accounts for the HSC defect in Foxo-deficient mice. Importantly, aged germline Foxo3a knockout animals exhibit a similar phenotype to the young adult mice with the Foxo-null hematopoietic system, suggesting that Foxo3a might be the primary Foxo involved in the regulation of HSC self renewal. In addition, loss of Foxo3a specifically in neuronal stem cells revealed that besides HSCs, Foxo3a is also important for maintenance of these cells in the quiescent state [13-15].

In contrast to the essential role of Foxo3a, there is no distinctive phenotype associated with loss of Foxo4 expression [6]. This apparent lack of any role *in vivo* is rather surprising as in a number of cellular studies Foxo4 has been proven to have important and possibly unique functions. Therefore, to acquire a better understanding of the specific role of Foxo4 in the whole animal we decided to determine the precise expression pattern of Foxo4 in the adult mouse. A number of attempts have been made before to elucidate the expression pattern of Foxo proteins, however these were mostly based on mRNA analysis. Since mRNA expression levels do not always correlate with protein expression levels we decided to generate GFP-Foxo4 knock-in mice. Taking advantage of homologous recombination we targeted the cDNA of EGFP in front of the coding region of the Foxo4 locus. Thus, knock-in mice would express the GFP-Foxo4 fusion protein driven by the endogenous Foxo4 promoter and therefore allow us to demonstrate the spatiotemporal pattern of Foxo4 protein expression.

Materials and methods

Reagents and materials

The following materials and reagents were purchased: Hybond-XL (20 cm × 3 m) RPN203S (GE Healthcare); collagenase Type I (Invitrogen); hyaluronidase from bovine testes, Type IV-S, (Sigma); trypsin 2.5% (Lonza); Cell Strainer (BD Falcon Bioscience); Tissue-Tek O.C.T™ Compound (Sakura Finetek); GFP ab290 antibody (Abcam) GFP (B-2) (Santa Cruz Biotechnology). All antibodies used for FACS analysis were purchased from BD Bioscience.

Molecular biology

DNA isolation has been essentially performed using standard phenol/chloroform extraction procedures (J. Sambrook Molecular Cloning: A Laboratory Manual). Southern blot analysis was performed as described [16]. PCR analysis was done using the primers indicated in Figure 1C with the following sequences: 1 Fwd: 5′-CTCCACTGATCGCTGAGCTG-CAG-3′, 1 Rev: 5′-GTTCGTGTGGCTC-CGTAGCGAGG-3′, 2 Rev: 5′-GTCGTG-CTGCTTCATGTGGTCGG-3′, 3 Fwd: 5′-GCGTGCAATCCATCTTGTTCAA-TG-3′ ,4 Fwd: 5′-GACAAGCAGAAGA-ACGGCATCAAGGTGAAC-3′ ,5 Rev: 5′-CGGACCATCCATTCGTAGATC-TG-3′, RNA isolation has been performed using RNeasy Mini kit according to the protocol of the manufacturer (Qiagen). The expression of endogenous GFP-Foxo4 was examined by reverse transcription of total RNA using the BIO-RAD iScript cDNA synthesis kit followed by PCR using primers indicated in Figure 3A with the following sequences: Fwd: 5′-GATCACATGGTCCTGCTG-GAG-3′, Rev Ex1/2: 5′-GACGGATGGA-GTTCTTCCATC-3′, Rev Ex2: 5′-TGTG-CAAGGACAGGTTGTGAC-3′ Immunoprecipitations, Western blot analysis and luciferase reporter assays were performed as described [17].

Targeting vector construction and generation of GFP-Foxo4 knock-in mice

Based on a previously published strat-

egy [18] we generated the *Foxo4*^{KI} allele, in which GFP-Foxo4 is expressed instead of Foxo4. The mouse BAC genomic library (Children's Hospital Oakland Research Institute), which was prepared from female 129S6/SvEvTac mouse spleen genomic DNA [19] was screened by PCR using primers specifically designed to amplify a fragment of genomic DNA corresponding to exon 1 of *Foxo4*. One positive clone (RP22-369H10) was subsequently chosen to generate the targeting construct. The schematic representation of the *Foxo4* knock-in construct is shown in Figure 1. Targeting and selection of embryonic stem (ES) cells as well as identification of positive clones was performed as described previously [16]. Two targeted ES cell clones with correct karyotypes were chosen to generate germline chimeric mice by injecting them into C57Bl/6 blastocysts. Chimeric males were mated with C57Bl/6 females to obtain germline transmission of the modified allele. Subsequently, germline-positive mice were crossed with CAG-Cre transgenic mice to remove the *neo* cassette and thereby convert the *Foxo4* knockout allele into the *Foxo4* knock-in allele. After establishing *Foxo4*^{KI} and *Foxo4*^{KO} transgenic mouse lines the correct genotype was verified using southern blot analysis. Routinely mouse genotyping was performed by PCR on genomic DNA (tail tips).

Isolation and culturing of GFP-Foxo4 expressing mouse adult fibroblasts (MAFs)

Mice were sacrificed by cervical translocation and the skin from the back of the mice was dissected after removal of the hairs. Skin was cut into small pieces and incubated in TCH solution containing 0.125% trypsin, 1 mg/ml collagenase type I and 0.3 mg/ml hyaluronidase in PBS for about 4 hours in 37 °C. The cell suspension was filtered through a 40-μm cell strainer to remove tissue debris. The cells were centrifuged at 1000 rpm for 5 min, washed with PBS and resuspended in DMEM/F10 (1:1) medium supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin and 2 mM L-glutamine, and seeded onto a 6 cm dish.

Live cell microscopy and immunofluorescence analysis

To analyze expression of GFP-Foxo4 in life cells, MAFs were seeded onto glass bottom WillCo-dishes and analyzed at 37 °C as published previously [7] using a Zeiss LSM 510 confocal microscope.

For immunofluorescence analysis MAFs were fixed in freshly prepared 4% paraformaldehyde (PFA) for 15 minutes, permeabilized in 0.2% Triton X-100 for 10 minutes and blocked in PBS containing 2% bovine serum albumin (BSA) for 1 hour at room temperature. 50 mM glycine solution (pH 7.4) was added to reduce aldehyde-related auto-fluorescence. As a washing/dilution solution 2% gelatin in PBS was used. Cells were incubated with polyclonal GFP antibody (ab290) for two hours, followed by incubation with Alexa Fluor 488-conjugated secondary antibody (Invitrogen) for 1 hour at room temperature. Nuclei were visualized using 4',6-diamidino-2-phenylindole (DAPI). Images were acquired using an Axioskop2 CLSM microscope (Zeiss) (63x magnification lenses, N.A. 1.4). Post-acquisition image adjustments were performed using LSM Image Browser software (Zeiss).

Histology

For cryosections, heart tissues were collected in Tissue-Tec embedding medium

(Sakura), snap-frozen in a liquid nitrogen bath and sectioned at 10 μm. For paraffin sections, various mouse tissues were isolated and fixed in 4% PFA in PBS at 4 °C overnight. Next, tissues were dehydrated through an ethanol series, embedded in paraffin and sectioned at 5 μm.

Immunohistochemistry

Cryosections were fixed for 30 minutes in 4% PFA in PBS and washed three times for 5 min in PBS. Sections were permeabilized in 0.2% Triton X-100 (1 h), blocked in 2% BSA (30 min) and incubated overnight in the presence of 10% normal goat serum (NGS) with primary GFP ab290 or GFP (B-2) antibody. After blocking again with 2% BSA (30 min) secondary labeling was performed with the appropriate fluorescein isothiocyanate (FITC)-conjugated antibodies (2 hours in the presence of 10% NGS). Finally, sections were mounted in Vectashield (Vector Laboratories). Nuclei were visualized using DAPI staining. PBS was used to wash sections between the subsequent incubations.

After deparaffinization with xylene and rehydration with an ethanol series, paraffin-embedded sections were microwave heated in 0.01M citrate buffer (pH 6.0) to retrieve the antigen. Slides were blocked with 10% normal goat serum and incubated for 2 hours with primary GFP ab290 antibody. Quenching of endogenous peroxidase activity was performed by incubating sections in 1.5% H_2O_2. To detect primary antibody the HRP-conjugated goat antibody (DAKO) was used and the diaminobenzidine (DAB) reaction was performed. Additionally, sections were counterstained with haematoxylin.

The cryosections were analyzed using an Axioskop2 CLSM microscope (Zeiss) (40x magnification lenses, N.A. 1.4). Post-acquisition image adjustments were performed using LSM Image Browser software (Zeiss). The paraffin sections were analyzed and photographed with an Olympus BX40 light microscope.

Multiparameter FACS analysis

Bone marrow was isolated from femoral and tibial bones. The bone marrow was expelled by flushing bones with PBS supplemented with 10% FBS. Collected bone marrow was filtered through a 70-μm cell strainer and subsequently centrifuged at 1000 rpm for 10 min. Cells were counted and 5 x 10^6 cells were used to perform staining for various cell surface markers. Cells were incubated with the following antibodies (each labeled with a specific fluorescent group): Sca1-PE/c-Kit1-APC or CD48-PE/CD150-APC, both in combination with a lineage cocktail (containing B220, CD4, CD8, CD3, Gr-1, Mac-1, and TER119), for 30 minutes at 4 °C. To detect lineage-specific antibodies, the PerCP-Cy55-conjugated secondary antibody was applied. Each antibody was used at 1:200 dilution and cells were washed one time with PBS/FBS between incubations. To mark dead cells, Hoechst was added. Samples were analyzed on a BD FACS Aria II flow cytometer using CellQuestTM (BD, Sunnyvale, CA) or FlowJo™ (Tree Star Inc., Ashland, OR) research software.

Results

Generation of the GFP-Foxo4 knock-in mice

To analyze the expression pattern of FOXO4 we generated a *Foxo4* knock-in allele (*Foxo4*^{KI}) in which GFP-Foxo4 is expressed instead of FOXO4 (Figure 1A). We have used a strategy similar

to one applied previously to generate GFP-CLIP170 knock-in mice [18]. In brief, we inserted a GFPloxP–pMC1neo-loxP cassette into the ATG translation initiation codon in the first exon of *Foxo4*, using homologous recombination in embryonic stem (ES) cells. Southern blot analysis confirmed correct targeting of the *Foxo4* gene in the ES cells (Figure 1B). The targeting efficiency was about 1%. In addition, karyotype analysis of positive ES cell clones was performed to ensure proper chromosome numbers. Subsequently, two positively targeted ES cell clones were injected in to recipient blastocysts to generate chimeric animals. These chimeras were mated with C57BL/6 mice to allow germline transmission and obtain a mouse line carrying the GFPloxP– pMC1neo-loxP cassette. The genotype of this F1 generation was verified by PCR analysis (Figure 1C).

The neomycin-resistance gene-containing casette (neo^r) abrogates the endogenous *Foxo4* gene, moreover neo^r transcription is antisense with respect to the *Foxo4* gene. Consequently, mice positive for the GFP-loxP–pMC1neo-loxP cassette are knockouts for *Foxo4* and therefore referred to as $Foxo4^{KO}$ in the text. To generate $Foxo4^{KI}$ mice, we crossed $Foxo4^{KO}$ mice with transgenic *CAG-Cre* mice, which express the Cre recombinase ubiquitously, resulting in deletion of the loxP flanked neo^r gene in virtually all tissues. The removal of the neo^r gene together with one loxP site was verified by PCR analysis and Southern blot analysis (Figure 1C and D). Subsequently, $Foxo4^{KI}$ and $Foxo4^{KO}$ mice were backcrossed with C57BL/6 mice for ten generations.

GFP-FOXO4 is a functional protein

To analyze whether GFP-FOXO4 is functional, we performed three experiments. First, we performed a luciferase reporter assay using the FOXO-responsive 6xDBE-luciferase construct where we compared the activity of GFP-FOXO4 to HA-FOXO4 (Figure 2A). Ectopic expression of both versions of FOXO4 induced expression of luciferase to comparable levels. Thus, we conclude that linking GFP to FOXO4 does not interfere with FOXO4 functions in a luciferase reporter assay.

To further explore the activity of GFP-FOXO, we analyzed the endogenous expression of $p27^{kip1}$, a well-established FOXO transcriptional target gene (Figure 2B). We observed that $p27^{kip1}$ protein levels were strongly induced by exogenous expression of GFP-FOXO4 meaning that GFP-FOXO4 is able to increase the rate of gene expression.

Our third approach to demonstrate the functionality of GFP-FOXO4 was to analyze its ability to translocate out of the nucleus in response to growth factors. Therefore, we cultured U2OS cells in serum free conditions to accumulate GFP-FOXO4 in the nucleus and we observed that upon treatment of these cells with insulin, GFP-FOXO4 was mainly localized in the cytoplasm (data not shown). Thus, GFP-FOXO4 is sensitive to PKB-dependent regulation. Taken together, we conclude that GFP-FOXO4 is a functional transcription factor.

Verification of the expression of the GFP-Foxo4 chimeric protein

To confirm the expression of the GFP-Foxo4 chimeric protein we derived MAFs from wild-type and homozygous $Foxo4^{KI}$ mice. Subsequently, RT-PCR analysis proved the presence of mRNA encoding GFP-Foxo4 only in the cells derived from $Foxo4^{KI}$ mice (Figure 3A). In addition, we analyzed the cDNA by

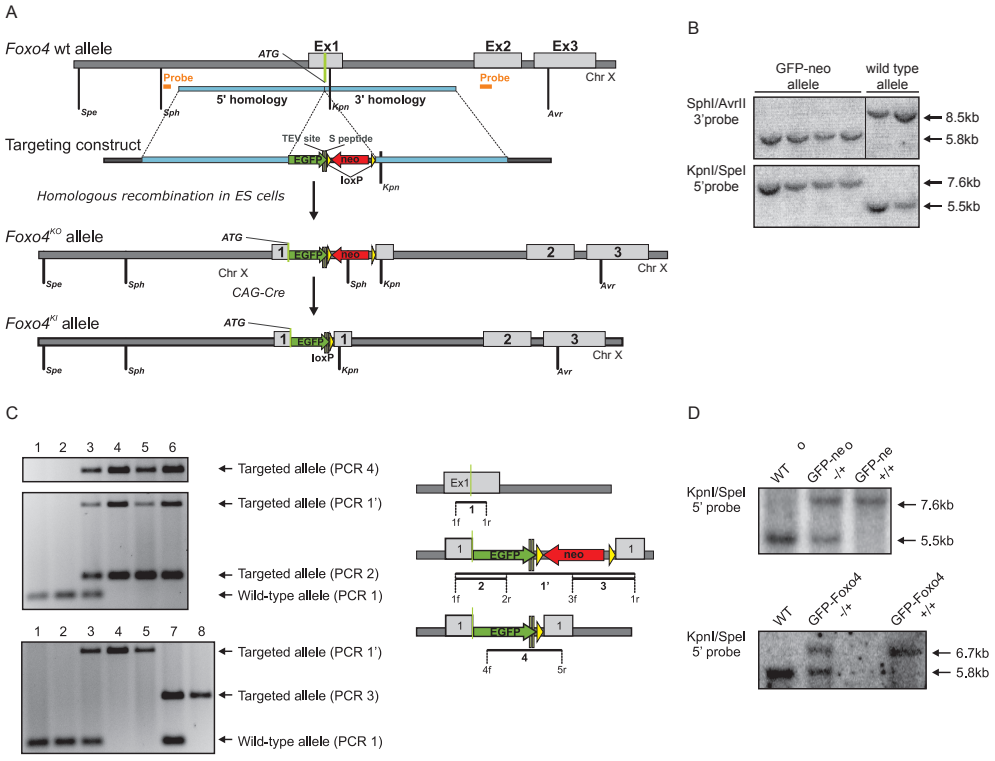


Figure 1. Characterization of *Foxo4* knock-in allele
(A) Murine *Foxo4* locus and gene targeting construct. Exons of the *Foxo4* gene (solid boxes) are numbered. Chr X indicates chromosome X. The Kpn/Spe restriction enzyme fragment hybridizes to the external 5′ probe; The Sph/Avr restriction enzyme fragment hybridizes to the external 3′ probe. Southern blot probes are shown below *Foxo4* gene. The targeting construct with loxP sites (small triangles) flanking a PMC1-neomycin cassette (*neo*) and GFP gene is shown with homologous regions. The *neo* gene is transcribed antisense to the *Foxo4* gene. The homologous recombination generates a $Foxo4^{KO}$ allele. Cre-mediated excision leaves GFP and one loxP site. As these are in frame with the Foxo4 encoding sequence, the $Foxo4^{KI}$ allele is generated. **(B)** Southern-blot analysis of the gene targeting event showing four positive ES clones. Southern-blot analysis of genomic DNA derived from wild-type (wt) and targeted ES cells (*Foxo4-T*) and digested with SphI/AvrII (upper blot) or KpnI/SpeI (lower blot). The 5′ external probe detects fragments of 7.6 kb (wild type allele) and 5.5 kb (*Foxo4-T* allele) and the 3′ external probe detects fragments of 5.8 kb (wild type allele) and 8.5 kb (*Foxo4-T* allele). **(C)** PCR analysis of wild-type, $Foxo4^{KI}$ and $Foxo4^{KO}$ alleles. Genomic tail DNA of wild-type, heterozygous and homozygous $Foxo4^{KI}$ and $Foxo4^{KO}$ mice was subjected to PCR with the indicated cocktails of primers, which yielded the expected fragments in the different genotypes. The sex and the genetic background of screened mice were as followed – wild-type mice: (1 – male, 2 – female); $Foxo4^{KI}$ mice: (3 - female$^{+/-}$, 4 - female$^{+/+}$, 5, 6 - male$^{+/y}$); $Foxo4^{KO}$ mice: (7 - female$^{+/-}$, 8 - male$^{+/y}$). The positions of PCR primers are indicated (right panel). **(D)** Southern-blot analysis of genomic tail DNA digested with KpnI and SpeI. The blot was hybridized with 5′ external probe, which detects fragments of 5.8 kb (wild type allele) and 6.7 kb ($Foxo4^{KI}$ allele) and 8.5 kb ($Foxo4^{KO}$ allele).

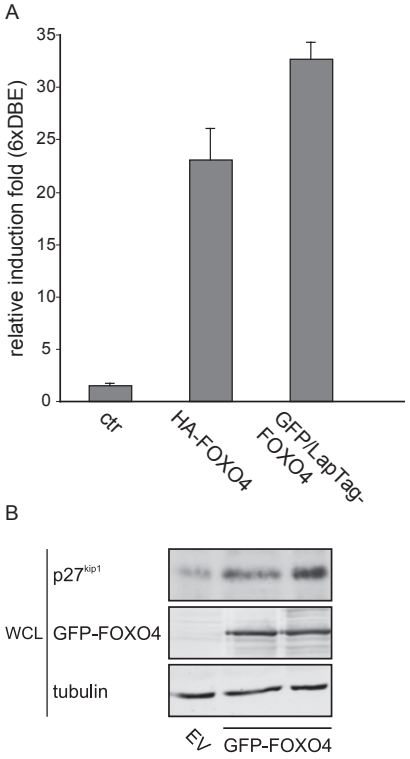


Figure 2. Functionality of GFP-FOXO4
(A) GFP-FOXO4 induces expression of luciferase gene in the dual reporter assay. A14 cells were transfected with FOXO-responsive 6xDBE-luciferase and Tk-*Renilla* constructs together with the indicated FOXO4 constructs. The expression of HA-FOXO4 and GFP-FOXO4 in whole cell extracts was verified by immunoblotting using the FOXO4 antibody. Representative data are shown as a mean ± s.d. of triplicates. **(B)** GFP-FOXO4 induces expression of $p27^{kip1}$. Western blot analysis of $p27^{kip1}$ expression of puromycin-selected HEK293T cells expressing the indicated proteins

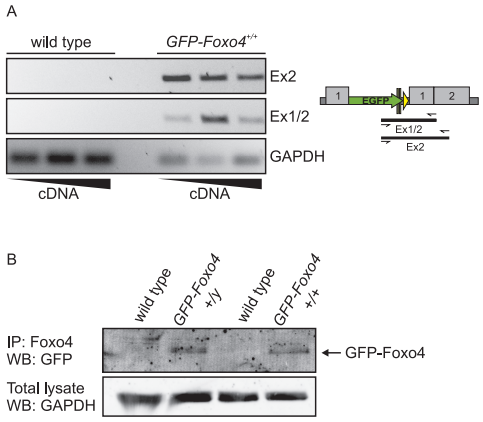


Figure 3. GFP-Foxo4 expression in mouse adult fibroblasts
(A) RT-PCR analysis of mRNA from MAFs. To verify the expression of GFP-Foxo4 total mRNA was isolated from wild-type and *Foxo4*^{KI} MAFs. Next, mRNA was copied to cDNA by reverse transcriptase and the PCR was performed using GFP-forward and Foxo-reverse primers. The positions of the PCR primers are indicated (right panel). **(B)** Western blot analysis of MAFs. Cell lysates from wild type and *Foxo4*^{KI} MAFs were subjected to immunoprecipitation using FOXO4-N19 antibody. Immunoprecipitated fractions were analyzed by SDS-PAGE and immunoblotting using the monoclonal GFP antibody. GFP-Foxo4$^{+/Y}$ indicates homozygous male and GFP-Foxo4$^{+/+}$ indicates homozygous female.

direct sequencing. Moreover, utilizing an anti-Foxo4 antibody for immunoprecipitation and a GFP antibody for detection, a protein of ~90kDa was visualized from cellular extracts of MAFs derived from the *Foxo4*^{KI} but not from wild-type mice (Figure 3B). However, we were able to detect this band on Western blot only when using an anti-GFP antibody but not with several Foxo4 antibodies, possibly due to poor quality of the latter antibodies, or their low affinity towards mouse Foxo4. For that reason we were also not able to compare the expression levels of Foxo4 and GFP-Foxo4. Nevertheless, we conclude that GFP-Foxo4 is expressed in the *Foxo4*^{KI} mouse line.

Determination of the expression of GFP-Foxo4 in mouse tissues

We attempted to visualize the expression of GFP-Foxo4 in live MAFs using confocal microscopy (Figure 4A). However, we did not observe a distinctive GFP-Foxo4 signal in *Foxo4*^{KI} MAFs compared to cells derived from wild-type mice. Thus, we performed an analysis

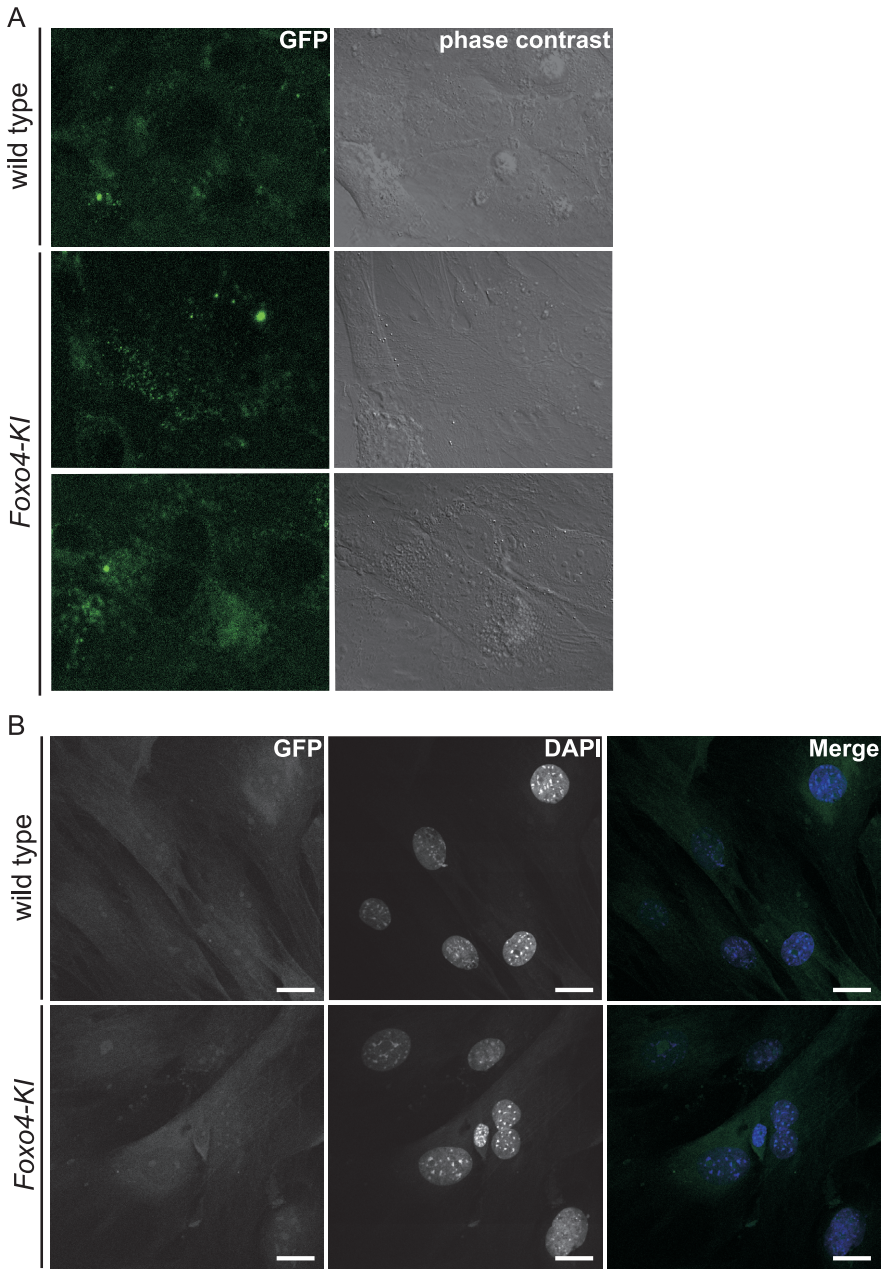


Figure 4. Imaging of GFP-Foxo4 expression in mouse adult fibroblasts
(A) Live imaging of GFP-Foxo4 in wild-type and Foxo4KI MAFs. The expression of GFP-Foxo4 was analyzed using confocal microscopy. Wild-type cells were used to determine the autofluorescence background signal. (B) Immunofluorescence analysis of wild-type and Foxo4KI MAFs. Cells were grown on coverslips and stained with GFP antibody to detect the expression of GFP-Foxo4. In the merge, anti-GFP staining is shown in green. Wild type cells were used to determine the autofluorescence background signal. Scale bars (all images in B), 20 μm.

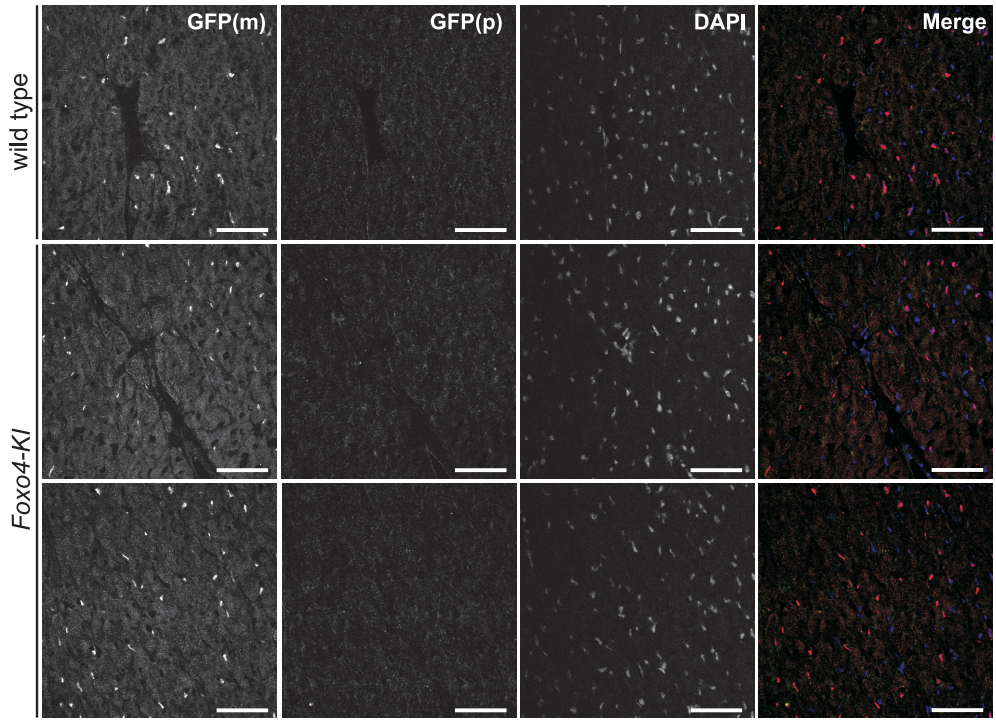


Figure 5. The expression of GFP-Foxo4 in heart tissue
Immunohistochemical analysis of GFP-FOXO4 expression in mouse heart tissue. Heart cryosections from wild-type and *Foxo4*^{KI} mice were incubated with two different GFP antibodies and counterstained with DAPI to visualize nuclei. In the merge, anti-GFP staining is shown in green for polyclonal anti-GFP antibody and in red for monoclonal anti-GFP antibody. Scale bars (all images) 50 μm.

on fixed cells (Figure 4B). Despite additional staining of these cells with anti-GFP antibody to amplify the signal, we were still not able to distinguish any GFP-Foxo4 specific signal from the background staining. Since it is possible that MAFs express FOXO4 at low levels we isolated several tissues from wild-type and *Foxo4*^{KI} mice. Subsequently, we performed staining of frozen tissue sections using anti-GFP antibody to visualize expression of GFP-Foxo4 in mouse heart tissues (Figure 5). In parallel to the frozen sections, paraffin-embedded sections were prepared and analyzed by immunohistochemistry using anti-GFP antibody (Figure 6A-C). Nevertheless, we were not able to detect any GFP-Foxo4 specific staining in both frozen and paraffin-embedded sections. Therefore, we conclude that GFP-Foxo4 is expressed at very low levels even in tissues such as muscle and heart, which have previously been shown to contain high FOXO4 mRNA levels. We conclude that the expression levels of GFP-Foxo4 are below the detection limit of our assay

Expression of GFP-Foxo4 in mouse hematopoietic cells

Since Foxo proteins have been shown to perform multiple functions in mouse hematopoietic cells and the immune system, we analyzed the expression of GFP-Foxo4 in these cells. We isolated bone marrow (BM) from the tibias and

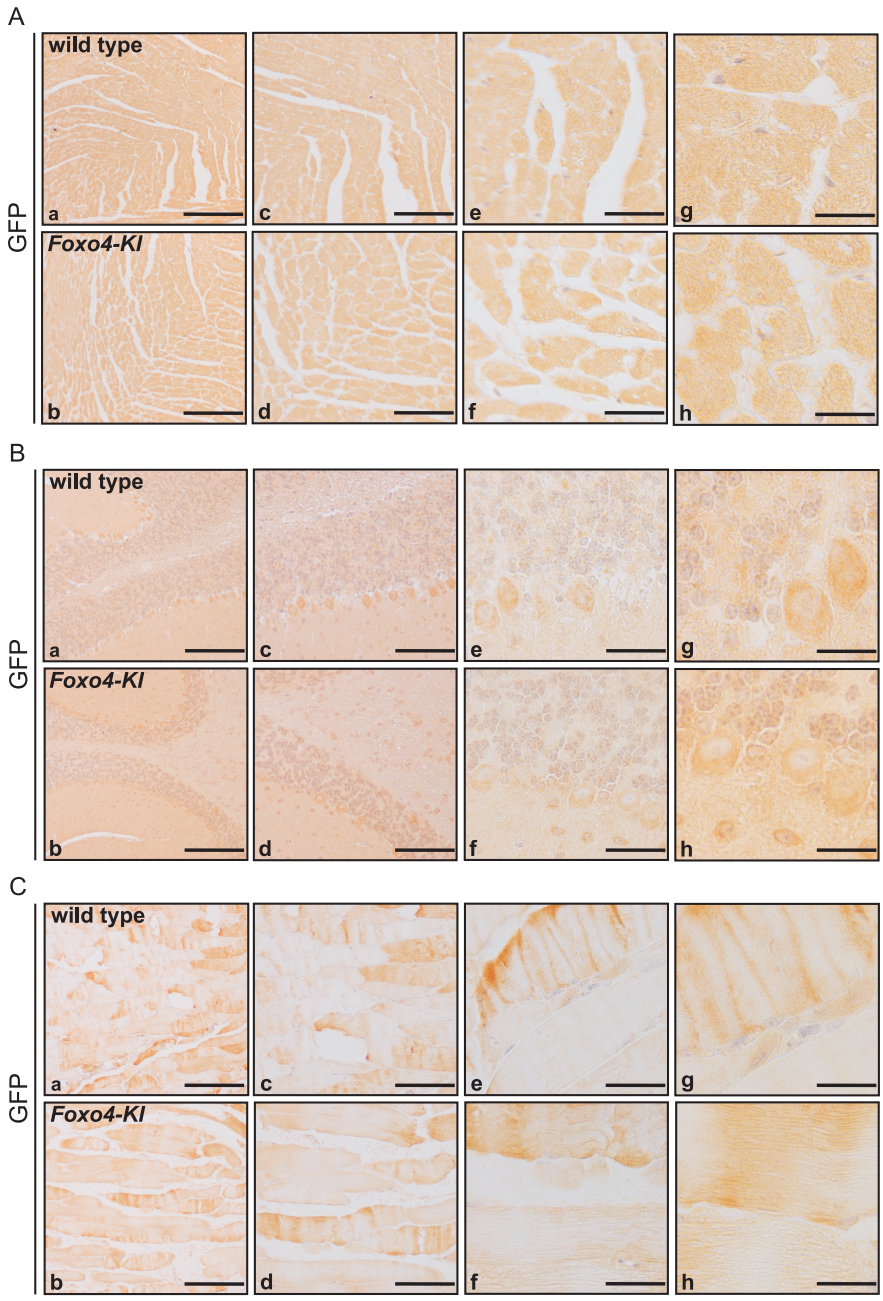
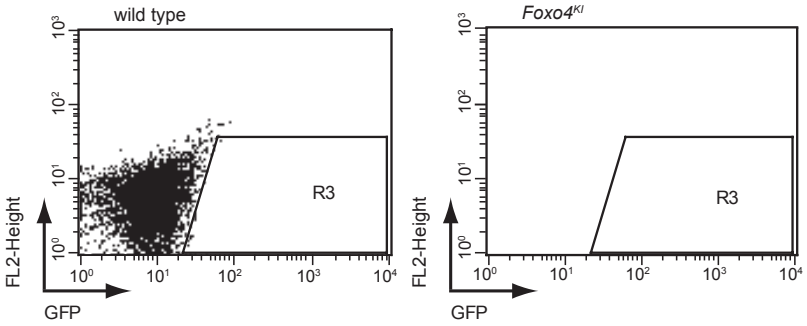


Figure 6. The expression of GFP-Foxo4 in various mouse tissues
(A-C) Immunohistochemical analysis of GFP-Foxo4 expression in mouse brain, heart and muscle tissues. Paraffin-embedded heart (A), brain (B) and muscle (C) sections from wild-type and Foxo4^{KI} mice were incubated with the polyclonal anti-GFP antibody and counterstained with haematoxylin. Left panels (**a** and **b**) show a low magnification view, whereas middle and right panels represent high magnification views. Scale bars for a, b - 200 μm; c,d - 100 μm; e,f - 50 μm; g,h - 20 μm.



Region	% Gated	% Total
R1	100.00	10.92
R2	100.00	10.92
R3	0.24	**0.03**

Region	% Gated	% Total
R1	100.00	10.09
R2	100.00	10.09
R3	2.85	**0.29**

Figure 7. GFP-Foxo4 expression in bone marrow from adult mice
(A) Flow cytometric analysis of mouse bone marrow. Total bone marrow cells of WT mice and *Foxo4*^{KI} mice were isolated, stained with 7 AAD solution to discriminate dead cells from live cells, and were analyzed by flow cytometry for GFP-Foxo4 expression.

femurs of 10-12 weeks old mice and examined the expression of GFP-Foxo4 by flow cytometry (Figure 7 and data not shown). We were able to identify a small population of GFP-positive cells. This was specific for bone marrow as we could not detect any GFP-positive cells in several other lymphoid tissues. Since the population of cells expressing GFP-Foxo4 was very small, we hypothesized that GFP-positive cells could represent hematopoietic stem cells and progenitors (HSPCs). Since HSCs can be identified using characteristic cell-surface markers [20], we further analyzed whether cells expressing GFP-Foxo4 are indeed stem cells (Figure 8A). Therefore, we stained bone marrow for the Sca-1 and c-Kit markers, which identify HSPCs, and for markers of mature hematopoietic cell lineages, referred to as lineage-positive (Lin$^+$), which collectively represent cells committed to major hematopoietic lineages including T lymphocytes, B lymphocytes, monocytes/macrophages, granulocytes, and erythrocytes. The lineage-negative (Lin$^-$) cell population was subdivided into four populations consisting of cells positive for Sca-1 and c-Kit, referred to as double positive (Lin$^-$/ Sca-1$^+$/c-Kit$^+$ or LSK), cells positive for only Sca-1 and c-Kit (Lin$^-$/Sca-1$^-$/c-Kit$^+$ or Lin$^-$/Sca-1$^+$/c-Kit$^-$ respectively) and a double negative cell population (Lin$^-$/Sca-1$^-$/c-Kit$^-$). Using FACS, we examined the expression of GFP-Foxo4 in these five populations of hematopoietic cells (Figure 8B). We were able to detect GFP-Foxo4 in the LSK population of cells derived from *Foxo4*^{KI} mice but not from their wild-type littermates. In addition, GFP-Foxo4 was expressed in cells positive only for Sca-1 or c-Kit staining. Strikingly, the Lin$^+$ cells appeared to be negative for GFP-Foxo4 expression.

The LSK compartment of the bone marrow contains progenitors in addition to HSCs. To determine the expression of GFP-Foxo4 specifically in HSCs we identified the Lin$^-$/CD150$^+$/CD48$^-$ cells, as it has been reported previously

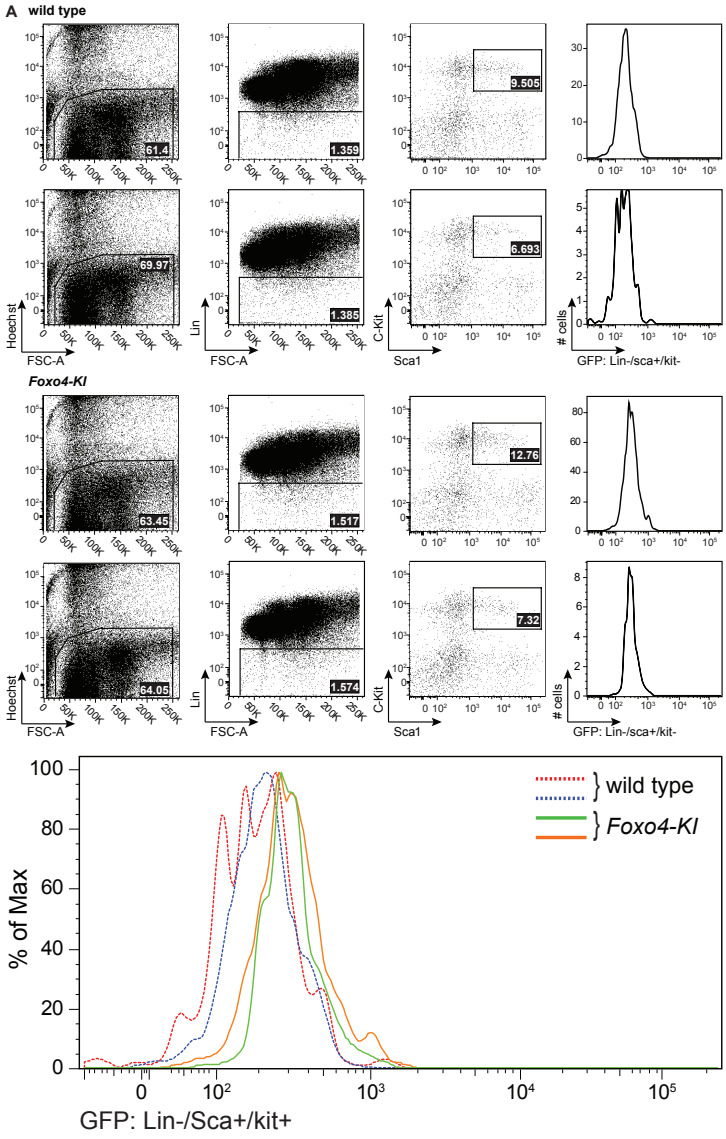
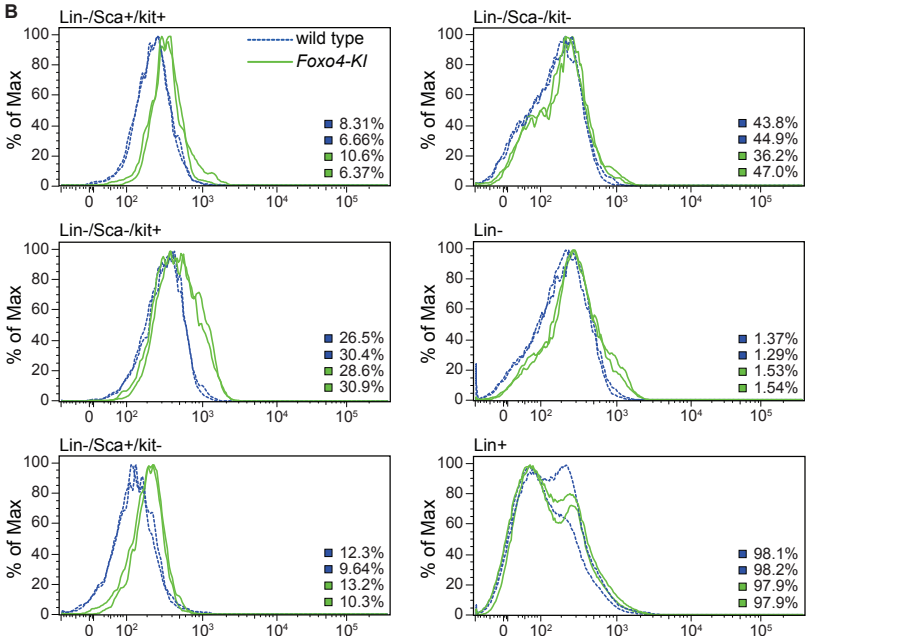


Figure 8. Expression of GFP-Foxo4 in the LSK population of cells from bone marrow of adult mice
(A) Identification of GFP-Foxo4 positive cells in the bone marrow from young adult mice. Cells that stained in the negative to low range for lineage markers were selected and analyzed for expression of c-kit/Sca1. Next, the LSK population was analyzed for expression of GFP-Foxo4 (lower panel). **(B)** Next several other populations of cells that have been identified based on the differences in the expression of surface markers were analyzed for expression of GFP-Foxo4. Expression profiles of surface markers are shown as dot plots and the percentage of cells within quadrants of gates are given. GFP-Foxo4 profiles of identified populations from wild-type mice in comparison to populations from *Foxo4*^{KI} mice are displayed as histograms.



that HSCs specifically express CD150 receptor whereas expression of CD48 is restricted to progenitors. However, analysis of those cells did not reveal any expression of GFP-Foxo4 (Figure 9). Taken together this suggests that GFP-Foxo4 is mainly expressed in multipotent progenitors (MPP) and seems to be excluded from HSCs.

Since it has been suggested previously that expression of Foxo4 might increase with the age of the animal, we performed the same multiparameter flow cytometry analysis of the bone marrow isolated from mice of ~64 weeks old (Figure 10A and B). We detected expression of GFP-Foxo4 in all four Lin- populations of cells isolated from *Foxo4*^{KI} mice but not from a *Foxo4*^{KO} or from wild-type mice. However, it appeared that the expression of GFP-Foxo4 in the LSK was reduced whereas it was increased in the double negative cell population. Thus, we did not observe an increased expression of GFP-Foxo4 in old mice but rather a shift from one bone marrow population to another. Therefore, we conclude that GFP-Foxo4 is expressed in the lineage-negative compartment of adult bone marrow isolated from both young and old mice.

Discussion

Recent *in vivo* studies have shown that Foxo1 and Foxo3a transcription factors exhibit specific physiological functions in the context of the whole organism, being the regulation of metabolism and stem cells maintenance, respectively [21, 22]. However, even though Foxo4 has been proven to play a significant role in the regulation of cellular functions in *in vitro* studies, the *in vivo* data indicate thus far that besides being a redundant tumor suppressor [5], Foxo4 does not have any distinct function in the regulation of physiological processes [6]. Here, we attempted to elucidate the spatial and temporal expression pattern

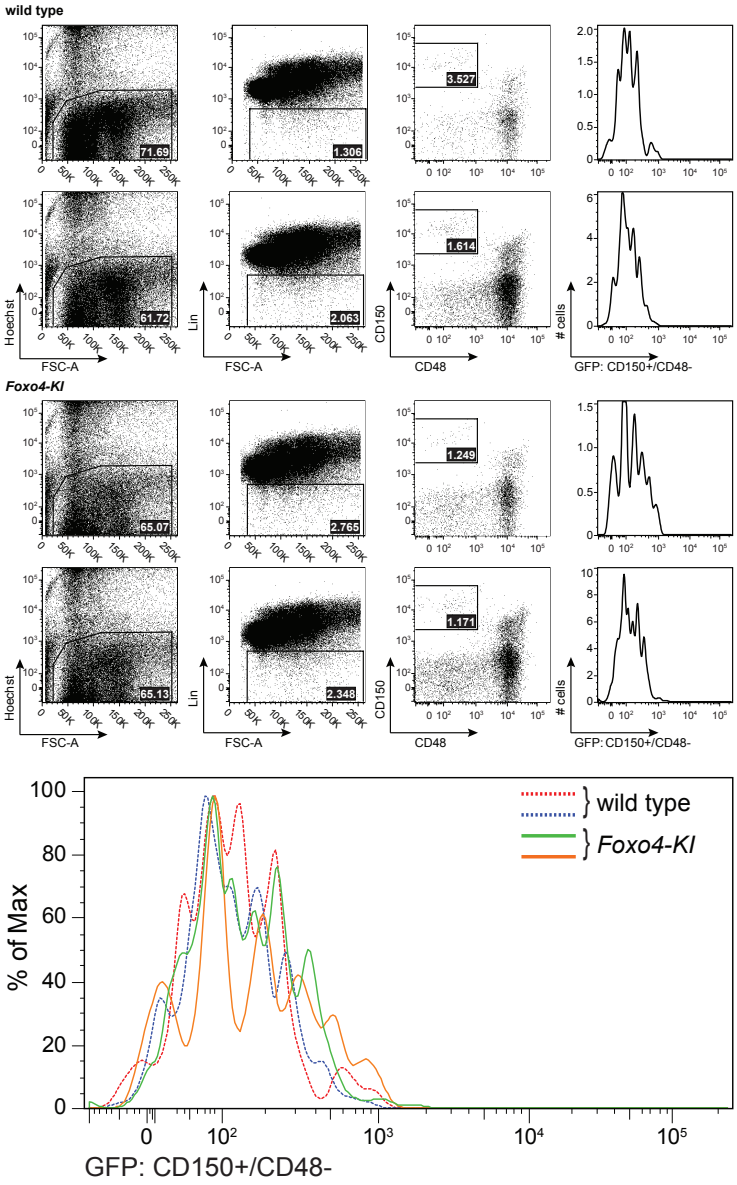


Figure 9. Expression of GFP-Foxo4 in the CD150^{+}/CD48^{-} population of cells from bone marrow of adult mice

(A) Identification of GFP-Foxo4 positive cells in bone marrow from young adult mice. Cells that stained in the negative to low range for lineage markers were selected and analyzed for expression of CD150/CD48 surface markers to identify populations of hematopoietic stem cells and progenitors. Next, the population of cells positive for expression of CD150 and negative for expression of CD48 marker was analyzed for the presence of GFP-Foxo4. Expression profiles of surface markers are shown as dot plots and the percentage of cells within quadrants of gates are given. GFP-Foxo4 profiles of identified populations from wild-type mice in comparison to populations from *Foxo4^{KI}* mice are displayed as histograms.

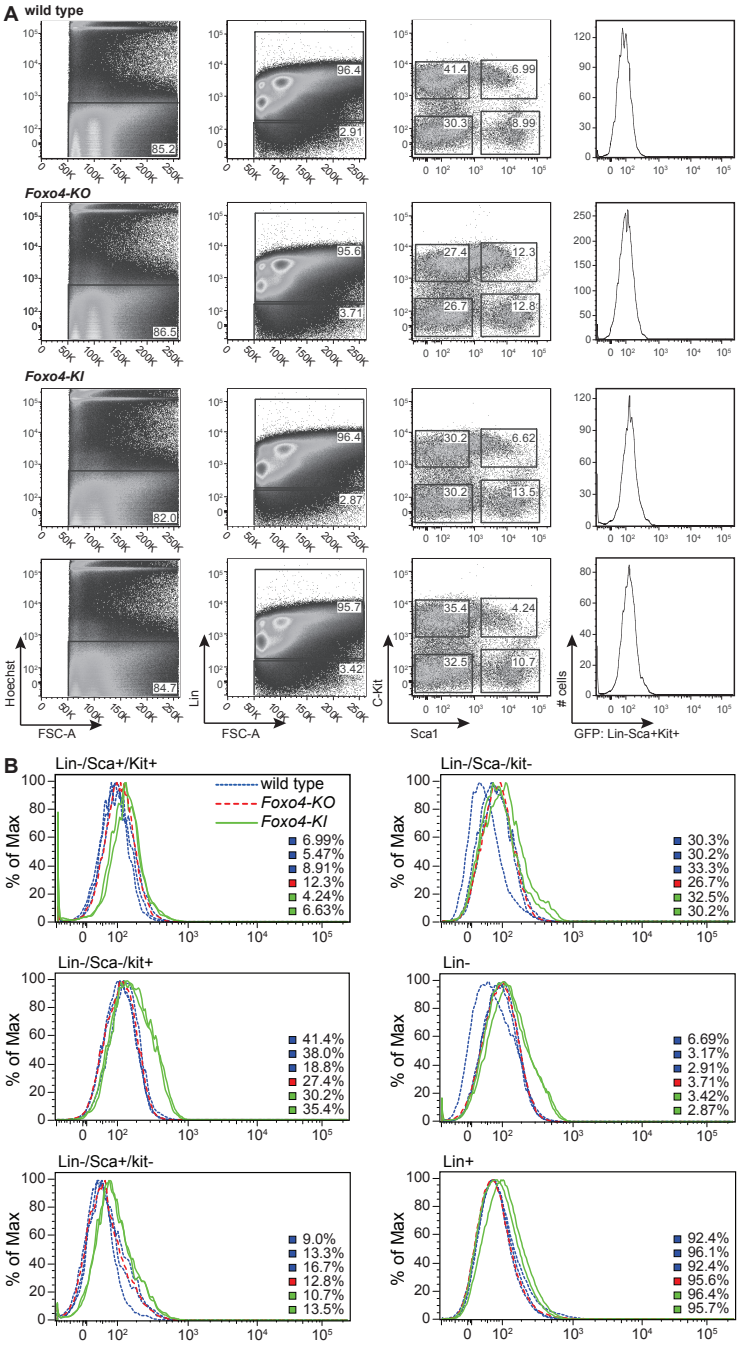


Figure 10. Expression of GFP-Foxo4 in the LSK population of cells from the bone marrow of aged mice
(A,B) Identification of GFP-Foxo4 positive cells in the bone marrow from aged mice. The cells were characterized as in (Figure 8) and additionally bone marrow from *Foxo4*^{KO} mouse was included.

of Foxo4 to get a better understanding of its relevance for the physiology of the whole organism.

A number of studies indicate that the mRNAs encoding Foxo1, Foxo3a and Foxo4 are expressed in virtually all tissues, however at variable levels. Foxo1 mRNA is especially abundant in adipose tissues, Foxo3a mRNA is highly expressed in the brain, whereas Foxo4 mRNA is most abundant in heart and skeletal muscles. In addition, expression of Foxo6 mRNA is restricted to the developing brain [4]. However, there are quite some discrepancies among different studies [23, 24]; therefore it is difficult to draw a final conclusion about the differential and overlapping expression patterns of different Foxo proteins in distinct tissues.

Moreover, regardless of the overall positive correlation between mRNA and protein expression levels, in many cases this correlation is moderate and even variable. This indicates that mRNA levels are not always correctly representing protein expression levels [25]. This is most probably due to multiple levels of posttranscriptional regulation of protein expression.

Besides mRNA analysis, the expression of Foxo proteins was verified using immunohistochemical approaches in some studies. However, these studies were restricted to analysis of Foxo expression in the human uterus [26]. Therefore, we generated Foxo4 knock-in mice in which GFP-Foxo4 is expressed instead of Foxo4. This gives the opportunity to follow the expression of Foxo4 at various time points of animal life and in effectively all mouse tissues. The *Foxo4*^{KI} mice are morphologically and histochemically indistinguishable from wild-type littermates. We performed immunohistochemical analysis of various mouse tissues using anti-GFP antibody; however, we did not observe any GFP-Foxo4 specific staining, even in the tissues that were characterized before by relatively high Foxo4 mRNA levels including heart and skeletal muscles. This is rather unexpected although it can simply mean that *in vivo* Foxo4 is expressed at very low levels. Nevertheless, we cannot exclude that GFP-Foxo4 is less stable than Foxo4 and therefore its expression level is lower. However, due to the lack of antibodies that are able to recognize mouse Foxo4 by Western blot analysis, we could not address this issue directly. Additionally, since the *in vivo* expression levels of Foxo proteins were not analyzed before, we have no references for comparison.

Assuming that Foxo4 is expressed at very low levels in mouse tissues, would this explain the lack of any phenotype in the *Foxo4* knockout mice? mRNA analysis suggests that Foxo1 and Foxo3a are generally expressed at much higher levels in comparison to Foxo4 [23]. Therefore, simply due to their relative high abundance they might be the ones that will predominantly occupy the promoters of target genes. Thus, in this hypothetical situation, the lack of Foxo4 expression would have no significant impact on the regulation of any physiological functions. Simultaneously, the functional diversity of Foxo1 and Foxo3a in mammals is possibly caused by different affinity of these transcription factors towards various target genes or binding partners and that is why these specific functions cannot be fulfilled by other Foxos.

Foxo transcription factors have been shown to play an important role in the regulation of stress resistance [27, 28]. Thus, it is possible that under stress conditions the expression of Foxo4 increases, thereby supporting its specific functions in

the activation of cellular stress responses. Therefore, it would be interesting to investigate the expression of GFP-Foxo4 in the knock-in mice after exposure of these mice to various stresses.

Importantly, there is a functional redundancy among Foxos with respect to their tumor suppressive functions. Thus, in the absence of Foxo1 and Foxo3a the expression of Foxo4 is sufficient to inhibit the development of tumors [5]. This indicates that all Foxos have the same affinity towards target genes, whose transactivation is crucial for the inhibition of tumor development. Plausibly, this is a safety mechanism to ensure that even if the expression or activity of two Foxo proteins is lost, the one that is still left is sufficient for the suppression of tumor growth.

A significant number of studies during the last few years revealed that Foxo transcription factors play crucial roles in the maintenance of immune cell homeostasis and the HSC pool [29]. Therefore, we also analyzed expression of GFP-Foxo4 in bone marrow as well as several lymphoid tissues obtained from wild-type and *Foxo4*^{KI} mice. Interestingly, using flow cytometry we could detect a small population of GFP-positive cells in the bone marrow of *Foxo4*^{KI} mice but not wild-type mice. Concurrently, we did not detect any GFP signal in the analyzed lymphoid tissues. This encouraged us to further identify, which type of cells expresses GFP-Foxo4. Since the population of GFP-positive cells was very small and absent in peripheral lymphoid tissues, we hypothesized that those cells represent HSPCs as they are especially abundant in bone marrow. Using multiparameter flow cytometry we identified several compartments of bone marrow and examined these for the expression of GFP-Foxo4. To define a population of HSPCs we labeled cells with antibodies recognizing markers of mature hematopoietic cell lineages in combination with antibodies specific for c-Kit and Sca-1, which are exclusively expressed by pluri/multi-potent cells of the bone marrow. Thus, we were able to identify two different compartments containing lineage-positive cells (Lin$^+$), which correspond to mature blood cells, and lineage-negative cells (Lin$^-$), which represent HSPCs. Since Lin$^-$ cells differentially express c-Kit and Sca-1 we subdivided the Lin$^-$ population of cells into four groups corresponding to double negative (no expression of Sca-1 and c-Kit), single positive (expressing only Sca-1 or c-Kit) and double positive (expressing both Sca-1 and c-Kit). The double positive population of cells is often referred to as LSK.

Interestingly, we were able to detect expression of GFP-Foxo4 only in the Lin$^-$ compartment of bone marrow from young adult *Foxo4*^{KI} mice but not in the Lin$^+$ compartment. This suggests that expression of GFP-Foxo4 is restricted to pluri/multi-potent cells of the bone marrow. Therefore, it would be interesting to analyze each lineage separately. Further analysis of the Lin$^-$ compartment revealed that GFP-Foxo4 is expressed in all subpopulations except Sca-1/c-Kit double negative cells. This again indicates that expression of GFP-Foxo4 is specific for HSPCs.

Since LSK cells are highly enriched for hematopoietic reconstituting activity, this bone marrow compartment contains several types of progenitor cells in addition to long-term HSCs. In fact only ~10% of the LSK cells are truly long-term HSCs. Therefore, to more accurately define the HSCs pool, we sorted bone marrow cells based on the HSC-marker marker CD150, and the

B lineage and myeloerythroid lineage progenitor marker CD48. However, this analysis did not reveal any expression of GFP-Foxo4 and we therefore assumed that expression of GFP-Foxo4 is associated with progenitors and does not take place in HSCs.

Since Foxo proteins are implicated in the regulation of longevity we were wondering whether expression of Foxo4 increases with the age of the animal. Therefore, we performed the same FACS analysis on bone marrow obtained from mice older than 60 weeks. Indeed, we observed the appearance of GFP-positive cells in both the Lin^+ and Lin^- compartment. On the other hand, the expression of GFP-Foxo4 seemed to decrease in the LSK population. This indicates that there is a shift in expression of GFP-Foxo4 between different compartments of bone marrow in aged animals. Consequently, it might be related to the changes in the HSCs during their aging.

Several studies indicate that the quality of HSCs declines during aging, which is reflected by the decreased repopulation capacity of these cells. In addition, this is associated with elevated numbers of HSCs in C57BL/6 aged mice and a decrease in the number of HSCs in other mouse strains including DBA/2 and Balb/c [30]. Nevertheless, the decline in HSC quality dominates over the increase in the number of HSCs in the C57Bl/6 strain. Since Foxo transcription factors have been shown to regulate HSC numbers and their self-renewal capacity, it is possible that reduced Foxo4 expression in aged HSCs might at least partially account for the decrease in the quality of these cells. Moreover, even though aged Foxo3a-null mice display a decrease in the frequency of LSK cells similarly to mice lacking all Foxos specifically in adult hematopoietic system, the loss of Foxo3a does not effect proliferation or apoptosis of those cells indicating that these processes might be regulated by other Foxos [15]. Thus, it is possible that expression of Foxo4 in LSK cells is relevant for the maintenance of normal functions of these cells. The age-related increase in the expression of GFP-Foxo4 in Lin^+ and Lin^- cells indicates that besides regulation of LSK cell homeostasis, Foxo4 plays a function in more mature hematopoietic cells.

Deficiency of Foxo proteins in the hematopoietic system has been associated with increased cellular ROS levels in HSPCs [12]. In addition, increased levels of ROS have been shown to impair self-renewal of HSCs as well as a significant shortening of their lifetime [29, 31, 32]. Therefore, the ability of Foxos to induce the expression of genes involved in detoxification of ROS is crucial for maintenance of the HSC (LSK) pool. Thus, the relatively high expression of GFP-Foxo4 in LSK cells observed in our experiments is in accordance with the protective role of Foxos. On the other hand cellular ROS levels in myeloid progenitors as well as terminally differentiated myeloid lineage cells are much higher than in HSCs, without being harmful to these cells [29]. Moreover, it has been suggested that the amount of ROS within hematopoietic cells positively correlates with their differentiation state [31, 32]. However, terminally differentiated myeloid cells represent one of the shortest-lived cells in mammals, indicating that sustained high ROS levels attenuate their lifespan. Nevertheless, it is possible that an age-related decrease in the quality of HSCs suppresses their ability to replenish blood cells as well as the accumulation of oxidative damage in the blood cells themselves would increase the demand

for protection of these cells. Thus, it is tempting to speculate that induction of Foxo expression in lineage-positive cells can be a part of a mechanism preserving blood cells in old animals. On the other hand, the appearance of GFP-Foxo4 in the Lin^- population might result from the age-related increase in the mobilization of HSPCs due to attenuation of their ability to adhere to stromal cells and thereby altered interaction with their niche [33, 34].

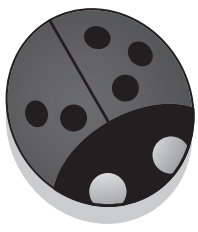
References

1. Greer, E.L. and A. Brunet, FOXO transcription factors at the interface between longevity and tumor suppression. Oncogene, 2005. 24(50): p. 7410-25.
2. van den Berg, M.C. and B.M. Burgering, Integrating Opposing Signals Toward Forkhead Box O. Antioxid Redox Signal.
3. Calnan, D.R. and A. Brunet, The FoxO code. Oncogene, 2008. 27(16): p. 2276-88.
4. Salih, D.A. and A. Brunet, FoxO transcription factors in the maintenance of cellular homeostasis during aging. Curr Opin Cell Biol, 2008. 20(2): p. 126-36.
5. Paik, J.H., et al., FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell, 2007. 128(2): p. 309-23.
6. Hosaka, T., et al., Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. Proc Natl Acad Sci U S A, 2004. 101(9): p. 2975-80.
7. Matsumoto, M., et al., Impaired regulation of hepatic glucose production in mice lacking the forkhead transcription factor Foxo1 in liver. Cell Metab, 2007. 6(3): p. 208-16.
8. Castrillon, D.H., et al., Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science, 2003. 301(5630): p. 215-8.
9. Lin, L., J.D. Hron, and S.L. Peng, Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. Immunity, 2004. 21(2): p. 203-13.
10. Su, H., N. Bidere, and M. Lenardo, Another fork in the road: Foxo3a regulates NF-kappaB activation. Immunity, 2004. 21(2): p. 133-4.
11. Freitas, A.A. and B. Rocha, Homeostasis of naive T cells: the Foxo that fixes. Nat Immunol, 2009. 10(2): p. 133-4.
12. Tothova, Z., et al., FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell, 2007. 128(2): p. 325-39.
13. Paik, J.H., et al., FoxOs cooperatively regulate diverse pathways governing neural stem cell homeostasis. Cell Stem Cell, 2009. 5(5): p. 540-53.
14. Renault, V.M., et al., FoxO3 regulates neural stem cell homeostasis. Cell Stem Cell, 2009. 5(5): p. 527-39.
15. Miyamoto, K., et al., Foxo3a is essential for maintenance of the hematopoietic stem cell pool. Cell Stem Cell, 2007. 1(1): p. 101-12.
16. Hoogenraad, C.C., et al., Targeted mutation of Cyln2 in the Williams syndrome critical region links CLIP-115 haploinsufficiency to neurodevelopmental abnormalities in mice. Nat Genet, 2002. 32(1): p. 116-27.
17. Szypowska, A.A., et al., Oxidative stress-dependent regulation of Forkhead box O4 activity by nemo-like kinase. Antioxid Redox Signal. 14(4): p. 563-78.
18. Akhmanova, A., et al., The microtubule plus-end-tracking protein CLIP-170 associates with the spermatid manchette and is essential for spermatogenesis. Genes Dev, 2005. 19(20): p. 2501-15.
19. Osoegawa, K., et al., Bacterial artificial chromosome libraries for mouse sequencing and functional analysis. Genome Res, 2000. 10(1): p. 116-28.
20. Challen, G.A., et al., Mouse hematopoietic stem cell identification and analysis. Cytometry A, 2009. 75(1): p. 14-24.
21. Chen, E. and T. Finkel, Preview. The Tortoise, the hare, and the FoxO. Cell Stem Cell, 2009. 5(5): p. 451-2.
22. Gross, D.N., M. Wan, and M.J. Birnbaum, The role of FOXO in the regulation of metabolism. Curr Diab Rep, 2009. 9(3): p. 208-14.
23. Furuyama, T., et al., Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. Biochem J, 2000. 349(Pt 2): p. 629-34.
24. Yang, Z., et al., An mRNA splice variant of the AFX gene with altered transcriptional activity. J Biol Chem, 2002. 277(10): p. 8068-75.
25. Guo, Y., et al., How is mRNA expression predictive for protein expression? A correlation study on human circulating monocytes. Acta Biochim Biophys Sin (Shanghai), 2008. 40(5): p. 426-36.
26. Lappas, M., et al., Expression and localisation of FoxO3 and FoxO4 in human placenta and fetal membranes. Placenta. 31(12): p. 1043-50.
27. Keizer, P.L., B.M. Burgering, and T.B. Dansen, Forkhead Box O as a Sensor, Mediator, and Regulator of Redox Signaling. Antioxid Redox Signal.
28. Storz, P., Forkhead Homeobox Type O Transcription Factors in the Responses to Oxidative Stress. Antioxid Redox Signal.
29. Tothova, Z. and D.G. Gilliland, FoxO transcription factors and stem cell homeostasis: insights from the hematopoietic system. Cell Stem Cell, 2007. 1(2): p. 140-52.
30. Waterstrat, A. and G. Van Zant, Effects of aging on hematopoietic stem and progenitor cells. Curr Opin Immunol, 2009. 21(4): p. 408-13.
31. Eliasson, P. and J.I. Jonsson, The hematopoietic stem cell niche: low in oxygen but a nice place to be. J Cell Physiol. 222(1): p. 17-22.
32. Jang, Y.Y. and S.J. Sharkis, A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. Blood, 2007. 110(8): p. 3056-63.
33. Xing, Z., et al., Increased hematopoietic stem

cell mobilization in aged mice. Blood, 2006. 108(7): p. 2190-7.
34. Geiger, H. and K.L. Rudolph, Aging in the lympho-hematopoietic stem cell compartment. Trends Immunol, 2009. 30(7): p. 360-5.

Chapter 5

General Discussion



Summary

FOXO transcription factors are downstream targets of IIS pathway; therefore, they are activated in response to nutrient deprivation and play an important role in the regulation of metabolism. Furthermore, the activity of FOXOs is stimulated by oxidative stress and due to their ability to transactivate several antioxidants, FOXO proteins are recognized as an important element of the cellular protective mechanisms against elevated levels ofreactive oxygen species (ROS). Simultaneously, they are capable of inducing cell cycle arrest, thereby providing time for the detoxifying processes to take place. Additionally, FOXOs can also trigger apoptosis, especially in certain cell types including hematopoietic cells and neurons, as for unknown reasons, it is apparently more beneficial to eliminate these cells under stress conditions. The mastery of FOXO to lower cellular ROS levels and restrict cell cycle progression enables them to function as tumor suppressors.

The engagement of FOXOs in the regulation of diverse biological processes is associated with precise governing of their activity. This is executed via multiple posttranslational modifications (PTMs), which together form a complex regulatory code. However, due to this complexity the PTM-dependent regulations as well as their functional outcomes are not always fully understood. Therefore, to obtain further insight into how different PTMs regulate FOXO functions under oxidative stress conditions we initiated the projects presented in Chapter 2 and 3.

In addition, it is evident now that besides several redundant functions, different FOXO proteins display distinct tissue- and cell-specific roles. Thus, in order to reveal FOXO4 specific functions in the context of a whole organism, we focused upon studies where the spatial and temporal expression pattern of FOXO4 has been examined. These are described in Chapter 4

NLK-mediated regulation of FOXO transcription factors.

Nemo-like kinase (NLK) is an evolutionarily conserved serine/threonine kinase, originally identified as a suppressor of β-catenin/ T-cell factor (TCF)-driven transcription [1-4]. Subsequently, it has been demonstrated that NLK inhibits the activity of several other transcription factors including Myb proteins, Notch and peroxisome proliferator-activated receptor γ (PPARγ) [5-8]. Interestingly, it has been shown that although the activation of NLK results in the inhibition of these proteins, it utilizes different mechanisms to exert its effect (Figure 1). For example, it has been demonstrated that even in case of closely related proteins like c-Myb and a-Myb, NLK suppresses their activity via two distinct mechanisms. In the case of c-Myb, NLK-dependent phosphorylation triggers degradation of c-Myb, whereas inhibition of a-Myb depends on the NLK-induced dissociation of the coactivator CBP [5, 6]. Furthermore, NLK has been shown to negatively regulate Notch-dependent transcriptional activation by decreasing the formation of its active ternary complex [7]. Finally, NLK can transrepress PPARγ functions via yet another molecular mechanism, which consists of enhanced histone methylation followed by transcriptional repression [8]. A similar observation has been made during studies on a-Myb where it has been shown that ectopic expression of NLK also induces histone methylation. Thus the ability of NLK to coordinate chromatin silencing might indicate a general

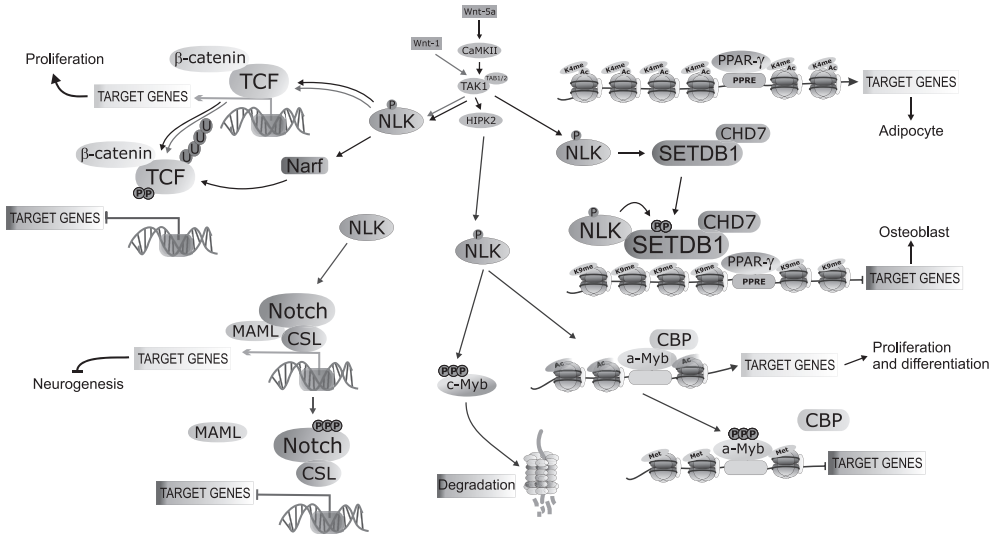


Figure 1. The NLK signaling network
NLK is mostly involved in the inhibition of several transcription factors. This is achieved through a variety of molecular mechanisms, including the induction of dissociation of co-activators, degradation of transcription factors themselves or histone modifications.

mechanism for NLK-induced repression of transcription. However, at present it is not clear whether the NLK-mediated stimulation of histone methylation is always accompanying the other mechanisms of regulation related to direct phosphorylation of transcription factors.

The activation of NLK via the noncanonical Wnt/Ca^{2+} pathway antagonizes β-catenin/TCF signaling due to NLK-mediated TCF phosphorylation, which hinders its ability to bind DNA [9]. In addition, NLK has been also revealed to inhibit β-catenin/TCF activity downstream of the canonical Wnt signaling pathway [10], which implicates the presence of negative feedback signaling. Since β-catenin interacts with FOXO4 in response to elevated cellular ROS levels [11], this creates the possibility for NLK-dependent regulation of β-catenin/FOXO4 signaling. Moreover, FOXO4 has previously been shown to be phosphorylated on multiple S/T-P motifs in response to oxidative stress [12] and these sites correspond to the NLK recognition motif. Indeed, in Chapter 2 we demonstrate that FOXO proteins are substrates for NLK. Ectopic expression of wild type NLK, but not a kinase dead version results in phosphorylation of FOXO proteins on multiple residues. However, we were not able to observe any consistent NLK-dependent regulation of the binding between FOXO4 and β-catenin. It is still possible that even though NLK has no effect on β-catenin/FOXO4 complex formation, it hinders its binding to DNA. We address this issue by performing a chromatin fractionation assay but also there we did not see that NLK alters the amount of FOXO4 bound to DNA (unpublished data). Similarly, even though NLK has been reported to modulate the interaction of the coactivator CBP with transcription factors a-Myb and NF-κB [6, 18], we did not see any reliable changes

in its binding to FOXO4. Furthermore, ectopic expression of NLK did not alter the acetylation state of FOXO4. It is still plausible that exogenous expression of several proteins simultaneously does not mimic the endogenous proportions and therefore some effects that normally occur in the cell might be not visible. However, the NLK-mediated regulation of transcription relies on its kinase activity and not on the competitive binding between proteins. Since we observed that FOXO4 was effectively phosphorylated by NLK, this would most likely be sufficient to see an NLK-driven effect on binding between FOXO4 and CBP or β-catenin.

The interaction between FOXO proteins and NLK is stimulated by elevated cellular ROS levels. However, treatment of the cells with hydrogen peroxide has no effect on NLK activity. This suggests that oxidative stress signaling might stimulate NLK-mediated phosphorylation of FOXO proteins via recruitment of the kinase to its substrate rather than promoting the activity of NLK. Since NLK negatively regulates the transcriptional activity of FOXO1 and FOXO4, this might constitute ROS-dependent negative feedback signaling similar to the NLK-mediated regulation of β-catenin/TCF in response to Wnt signaling.

The molecular basis for the NLK-mediated regulation of FOXO4 transcriptional activity involves modulation of its ubiquitination. Previously, it has been shown that FOXO4 is monoubiquitinated in response to hydrogen peroxide treatment of cells, which correlates with nuclear accumulation of FOXO4 and promotes FOXO4-dependent transactivation [13]. Murine double minute 2 (Mdm2) has been identified as an E3 ubiquitin ligase for FOXO4 that monoubiquitinates FOXO4 in response to oxidative stress [14]. Conversely, ubiquitin-specific peptidase 7 (USP7) deubiquitinates FOXO4, which attenuates its transcriptional activity [13]. The ectopic expression of NLK abolishes monoubiquitination of FOXO4, which can possibly account for the NLK-mediated inhibition of its activity. However, we excluded that NLK mediates its effect on monoubiquitination of FOXO4 via inhibition of Mdm2 or activation of USP7. Importantly, NLK has previously been reported to increase polyubiquitination of TCF and c-Myb via two different E3 ligases designated Nuclear prelamin A recognition factor (NARF) and F-box/WD repeat-containing protein 7 (Fbxw7), respectively [15, 16]. This might seem contradictory to our observation; however, FOXO4 is shown to be a relatively stable protein and it does not seem that FOXO4 is regulated via protein degradation. Therefore, it is not necessarily surprising that inhibition of monoubiquitination rather than induction of polyubiquitination and therefore degradation constitute the predominant mechanism used to repress the activity of FOXO4. Moreover, it is apparent that Mdm2 is not the only E3 ligase that ubiquitinates FOXO4 and we obtained some preliminary data indicating that another E3, β-TRCP can also target FOXO4 (unpublished data). In addition, NLK has been demonstrated to interact with several E3 ligases including Fbxw4, Fbxw5, and S-phase kinase-associated protein 2 (Skp2) [16], so it is possible that NLK can diminish monoubiquitination of FOXO4 via inhibition of β-TRCP or any other E3 that is able to modify FOXO4. Furthermore, NLK has been also demonstrated to interact with the deubiquitinating enzyme (DUB) USP4 and promote its nuclear accumulation [17]. USP4 appears to negatively regulate Wnt signaling.

Thus, it is possible that NLK promotes the activity of USP4 towards Wnt pathway components, which constitute yet another mechanism inhibiting the activity of the β-catenin/TCF complex. Taken together, it appears that NLK can influence the activity of a broad range of proteins involved in ubiquitin-dependent protein modifications and it can both enhance and abolish protein ubiquitination. Therefore, further studies are required to establish the mechanism underlying NLK-dependent inhibition of FOXO4 ubiquitination.

In parallel to our studies another group provided evidence that NLK negatively regulates the activity of FOXO1 [19]. In spite of convergence between our and their results with respect to the effect of NLK on the FOXO functions, it appears that the mechanisms of the regulation are different. Dissimilarly to FOXO4, NLK induces translocation of FOXO1 out of the nucleus and by doing so it inhibits FOXO1-dependent transactivation of its target genes [19].

It might appear surprising that NLK utilizes distinct mechanisms to regulate different but closely related FOXO proteins. However, several other examples have been reported that illustrate differential regulation of FOXOs. This includes c-Jun N-terminal kinase (JNK)-mediated activation of FOXO proteins, Mdm2- and Skp2-dependent ubiquitination of different FOXOs as well as regulation of FOXO phosphorylation by PP2 [20-23].

Moreover, Kim et al. suggest that Transforming growth factor β-activated kinase 1 (TAK1) can promote NLK activity towards FOXO1. This seems to be different for FOXO4 as ectopic expression of TAK1 promotes monoubiquitination of FOXO4 and its activation. Since TAK1, in addition to NLK, can activate JNK [24] it is possible that under stress conditions TAK1 acts predominantly towards JNK, which results in activating phosphorylation of FOXO4. Thus, another stress responsive factor as a part of negative feedback signaling must trigger the binding between FOXO4 and NLK and thereby inhibit FOXO4 activity. It is not clear why TAK1 would inhibit FOXO1 in a NLK-dependent manner instead of activating it via JNK-mediated phosphorylation. One possible explanation might be that FOXO1 is simply not a substrate of JNK as it has been shown to be phosphorylated by p38 mitogen-activated protein kinase (p38MAPK) and Extracellular signal-regulated kinase (ERK), but not by JNK [25]. Still, it has been demonstrated that JNK is required for ROS-induced FOXO1 nuclear translocation [26]. This might depend on the phosphorylation of 14-3-3 protein rather than FOXO1. Furthermore, since studies addressing the TAK1/NLK-mediated regulation of FOXO1 omitted oxidative stress, it will be interesting to see whether, in response to elevated ROS levels, TAK1 also inhibits activity of FOXO1.

One question that remains is whether the NLK-induced increase in histone methylation (associated most probably with a decrease in the histone acetylation) plays any role in inhibition of the FOXO-driven transcription. We did not address this issue as our main focus of interest was the direct regulation of FOXO4 itself. Similarly, NLK-dependent histone modification is not examined in most other studies relevant to the regulation of transcription by NLK. Therefore, further studies are required to establish whether NLK exerts its effect through modulation of the activity of transcription factors in concert with alteration of the histone code or whether these two activities of NLK are occur-

ring independent of each other. In addition, our genome-wide microarray analysis (data not shown) showed that ectopic expression of NLK can have both inhibitory and stimulatory effect on expression of genes. This indicates that overexpression of NLK is not always associated with silencing of chromatin and it would be interesting to see whether NLK can also induce other histone modifications that correlate with gene expression. Interestingly, the same data revealed that ectopic NLK has an inhibitory effect only towards certain FOXO4 target genes, whereas it simultaneously stimulates the expression of a variety of other genes in a FOXO4-dependent manner. This is quite interesting and indicates that NLK does not simply inhibit FOXO4 activity but rather modulates it and apparently requires transactivation of FOXO4 to exert some of its effects. However, further analysis is necessary to draw extensive biological conclusions from this experiment.

Why would NLK modulate FOXO activity? The binding between FOXO4 and NLK is specifically induced after hydrogen peroxide treatment of cells, as applying several other stimuli including Transforming growth factor β TGFβ, Tumor necrosis factor α (TNFα) and DNA damaging agents did not promote this interaction (unpublished data). Thus, it is possible that NLK has a role in negative feedback signaling to downregulate some of the FOXO4 target genes under prolonged and severe stress conditions. This might be crucial for the termination of the protective mechanisms stimulated by FOXO signaling. Simultaneously, activation of other FOXO-regulated genes might be important for induction of cell death as NLK has been demonstrated to trigger apoptosis (unpublished data and [27]).

NLK has been shown to negatively regulate several signaling pathways that, when iteratively activated, are associated with the development of cancer. This suggests that NLK can have antiproliferative functions, which is further supported by the observation that it can suppress growth of several human cancer cell lines when ectopically expressed [27-29]. However, NLK also appears to have a neoplastic potential. It has been reported that expression of NLK is induced in several human liver cancer cell lines and targeted disruption of NLK forces these cells to arrest in the G1 phase of the cell cycle [30]. In addition, it has been shown that lack of NLK correlates with decreased expression of cyclin D1 and Cyclin-dependent kinase 2 (CDK2) in Hep3B cells. Interestingly, FOXOs negatively regulate the expression of cyclin D [31] and therefore it is tempting to speculate that decreased levels of cyclin D1 triggered by the lack of NLK, result from the release of the inhibitory effect that NLK has on the activity of FOXOs. Thus, it will be interesting to see to which other cellular processes NLK might be linked through regulation of FOXO proteins.

Importantly, NLK knockout mice have been generated; however, these mice exhibit a complex phenotype, which varies significantly with the genetic background. Nevertheless, NLK deficiency leads to abnormalities in the stromal compartment of the bone marrow, which results from the aberrant differentiation of stromal cells [32]. This phenotype associated with the lack of NLK is consistent with several other studies indicating involvement of NLK in the regulation of mesenchymal stem cell (MSC) differentiation [8, 33] Moreover, NLK has been proven to suppress Bone morphogenetic protein (BMP) signaling

in *Drosophila melanogaster* [34] , which acts as a major osteogenic inducer in vertebrate organisms. Therefore, NLK can influence adipogenesis and osteoblastogenesis via several pathways.

Interestingly, it has been recently reported that oxidative stress suppresses osteoblast differentiation by diverting β-catenin from TCF to FOXO3a and thereby antagonizing the Wnt/TCF pathway [35, 36]. The activity of β-catenin/TCF is crucial for the commitment of MSCs to the osteoblast lineage, which in turn is essential for the regeneration of the skeleton [37]. Therefore, the elevated ROS levels associated with aging might gradually inhibit Wnt signaling by promoting FOXO/β-catenin functions, which in turn would result in the age-associated bone loss and strength in mice and possibly humans. Even though we did not observe NLK-dependent regulation of the interaction between FOXO4 and β-catenin, it is still possible that NLK might be involved in the regulation of FOXO functions in osteoblastic cells. Therefore, additional studies are required to determine the relationship between NLK and FOXOs in those cells. This in turn will be beneficial for an unequivocal understanding of FOXO functions in bone biology. This function appears to be complex, as recently it has also been reported that FOXOs are required for the protection against oxidative stress in osteoblasts and therefore for the maintenance of skeletal homeostasis in mice [38, 39].

MYBBP1a, a novel FOXO4 interaction partner

The decoration of FOXO proteins with PTMs requires their association with protein modifying enzymes. Thus, any changes in the activities of these enzymes will result in an altered pattern of PTMs on FOXOs. Another way to regulate this process involves the proteins that do not have enzymatic activities themselves but can compete with modifying enzymes from binding to their substrates or introduce a steric hindrance that prevents the covalent attachment of modifying moieties.

In Chapter 3 we enlighten a novel interaction between FOXO4 and Myb binding protein 1a (MYBBP1a) and its consequence for the activity of FOXO4. Interestingly, ectopic expression of MYBBP1a impairs acetylation of FOXO4 triggered by overexpression of p300 or CBP. However, MYBBP1a does not hamper the binding between FOXO4 and CBP/p300 and even though MYBBP1a can associate with p300, it has no effect on the intrinsic activities of CBP and p300. Thus, most probably MYBBP1a binds to FOXO4 and its presence interfere with acetylation of FOXO4 by introducing the steric protection of lysine residues that are normally targeted by CBP/p300. Because acetylation of FOXO proteins takes place in the DNA binding domain, it interferes with their ability to bind DNA, thereby inhibiting FOXO-dependent transcription [40-42]. Thus, MYBBP1a, by impeding acetylation, promotes its binding to DNA and induces FOXO4-driven expression of a luciferase gene from an artificial promoter. Interestingly, MYBBP1a cannot promote the activity of FOXO4ΔCys in the reporter assay. Since acetylation of FOXO4ΔCys is dramatically impaired, this supports that MYBBP1a exerts its effect on FOXO4 activity via inhibition of FOXO4 acetylation and because acetylation of FOXO4ΔCys is already inhibited, MYBBP1a has no effect on its activity. Strikingly, ectopic expression of MYBBP1a inhibits FOXO4-induced transactivation of the endogenous

$p27^{kip1}$ promoter. Thus, MYBBP1a affects FOXO4 functions in an ambiguous way as it can promote the activity of FOXO4 in a luciferase reporter assay but it inhibits FOXO4 activity towards at least some of its target genes. This is in line with previous studies showing that acetylation has dual yet opposing effects on the activity of FOXO proteins [43].

Importantly, acetylation is not restricted to FOXOs and occurs on histone proteins as well. Histone acetylation is well known to generally stimulate transcriptional activity, as it opens the chromatin structure and facilitates binding of transcription factors to their target sequences [44]. Therefore, interference with the acetylation of histones has a negative effect on transcription and this might explain the MYBBP1a-dependent inhibition of the FOXO4-dependent induction of $p27^{kip1}$ protein levels. This is further supported by the fact that MYBBP1a also suppresses FOXO4ΔCys-dependent transactivation of the $p27^{kip1}$ promoter and suggests that the acetylation state of histones might overrule the effect that acetylation of FOXOs themselves has on their transcription. Nevertheless, further studies are required to establish whether MYBBP1a can indeed influence the acetylation state of histones.

Importantly, acetylation can also modulate FOXO specificity, as it has been shown that p300 promotes FOXO-dependent expression of apoptosis-related genes, whereas Sirt1 stimulates the expression of FOXO target genes involved in cell cycle regulation and stress resistance [45, 46]. Thus, it is proposed now that acetylation of FOXOs does not inhibit their functions but it rather determines their specificity for particular genes. Therefore, it will be interesting to investigate whether MYBBP1a can also shift specificity of FOXO4 from one subset of genes to another. As presented in Figure 7 of Chapter 3, ectopic expression of MYBBP1a does not seem to influence FOXO4-induced apoptosis. However, it has been shown that MYBBP1a is expressed at particularly high levels in Jurkat T cells [47]. Interestingly, forced expression of FOXOs in immune cells predominantly induces apoptosis [48]. Therefore, it might be especially relevant to evaluate how MYBBP1a regulates apoptosis-related functions of FOXOs in these cells.

The inhibition of protein acetylation is generally accompanied by increased ubiquitination, as these PTMs often occur on the same lysine residues. Therefore, ectopic expression of MYBBP1a inhibits acetylation of FOXO4 but in turn it promotes its monoubiquitination. Previously, monoubiquitination of FOXO4 has been associated with enhanced activation of FOXO4 [13]. Thus it is possible that the MYBBP1a-dependent regulation of the activity of FOXO4 relies on the combination between decreased acetylation of specific lysine residues and increased monoubiquitination of others. Interestingly, MYBP1a also promotes ubiquitination of FOXO4ΔCys (unpublished data) suggesting that release of lysine residues from acetylation is not inducing ubiquitination per se but that MYBBP1a additionally activates the attachment of ubiquitin moieties to FOXO4. Since, MYBBP1a can affect ubiquitination of FOXO4 it is also possible that it can modulate histone ubiquitination, adding yet another level of complexity to the MYBBP1a-dependent modulation of transcription.

The binding between FOXO4 and MYBBP1a is stimulated by elevated cellular ROS levels; however, at present it is not known how the ROS-dependen-

cy is mediated at the molecular level. It is possible that phosphorylation of FOXO4 might be relevant for binding of MYBBP1a as it has been demonstrated that the interaction between MYBBP1a and PGC1α is regulated by p38MAPK [49].

Interestingly, MYBBP1a is localized within the nucleolus and it translocates to the nucleus upon ribosomal stress induction. It is unclear whether MYBBP1a plays any role in the ribosomal stress response. Nevertheless, the ribosomal stress-induced translocation of MYBBP1a to the nucleus allows its colocalization with FOXO4, which localizes in the nucleus but is excluded from the nucleoli. This implies that MYBBP1a might regulate the activity of FOXO4 under ribosomal stress conditions. Since our data indicates that MYBBP1a inhibits the expression of at least some of the target genes of FOXO4, it will be interesting to determine whether it has any role in ribosomal stress.

Recently, MYBBP1a has been identified as a novel substrate of aurora B kinase. Depletion of MYBBP1a leads to prolongation of mitosis and mitotic spindle defects [50], suggesting an essential role of MYBBP1a in normal progression of mitosis. Moreover, MYBBP1a-mediated inhibition of $p27^{kip1}$ suggests that MYBBP1a also promotes progression through other phases of the cell cycle. Interestingly, FOXO proteins have also been shown to induce expression of Polo-like kinase and cyclin B [51, 52], thereby promoting mitotic progression. Thus, it would be interesting to see whether MYBBP1a promotes FOXO-dependent induction of genes related to cell cycle progression.

GFP-Foxo4 knock-in mice, *in vivo* expression of Foxo4

Several studies employing transgenic mice revealed that besides sharing various redundant functions, FOXO transcription factors are also characterized by their unique roles in the regulation of different physiological processes [53-56]. This might be associated with diversity within the structure of distinct FOXOs, which in combination with different expression patterns might result in specific recognition of certain target genes by only a particular FOXO and not by other members of the FOXO family. Nevertheless, the understanding of specific functions of FOXO4 is rather limited. This most probably results from the fact that FOXO4 knockout mice did not show any phenotype [53], which might have discouraged subsequent studies on this FOXO protein. In order to get better insight into specific FOXO4 functions, we decided to examine its *in vivo* expression pattern, the results of which are described in Chapter 4. Therefore, we generated transgenic mice in which GFP is incorporated in-frame at the endogenous Foxo4 translation initiation site. Unexpectedly, the immunohistochemical analysis of several tissues coming from young adult mice including brain, heart and muscle revealed that the expression of Foxo4 is remarkably low, as we were unable to observe any specific staining of GFP-Foxo4. This might explain the lack of phenotype associated upon Foxo4 depletion, as apparently the presence of other Foxo family members might compensate for loss of the low level of Foxo4. However, the significance of Foxo4 becomes apparent in the absence of two other Foxo proteins, since only simultaneous deletion of all three Foxo proteins is permissive for the spontaneous development of tumor in mice [57]. This indicates that all Foxo modulate the expression of genes that are crucial for the suppression of tumorigenesis, with the same, relatively

high affinity, and that is the reason why they can substitute for each other. Moreover, it is likely that expression of Foxo4 increases in the absence of other Foxo proteins, thereby becoming sufficient in suppressing tumor formation. Besides, since FOXO proteins are activated under stress conditions it is still possible that also their expression increases in response to stress. However, further studies are required to address this issue.

Using FACS analysis we were able to detect expression of GFP-Foxo4 in mouse hematopoietic stem cells (HSCs) and their progenitors. This is in line with the previously reported pivotal role of Foxo transcription factors in maintenance of the HSC pool [58]. It is not clear whether expression of Foxo4 has any relevance for the functions of HSCs and it has been demonstrated that Foxo3a deficiency is sufficient to impair the self-renewal capacity of HSCs [54]. Moreover, the data presented in Figure 9 (Chapter 4) suggest that expression of FOXO4 might be rather restricted to progenitors than HSCs. Therefore, it is possible that Foxo3a is important for stemcellness of HSCs, whereas Foxo4 might function in maintenance of the progenitor pool. In addition, we observed age-related changes in the expression of Foxo4 in the hematopoietic compartment, reflected by induction of expression of GFP-Foxo4 in lineage positive cells. At present it is not known whether this has any biological significance and therefore further studies are necessary to elucidate this issue.

General remarks

Even though a great number of studies demonstrate the essential role of FOXO transcription factors in the regulation of oxidative stress resistance, we are still far from understanding how exactly FOXO functions are regulated under stress conditions. This is most probably due to the enormous complexity of oxidative stress signaling as it can both activate and inhibit the transcriptional activity of FOXOs. This in turn is associated with ROS-induced convergence of numerous signaling pathways that alter the activity of FOXOs through various PTMs. Importantly, it is appearing now that a number of signaling pathways that were not implicated in oxidative stress signaling before, might regulate FOXO functions in response to elevated cellular ROS levels. Those pathways are often associated with the overall regulation of transcription, suggesting that oxidative stress employs general transrepressors and transactivators in order to modulate FOXO-dependent transcription.

It is important to realize that the co-existence of ROS-dependent, yet often opposing, feed-forward and feedback loops towards FOXOs need to be precisely regulated in time and space. Since it has been shown for p53 that average responses of a whole population of cells do not always represent the dynamic behavior of individual cells, studies that follow responses of a single cell might be more relevant for understanding how opposing regulatory signals converge on FOXO proteins [59].

Furthermore, it is evident that *in vivo* studies employing transgenic mice are equally important as they reveal which physiological processes depend on the FOXO-mediated protection against oxidative stress.

References

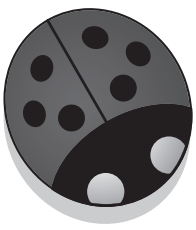
1. Ishitani, T., J. Ninomiya-Tsuji, and K. Matsumoto, Regulation of lymphoid enhancer factor 1/T-cell factor by mitogen-activated protein kinase-related Nemo-like kinase-dependent phosphorylation in Wnt/beta-catenin signaling. Mol Cell Biol, 2003. 23(4): p. 1379-89.
2. Shin, T.H., et al., MOM-4, a MAP kinase kinase kinase-related protein, activates WRM-1/LIT-1 kinase to transduce anterior/posterior polarity signals in C. elegans. Mol Cell, 1999. 4(2): p. 275-80.
3. Meneghini, M.D., et al., MAP kinase and Wnt pathways converge to downregulate an HMG-domain repressor in Caenorhabditis elegans. Nature, 1999. 399(6738): p. 793-7.
4. Ishitani, T., et al., The TAK1-NLK-MAPK-related pathway antagonizes signalling between beta-catenin and transcription factor TCF. Nature, 1999. 399(6738): p. 798-802.
5. Kanei-Ishii, C., et al., Wnt-1 signal induces phosphorylation and degradation of c-Myb protein via TAK1, HIPK2, and NLK. Genes Dev, 2004. 18(7): p. 816-29.
6. Kurahashi, T., et al., The Wnt-NLK signaling pathway inhibits A-Myb activity by inhibiting the association with coactivator CBP and methylating histone H3. Mol Biol Cell, 2005. 16(10): p. 4705-13.
7. Ishitani, T., et al., Nemo-like kinase suppresses Notch signalling by interfering with formation of the Notch active transcriptional complex. Nat Cell Biol. 12(3): p. 278-85.
8. Takada, I., et al., A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-gamma transactivation. Nat Cell Biol, 2007. 9(11): p. 1273-85.
9. Ishitani, T., et al., The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca(2+) pathway to antagonize Wnt/beta-catenin signaling. Mol Cell Biol, 2003. 23(1): p. 131-9.
10. Smit, L., et al., Wnt activates the Tak1/Nemo-like kinase pathway. J Biol Chem, 2004. 279(17): p. 17232-40.
11. Essers, M.A., et al., Functional interaction between beta-catenin and FOXO in oxidative stress signaling. Science, 2005. 308(5725): p. 1181-4.
12. Brenkman, A.B., et al., The peptidyl-isomerase Pin1 regulates p27kip1 expression through inhibition of Forkhead box O tumor suppressors. Cancer Res, 2008. 68(18): p. 7597-605.
13. van der Horst, A., et al., FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP. Nat Cell Biol, 2006. 8(10): p. 1064-73.
14. Brenkman, A.B., et al., Mdm2 induces mono-ubiquitination of FOXO4. PLoS One, 2008. 3(7): p. e2819.
15. Yamada, M., et al., NARF, an nemo-like kinase (NLK)-associated ring finger protein regulates the ubiquitylation and degradation of T cell factor/lymphoid enhancer factor (TCF/LEF). J Biol Chem, 2006. 281(30): p. 20749-60.
16. Kanei-Ishii, C., et al., Fbxw7 acts as an E3 ubiquitin ligase that targets c-Myb for nemo-like kinase (NLK)-induced degradation. J Biol Chem, 2008. 283(45): p. 30540-8.
17. Zhao, B., et al., The ubiquitin specific protease 4 (USP4) is a new player in the Wnt signalling pathway. J Cell Mol Med, 2009. 13(8B): p. 1886-95.
18. Yasuda, J., et al., Nemo-like kinase suppresses a wide range of transcription factors, including nuclear factor-kappaB. Cancer Sci, 2004. 95(1): p. 52-7.
19. Kim, S., et al., Regulation of FOXO1 by TAK1-Nemo-like kinase pathway. J Biol Chem. 285(11): p. 8122-9.
20. Huang, H., et al., Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation. Proc Natl Acad Sci U S A, 2005. 102(5): p. 1649-54.
21. Yan, L., et al., PP2A regulates the pro-apoptotic activity of FOXO1. J Biol Chem, 2008. 283(12): p. 7411-20.
22. Sunayama, J., et al., JNK antagonizes Akt-mediated survival signals by phosphorylating 14-3-3. J Cell Biol, 2005. 170(2): p. 295-304.
23. Essers, M.A., et al., FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. EMBO J, 2004. 23(24): p. 4802-12.
24. Fanger, G.R., et al., MEKKs, GCKs, MLKs, PAKs, TAKs, and tpls: upstream regulators of the c-Jun amino-terminal kinases? Curr Opin Genet Dev, 1997. 7(1): p. 67-74.
25. Asada, S., et al., Mitogen-activated protein kinases, Erk and p38, phosphorylate and regulate Foxo1. Cell Signal, 2007. 19(3): p. 519-27.
26. Kawamori, D., et al., The forkhead transcription factor Foxo1 bridges the JNK pathway and the transcription factor PDX-1 through its intracellular translocation. J Biol Chem, 2006. 281(2): p. 1091-8.
27. Yasuda, J., et al., Nemo-like kinase induces apoptosis in DLD-1 human colon cancer cells. Biochem Biophys Res Commun, 2003. 308(2): p. 227-33.
28. Emami, K.H., et al., Nemo-like kinase induces apoptosis and inhibits androgen receptor signaling in prostate cancer cells. Prostate, 2009. 69(14): p. 1481-92.
29. Cui, G., et al., Clinical and biological signifi-

cance of Nemo-like kinase expression in glioma. J Clin Neurosci.
30. Jung, K.H., et al., Targeted disruption of Nemo-like kinase inhibits tumor cell growth by simultaneous suppression of cyclin D1 and CDK2 in human hepatocellular carcinoma. J Cell Biochem. 110(3): p. 687-96.
31. Schmidt, M., et al., Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. Mol Cell Biol, 2002. 22(22): p. 7842-52.
32. Kortenjann, M., et al., Abnormal bone marrow stroma in mice deficient for nemo-like kinase, Nlk. Eur J Immunol, 2001. 31(12): p. 3580-7.
33. Nifuji, A., et al., Nemo-like kinase (NLK) expression in osteoblastic cells and suppression of osteoblastic differentiation. Exp Cell Res. 316(7): p. 1127-36.
34. Zeng, Y.A., et al., *Drosophila* Nemo antagonizes BMP signaling by phosphorylation of Mad and inhibition of its nuclear accumulation. Development, 2007. 134(11): p. 2061-71.
35. Almeida, M., et al., Oxidative stress antagonizes Wnt signaling in osteoblast precursors by diverting beta-catenin from T cell factor- to forkhead box O-mediated transcription. J Biol Chem, 2007. 282(37): p. 27298-305.
36. Almeida, M., et al., Increased lipid oxidation causes oxidative stress, increased peroxisome proliferator-activated receptor-gamma expression, and diminished pro-osteogenic Wnt signaling in the skeleton. J Biol Chem, 2009. 284(40): p. 27438-48.
37. Glass, D.A., 2nd and G. Karsenty, In vivo analysis of Wnt signaling in bone. Endocrinology, 2007. 148(6): p. 2630-4.
38. Ambrogini, E., et al., FoxO-mediated defense against oxidative stress in osteoblasts is indispensable for skeletal homeostasis in mice. Cell Metab. 11(2): p. 136-46.
39. Rached, M.T., et al., FoxO1 is a positive regulator of bone formation by favoring protein synthesis and resistance to oxidative stress in osteoblasts. Cell Metab. 11(2): p. 147-60.
40. Brent, M.M., R. Anand, and R. Marmorstein, Structural basis for DNA recognition by FoxO1 and its regulation by posttranslational modification. Structure, 2008. 16(9): p. 1407-16.
41. Hatta, M., F. Liu, and L.A. Cirillo, Acetylation curtails nucleosome binding, not stable nucleosome remodeling, by FoxO1. Biochem Biophys Res Commun, 2009. 379(4): p. 1005-8.
42. Tsai, K.L., et al., Crystal structure of the human FOXO3a-DBD/DNA complex suggests the effects of post-translational modification. Nucleic Acids Res, 2007. 35(20): p. 6984-94.
43. van der Heide, L.P. and M.P. Smidt, Regulation of FoxO activity by CBP/p300-mediated acetylation. Trends Biochem Sci, 2005. 30(2): p. 81-6.
44. Villar-Garea, A. and A. Imhof, The analysis of histone modifications. Biochim Biophys Acta, 2006. 1764(12): p. 1932-9.
45. Brunet, A., et al., Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science, 2004. 303(5666): p. 2011-5.
46. Dansen, T.B., et al., Redox-sensitive cysteines bridge p300/CBP-mediated acetylation and FoxO4 activity. Nat Chem Biol, 2009. 5(9): p. 664-72.
47. Owen, H.R., et al., Identification of novel and cell type enriched cofactors of the transcription activation domain of RelA (p65 NF-kappaB). J Proteome Res, 2005. 4(4): p. 1381-90.
48. Birkenkamp, K.U. and P.J. Coffer, FOXO transcription factors as regulators of immune homeostasis: molecules to die for? J Immunol, 2003. 171(4): p. 1623-9.
49. Fan, M., et al., Suppression of mitochondrial respiration through recruitment of p160 myb binding protein to PGC-1alpha: modulation by p38 MAPK. Genes Dev, 2004. 18(3): p. 278-89.
50. Perrera, C., et al., Identification of Myb-binding protein 1A (MYBBP1A) as a novel substrate for aurora B kinase. J Biol Chem. 285(16): p. 11775-85.
51. Alvarez, B., et al., Forkhead transcription factors contribute to execution of the mitotic programme in mammals. Nature, 2001. 413(6857): p. 744-7.
52. Yuan, Z., et al., Activation of FOXO1 by Cdk1 in cycling cells and postmitotic neurons. Science, 2008. 319(5870): p. 1665-8.
53. Hosaka, T., et al., Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. Proc Natl Acad Sci U S A, 2004. 101(9): p. 2975-80.
54. Miyamoto, K., et al., Foxo3a is essential for maintenance of the hematopoietic stem cell pool. Cell Stem Cell, 2007. 1(1): p. 101-12.
55. Renault, V.M., et al., FoxO3 regulates neural stem cell homeostasis. Cell Stem Cell, 2009. 5(5): p. 527-39.
56. Gross, D.N., M. Wan, and M.J. Birnbaum, The role of FOXO in the regulation of metabolism. Curr Diab Rep, 2009. 9(3): p. 208-14.
57. Paik, J.H., et al., FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell, 2007. 128(2): p. 309-23.
58. Tothova, Z., et al., FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell, 2007. 128(2): p. 325-39.
59. Lahav, G., et al., Dynamics of the p53-Mdm2 feedback loop in individual cells. Nat Genet, 2004. 36(2): p. 147-50.

Chapter 6

The peroxide dilemma: Opposing and mediating insulin action

Anna A. Szypowska and Boudewijn M.T. Burgering



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The peroxide dilemma: Opposing and mediating insulin action

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Abstract

Recent compelling data show that reactive oxygen species (ROS) are not only a harmful byproduct of aerobic metabolism, but are also utilized as signaling molecules to regulate various cellular processes. In mammalian cells ROS are produced transiently in response to many extracellular stimuli, including insulin and specific inhibition of the ROS suppresses insulin-dependent signaling. Initially, this finding rationalized the concept of ROS acting as insulin mimetic. However, it is becoming evident that ROS are also causal to diabetes, a metabolic disorder characterized by insufficiency of secretion of, or receptor insensitivity to, endogenous insulin. This notion underlines a dual role for ROS in insulin signaling as both deleterious and beneficiary. Moreover, it strongly suggests that a delicate redox balance is required for insulin signaling to remain 'healthy' for an organism.

Introduction

Following the discovery of insulin in 1922 by Banting and Best, and the establishment that insulin is the causative link in diabetes, it became essential to understand the cellular consequences of insulin action. By now, it is well documented how insulin through activation of complex signaling network regulates glucose and lipid metabolism. In the presence of insulin, the insulin receptor (IR) triggers a series of downstream events leading to activation of two major signaling pathways: the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB, also called c-Akt) pathway and the Ras–mitogen-activated protein kinase (MAPK) pathway (reviewed in [1-3]). In addition, other signaling events e.g. atypical protein kinase C (PKC) signaling also contribute to the action of insulin [1].

The PI3K/PKB pathway is responsible for most of the metabolic actions of insulin, which is illustrated by the observation that an inactivating mutation in the PKBβ/AKT2 gene leads to the development of severe insulin resistance and diabetes mellitus [4]. Whereas loss of function of PKB may confer insulin resistance, the opposite i.e. gain of function of PKB contributes to tumorigenesis [5]. Hence, PKB is not only involved in the regulation of glucose homeostasis, but it also mediates the proliferative effects of insulin (Figure 1).

Besides insulin, many growth factors, especially those acting through tyrosine kinase receptors, employ PI3K/PKB pathway [6]. Thus a pertinent question is how does insulin or any other growth factor in that respect gain specificity?

Recent progress suggests that reactive oxygen species (ROS) may act as a signaling molecule to integrate multiple signaling pathways by simultaneously affecting the activity of various signal-

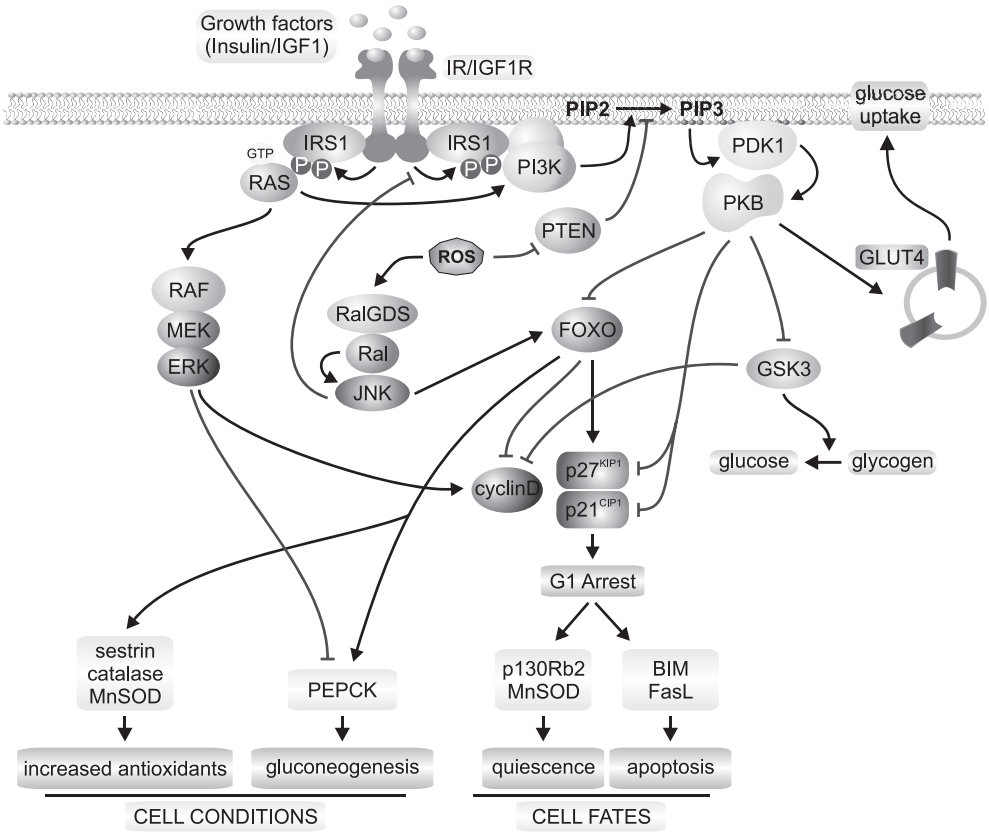

Figure 1. Insulin signaling in regulation of the cell cycle and glucose homeostasis
Both the Ras and PKB arm of insulin-induced signaling regulate a multitude of events involved in cell cycle progression. Proper cell cycle progression requires sufficient energy resources, minimal cellular protein mass and cell size. Thus the ability of insulin to regulate glucose metabolism fits into its broader role to regulate cell proliferation as it provides an energy resource for cell cycle progression. Insulin influences cell cycle progression via transcription (FOXO) and protein stability (PKB/GSK3). Important in this scheme is that through a regulated G1 progression, cells can decide based on intra- and extracellular cues (most importantly ROS and stress status) how to proceed (e.g. apoptosis and quiescence but also others like differentiation are a possibility). Importantly, FOXO–mediated transcription of genes important for ROS protection and energy supply provides secondary requirements for these cell fate decisions.

ing components of different pathways [7]. Therefore, it creates a possibility that the differential impact of growth factors on cellular redox, and vice-versa, may determine strength and localization and thereby specificity of signaling.

Cellular locations of ROS generation

For a long time, ROS were considered to be toxic by-products of certain metabolic systems, causing damage to the cellular content and eventually leading to cell death. However, by now it is known that ROS can function as signaling molecules regulating diverse cellular processes and are even produced intentionally in response to specific stimuli including hormones, growth factors and proinflammatory cytokines [8]. Under physiological conditions cellular ROS is

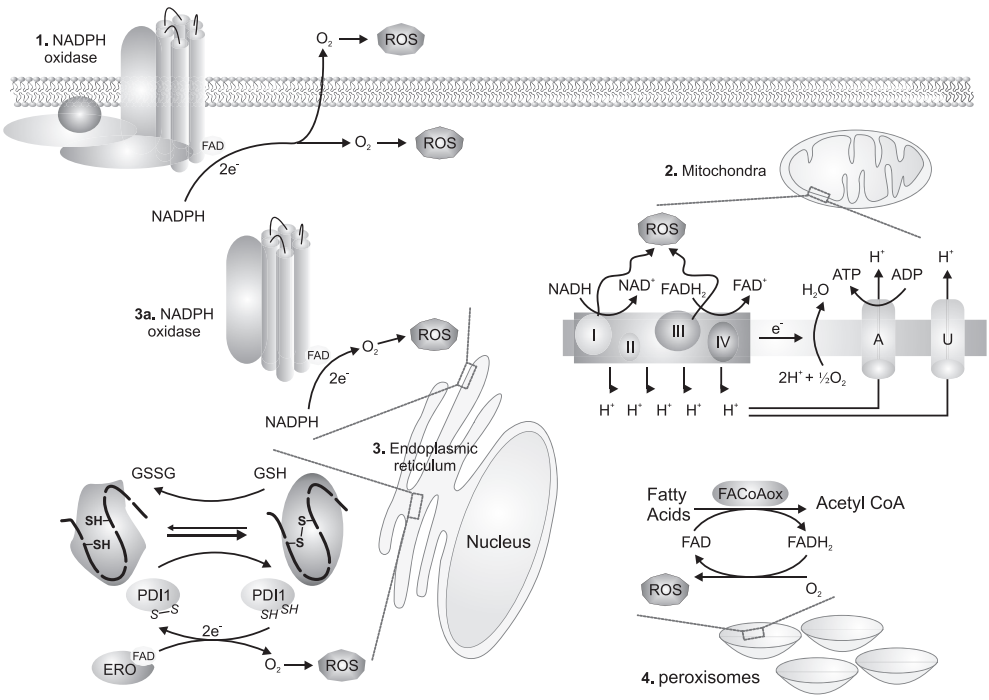

Figure 2. Major cellular sources of ROS under physiological conditions
Generation of ROS occurs at numerous cellular locations including plasma membrane, mitochondria, peroxisomes and endoplasmic reticulum. ROS are primarily produced intracellularly by four metabolic sources: the mitochondrial ETC **(1)** and NADPH oxidases **(2, 3a)**, oxidative protein folding **(3)** and β-oxidation of fatty acids **(4)**.

primary produced by several enzymatic systems (Figure 2).

These include the mitochondrial electron transport chain (ETC), NADPH oxidases (NOXs), oxidative protein folding in the endoplasmic reticulum (ER) and β-oxidation of fatty acids within peroxisomes [9-12].

As ROS are diffusible and short-lived, it is important to precisely localize their production to guarantee specific oxidative regulation of certain proteins without imposing unwanted changes on others. NADPH oxidases are recognized as the major source of localized ROS production due to their specific localization within distinct cellular compartments (reviewed in [12, 13]). They are multi-subunit enzymes composed of a core catalytic subunit (NOX1 to 5 and DUOX1, and DUOX2), regulatory subunit $p22^{phox}$ and cytosolic cofactors including $p47^{phox}$, $p40^{phox}$, $p67^{phox}$ and the small GTPase Rac. The requirement for $p22^{phox}$ and cytosolic subunits varies between different NOXs; however it has been shown that $p22^{phox}$ is an essential regulatory component of NOX1-4 complexes [12].

In addition, phosphorylation of both core and regulatory subunits of various NADPH oxidases may play an important role in positive and negative regulation of NOX activity [14]. A large number of kinases including PKC, extracellular signal-regulated kinase 1/2 (ERK1/2),

p38MAPK, p21-activated protein kinase 1 (PAK1), and PKB have been reported to mediate phosphorylation of the NOX2 complex. Direct phosphorylation of NOX2 by PKC in response to neutrophil stimulation enhances the catalytic activity of NOX2 and its association with cytosolic cofactors. Moreover, in the same system it has also been shown that PKC-mediated phosphorylation of $p47^{phox}$ is essential for release of the autoinhibitory conformation of $p47^{phox}$ and therefore activation of the NOX2 complex. In addition, PKB directly binds and phosphorylates $p47^{phox}$ upon neutrophil stimulation [14]. Whether insulin triggers phosphorylation of NOX complexes and thereby contributes to their regulation is currently unknown.

Besides mitochondria- and NADPH oxidases-derived ROS, it has been estimated that about 25% of cellular ROS may be generated in the ER. This is associated with the activity of two enzymes: protein disulfide isomerase (PDI1) and ER oxidoreductin (ERO1), which are involved in oxidative protein folding [10]. Overload of the ER folding capacity is inevitably associated with increased ROS generation and leads to induction of ER stress and secondary activation of an adaptive signaling cascade known as the unfolded protein response (UPR). The UPR coordinates many biological processes to restore ER homeostasis. One of the UPR effectors is double-stranded RNA-activated protein kinase-like ER kinase (PERK). Active PERK transiently attenuates mRNA translation, thereby preventing further influx of newly synthesized polypeptides into the stressed ER lumen. PERK can also phosphorylate and activate nuclear respiratory factor 2 (NRF2). Cells depleted of NRF2 are highly sensitive to ER stress-induced apoptosis. NRF1 and NRF2 are transcription factors that regulate transcription of genes encoding ROS detoxifying enzymes. Therefore, activation of PERK protects cells from oxidative stress, which is supported by the fact that $PERK^{-/-}$ cells accumulate ROS when exposed to ER stress [15].

Another branch of the UPR, consisting of inositol-requiring kinase 1 (IRE1) and X-box binding protein (XBP1), leads to the activation of ER-associated degradation (ERAD) of malfolded proteins [16]. During ERAD, improper folded proteins are redirected to the cytoplasm and degraded, which leads to reduction in ER-protein load, thereby preventing unnecessary consumption of glutathione (GSH) that is indispensible for counterbalancing elevated ROS levels [16].

ER stress also increases the concentration of Ca^{2+} in the cytosol due to leakage of Ca^{2+} from the ER lumen. This released Ca^{2+} may subsequently enter mitochondria where the increased Ca^{2+} concentration can induce generation of ROS. This occurs due to inhibition of complex III (by release of cytochrome c) or complex IV (by stimulation of nitric oxide synthase and generation of NO•). In addition, ROS is generated due to Ca^{2+}-mediated stimulation of the tricarboxylic acid (TCA) cycle, thereby increasing O_2 consumption, and finally by Ca^{2+}-induced permeability transition pore opening causing GSH leakage [16, 17].

Furthermore, all ER processes require a vast amount of energy supplied by mitochondria. Thus, any rise in ER activity increases mitochondrial oxidative phosphorylation and consequently elevates ROS. In addition, elevated Ca^{2+} levels in the cytosol might activate PKC and by that promote the activity of NADPH oxidase and p66SHC even further, thereby enhancing ROS production. Therefore, increased ROS level in

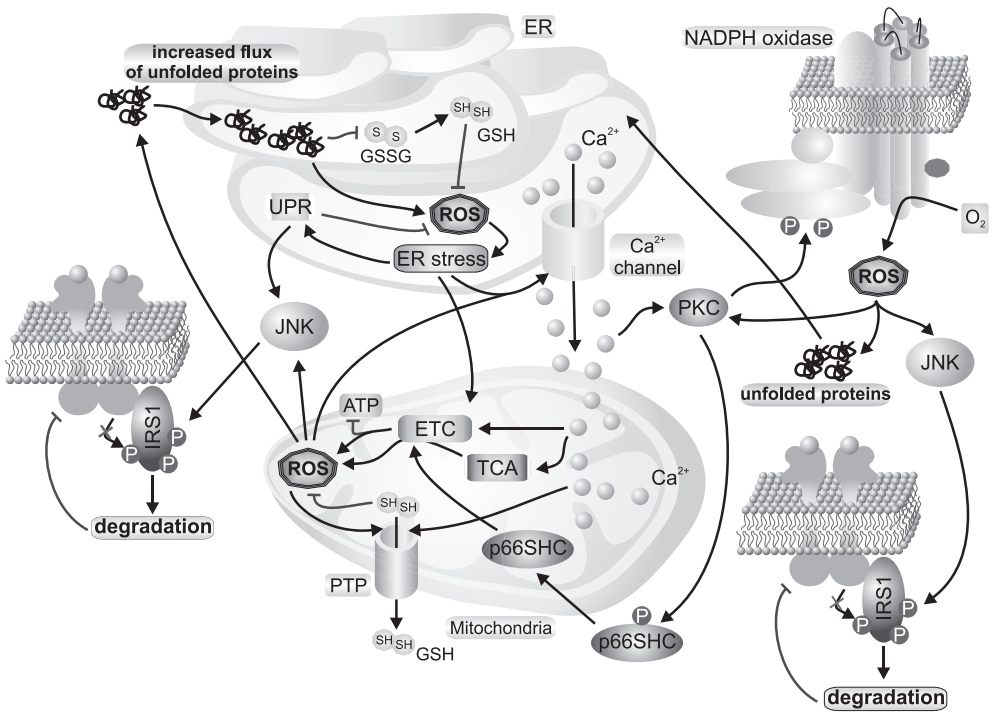

Figure 3. The vicious cycle of ER stress and overall ROS production
Simplified model illustrating how ER stress induces mitochondrial-mediated ROS production and enhances the activity of NADPH oxidase and p66SHC. This is mainly mediated by ER stress-induced release of Ca^{2+} from the ER lumen. The unresolved ER stress induces production of ROS in other cellular compartments, leading to further oxidative stress induction. This results in an increased degree of misfolded proteins and additional release of Ca^{2+} from the ER, which sustains ER stress. Oxidative stress and ER stress both inhibit insulin signaling through activation of the protein kinase JNK.

the ER can stimulate production of ROS within other cellular compartments as depicted in Figure 3.

The friend: ROS as signaling molecule for insulin

Insulin stimulates ROS production

Insulin is one of numerous extracellular stimuli that increase intracellular H_2O_2 concentration. Increased H_2O_2 is essential for efficient insulin signaling, as blocking H_2O_2 production reduces insulin signaling [18]. At the plasma membrane NOX4 and NOX3 have been implicated in insulin induced H_2O_2 generation and signaling. Ectopic expression of dominant-negative NOX4 in differentiated 3T3-L1 adipocytes attenuated insulin-stimulated H_2O_2 generation, tyrosine phosphorylation of IR and insulin receptor substrate-1 (IRS-1), activation of downstream kinases, and glucose uptake. In accordance, siRNA-mediated knockdown of NOX4 inhibited insulin signaling [19]. Conversely, in HepG2 cells ablation of NOX3 expression abrogates H_2O_2 production following insulin stimulation and modulated insulin-induced MAPK/ERK phosphorylation [20]. Thus, either both NOX3 and/or NOX4 may mediate H_2O_2 involvement

in insulin signaling or alternatively the experimental conditions affect the role of $p22^{phox}$, which is a regulatory component of NOX4, but also interacts with NOX1, NOX2 and NOX3. The requirements for $p22^{phox}$ to function in NOX4 versus NOX2 and 3 regulation apparently differ [21]. Thus manipulating either NOX isoform may have consequences for the ability of $p22^{phox}$ to regulate other isoforms.

Insulin-mediated regulation of p66SHC may also directly affect the cellular ROS status. The SHC protein family consists of 3 members, i.e. p46, p52 and p66SHC. All three SHC isoforms become rapidly tyrosine phosphorylated following insulin treatment of cells. However, p46/52SHC are involved in mediating MAPK/ERK activation, whereas p66SHC appears to inhibit this role of p46/p52SHC [22]. In addition, p66SHC may function as a redox enzyme through oxidation of cytochrome c [23]. This function requires its unique N-terminus and interestingly may be redox dependent. It has been shown that activation of p66SHC requires its tetramerization via formation of disulfide bonds between its N-terminal parts. In turn, GSH and thioredoxin (TRX) can reduce and inactivate p66SHC, which results in a thiol-based redox sensor system [24]. Furthermore, insulin via redox sensitive PKCβ was shown to phosphorylate p66SHC. However, this phosphorylation enhances apoptosis through mitochondrial dysfunction. This suggests a role for p66SHC under pathological rather than normal conditions, and indeed loss of PKCβ protects mice against high fat diet (HFD)-induced diabetes [25]. Based on these observations, it is likely that insulin through p66SHC increases mitochondrial derived H_2O_2. However, it is unclear what the relative contribution (quantitative and qualitative) of p66SHC-mediated ROS changes is compared to NOX-mediated changes in insulin signaling.

The Forkhead Box O (FOXO) transcription factors regulate expression of numerous genes encoding anti-oxidants, including superoxide dismutase enzymes (MnSOD), catalase, sestrins and selenoprotein P [26]. Consequently, insulin-induced inhibition of FOXOs will reduce the expression of these anti-oxidants. The consequence of this is currently unknown. However, cell cycle progression is known to depend in part on ROS [27] and insulin-induced FOXO inhibition may thus result in downregulation of those anti-oxidants that otherwise would impair cell cycle progression. Thus, FOXOs regulate anti-oxidants in the context of additional functions.

Insulin controls mitochondrial function through numerous pathways. Insulin controls mitochondrial biogenesis by controlling transcription of the PPARγ coactivator 1α (PGC1α), in part through direct PGC1α/FOXO interaction [28]. Furthermore, by regulating glucose availability and other means it potently stimulates mitochondrial oxidative capacity and ATP production [28]. Thus, insulin treatment of cells will enhance mitochondrial O_2 consumption and hence ROS production.

In conclusion, although the paradigm of ROS involvement in insulin signaling was set by H_2O_2 generation through NOX activation it appears that insulin can lead to ROS generation at multiple cellular locations, all of which may directly or indirectly affect insulin signaling.

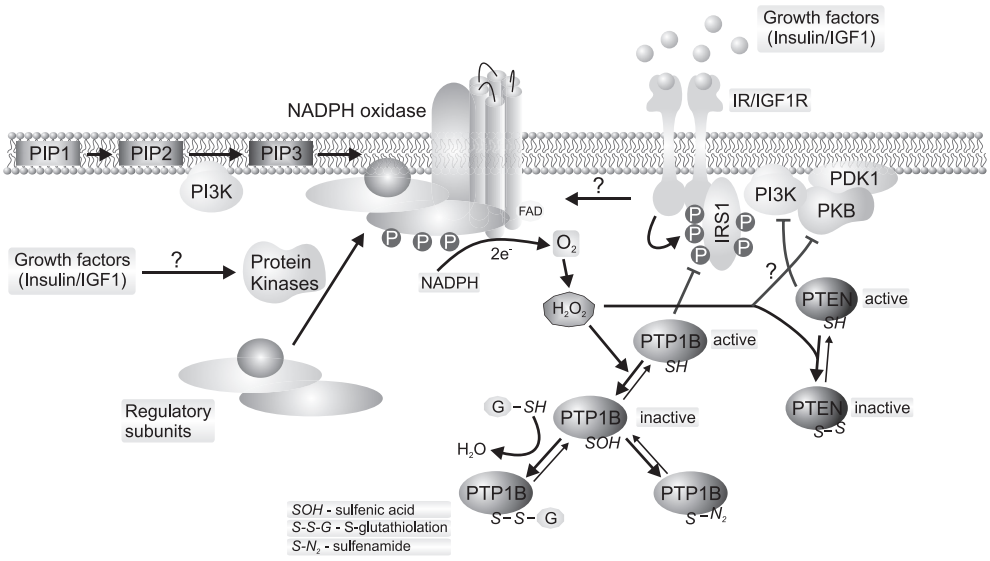

Figure 4. Insulin stimulates oxidative inactivation of PTPs
Upon activation of insulin signaling, the NADPH oxidase-mediated generation of ROS increases. The recruitment of PTPs to the plasma membrane stimulates oxidation of their catalytically active cysteine residues resulting in inactivation of PTP. This promotes Tyr phosphorylation of IRS or activation of PKB and augments insulin action (see text for further details).

Targets of ROS in insulin signaling

Phosphatases (Figure 4)

The ability of H_2O_2 to oxidize thiols of cysteine residues provides a mechanism for the regulation of protein activity. The sensitivity of cysteine residues to oxidation depends on the pK_a value of their thiol groups, which is usually around 8.5 at neutral pH. However, the electrostatic interaction of the thiol group with adjacent polar or positively charged amino acids lowers its pK_a and enhances reactivity with H_2O_2. In addition, cysteine oxidation also depends on the accessibility of H_2O_2 to possible reactive cysteine residues. [23]

Oxidation of the cysteine residue results in formation of sulfenic acid (SOH), which can further react with a second cysteine either in the same or another protein to yield a disulphide. Alternatively, sulfenic acid can form a disulphide bond with GSH that results in S-glutathionylation or be targeted by amide nitrogen of the neighboring residue and form a sulfenamide. All these modifications are reversible and can be reduced by reductase systems to regenerate the thiols [23]. It has been postulated that formation of sulfenamide functions as a protective intermediate preventing from irreversible oxidation of protein tyrosine phosphatases (PTPs) and might facilitate reactivation of PTPs once their activity is required [29]. Nevertheless, when levels of H_2O_2 are greatly elevated the labile sulfenic acid may be further oxidized by H_2O_2, which results in irreversible modification and in most cases inactivation of the proteins. Thus, cysteine oxidation can be employed to modulate protein functions and indeed in insulin signaling, H_2O_2 is used to signal through cysteine oxidation of path-

way components.

PTPs limit the rate and duration of insulin signaling. The signature motif of the family of PTPs, [I/V]HCxxGxxR[S/T], contains an invariant cysteine which functions as a nucleophile in catalysis. The catalytic pocket of the PTPs lowers the normally high pK_a of the cysteine residue, thereby enhancing its nucleophilic property but at the cost of becoming more sensitive to oxidation. Initial work from several laboratories showed that PTPs are oxidized after treatment of cells with H_2O_2. This reduces PTP activity resulting in increased tyrosine phosphorylation of IR [30]. Importantly, this relies on the activity of NOX but also the most probably requires local inactivation of antioxidant defenses. It has been shown recently that upon growth factor stimulation, peroxiredoxin 1 (PRX1) is inactivated, allowing transient high concentration of H_2O_2 to accumulate locally. This inhibition is restricted to the plasma membrane associated pool of PRX1 and depends on the activity of c-Src protein tyrosine kinase and NOX1. In summary, stimulation with growth factors triggers the generation of H_2O_2 by activating membrane-associated NOX1. Simultaneously, activation of c-Src results in phosphorylation and inactivation of PRX1, therefore, preventing newly synthesized H_2O_2 from being converted into nonreactive species. Increase in H_2O_2 may further promote phosphorylation and subsequent inactivation of PRX1 due to ROS-dependent stimulation of c-Src activity and inactivation of PTPs [31]. The predisposition of PRXs to transient inactivation has been proposed as a 'floodgate' that permits H_2O_2 to accumulate and to act as a signaling molecule [32]. Although this is a possible explanation, it should be noted that PRXs may also function as pro-oxidants i.e. whereas normally PRX removes H_2O_2 by coupling the oxidation to TRX, it may also couple oxidation to other proteins involved in signaling and as such 'transmit' the H_2O_2 signal to target proteins to oxidize cysteines present in these proteins [33].

Several phosphatases employ a catalytic cysteine regulate insulin signaling. These include phosphatase and tensin homolog (PTEN) and PTPs, amongst which protein tyrosine phosphatases 1B (PTP1B) is the major one [2]. PTP1B is expressed in all insulin-responsive tissues. Overexpression studies have shown that upon insulin stimulation, PTP1B interacts directly with IR and mediates its dephosphorylation, which results in attenuation of insulin signaling and impaired glucose incorporation into glycogen [34, 35]. Accordingly, PTP1B$^{-/-}$ mice exhibited increased IR phosphorylation in liver and muscle tissue after insulin injection, enhanced insulin sensitivity and were resistant to obesity [36]. Several studies in rodents and humans with insulin resistance, diabetes, and obesity showed increased expression of PTP1B in these conditions [37]. PTP1B becomes reversible oxidized and inactivated upon stimulation with epidermal growth factor (EGF) and insulin [38-40]. It has been shown in endothelial cells that oxidation of PTP1B in response to EGF depends on its colocalization with NOX4 in the ER. Moreover, targeting of PTP1B to the cytosol increases its activity and abolishes the ability of NOX4 to oxidize PTP1B, and to stimulate EGF signaling [40]. Thus, colocalization of NOX complexes with redox-sensitive targets provides a means to determine selectivity of intracellular ROS signaling.

In addition to PTP1B, oxidative regulation was reported also for other members of the PTP superfamily involved in

insulin signaling, including PTEN and T-cell PTP (TCPTP).

Two isoforms have been described for human TCPTP, an endoplasmic reticulum-targeted 48-kDa form (TC48) and a nuclear 45-kDa form (TC45). TCPTP can interact with IR and dephosphorylates its β-subunit, both *in vitro* and in cellular context, and therefore augments insulin signaling. It has been shown that insulin stimulates nuclear exit of TC45, colocalization with the IR and rapid oxidation of TC45 resulting in inactivation of TC45. [41]. Accordingly, cells lacking TCPTP show enhanced insulin-induced activation of PKB [42]. This indicates that TCPTP, similarly to PTP1B, acts to inhibit insulin signaling and that insulin-induced oxidation relieves this inhibition. However, PTP1B and TCPTP have been also shown to display substrate specificity. For example it has been reported for JAK/STAT signaling that JAK2 is dephosphorylated by PTP1B whereas TCPTP shows preference for JAK1/3, thus these two phosphatases may have both a redundant and a complementary role in regulating insulin signaling.

Two other PTPs, i.e. low molecular weight-PTP (LMWPTP) and a Src homology 2 (SH2) domain containing phosphatase (SHP2), have also been suggested as negative regulators of insulin signaling. However, $SHP2^{-/-}$ mice do not display obvious deregulation of insulin signaling [43] and knowledge on LMWPTP is lacking in this respect. Also, LMWPTP and SHP2 are oxidized in response to platelet-derived growth factor (PDGF) stimulation [44]. Considering that PTP1B and TCPTP are oxidized in response to insulin and EGF and this oxidation has no impact on PDGF signaling, it is equally possible that LMWPTP is specific to PDGF rather than insulin signaling.

Studies from the groups of Rhee and Downes showed that treatment of cells with EGF, PDGF or insulin all result in inactivation of PTEN due to formation of an intramolecular disulphide between the catalytically active cysteine and a proximal 'backdoor' cysteine [45, 46]. Subsequently, studies employing glutathione peroxidase (GPX1) knockout mice reveal that these mice exhibit increased insulin sensitivity and were protected from high-fat-diet-induced insulin resistance due to enhanced glucose uptake in muscles [47]. Prolonged and elevated oxidation of PTEN is at least partly responsible for this phenotype. At the cellular level, GPX1 deficiency enhances insulin-induced ROS levels, which promotes oxidation of PTEN resulting in its inactivation. This in turn elevates PI3K/Akt signaling and improves insulin sensitivity. In agreement, the phenotype exhibited by $GPX1^{-/-}$ mice can be reversed by treatment with the antioxidant N-acetyl cysteine [47]. Taken together, these studies further support the promoting role of ROS in insulin signaling due to reversible oxidation and inhibition of phosphatases. Thus, activation of insulin signaling leads to elevation in H_2O_2 levels that potentiates signal transduction due to oxidative inactivation of PTPs, which otherwise counteract insulin actionKinases (Figure 5)

Besides phosphatases, ROS can also alter the activity of other proteins involved in insulin action and consequently, suppress or stimulate transmission of insulin signaling.

The crystal structure of PKB revealed an intermolecular disulphide bond between two cysteine residues within the kinase domain, making PKB a possible target of redox regulation. Indeed, increase in ROS may result in cysteine oxidation within PKB to form an inter-

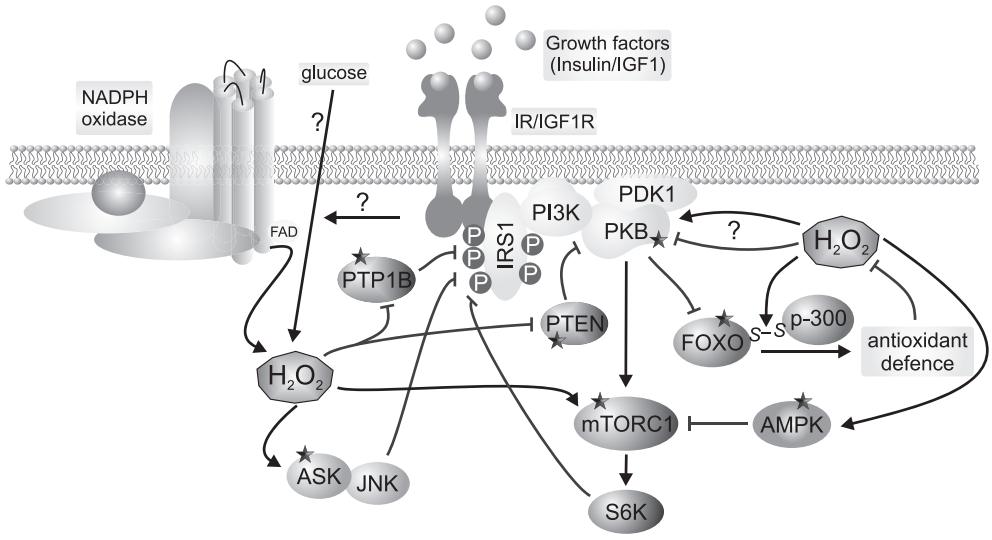

Figure 5. ROS-mediated feed-forward and feedback signaling regulating insulin action
Cysteine oxidation is a major way by which H2O2 can modulate protein function. The known mediators of insulin signaling that are directly sensitive to cysteine oxidation are indicated by 'stars'. ROS can have stimulatory effects on insulin signaling due to inactivation of PTPs (e.g. PTP1B and PTEN). In turn ROS promotes feedback signaling via e.g. JNK and mTOR, which attenuates insulin action. In addition ROS-mediated activation of FOXO is important for antioxidant defences decreasing cellular ROS levels and therefore diminishes negative feedback signaling. Whether ROS promotes or demotes insulin signaling possibly depends on ROS levels and duration.

nal disulphide bond. This promotes the binding of protein phosphatase 2 (PP2A) to PKB and inhibition of its activity. However, PP2A itself contains redox-sensitive cysteine residue and is potentially susceptible to inhibition by H_2O_2. Moreover, oxidized PKB can be reduced by glutaredoxins (GRX) and therefore overexpression of GRX sustains PKB activity [48]. Thus, ROS can both activate or inhibit PKB activity. This raises the possibility that during insulin signaling PKB is differentially regulated depending on the duration and strength of ROS signaling.

Besides PKB, also other kinases directly or indirectly involved in insulin signaling can be subjected to regulation by cysteine oxidation. H_2O_2 treatment of cells results in activation of AMP-dependent protein kinase (AMPK) and this can occur independently of changes in the AMP/ATP ratio. In agreement with this, both *in vitro* as in cells, ROS induced S-glutathionylation of both α and β subunits of AMPK and mutation of Cys^{299} of AMPK resulted in loss of AMPK responsiveness to ROS [49]. Insulin activates numerous PKC isoforms including PKCβ and this isoform has also been shown to become active after increase in ROS [50]. However, the mechanism underlying PKCβ activation by ROS is still unresolved. Finally, structural analysis of mammalian target of rapamycin (mTOR) revealed a disulphide bond apparently involved in regulation of protein stability [51]. This may be a mechanism via which the cellular redox state would regulate the abundance and thus the activity of mTOR.

Importantly, cellular ROS can also

have an impact on insulin signaling via stress responsive pathways. For example, it has been shown that tumor necrosis factor α (TNFα) treatment of Huh7 cells increases mitochondrial ROS levels and activates apoptosis signal-regulating kinase 1 (ASK1), which resulted in impaired insulin signaling [52]. In unstimulated cells ASK1 is in complex with TRX1, which prevents it from becoming active. H_2O_2 induces dissociation of TRX1 and formation of intermolecular disulfide bond between ASK1 monomers resulting in di/multimerization and activation of ASK1 [53]. The ASK1-dependent negative regulation of insulin signaling is most probably mediated by its downstream target c-Jun NH_2-terminal kinase 1 (JNK1), which can target IRS for degradation. ASK1-mediated activation of JNK1 can be reversed by MAPK phosphatases (MKPs), which similarly to other PTPs can be negatively regulated due to oxidation of their catalytic cysteine. Thus, elevated ROS levels promote phosphorylation of JNK1 that is necessary for its enzymatic activity by activating upstream kinases and inhibiting phosphatases.

In addition to targeting IRS, JNK can potentially regulate the activity of several other insulin signaling intermediates such as PKB and FOXO (for a recent review see [54]). In case of FOXOs, it has been shown that in response to H_2O_2 treatment JNK directly phosphorylates FOXO4 in a Ral-dependent manner [55]. This phosphorylation counteracts the PKB mediated export of FOXO4 from the nucleus, promoting FOXO4 activation under oxidative stress conditions (reviewed in [26, 54, 56]). Besides being a target of upstream redox sensitive pathways, FOXO4 itself functions as a redox sensor. Increased ROS triggers disulfide bond formation between FOXO4 and regulatory proteins i.e. E1A binding protein p300 (p300)/CREB-binding protein (CBP) acetyl transferases and this is indispensable for FOXO4 acetylation [57]. As FOXOs also direct transcription of antioxidant genes this makes them true redox sensors.

In summary, it is apparent that ROS regulates multiple transducers of insulin signaling and by that can inhibit or sustain signaling. This suggests that ROS concentrations and localization are important determinants for the outcome of the interplay between ROS and insulin signaling.

The enemy: ROS deregulating insulin signaling

As discussed above, ROS play an important role in proper tuning of insulin signal transduction and the importance of ROS is further emphasized, albeit paradoxically, by substantial evidence that implies ROS in the development of diabetes, but also in the complications accompanying diabetes, such as cardiovascular problems, kidney disease, stroke etc. [58]. Aberrant ROS signaling plays a role in both type 1 diabetes, characterized by loss of insulin production due to loss of pancreatic β-cells, and type 2 diabetes, characterized by progressive loss of insulin responsiveness combined with β-cell dysfunction during disease progression [58]. A variety of conditions can cause diabetes, including hyperinsulinemia, hyperglycemia, obesity, inflammatory signaling etc., and although the mechanism(s) employed by these conditions to induce diabetes is in part unknown, the possibility emerges that ROS play a unifying causal role under all these conditions.

Initially, it was shown that in TNFα- and dexamethasone-induced insulin resistance, cellular redox changes and

ROS levels increase [59]. Reversal of ROS increase, by MnSOD, and catalase expression, or administration of SOD mimetics, all relieved insulin resistance to some extent [59, 60]. Also, in obese mice as well as insulin-resistant mice reducing ROS levels increased insulin sensitivity and glucose homeostasis [59, 61].

In agreement, TNFα-mediated insulin resistance results from TNFα-induced activation of the stress kinase JNK [62] and TNFα induction of JNK requires ROS, and JNK activity is controlled by cellular redox in general. Numerous studies showed that JNK1 mediates phosphorylation of IRS-1 on Ser^{307} (corresponding to Ser^{312} in human) [63]. This residue is adjacent to the phosphotyrosine binding domain and its phosphorylation disrupts the interaction between IRS-1 and IR, and thereby hinders Tyr phosphorylation and promotes the degradation of IRS-1 [64]. Moreover, JNK can phosphorylate IRS-2 on Thr^{347}, which likely is an inhibitory residue as well [65]. The significance of JNK in the regulation of insulin signaling has been confirmed in $JNK^{-/-}$ mice where genetic disruption of JNK1 (but not JNK2) results in resistance to obesity and reduction in insulin resistance, accompanied by a decrease in IRS-1 serine phosphorylation [62, 63]. In addition, total JNK activity is often significantly elevated in various tissues in type 2 diabetic patients and in animal models of obesity and diabetes [62].

Finally, inhibition of JNK signaling using competitive inhibitors disrupting the interaction between JNK and the scaffolding protein JNK-interacting protein-1 (JIP1) restores insulin sensitivity in db/db mice [66, 67]. Similarly, mice expressing a mutant form of JIP1, unable to activate JNK, are protected against obesity-induced insulin resistance [68].

Besides regulating JNK activity, TNFα is critical in the induction of inflammatory responses, and chronic low-level inflammation is a key pathogenic mechanism underlying type 2 diabetes [69]. The so-called metabolic inflammation is induced by accumulation of fat tissue and exhaustion of the lipid storage capacity of adipocytes as depicted in Figure 6. This triggers infiltration of fat tissue by immune cells e.g. neutrophils, eosinophils and macrophages. Once recruited, macrophages express TNFα and other inflammatory cytokines, which sustains the inflammatory state interfering with insulin signaling. Interestingly, adipocytes can also initiate inflammatory responses, which is most probably primary to the recruitment of macrophages [69]. Adipocyte-induced inflammation is possibly mediated by ER- and oxidative-stress, both associated with obesity, (see also Figure 7). In addition, TNFα-mediated inhibition of peroxisome proliferator-activated receptor γ (PPARγ) activity resulting in increased levels of circulating free fatty acids (FFA) and deposition of triglycerides (TGs) in nonfat tissues is also crucial to the induction of systemic inflammation [70].

Another important mediator of the inflammatory functions of TNFα is IκB kinase β (IKKβ). Importantly, IKKβ can interact with IRS-1 and phosphorylate the inhibitory Ser^{307}[64], and reduced signaling through the IKKβ pathway, either by salicylate inhibition or decreased IKKβ expression is accompanied by improved insulin sensitivity *in vivo* [71]. Furthermore, selective deletion of IKKβ in myeloid cells preserves whole body insulin sensitivity and protects against HFD induced resistance to insulin. In contrast, liver-specific IKKβ deficiency leads to the development of insulin resistance in muscle and fat in response

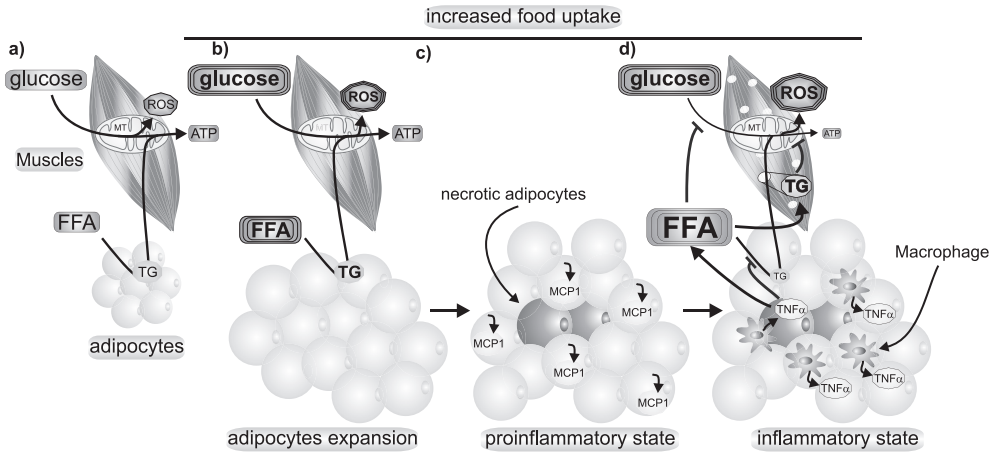

Figure 6. Obesity-induced inflammation as an underlying mechanism of insulin resistance
In the lean state **(a)**, FFA are primary stored by adypocytes as TGs, which provides energy supply for muscles. **(b)** Overnutrition (elevated levels of circulating FFA) leads to increased TG levels and adipocyte enlargement. **(c)** Further influx of FFA promotes necrosis of adipocytes and secretion of chemoattractants (e.g. MCP1), which all together result in the infiltration of adipose tissue by macrophages. **(d)** The macrophages-mediated secretion of proinflammatory cytokines (e.g.TNFα) impairs storage of TGs and enhances lypolisis, thereby further increasing levels of circulating FFA, which eventually accumulate in muscles. This hinders mitochondrial respiration and insulin-stimulated glucose transport. In addition, TNFα induces systemic inflammation, which all together leads to insulin resistance in peripheral tissues (e.g. muscle). The figure was adapted from [70].

to HFD, obesity or aging [72]. These contradictory results may indicate that phosphorylation on IRS-1 has tissue specific consequences. Additionally, the notion that multiple serine residues in IRS-1 are phosphorylated suggests the possibility that the co-occurrence of a specific set of multiple phosphorylations may differentially regulate signal transduction [64]. Moreover, insulin itself stimulates serine phosphorylation on IRS-1 through multiple kinases including PKB, PKCζ, MAPK, glycogen synthase kinase 3 (GSK) and p70 S6 kinase (S6K) [64]. It remains to be established whether phosphorylation of IRS-1 by these kinases has an inhibitory effect on insulin signaling. However, similar to JNK, S6K deletion in mice protects against age- and diet-induced obesity while enhancing insulin sensitivity [64], suggesting negative feedback by S6K. In agreement with their role in regulating S6K activity, pharmacological inhibition of mTOR (rapamycin) or activation of AMPK (metformin or aminoimidazole carboxamide ribonucleotide (AICAR)) provides similar protection against development of insulin resistance in response to HFD, obesity or aging [64]. As discussed above, besides JNK, also AMPK and mTOR are potential direct targets of cellular ROS and as such ROS may also signal through these proteins to mediate insulin resistance.

The above illustrates the importance of serine phosphorylation of IRS proteins in determining the outcome of insulin signaling and clearly ROS are important but not unique regulators of IRS phosphorylation. However, many studies confirmed a role for ROS in inducing insulin resistance and/or β-cell failure, which we in brief summarize below.

Hyperglycemia and ROS

Diabetes is characterized by hyperglycemia. Hyperglycemia induces an increase in cellular ROS and cellular damage [73] and this involves at least four metabolic pathways, i.e. increased polyol pathway flux, increased advanced glycation end product (AGE) formation, activation of PKC isoforms and increased hexosamine pathway flux [73].

Hyperglycemia results in enhanced shunting of glucose into the hexosamine pathway, generating excess NADH and $FADH_2$ and a subsequent increase in mitochondrial membrane potential. This inhibits electron transfer at complex III resulting in a marked increase in $O_2^{\bullet-}$ production [73]. Increased $O_2^{\bullet-}$ levels inhibit glucose-6-phosphate dehydrogenase that is essential for providing reducing equivalents (NADPH) to the antioxidant defense system. Hyperglycemia also elevates sorbitol, which is metabolized to fructose, thereby increasing the ration $NADH/NAD^+$ [74]. This results in *de novo* synthesis of diacylglycerol followed by activation of PKC, IKKβ and NADPH oxidases. PKCβ activation by hyperglycemia may be important in this respect since it has been shown that $PKC\beta^{-/-}$ mice are protected against diet-induced obesity and insulin resistance [25].

In agreement with the above, decreasing $O_2^{\bullet-}$ production by depolarization of the mitochondrial membrane was sufficient to abolish the activation of pathways downstream of hyperglycemia. Activation of uncoupling proteins (UCPs) lowers the mitochondrial membrane potential due to their ability to facilitate proton leakage across the membrane, thereby inducing partial depolarization and decrease in membrane potential. Consequently, this leads to reduced electron flow via the ETC and a decline in superoxide production [73]. It has been shown that endogenously produced mitochondrial $O_2^{\bullet-}$ enhances expression of UCP2 and activates UCP2-mediated proton leakage followed by a reduction in ATP levels and an impairment in glucose-stimulated insulin secretion [73]. $UCP2^{-/-}$ mice exhibit a higher level of ROS production and ectopic expression of UCPs in cultured neurons blocks glucose-induced apoptosis by preventing mitochondrial hyperpolarization and formation of ROS [73, 75]. Similarly, it has been shown that UCP2 is important for protection of β-cell against oxidative stress [73]. Thus, UCPs play an essential role in the regulation of ROS levels generated by mitochondria and deregulation of their activity might be a key factor involved in glucotoxicity.

Obesity and ROS (Figure 6)

Obesity caused by excessive intake of nutrients including glucose and fat is rapidly becoming the major cause of diabetes. Several mechanisms by which nutrient excess contributes to diabetes onset have been proposed. For example, enhanced fat intake may account for increased FFA flux into circulation with ectopic accumulation of fat in the skeletal muscle and this is associated with insulin resistance in human [70]. This is supported by the observation that both excessive fat tissue, as seen in common obesity, or the inability of store fat, as seen in congenital or acquired lipodystrophy, are associated with insulin resistance [70, 76]. FFA and intermediates have been shown to activate the immune responses (see above) but also PKC-θ, a serine/threonine kinase that can phosphorylate serine residues of IRS and thus attenuate insulin signaling [64]. Recent data also connect chronic increased nutrient intake to ROS production. Ex-

cessive nutrients, through metabolic pathways generate a surplus of reducing equivalents and augment the rate of electron flux through the mitochondrial ETC. Electron leakage from complex I and III of the ETC will increase accordingly, leading to increased production of $O_2^{\bullet-}$ and subsequently H_2O_2 [70]. As discussed above this activates JNK, which in turn phosphorylates IRS to inhibit insulin signaling.

There is also substantial evidence for ER stress as an underlying mechanism for obesity-mediated deterioration in insulin signaling and the development of diabetes. Using cell culture and mouse models it has been shown that obesity causes ER stress in liver and adipose tissue, which subsequently leads to suppression of insulin signaling via hyperactivation of JNK [77]. In addition, mice haploinsufficient for XBP1 are prone to ER stress and eventually develop diet-induced peripheral insulin resistance and type 2 diabetes [77]. In accordance, treatment of obese and diabetic mice with chemical or pharmaceutical chaperones results in normalization of hyperglycemia, restoration of systemic insulin sensitivity, resolution of fatty liver disease, and enhancement of insulin function [78]. In addition, a direct link between PI3K signaling and the regulation of the cellular response to ER stress has been proposed. The p85α regulatory subunit of PI3K was shown to interact with XBP1 in an ER stress-dependent manner. This interaction is abolished in ob/ob mice, resulting in a severe defect in XBP-1 translocation to the nucleus. Similarly, liver specific p85α deletion inhibits nuclear translocation of XBP1 leading to attenuation of UPR [79]. These studies directly link UPR to PI3K pathway suggesting that insulin itself may support UPR activity.

β-cell failure due to ROS (Figure 7)

Pancreatic β-cells are highly specialized to produce and secrete insulin in response to elevated glucose levels. This imposes a high oxidative protein folding demand on β-cells. Therefore, β-cells contain a relative low level of antioxidants, the downside being vulnerability to changes in cellular redox. In agreement, mice deficient for PERK, an UPR transducer responsible for attenuating global protein translation, display reduced β-cell mass [80]. This phenotype is reminiscent of the Wolcott-Rallison syndrome in human, an autosomal recessive disorder resulting from PERK mutation and characterized by severe infantile diabetes [81]. Adversely, the transcription factor C/EBP homologous protein (CHOP) also involved in UPR, can mediate β-cell apoptosis under ER stress. Therefore, mice lacking CHOP were protected from ER stress-induced β-cell apoptosis [82]. Furthermore, overexpression of the ER chaperone, BiP/GRP78, reverses hyperglycemia-induced inhibition of insulin synthesis and secretion [83]. Thus, ER-stress is an important parameter of β-cell function.

Whereas in type 1 diabetes β-cells are depleted due to auto-immune reactions, defective proliferation is a major cause of β-cell dysfunction in type 2 diabetes. This is illustrated by the diabetic phenotype of numerous transgenic mice with alterations in cell cycle regulatory genes. For example, $p27^{kip1-/-}$ mice show increased β-cell proliferation and suppressed hyperglycemia, whereas $p27^{kip1}$ overexpression resulted in severe diabetes [84]. In addition, $cyclinD2^{-/-}$ mice develop severe early onset diabetes because of defective β-cell replication [85]. A common feature of these perturbations is that these are all under the control of PKB/FOXO signaling (Figure 1). In

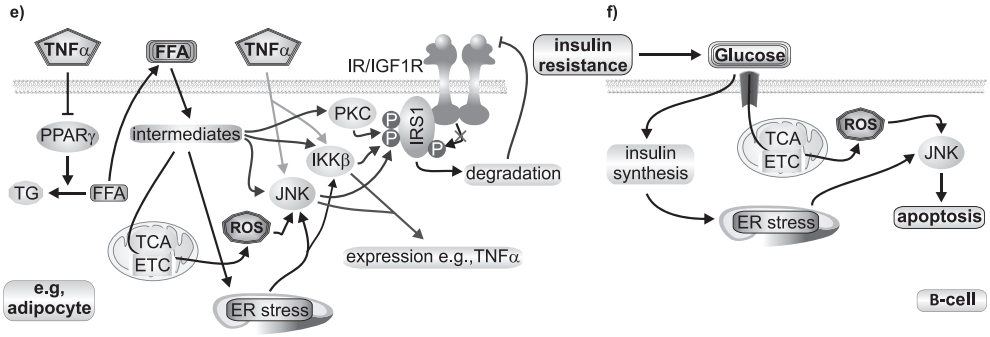

Figure 7. Molecular mechanism of obesity-induced insulin resistance and β-cell failure
(a) At the molecular level, TNF signaling and accumulation of intracellular lipid metabolites induce the activity of various serine kinases that phosphorylate IRS, thereby triggering its degradation. This leads to decrease in glucose uptake and insulin resistance. **(b)** Elevated glucose levels, due to overnutrition or insulin resistance, increase the demand for insulin production. This induces ER stress in β-cells. Simultaneously, overload of mitochondria enhances ROS production and triggers mitochondrial stress, thereby decreasing ATP production. Together this may cause depletion of β-cells due to induction of apoptosis.

agreement, FOXOs regulate β-cells proliferation in addition to gluconeogenesis and gain-of-function mutation of FOXO1 in mice causes diabetic phenotype arising from increased hepatic glucose output and reduced β-cell mass [86].

Diabetes and mitochondrial dysfunction

Insulin regulates mitochondrial metabolism in numerous ways (for a recent review see [28]) and insulin resistance is associated with mitochondrial dysfunction. Recently, it was found that FOXO1 integrates insulin signaling with mitochondrial functions [87]. Liver specific deletion of IRS-1 and IRS-2 causes insulin resistance, but also severe mitochondrial abnormalities followed by disruption in integrity of mitochondrial ETC. As a consequence, ATP generation and fatty acid oxidation is impaired. IRS-1/2 deletion as well as obesity-induced hepatic insulin resistance was shown to correlate with hyperactive FOXO1 resulting in deregulated expression of many hepatic genes including heme oxygenase (decycling) 1 (*Hmox1)*. HMOX1 can disrupt ETC, which attenuates the concentration of NAD^+ leading to inhibition of the NAD-dependent deacetylase sirtuin-1 (SIRT1). SIRT1-mediated acetylation of PGC1α abolishes its ability to promote mitochondrial biogenesis [87]. Thus, hyperactivated FOXO1, due to decreased insulin signaling, is central in deregulating mitochondrial function during hepatic insulin resistance. Importantly, dysfunctions in mitochondrial ETC may also result in elevated ROS production leading to oxidative stress and progression to diabetes by JNK and other stress kinases as discussed above. Importantly, under these conditions ROS contributes to FOXO activation whereas SIRT1 supports deacetylation of FOXO1 yet this increases FOXO1 transcriptional activity. Hence, inhibition of SIRT1 not only increases the acetylation of PGC1α but also FOXO1, raising the question why increased acetylation of FOXO1 cannot counteract the increased activity of FOXO observed due to aberrant insulin signaling. One possible explanation may be that acetylation of FOXO rather

regulates substrate specificity as previously suggested [57].

Recent studies have also indicated an important role for mTOR in regulating mitochondrial function. Pharmacological inhibition of mTOR results in decreased expression of the PGC1, followed by a reduction in mitochondrial gene expression and oxygen consumption [88]. In agreement, mice specifically lacking raptor in muscle and therefore a functional mTOR complex 1 (TORC1), exhibit severe attenuation of the number of mitochondria, paralleled by an increase in glycogen content [89]. This results in impaired oxidative capacity of muscles and a compensatory increase in glycolytic activity. Interestingly, targeted deletion of raptor in adipose tissue does not affect the number of mitochondria but it increases expression of UCP1 [89]. This is most probably the underlying mechanism for elevated energy expenditure observed in these mice. Probably therefore, adipose knockout mice are resistant to diet-induced obesity and exhibit insulin hypersensitivity with enhanced glucose tolerance. The insulin sensitivity is due to increased insulin signaling via PKB in both adipose tissue and skeletal muscle [89]. Taken together, these studies delineate a role of mTOR in metabolic and mitochondrial control in adipose and muscle tissues. Nevertheless, the details hereof are not yet fully understood and at least appear tissue specific.

ROS, ageing and the insulin axis

In the nematode *Caenorhabdites elegans*, an insulin-like signaling pathway controls the FOXO homolog abnormal dauer formation protein 16 (DAF-16). Remarkably, insulin signaling to DAF-16 has been shown to be a key component in controlling adult *C. elegans* lifespan [90]. One of the prevailing theories in ageing is the 'free-radical theory of ageing', which states that the life-time accumulation of cellular damage caused mostly by ROS, drives the process of ageing. Along this line, it has been suggested that the ability of the insulin/DAF-16 pathway to lower oxidative stress rationalizes this concept. In contrast, recent evidence opposes this concept or at least argues it not to be as straightforward. For example, glucose restriction alters mitochondrial metabolism and elevates ROS levels yet increasing lifespan of *C. elegans* [91]. However, the primary biological role of insulin/DAF-16(FOXO) signaling is to allow adaptation to hostile conditions and apparently adaptation can also be triggered by mild conditions of stress. The phenomenon of stress tolerance induced by a mild stress condition is generally referred to as 'hormesis' (for a balanced review see [92]). Hormesis can be induced by various forms of stress, including ER stress [93], and in case of oxidative tolerance due to improved mitochondrial function this is also called mitohormesis. Hormesis or mitohormesis may result in increased lifespan [91, 92]. Thus, the common denominator for ageing remains increased stress resistance whether it is induced due to loss of insulin signaling or mild preconditioning by stress. Interestingly, we have shown FOXO to be activated by ROS due to JNK-mediated phosphorylation [55] and JNK extends lifespan in a DAF-16/FOXO dependent manner [94]. Consequently, DAF-16/FOXO can be activated by loss of insulin signaling or increased ROS. Indeed, DAF-16 has been implied in mediating some forms of hormesis [92].

The above reinforces the central role of ROS in tailoring insulin signaling in ageing and disease but also indicates

that our understanding of this interplay is still far from complete.

Concluding remarks

The insulin-dependent production of ROS is indispensable for the proper propagation of insulin signaling. However, excessive ROS generation also poses a threat to insulin signaling and may cause disruption leading to diabetes. The physiological function of ROS in the regulation of insulin signaling can be simplified to the generation of $O^{2\bullet-}$ and H_2O_2 by NADPH oxidases and cell organelles (Figure 4). H_2O_2 promotes signal transduction predominantly due to oxidative inactivation of inhibitory phosphatases. Remarkably, ROS through other targets e.g. JNK also potently downregulates insulin signaling. Thus, for this dual activity of ROS to result in sensible signaling location of ROS production, level of ROS concentration, ROS lifetime etc. will be important determinants. Low levels of ROS existing over a short period of time most likely will have a stimulatory effect on insulin signaling and promote proliferation. Conversely, high and sustained ROS levels due to possible induction of oxidative stress would inhibit insulin activity to stop proliferation and allow activation of stress responses. Further understanding of this intricate interplay between ROS and insulin may not only result in the development of effective treatments for diabetes, but may also expand our understanding as to how ROS and insulin cooperatively affect lifespan and possibly also other age-related disease. As such this will remain an important paradigm for further studies.

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References

1. Avruch, J., Insulin signal transduction through protein kinase cascades. Mol Cell Biochem, 1998. 182(1-2): p. 31-48.
2. Taniguchi, C.M., B. Emanuelli, and C.R. Kahn, Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol, 2006. 7(2): p. 85-96.
3. Maassen, J.A., et al., The role of ras proteins in insulin signal transduction. Horm Metab Res, 1992. 24(5): p. 214-8.
4. George, S., et al., A family with severe insulin resistance and diabetes due to a mutation in AKT2. Science, 2004. 304(5675): p. 1325-8.
5. Altomare, D.A. and J.R. Testa, Perturbations of the AKT signaling pathway in human cancer. Oncogene, 2005. 24(50): p. 7455-64.
6. Katso, R., et al., Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. Annu Rev Cell Dev Biol, 2001. 17: p. 615-75.
7. Stone, J.R. and S. Yang, Hydrogen peroxide: a signaling messenger. Antioxid Redox Signal, 2006. 8(3-4): p. 243-70.
8. Giorgio, M., et al., Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? Nat Rev Mol Cell Biol, 2007. 8(9): p. 722-8.
9. Antonenkov, V.D., et al., Peroxisomes are oxidative organelles. Antioxid Redox Signal. 13(4): p. 525-37.
10. Malhotra, J.D. and R.J. Kaufman, Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal, 2007. 9(12): p. 2277-93.
11. Zhang, D.X. and D.D. Gutterman, Mitochondrial reactive oxygen species-mediated signaling in endothelial cells. Am J Physiol Heart Circ Physiol, 2007. 292(5): p. H2023-31.
12. Leto, T.L., et al., Targeting and regulation of reactive oxygen species generation by Nox family NADPH oxidases. Antioxid Redox Signal, 2009. 11(10): p. 2607-19.
13. Ushio-Fukai, M., Compartmentalization of redox signaling through NADPH oxidase-derived ROS. Antioxid Redox Signal, 2009. 11(6): p. 1289-99.
14. Bokoch, G.M., et al., Emerging evidence for the importance of phosphorylation in the regulation of NADPH oxidases. Antioxid Redox Signal, 2009. 11(10): p. 2429-41.
15. Cullinan, S.B. and J.A. Diehl, Coordination of ER and oxidative stress signaling: the PERK/Nrf2 signaling pathway. Int J Biochem Cell Biol, 2006. 38(3): p. 317-32.
16. Santos, C.X., et al., Mechanisms and implications of reactive oxygen species generation during the unfolded protein response: roles of endoplasmic reticulum oxidoreductases, mitochondrial electron transport, and NADPH oxidase. Antioxid Redox Signal, 2009. 11(10): p. 2409-27.
17. Csordas, G. and G. Hajnoczky, SR/ER-mitochondrial local communication: calcium and ROS. Biochim Biophys Acta, 2009. 1787(11): p. 1352-62.
18. Goldstein, B.J., et al., Role of insulin-induced reactive oxygen species in the insulin signaling pathway. Antioxid Redox Signal, 2005. 7(7-8): p. 1021-31.
19. Mahadev, K., et al., The NAD(P)H oxidase homolog Nox4 modulates insulin-stimulated generation of H2O2 and plays an integral role in insulin signal transduction. Mol Cell Biol, 2004. 24(5): p. 1844-54.
20. Carnesecchi, S., et al., Insulin-induced vascular endothelial growth factor expression is mediated by the NADPH oxidase NOX3. Exp Cell Res, 2006. 312(17): p. 3413-24.
21. von Lohneysen, K., et al., Mutational analysis reveals distinct features of the Nox4-p22 phox complex. J Biol Chem, 2008. 283(50): p. 35273-82.
22. Ravichandran, K.S., Signaling via Shc family adapter proteins. Oncogene, 2001. 20(44): p. 6322-30.
23. Paulsen, C.E. and K.S. Carroll, Orchestrating redox signaling networks through regulatory cysteine switches. ACS Chem Biol. 5(1): p. 47-62.
24. Gertz, M., et al., Activation of the lifespan regulator p66Shc through reversible disulfide bond formation. Proc Natl Acad Sci U S A, 2008. 105(15): p. 5705-9.
25. Huang, W., et al., Loss of protein kinase Cbeta function protects mice against diet-induced obesity and development of hepatic steatosis and insulin resistance. Hepatology, 2009. 49(5): p. 1525-36.
26. de Keizer, P., B. Burgering, and T.B. Dansen, FOXO as a sensor, mediator and regulator of redox signaling. Antioxid Redox Signal.
27. Sarsour, E.H., et al., Redox control of the cell cycle in health and disease. Antioxid Redox Signal, 2009. 11(12): p. 2985-3011.
28. Cheng, Z., Y. Tseng, and M.F. White, Insulin signaling meets mitochondria in metabolism. Trends Endocrinol Metab. 21(10): p. 589-98.
29. van Montfort, R.L., et al., Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B. Nature, 2003. 423(6941): p. 773-7.
30. Meng, T.C., T. Fukada, and N.K. Tonks, Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo. Mol Cell, 2002. 9(2): p. 387-99.
31. Woo, H.A., et al., Inactivation of peroxiredoxin

I by phosphorylation allows localized H(2)O(2) accumulation for cell signaling. Cell. 140(4): p. 517-28.
32. Wood, Z.A., L.B. Poole, and P.A. Karplus, Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. Science, 2003. 300(5619): p. 650-3.
33. Fourquet, S., et al., The dual functions of thiol-based peroxidases in H2O2 scavenging and signaling. Antioxid Redox Signal, 2008. 10(9): p. 1565-76.
34. Kenner, K.A., et al., Protein-tyrosine phosphatase 1B is a negative regulator of insulin- and insulin-like growth factor-I-stimulated signaling. J Biol Chem, 1996. 271(33): p. 19810-6.
35. Seely, B.L., et al., Protein tyrosine phosphatase 1B interacts with the activated insulin receptor. Diabetes, 1996. 45(10): p. 1379-85.
36. Elchebly, M., et al., Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. Science, 1999. 283(5407): p. 1544-8.
37. Zhang, Z.Y. and S.Y. Lee, PTP1B inhibitors as potential therapeutics in the treatment of type 2 diabetes and obesity. Expert Opin Investig Drugs, 2003. 12(2): p. 223-33.
38. Mahadev, K., et al., Insulin-stimulated hydrogen peroxide reversibly inhibits protein-tyrosine phosphatase 1b in vivo and enhances the early insulin action cascade. J Biol Chem, 2001. 276(24): p. 21938-42.
39. Lee, S.R., et al., Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. J Biol Chem, 1998. 273(25): p. 15366-72.
40. Chen, K., et al., Regulation of ROS signal transduction by NADPH oxidase 4 localization. J Cell Biol, 2008. 181(7): p. 1129-39.
41. Meng, T.C., et al., Regulation of insulin signaling through reversible oxidation of the protein-tyrosine phosphatases TC45 and PTP1B. J Biol Chem, 2004. 279(36): p. 37716-25.
42. Galic, S., et al., Regulation of insulin receptor signaling by the protein tyrosine phosphatase TCPTP. Mol Cell Biol, 2003. 23(6): p. 2096-108.
43. Asante-Appiah, E. and B.P. Kennedy, Protein tyrosine phosphatases: the quest for negative regulators of insulin action. Am J Physiol Endocrinol Metab, 2003. 284(4): p. E663-70.
44. Chiarugi, P., PTPs versus PTKs: the redox side of the coin. Free Radic Res, 2005. 39(4): p. 353-64.
45. Kwon, J., et al., Reversible oxidation and inactivation of the tumor suppressor PTEN in cells stimulated with peptide growth factors. Proc Natl Acad Sci U S A, 2004. 101(47): p. 16419-24.
46. Leslie, N.R., et al., Redox regulation of PI 3-kinase signalling via inactivation of PTEN. EMBO J, 2003. 22(20): p. 5501-10.
47. Loh, K., et al., Reactive oxygen species enhance insulin sensitivity. Cell Metab, 2009. 10(4): p. 260-72.
48. Murata, H., et al., Glutaredoxin exerts an antiapoptotic effect by regulating the redox state of Akt. J Biol Chem, 2003. 278(50): p. 50226-33.
49. Zmijewski, J.W., et al., Exposure to Hydrogen Peroxide Induces Oxidation and Activation of AMP-activated Protein Kinase. J Biol Chem. 285(43): p. 33154-64.
50. Giorgi, C., et al., Redox control of protein kinase C: cell- and disease-specific aspects. Antioxid Redox Signal. 13(7): p. 1051-85.
51. Dames, S.A., et al., The solution structure of the FATC domain of the protein kinase target of rapamycin suggests a role for redox-dependent structural and cellular stability. J Biol Chem, 2005. 280(21): p. 20558-64.
52. Imoto, K., et al., Impact of mitochondrial reactive oxygen species and apoptosis signal-regulating kinase 1 on insulin signaling. Diabetes, 2006. 55(5): p. 1197-204.
53. Nadeau, P.J., et al., Disulfide Bond-mediated multimerization of Ask1 and its reduction by thioredoxin-1 regulate H(2)O(2)-induced c-Jun NH(2)-terminal kinase activation and apoptosis. Mol Biol Cell, 2007. 18(10): p. 3903-13.
54. van den Berg, M.C. and B. Burgering, Integrating opposing signals towards FOXO. Antioxid Redox Signal.
55. Essers, M.A., et al., FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. EMBO J, 2004. 23(24): p. 4802-12.
56. Storz, P., FOXO transcription factors in the responses to oxidative stress. Antioxid Redox Signal.
57. Dansen, T.B., et al., Redox-sensitive cysteines bridge p300/CBP-mediated acetylation and FoxO4 activity. Nat Chem Biol, 2009. 5(9): p. 664-72.
58. Bloch-Damti, A. and N. Bashan, Proposed mechanisms for the induction of insulin resistance by oxidative stress. Antioxid Redox Signal, 2005. 7(11-12): p. 1553-67.
59. Houstis, N., E.D. Rosen, and E.S. Lander, Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature, 2006. 440(7086): p. 944-8.
60. Hoehn, K.L., et al., Insulin resistance is a cellular antioxidant defense mechanism. Proc Natl Acad Sci U S A, 2009. 106(42): p. 17787-92.
61. Furukawa, S., et al., Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest, 2004. 114(12): p. 1752-61.

62. Hirosumi, J., et al., A central role for JNK in obesity and insulin resistance. Nature, 2002. 420(6913): p. 333-6.
63. Sabio, G. and R.J. Davis, cJun NH2-terminal kinase 1 (JNK1): roles in metabolic regulation of insulin resistance. Trends Biochem Sci. 35(9): p. 490-6.
64. Boura-Halfon, S. and Y. Zick, Phosphorylation of IRS proteins, insulin action, and insulin resistance. Am J Physiol Endocrinol Metab, 2009. 296(4): p. E581-91.
65. Solinas, G., et al., Saturated fatty acids inhibit induction of insulin gene transcription by JNK-mediated phosphorylation of insulin-receptor substrates. Proc Natl Acad Sci U S A, 2006. 103(44): p. 16454-9.
66. Chen, T., et al., Identification of small-molecule inhibitors of the JIP-JNK interaction. Biochem J, 2009. 420(2): p. 283-94.
67. Stebbins, J.L., et al., Identification of a new JNK inhibitor targeting the JNK-JIP interaction site. Proc Natl Acad Sci U S A, 2008. 105(43): p. 16809-13.
68. Morel, C., et al., Requirement of JIP1-mediated c-Jun N-terminal kinase activation for obesity-induced insulin resistance. Mol Cell Biol. 30(19): p. 4616-25.
69. Hotamisligil, G.S., Inflammation and metabolic disorders. Nature, 2006. 444(7121): p. 860-7.
70. Guilherme, A., et al., Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. Nat Rev Mol Cell Biol, 2008. 9(5): p. 367-77.
71. Yuan, M., et al., Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. Science, 2001. 293(5535): p. 1673-7.
72. Arkan, M.C., et al., IKK-beta links inflammation to obesity-induced insulin resistance. Nat Med, 2005. 11(2): p. 191-8.
73. Rolo, A.P. and C.M. Palmeira, Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress. Toxicol Appl Pharmacol, 2006. 212(2): p. 167-78.
74. Brownlee, M., Biochemistry and molecular cell biology of diabetic complications. Nature, 2001. 414(6865): p. 813-20.
75. Arsenijevic, D., et al., Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production. Nat Genet, 2000. 26(4): p. 435-9.
76. Walker, U.A., Acquired and inherited lipodystrophies. N Engl J Med, 2004. 351(1): p. 103-4; author reply 103-4.
77. Ozcan, U., et al., Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science, 2004. 306(5695): p. 457-61.
78. Ozcan, U., et al., Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science, 2006. 313(5790): p. 1137-40.
79. Wek, R.C. and T.G. Anthony, Obesity: stressing about unfolded proteins. Nat Med. 16(4): p. 374-6.
80. Zhang, W., et al., PERK EIF2AK3 control of pancreatic beta cell differentiation and proliferation is required for postnatal glucose homeostasis. Cell Metab, 2006. 4(6): p. 491-7.
81. Delepine, M., et al., EIF2AK3, encoding translation initiation factor 2-alpha kinase 3, is mutated in patients with Wolcott-Rallison syndrome. Nat Genet, 2000. 25(4): p. 406-9.
82. Song, B., et al., Chop deletion reduces oxidative stress, improves beta cell function, and promotes cell survival in multiple mouse models of diabetes. J Clin Invest, 2008. 118(10): p. 3378-89.
83. Zhang, L., et al., GRP78, but Not Protein-disulfide Isomerase, Partially Reverses Hyperglycemia-induced Inhibition of Insulin Synthesis and Secretion in Pancreatic {beta}-Cells. J Biol Chem, 2009. 284(8): p. 5289-98.
84. Uchida, T., et al., Deletion of Cdkn1b ameliorates hyperglycemia by maintaining compensatory hyperinsulinemia in diabetic mice. Nat Med, 2005. 11(2): p. 175-82.
85. Kushner, J.A., et al., Cyclins D2 and D1 are essential for postnatal pancreatic beta-cell growth. Mol Cell Biol, 2005. 25(9): p. 3752-62.
86. Gross, D.N., A.P. van den Heuvel, and M.J. Birnbaum, The role of FoxO in the regulation of metabolism. Oncogene, 2008. 27(16): p. 2320-36.
87. Cheng, Z., et al., Foxo1 integrates insulin signaling with mitochondrial function in the liver. Nat Med, 2009. 15(11): p. 1307-11.
88. Cunningham, J.T., et al., mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. Nature, 2007. 450(7170): p. 736-40.
89. Shaw, R.J., Raptor swoops in on metabolism. Cell Metab, 2008. 8(5): p. 343-4.
90. Lee, S.S., et al., DAF-16 target genes that control C. elegans lifespan and metabolism. Science, 2003. 300(5619): p. 644-7.
91. Schulz, T.J., et al., Glucose restriction extends Caenorhabditis elegans lifespan by inducing mitochondrial respiration and increasing oxidative stress. Cell Metab, 2007. 6(4): p. 280-93.
92. Gems, D. and L. Partridge, Stress-response hormesis and aging: "that which does not kill us makes us stronger". Cell Metab, 2008. 7(3): p. 200-3.
93. Salminen, A. and K. Kaarniranta, ER stress and

hormetic regulation of the aging process. Ageing Res Rev. 9(3): p. 211-7.
94. Oh, S.W., et al., JNK regulates lifespan in Caenorhabditis elegans by modulating nuclear translocation of forkhead transcription factor/DAF-16. Proc Natl Acad Sci U S A, 2005. 102(12): p. 4494-9.

Summary

The 'Oxygen Paradox' is that higher eukaryotic aerobic organisms cannot exist without oxygen, yet oxygen is innately harmful to their existence. This is associated with the generation of free radicals during respiration, which in general are detrimental to the cell and therefore compromise health and age of the organism. However, reactive oxygen species (ROS) signaling is also involved in the regulation of fundamental biological processes including cell proliferation, survival and migration, along with metabolism, angiogenesis, development and aging. Nevertheless, when ROS levels increase above safe threshold this leads to induction of oxidative stress, which contributes to premature aging or the development of tumors as well as neurodegenerative and autoimmune disorders. Therefore, maintenance of redox homeostasis is crucial for organismal longevity. Under physiological conditions this is sustained through the balanced activation of pathways that produce and metabolize ROS within the cell. Thus, cells are equipped with a natural defense system consisting of antioxidants that can neutralize ROS. The activity of Forkhead Box O (FOXO) transcription factors correlates with the activation of cellular antioxidant defense mechanisms, as FOXOs are able to induce expression of several ROS scavenging enzymes including Manganese Superoxide dismutase (MnSOD) and Catalases. Therefore, precise understanding of how the activity of FOXO proteins is regulated is important for comprehension of ROS signaling.

Previously, it has been shown that FOXO activation in response to elevated cellular ROS levels is triggered by changes in posttranslational modifications (PTMs) on the FOXO proteins, including phosphorylation, acetylation, and ubiquitination. Importantly, there is crosstalk between different PTMs and therefore some modifications can influence others in a positive or negative way. Thus, it appears that specific combinations of PTMs can form a dynamic "FOXO code", which can alter the activity of FOXO proteins but also their localization as well as modulate their binding to other proteins. In this way, ROS signaling might not only stimulate FOXO activity but also modulate their specificity towards different target genes and even inhibit them due to feedback signaling. Interestingly, ROS are also indispensable for insulin signaling, which is established to negatively regulate the activity of FOXO proteins. On the other hand, ROS can also oppose insulin signaling. Thus, due to the great complexity of ROS signaling, the understanding how it regulates FOXO proteins is still incomplete. Therefore, the main goal of this thesis was to expand our knowledge about the molecular mechanisms underlying ROS-dependent regulation of FOXO transcription factors.

Previously, we observed that the FOXO family member FOXO4 is phosphorylated on multiple residues in response to increased ROS levels. Several of these sites are specifically phosphorylated by c-Jun N-terminal kinase (JNK), which stimulates the activity of FOXO4. However, the kinase(s) responsible for the other phosphorylations have not been identified and it has not been determined how do they influence the activity of FOXO4. In Chapter 2 we show that FOXO4 is a substrate for Nemo-like kinase (NLK), which has not been previously implicated in the regulation of oxidative stress responses. The binding between NLK and FOXO4 is strongly induced in response to elevated ROS levels. Moreover, NLK phosphorylates

FOXO4 on multiple residues and the activity of NLK correlates with inhibition of the monoubiquitination of FOXO4. Since, FOXO4 monoubiquitination is associated with nuclear retention and increased activity of FOXO4, ectopic expression of NLK suppresses FOXO4-dependent expression of $p27^{kip1}$, which is a well established FOXO target. This is in line with the fact that ubiquitin-specific-processing protease 7 (USP7), which can remove ubiquitin moieties from FOXO4, also inhibits expression of $p27^{kip1}$. Interestingly, genome wide profiling of gene expression in HEK293T cells ectopically expressing FOXO4 and NLK revealed that NLK besides inhibiting a subset of FOXO4-transctivated genes, is also activating a pool of genes in a FOXO4-dependent manner (data not shown). Since ubiquitination affects lysine residues it is possible that it can modulate the activity of FOXO4 in a way similar to acetylation, which has been shown to affect the specificity of FOXO proteins. This might explain why NLK would inhibit expression of certain FOXO4 target genes and activate others.

A common way to mediate ROS signaling is via oxidation of cysteine residues, which results in formation of intra- and intermolecular cysteine-thiol disulfide bridges. Recently, it has been shown that the acetylation of FOXO4 mediated by the p300 acetyltransferase is fully dependent on a ROS-induced intermolecular disulfide bridge between these proteins. In Chapter 3 we describe another way to regulate FOXO4 acetylation, which involves a nucleolar protein called Myb-binding protein 1a (MYBBP1a). In response to increased ROS levels MYBBP1a binds to FOXO4, which interferes with the acetylation status of FOXO4. This correlates with reduced activity of FOXO4 in a luciferase reporter assay. Interestingly, MYBBP1a is not able to inhibit the activity of a cysteine-free version of FOXO4, which is in line with the fact that acetylation of this mutant of FOXO4 is already greatly reduced. In contrast to the effect in the reporter assay, ectopic expression of MYBBP1a inhibits FOXO4-induced transactivation of endogenous $p27^{kip1}$. This is however in agreement with previous studies showing that acetylation affects the activity of FOXO proteins in an ambiguous way, as acetylation of FOXOs themselves can inhibit their binding to DNA, whereas the acetylation of histones is essential for FOXO-dependent transcription. Thus, it is plausible that MYBBP1a, in addition to inhibition of FOXO4 acetylation, impairs the acetylation of histones, which would provide an explanation for the MYBBP1a-dependent negative regulation $p27^{kip1}$ expression.

To obtain better understanding of the FOXO4 function in the context of a whole organism we decided to study the *in vivo* expression pattern of FOXO4 data of which is presented in Chapter 4. Thus, we generated transgenic knock-in mice expressing an EGFP-Foxo4 fusion protein from the endogenous Foxo4 locus. The overall expression levels of Foxo4 proved to be very low. Therefore, we were not able to detect it using immunofluorescence and histochemical methods. However, FACS analysis of whole bone marrow revealed that Foxo4 is predominantly expressed in the progenitors of hematopoietic stem cells (HPCs). This is in line with previous studies showing that simultaneous depletion of Foxo1, 3 and 4, specifically in the hematopoietic system, results in impairment of the long-term regenerative potential of the hematopoietic stem cell (HSC) compartment. Furthermore,

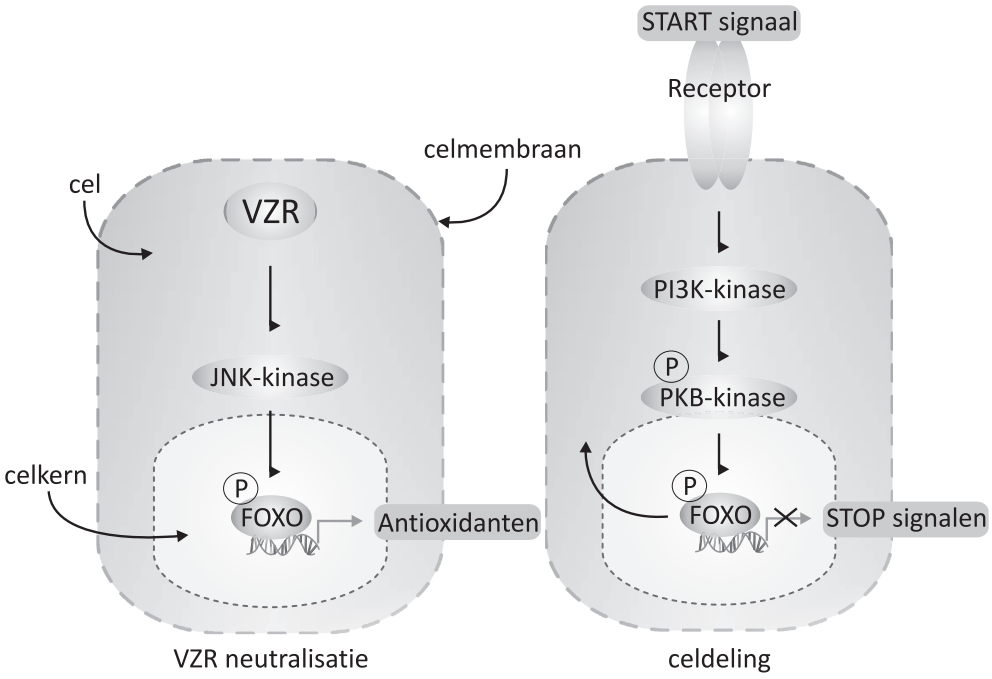
it has been shown that these changes in the HSC pool are due to elevated ROS levels in these cells, in addition to their increased cycling and enhanced apoptosis. A similar phenotype has been observed in mice lacking Foxo3a albeit with a later onset. Thus, the presence of Foxo4 in HPCs might indicate that Foxo4 is important specifically for the maintenance of these cells, whereas Foxo3a is essential for proper functions of HSCs.

In conclusion, this thesis advances our understanding about the *in vitro* and *in vivo* FOXO functions. We showed that in response to elevated ROS levels the activity of FOXO proteins is modulated by two proteins, which have not been implicated in stress signaling before. On other hand, this indicates that cellular responses to ROS might be even more complex that originally anticipated. Furthermore, we observed that in the context of the murine hematopoietic system Foxo4 is most abundantly expressed in the HPCs. This might indicate an important role for Foxo4 in the maintenance of these cells.

Nederlandse samenvatting voor niet-ingewijden

Het menselijk lichaam is een complexe, zeer georganiseerde structuur die bestaat uit verschillende organen zoals het hart, de longen en de nieren. Deze organen werken samen om de specifieke functies te vervullen die nodig zijn om in leven te blijven. Elk orgaan bestaat uit verschillende soorten weefsels en elk van die weefsels wordt gevormd door vele aaneengeregen cellen. Deze cellen zijn de functionele en structurele eenheden van alle levende dingen. De binnenkant van een cel bestaat uit twee grote compartimenten die we het cytoplasma en de celkern noemen. De celkern is in feite de hersenen van de cel. Het bevat het genetische materiaal in de vorm van DNA, welke is onderverdeeld in kleinere stukken, die genen worden genoemd. Elk gen bevat de informatie voor het maken van specifieke cellulaire componenten die we eiwitten noemen. Eiwitten zijn zeer belangrijke moleculen voor een cel zoals machines essentieel voor een fabriek zijn. Dus zonder eiwitten kunnen cellen niet functioneren en zouden ze zelfs helemaal niet kunnen bestaan. Ons lichaam is opgebouwd uit verschillende soorten cellen, zoals spiercellen, zenuwcellen, bloedcellen, etc., en elk type cel heeft een verschillende functie. Afhankelijk van de functie van een cel moet deze slechts een speciale reeks van eiwitten produceren die nodig zijn om deze specifieke functie te kunnen vervullen. Er zijn speciale eiwitten in de cel die we transcriptiefactoren noemen en door aan het DNA te binden zorgen deze eiwitten ervoor dat de informatie van specifieke genen gedecodeerd wordt. Dit proces heet transcriptie en zorgt op zijn beurt weer voor het induceren van de productie van specifieke eiwitten.

Het wetenschappelijk onderzoek dat in dit proefschrift beschreven is, richt zich op de regulatie van de FOXO transcriptiefactoren. Dus de vraag is hoe de activiteit van FOXO eiwitten gereguleerd is en waarom ze belangrijk zijn voor de cellen en het gehele organisme? Er zijn weefsels in ons lichaam, zoals de huid, waar cellen voortdurend moeten delen om de dode cellen te vervangen. Ons lichaam bepaalt zorgvuldig welke cellen delen en wanneer zij dit doen met behulp van moleculaire “start” en “stop” signalen. Dit is erg belangrijk omdat ongecontroleerde deling van cellen kan leiden tot de ontwikkeling van tumoren. De “start” signalen zijn kleine eiwitten die we groeifactoren noemen en zij geven de cel de boodschap dat de condities optimaal zijn om te gaan delen. Hoe doen ze dat? Groeifactoren kunnen binden aan gespecialiseerde eiwitten, genaamd receptoren, die op het membraan aanwezig zijn dat rond elke menselijke cel zit. Hierdoor wordt de receptor geactiveerd en deze zorgt ervoor dat de informatie naar de binnenkant van de cel overgebracht wordt. Dit proces heet signaaltransductie (Figuur 1). Het cytoplasma bevat vele eiwitten die belangrijk zijn voor de transductie van de signalen van membraanreceptoren naar de kern. Eigenlijk kan signaaltransductie het beste worden vergeleken met een spelletje poolbiljart, waarin het eerste signaal ontstaat wanneer de keu de witte bal raakt. Dit kan vergeleken worden met de groeifactor die aan de receptor bindt. Hierna raakt de witte bal een andere bal die vervolgens nog een bal raakt totdat deze laatste in de pocket verdwijnt. De ballen zijn in dit geval de eiwitten in het cytoplasma en het gat in de pocket is de celkern. Veel van deze eiwitten zijn speciale enzymen die we kinasen noemen en deze kunnen een signaal van de ene naar de andere doorgeven, zoals poolballen die elkaar



Figuur 1. Twee belangrijke signaaltransductie routes die de activiteit van FOXO eiwitten in de cel reguleren
Zie de tekst voor details.

raken. Dit wordt mogelijk gemaakt door het feit dat kinasen aan andere kinasen kunnen binden en een fosfaatgroep aan hen kunnen koppelen. Dit proces wordt fosforylering genoemd. Wanneer de laatste kinase binnen de keten van eiwitten gefosforyleerd is, gaat het de kern in waar het FOXO eiwitten kan fosforyleren. Hierdoor wordt FOXO de kern uitgestuurd, waardoor het weggehouden wordt van het DNA en het zijn werk dus niet kan doen. Dat FOXO zijn werk niet kan doen is op zijn beurt een vereiste voor het delen van cellen (Figuur 1). Echter, wanneer er geen groeifactoren aanwezig zijn, wordt dit signaal niet doorgegeven aan de kern en kan FOXO de productie van speciale eiwitten induceren die als "stop" signalen voor de celdeling functioneren. Dit is zeer belangrijk om het juiste aantal cellen binnen een weefsel te handhaven en we weten nu dat permanente inactiviteit van FOXO eiwitten kan leiden tot ongecontroleerde celdeling en daardoor kan resulteren in de ontwikkeling van tumoren.

Een andere belangrijke functie van FOXO eiwitten is gerelateerd aan het feit dat ons lichaam niet zonder zuurstof kan, maar dat deze zuurstof tegelijkertijd schadelijk is voor ons lichaam. Dit komt doordat er speciale vormen van zuurstof, genaamd vrije zuurstof radicalen (VZR), gegenereerd worden tijdens de ademhaling, en deze zijn in het algemeen schadelijk voor de cel. Deze VZR kunnen tot de ontwikkeling van bijvoorbeeld neurologische ziekten leiden en dragen bij aan de vroegtijdige veroudering van organismen. Gelukkig zijn cellen uitgerust met een natuurlijk verde-

digingssysteem dat bestaat uit speciale eiwitten die we antioxidanten noemen en deze kunnen de VZR neutraliseren. FOXO kan de productie van deze antioxidanten bevorderen en daardoor de cellen beschermen tegen beschadigingen door VZR. Het is daarom erg belangrijk om precies te weten hoe cellen deze FOXO eiwitten reguleren.

In Hoofdstuk 2 laten we zien dat een kinase, genaamd NLK, FOXO eiwitten reguleert. Wanneer het niveau van VZR binnen de cellen stijgt, bindt NLK aan FOXO en fosforyleert het deze. Dit blokkeert op zijn beurt de ubiquitinering van FOXO. Ubiquitinering is een proces waarbij hele kleine eiwitten, genaamd ubiquitine, worden vastgezet aan een ander eiwit. In het geval van FOXO bevordert ubiquitinering zijn activiteit, omdat het FOXO in de celkern houdt zodat het aan het DNA kan binden en zijn werk kan doen. Waarom zou NLK de ubiquitinering van FOXO voorkomen en er daarmee voor zorgen dat het zijn werk niet kan doen? FOXO beschermt cellen tegen beschadigingen die veroorzaakt worden door VZR, maar als het niveau van VZR te hoog wordt, kan zelfs FOXO de cellen niet goed meer beschermen. Deze cellen moeten worden weggehaald door een proces dat we apoptose noemen en daarvoor moeten de FOXO-afhankelijke beschermende mechanismen worden uitgeschakeld. Dit is belangrijk omdat beschadigde cellen niet meer goed kunnen functioneren of zelfs kankercellen kunnen worden.

In Hoofdstuk 3 beschrijven we de regulatie van FOXO acetylering, dat vergelijkbaar is met fosforylatie, maar in plaats van een fosfaatgroep wordt er een andere chemische groep, genaamd een acetylgroep, vastgemaakt aan het eiwit. Acetylering van FOXO eiwitten is belangrijk voor hun functie, omdat het bepaalt waar op het DNA de FOXO eiwitten binden. Hierdoor wordt beslist welke eiwitten er uiteindelijk worden gesynthetiseerd. We laten zien dat een eiwit, genaamd MYBBP1a, aan FOXO bindt en voorkomt dat FOXO wordt geacetyleerd. Dit kan belangrijk zijn voor de regulatie van de celdeling, omdat cellen die geen MYBBP1a in zich hebben niet goed meer kunnen delen. FOXO eiwitten kunnen de celdeling stoppen, maar zodra de cellen beginnen te delen, hebben ze FOXO nodig om dit proces te voltooien. Het is daarom mogelijk dat MYBBP1a de acetylering van FOXO zo reguleert dat het daardoor het delen van cellen toestaat.

Tot slot beschrijven we in Hoofdstuk 4 hoe we transgene muizen hebben gemaakt die een speciale vorm van FOXO in zich hebben dat aan een ander eiwit, genaamd GFP, is bevestigd. Dit eiwit kan worden gedetecteerd onder een speciale microscoop en daardoor kunnen we kijken welke weefsels FOXO eiwitten bevatten. We maakten de interessante observatie dat FOXO voornamelijk voorkomt in de stamcellen van ons bloed. Deze cellen zijn de meestercellen van ons lichaam omdat ze zichzelf kunnen vernieuwen, en tegelijkertijd ook een verscheidenheid aan andere soorten cellen kunnen vormen. Echter om een stamcel te blijven, moeten zij hun VZR niveau heel laag houden. Daarom is de aanwezigheid van FOXO in deze cellen heel belangrijk, omdat FOXO de expressie van antioxidanten induceert en daarbij de stamcellen helpt hun VZR niveau laag te houden. Dit is cruciaal voor de productie van onze bloedcellen die een zeer korte levensduur hebben daarom voortdurend moeten worden vervangen.

Summary for the outsider

The human body is a complex, highly organized structure made up of a number of organs such as the heart, lungs, and kidneys. These organs work together to accomplish the specific functions necessary for sustaining life. Each organ is made up of several types of tissue, whereas each tissue is formed by many cells that are joined together. Cells are the functional and structural units of all living things. The inside of the cell is divided into two major compartments called the cytoplasm and the nucleus. The nucleus is basically the brain of the cells. It contains the genetic material in the form of DNA, which is divided into smaller pieces that are called genes. Each gene contains information about how to make specific cellular components called proteins. Proteins are very important molecules for a cell like machines are essential for a factory. So without proteins, cells could not function or even exist. Our body is built up of different types of cells (e.g., muscle cells, nerve cells, blood cells, and so on) each having different functions. Depending on the function of the cell, it needs to produce only a special subset of proteins necessary to fulfill this specific function. There are special proteins inside the cell called transcription factors, which can bind to the DNA and allow decoding of the information of specific genes. This process is called transcription, which in turn induces the synthesis of specific proteins.

The scientific research described in this thesis focuses on the regulation of transcription factors called FOXO. So the question is how the activity of FOXO proteins is regulated and why they are important for the cells and the whole organism? There are some tissues in our body like skin where cells need to constantly divide in order to replace cells that died. Our bodies carefully control which cells divide and when they do so by using molecular "stop" and "go" signals. This is very important since uncontrolled division of cells might lead to the development of tumors. The "go" signals are small proteins called growth factors and they provide the cell with the information that the conditions are optimal for cell division. How do they do so? The growth factors can bind to specialized proteins, called receptors, which are present within the membrane surrounding each human cell. This activates the receptor and induces the transmission of the information to the inside the cell. This process is called signal transduction (Figure 1). The cytoplasm contains many proteins that are important for transduction of the signals from membrane receptors to the nucleus. Thus, signal transduction can be compared to a game of pool, which starts with an initial signal when the cue stick strikes the white ball. This corresponds to the growth factor binding the receptor. After that, the white ball hits another ball, which hits yet another one, until the last one gets into the pocket. The balls correspond to the proteins within the cytoplasm and the hole in the pocket corresponds to the nucleus. Many of these proteins are special enzymes called kinases and they can pass a signal from one to another like pool balls hitting each other. This is possible because kinases can bind other kinases and attach a phosphate group to them. This process is called phosphorylation. When the last kinase within the chain of the proteins is phosphorylated, it goes into the nucleus where it can phosphorylate FOXO proteins. This sends FOXO outside of the nucleus, keeping it away from the DNA so it cannot do its job. This in turn is required for cells to di-

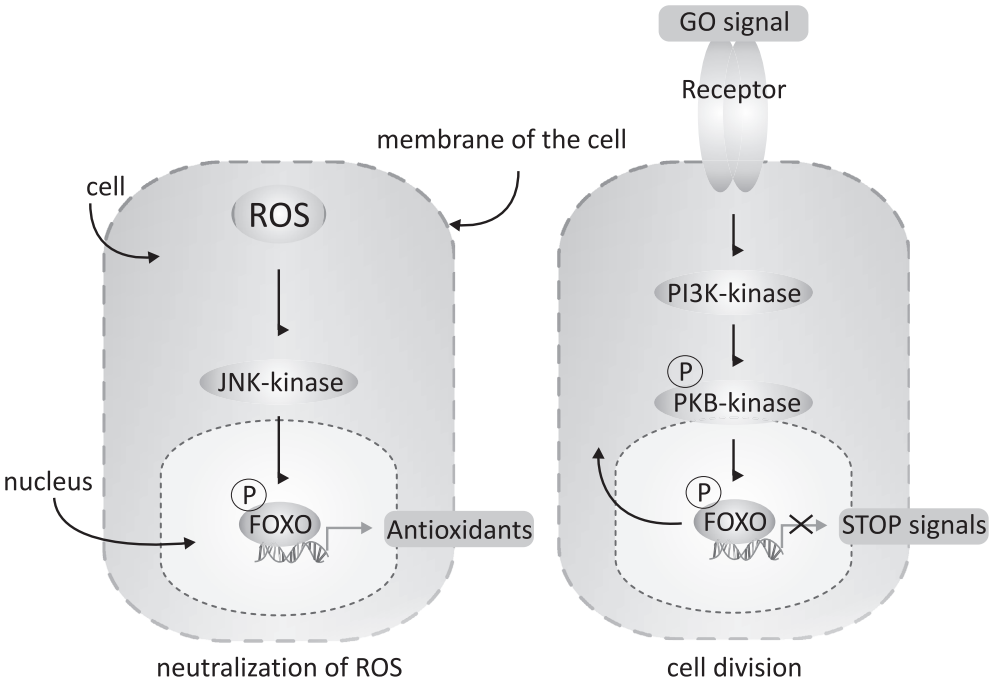


Figure 1. Two major signal transduction routes that regulate the activity of FOXO proteins inside the cell
See text for details.

vide (Figure 1). However, when growth factors are absent, the signal is not transmitted to the nucleus and FOXO induces the synthesis of special proteins that function as a "stop" signals for the division of the cell. This is very important for the maintenance of the proper cell number within the tissue and we know now that permanent inactivation of FOXO proteins leads to uncontrolled duplication of cells and the development of tumors.

Another important function of FOXO proteins is related to the fact that our body cannot exist without oxygen, yet oxygen is innately harmful to our existence. This is associated with the generation of special forms of oxygen called free radicals (ROS) during respiration, which in general are detrimental to the cell and can lead to the development of for example neurological diseases and premature ageing of the organism. Cells, however, are equipped with a natural defense system consisting of special proteins called antioxidants that can neutralize free radicals. Importantly, FOXO can promote the synthesis of antioxidants and by doing so protect the cells from being damaged by free radicals. Taken together, it is important to know precisely how cells regulate FOXO proteins.

In Chapter 2 we show that a kinase called NLK regulates FOXO proteins. When the level of ROS within the cells increases, NLK binds to FOXO and phosphorylates it. This in turn blocks ubiquitination of FOXO. Ubiquitination is a process where a small protein called

ubiquitin is attached to another protein. In the case of FOXO, ubiquitination promotes its activity because it keeps FOXO inside the nucleus so it can bind to DNA. Why would NLK prevent ubiquitination of FOXO? FOXO protects cells from being damaged by ROS. However, if the level of ROS is too high it is impossible to efficiently protect the cells. These cells need to be eliminated by a process called apoptosis and for that the FOXO-dependent protective mechanisms need to be switched off. This is important because damaged cells cannot function properly or can even become cancer cells.

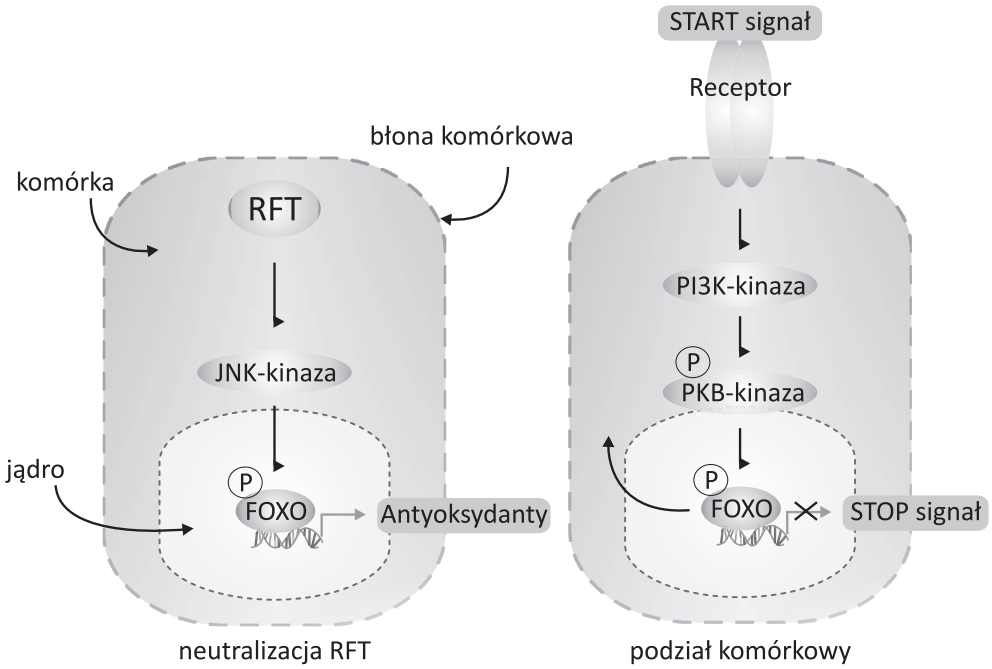
In Chapter 3 we describe the regulation of FOXO acetylation, which is similar to phosphorylation but instead of a phosphate group, another chemical group, called an acetyl group, is attached to the protein. Acetylation of FOXO proteins is important for their functions because it determines to which place on the DNA FOXO binds and by that it decides which proteins are synthesized. We showed that a protein called MYBBP1a binds to FOXO and prevents FOXO from being acetylated. This might be important for the regulation of the division of cells because cells that do not have MYBBP1a cannot divide properly. FOXO proteins can stop cell division, however, once cells start to divide they need FOXO to finish this process. Therefore, it is possible that the MYBBP1a regulates the acetylation of FOXO and allows cells to divide.

Finally, in Chapter 4 we describe transgenic mice that contain a special form of FOXO, which is fused to another protein called GFP. This protein is visible under the microscope and therefore we can look which tissues contain FOXO. Interestingly, we saw that FOXO is expressed in the stem cells of our blood. These cells are the master cells of our body because they can renew themselves and they can also make a variety of other kinds of cells. However, in order to stay a stem cell, they have to keep their ROS at very low levels. Therefore, the presence of FOXO in these cells is important because FOXO induces expression of antioxidants and helps stem cells to keep their ROS levels low. This is crucial for the production of our blood cells, which are very short lived and therefore need to be replaced constantly.

Streszczenie dla niebiologa

Ciało ludzkie jest złożoną, wysoce zorganizowaną strukturą, która składa się z wielu organów, takich jak serca, płuca i nerki. Narządy te współpracują, aby osiągnąć określone funkcje niezbędne do życia. Każdy narząd składa się z kilku typów tkanek, a każda tkanka składa się z wielu połączonych ze soba komórek. Komórki są funkcjonalnymi i strukturalnymi jednostkami wszystkich żywy organizmów. Wnętrze każdej komórki podzielone jest na dwa główne kompartmenty nazywane cytoplazma i jądro. Jądro jest nadzorcą komórki. Nadzoruje wszystkie procesy zachodzące w komórce poprzez posiadaną informację w postaci materiału genetycznego, upakowanego w formie DNA. Nici DNA podzielone są na mniejsze części, które nazywane są genami. Każdy gen zawiera informacje na temat produkcji specjalnych komórkowych cząsteczek zwanych białkami. Białka są niezwykle ważne dla komórek, podobnie jak maszyny są istotne dla fabryki. Oznacza to że bez białek komórki nie byłyby w stanie funkcjonować a nawet istnieć. Nasze ciało zbudowane jest z różnego rodzaju komórek (np. komórek mięśniowych, neuronów, komórek krwi i innych) i każde z nich wykonują inną funkcje. W zależności od funkcji komórka musi wyprodukować określony zestaw białek, który umożliwi jej realizację tej funkcji. Każda komórka wyposażona jest w specjalne białka, które nazwane są czynnikami transkrypcyjnymi. Białka te wiążą się z DNA i umożliwiają rozszyfrowanie informacji zakodowanej w genach. Proces ten jest nazywany transkrypcją i wywołuje syntezę specyficznych białek.

Badania naukowe opisane w tej pracy doktorskiej koncentrują się na regulacji czynników transkrypcyjnych z grupy FOXO. Dlaczego aktywność FOXO jest ważna dla prawidłowego funcjonowania komórek jak i całego organizm człowieka? Niektóre tkanki naszego organizmu jak na przykład skóra musza się stale odnawiać. Oznacza to że komórki.ciągle tam obumieraja i muszą być zastąpione przez nowe komórki, które produkowane są na drodze duplikacji (podziałów) żywych komórek skóry. Nasze ciało ostrożnie kontroluje kiedy i gdzie podziały komórkowe maja miejsce przy pomocy specjanych „stop" i „start" sygnałow molekularnych: Stanowi to bardzo ważny proces, ponieważ niekontrolowane podziały komórek mogą przyczynić się do powstawania nowotworów. Sygnały „start" to małe białka zwane czynnikami wzrostu, które informują komórkę o optymalnych warunkach do podziału komórki. W jaki sposób czynniki wzrostu stymulują podziały komórkowe? Czynniki wzrostu wiążą się do białek błonowych, zwanymi receptorami, które znajdują się w błonie komórkowej otaczającej poszczególne komórki ludzkie. Wiązanie czynnika wzrostu do receptora przekazuje informację do wnętrza komórki. Proces ten jest nazywany transdukcją sygnału (Rysunek 1). W cytoplaźmie komórkowej znajduje się cały szereg białek, które są ważne dla przekazywania sygnałów z receptorów błonowych do jądra. W związku z tym przewodnictwo sygnałów można porównać do gry w bilarda. Gra rozpoczyna się gdy gracz kijem bilardowym uderza w białą kulę (bilę), co odpowiada czynnikowi wzrostu, wiążącemu receptor. Po tym biała kula uderza kolejną kulę i kolejną, dopóki ostatnia kula wpadnie do kieszeni. Kule można porównać do białek w cytoplaźmie a otwór odpowiada jądru komórkowemu. Na drodze przekaźnictwa sygnału jedne z białek biorące udział w transdukcji sygnału to enzymy o nazwie kinazy. Ki-



Rysunek 1. Dwa główne szlaki transdukcyjne sygnał, które reguluja aktywność białek FOXO w komórce.
Szczegółowe wytłumaczenie w tekście.

nazy przekazują sygnał z jednego białka do drugiego, tak jak kule bilardowe. Jest to możliwe, dzięki temu, że kinazy mogą wiązać inne kinazy i dołączyać do nich grupy fosforanowe. Proces ten jest nazywany fosforylacją. Kiedy ostatnia kinaza w łańcuchu białek ulegnie fosforylacji przechodzi ona do jądra gdzie moze fosforylować FOXO. FOXO z dodatkową grupą fosforanową przechodzi z jądra do cytoplazmy pozostając z dala od DNA wskutak czego nie może wykonywać swojej praca. Czynnik transkrypcyjny FOXO znajdujący się w cytoplaźmie jest sygnałem dla komórki do podziału (Rysunek. 1). Jednakże gdy czynniki wzrostu są nieobecne, sygnał nie jest przekazywany do jądra i FOXO powoduje syntezę specjalnych białek, które pełnią rolę sygnału „stop" dla podziału komórki. Jest to bardzo ważne dla utrzymania właściwej ilości komórek w tkance. Dodatkowo już wiemy, że trwała inaktywacja białek FOXO, prowadzi do niekontrolowanego podziału komórek i rozwoju nowotworu.

Inną ważną funkcją białek FOXO jest utrzymanie naszego ciała w prawidłowych warunkach tlenowych. Brak jak i nadmiar tlenu może być szkodliwy dla naszego istnienia. Jest to związane z produkcją specjalnych form tlenu, nazywanych wolnymi rodnikami (RFT) podczas oddychania. Wolne rodniki są uznawane za niebezpieczne dla komórki, gdyż ich nadmiar może doprowadzić do jej uszkodzenia i rozwoju na przykład chorób neurodegeneracyjnych i przedwczesnego starzenia się organizmu. Jednak komórki, są wyposażone w system naturalnej ochrony składający się ze specjalnych białek nazywanych

przeciwutleniacze, które mogą zneutralizować wolne rodniki. Wiemy, że FOXO może promować syntezę przeciwutleniaczy i czyniąc to chroni komórki przed negatywnymi skutkiami wolnych rodników. Podsumowując, dla zrozumienia działania komórki bardzo ważne jest aby wiedzieć dokładnie jak w komórce regulowane jest białko FOXO.

W Rozdziale 2 pokazujęmy, że kinaza o nazywie NLK reguluje FOXO. Gdy poziom RFT wewnątrz komórki zwiększa się, NLK wiąże się do FOXO i je fosforyluje, blokując ubikwitynacje FOXO. Ubikwitynacja jest procesem przyłączenia małych białek o nazwie ubikwityna do innego białka. W odniesieniu do FOXO ubikwitynacja promuje jego aktywność, ponieważ umożliwia FOXO pozostanie w jądrze co z kolei umożliwia jego wiązanie sie do DNA. Dlaczego NLK hamuje ubikwitynacje FOXO? FOXO chroni komórki przed uszkodzeniem przez RFT. Jednakże jeżeli poziom RFT jest zbyt wysoki skuteczna ochrona komórki staje się niemożliwa. Te komórki muszą zostać wyeliminowane przez proces nazywany apoptozą i dlatego mechanizm ochronny zależny od FOXO musi być wyłączony. Jest to bardzo ważne, ponieważ komórki uszkodzone nie mogą funkcjonować prawidłowo lub mogą przekształcić się w komórki nowotworowe.

W Rozdziale 3 opisujemy regulacje FOXO przez acetylację, która jest podobna do foforylacji, ale zamiast grupy fosforanowej, inne grupy chemiczne, nazywane grupą acetylu, jest dołączony do białka. Acetylowanie białek FOXO jest ważne dla ich funkcji, ponieważ określa, do którego miejsca w nici DNA powinno się wiązać, a to z kolei determinuje które białka są syntetyzowane. Uzyskane przez nas wyniki pokazują, że białko o nazwie MYBBP1 może wiązać się do FOXO i zapobiegć jego acetylacji. Może to być ważne dla regulacji podziałów komórek, ponieważ komórki, które nie mają MYBBP1a nie mogą prawidłowo dzielić się. Białka FOXO mogą zatrzymać podziały komórkowe. Jednak gdy komórka rozpocznie podział potrzebuje FOXO do zakończenia tego procesu. W związku z tym istnieje możliwość, że MYBBP1a reguluje acetylację FOXO co jest ważne dla prawidłowego podzialu komórkowego.

Na koniec w Rozdziale 4 opisujemy transgeniczne myszy, zawierające specjalną formę FOXO, które jest związane z białkiem fluorescencyjnym GFP, tworząc białko fuzyjne GFP-FOXO. Białko GFP-FOXO jest widoczne pod mikroskopem, a zatem możemy spojrzeć które tkanki zawierają FOXO. Co ciekawe pokazujemy że FOXO jest produkowane w komórkach macierzystych krwi. Te komórki są ważnymi komórkami naszego ciała, ponieważ one jako jedyne mogą się odnawiają jak również „zmieniać się" w szereg innych rodzajów komórek. Jednakże aby pozostać komórką macierzystą, muszą zachować poziom RFT na bardzo niskim poziomie. W związku z tym obecność FOXO w tych komórkach jest bardzo ważna, ponieważ FOXO wywołuje produkcję przeciwutleniaczy i pomaga komórką macierzystym w utrzymania niskiego poziomu ROS. Jest to kluczowe dla produkcji naszych komórek krwi, które bardzo krótko żyją i dlatego muszą być ciagle na nowo produkowane.

List of abbreviations

Acyl-CoA, Acetyl coenzyme A
AGE, advanced glycation end product
AICAR, aminoimidazole carboxamide ribonucleotide
AMP, adenosine monophosphate
AMPK, AMP-dependent protein kinase
ASK1, apoptosis signal-regulating kinase 1
CCR7, C-C chemokine receptor type 7
CDK, cyclin-dependent kinase
CHOP, C/EBP homologous protein
CKI, CDK inhibitor
CHX, cycloheximide
DAF-16, abnormal dauer formation 16
DUOX, dual oxidase
DYRK1A, dual-specificity tyrosine-phosphorylated and -regulated kinase 1A
EGF, epidermal growth factor
ER, endoplasmic reticulum
ERAD, ER-associated degradation
ERK1/2, extracellular signal-regulated kinase1/2
ERO1, ER oxidoreductin 1
ETC, electron transport chain
FFA, free fatty acids
FOXO, forkhead Box O
GPX, glutathione peroxidase
GRX, glutaredoxins
GSH, glutathione
GSK3, glycogen synthase kinase 3
GST, glutathione S-transferase
HAT, histone acetyltransferase
HDAC, histone deacetylase acetyltransferase
HFD, high fat diet
IKKβ, IκB kinase β
KLF2, Kruppel-like factor 2
IL7R, Interleukin 7 receptor alpha chain
IR, Insulin receptor
IRE1, inositol-requiring kinase 1
IRS, Insulin receptor substrate
JAK/STAT, Janus kinase/signal transducers and activators of transcription
JIP1, JNK interacting protein 1
JNK1, c-Jun NH_2-terminal kinase 1
LMWPTP, low molecular weight-PTP
LEF/TCF, lymphoid enhancer factor/T-cell factor
MDM2, murine double minute 2
MKPs, mitogen-activated protein kinase (MAPK) phosphatases
MnSOD, superoxide dismutase enzymes
MYBBP1a, Myb binding protein 1a
mTOR, mammalian target of rapamycin
NAC, N-acetyl cysteine
NARF, NLK-associated ring finger protein
NES, nuclear export signal

NLK, Nemo like-kinase
NLS, nuclear localization signal
NOX, NADPH oxidases
NRF2, nuclear respiratory factor 2
PAK1, p21-activated protein kinase 1
p300/CBP, E1A binding protein p300/CREB-binding protein
p38MAPK, p38 mitogen-activated protein kinase
p53, protein 53
PDGF, platelet-derived growth factor
PDI1, protein disulfide isomerase
PDK1, 3-Phosphoinositide-dependent Protein Kinase-1
PEPCK, phosphoenolpyruvate carboxykinase
PERK, protein kinase RNA-like endoplasmic reticulum kinase
PGC1, PPARg coactivator 1
PI3K, Phosphoinositide 3-kinase
Pin1, peptidyl-prolyl isomerase 1
PKB, Protein kinase B (also c-Akt)
PKC, Protein kinase C
PLK-1, Polo-like kinase
PP2A, protein phosphatase 2
PPAR, peroxisome proliferator-activated receptor
PRX1, peroxiredoxin 1
PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-triphosphate
PTEN, phosphatase and tensin homolog
PTP, permeability transition pore
PTP1B, protein tyrosine phosphatases 1B
PTPs, protein tyrosine phosphatases
ROS, Reactive oxygen species
S6K, p70 S6 kinase
SETDB1, SET domain, bifurcated 1
SHP2, Src homology 2 (SH2) domain containing phosphatase
SIRT1, sirtuin 1
SMAD, similar to mothers against decapentaplegic
SOH, sulfenic acid
TAB1, TAK1-binding protein
TAK1, transforming growth factor β-activated kinase 1
TBP-2, thioredoxin binding protein-2
TCA, tricarboxylic acid cycle
TCPTP, T-cell protein tyrosine phosphatase
TG, triglyceride
TNFα, tumor necrosis factor α
TRX, thioredoxin
TSA, trichostatin A
UCP, uncoupling protein
UPR, unfolded protein response
USP7, ubiquitin-specific peptidase 7
USP10, ubiquitin-specific peptidase 10
XBP1, X-box binding protein 1

Curriculum Vitae

Anna Szypowska was born the 30th of November 1977, in Ząbkowice Śląskie, Poland. From 1992 she studied Natural Sciences at the third High school of Władysław Jagiełło in Ząbkowice Śląskie.

Upon receiving her diploma in 1996, she started her study Biotechnology at the University of Wrocław and graduated in July 2002 with a Masters Degree in Biology/ Biotechnology. During her study she completed an internship in the lab of Prof. Dr. Maria Malicka-Błaszkiewicz in the Department of Cell Pathology at the University of Wrocław.

From January 2004 she has been working as a PhD student in the laboratory of Prof. Dr. Boudewijn Burgering in the Department of Physiological Chemistry (Department of Molecular Cancer Research at present) at the University Medical Center Utrecht. The results of her work are described in this thesis

List of publications

A.A. Szypowska, H de Ruiter, L.A. Meijer, L.M. Smits, and B.M. Burgering
Oxidative stress-dependent regulation of Forkhead box O4 activity by nemo-like kinase.
Antioxid Redox Signal. 14(4): p. 563-78.

A.A. Szypowska and B.M. Burgering, **The peroxide dilemma: opposing and mediating insulin action.**
Antioxid Redox Signal. 2011 Jan 14. [Epub ahead of print]

P.L. de Keizer, L.M. Packer, A.A. Szypowska, P.E. Riedl-Polderman, N.J. van den Broek, A. de Bruin, T.B. Dansen, R. Marais, A.B. Brenkman, B.M. Burgering.
Activation of forkhead box O transcription factors by oncogenic BRAF promotes p21cip1-dependent senescence.
Cancer Res. 70(21): p. 8526-36.

T.B. Dansen, L.M. Smits, M.H. van Triest, P.L. de Keizer, D. van Leenen, M.G. Koerkamp, A. Szypowska, A. Meppelink, A.B. Brenkman, J. Yodoi, F.C. Holstege, B.M. Burgering BM. **Redox-sensitive cysteines bridge p300/CBP-mediated acetylation and FoxO4 activity.**
Nat Chem Biol, 2009. 5(9): p. 664-72.

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Veel plezier!

Anna