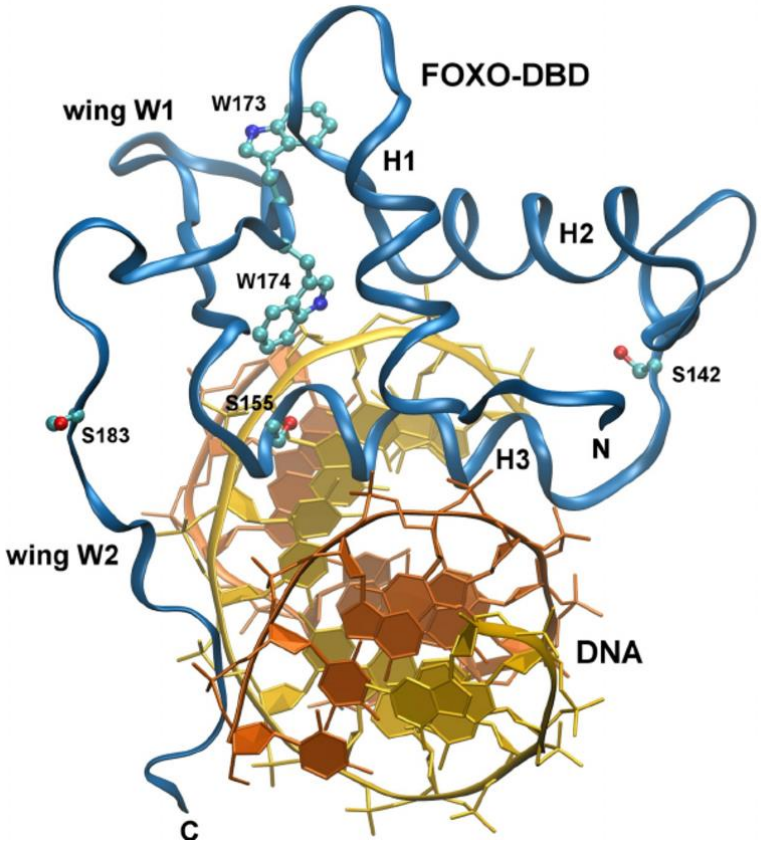
The paradox of FoxO transcription factors in cancer



(Silhan et al., 2009)

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Abstract

The Forkhead box O (FoxO) fulfil many functions, including the regulation of cell cycle arrest, apoptosis and oxidative stress resistance, which are all important in the regulation of cancer. Mostly, these functions make FoxO factors tumour suppressors by inhibition of proliferation and development of cancer cells. However, cancer cells may also benefit from the many functions of FoxO factors to use them for growth and homeostasis. It might be that FoxO factors can be tumour suppressive and tumour supportive, dependent on the level of FoxO and the tissue in which FoxO factors are present.

The FoxO factors are regulated by post-translational modifications, mainly by phosphorylation. The phosphoinositide 3-kinase (PI3K)-protein kinase B (PKB)/Akt pathway negatively regulates FoxO factors. On the other hand, FoxO factors can be stimulated by the c-jun terminal kinase (JNK) or Mst1 pathway.

In the future, FoxO factors may be a good target in different types of cancer, but they have to be handled with care because of its unknown function in cancer.

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Introduction

Cancer is a severe disease that is present all over the world and it is in the United States the second leading cause of death (Siegel, Miller, & Jemal, 2016). Epithelial cancer or carcinoma is the most common type of cancer. In 2017 the United States counted 1,6 million new patients and around 6 million cases of death (Islami et al., 2017). The incidence of cancer is already decreasing, 23% from 1991 until 2016 and measuring from 2010 until 2014 a decrease around 1,6% is estimated (Siegel et al., 2016). The decline in cancer patients is due to improved detection methods and treatment (Jemal et al., 2018). Still, many people are diagnosed with cancer and do not survive, because of the complexity and the multiple types of the disease.

Cancer is a very seriously disease where a mutation occurs in a cell, causing the cell to become resistant against control systems of the cell cycle and allow for uncontrolled proliferation. Cancer cells can also evade apoptosis or programmed cell death, what can lead to cells growing out to become a tumour.

There is not yet a cure for cancer, but there might be a transcription factor that can give some more insight in the regulation of cancer.

This transcription factor is involved in the regulation of cancer on or offset and is part of the Forkhead box class O (FoxO) subgroup of the Forkhead family of transcription factors (Daitoku, Sakamaki, & Fukamizu, 2011).

The factor is a regulator of cellular homeostasis and is a possible tumour suppressor, which includes a role in cell cycle arrest, DNA repair, glucose and fatty acid metabolism, reactive oxygen detoxification and the factor can cause oxidative stress resistance (Calnan & Brunet, 2008).

The family name Forkhead is derived from the invertebrates Caenorhabditis elegans and Drosophila melanogaster (Fu & Tindall, 2008). In the C. elegans the homolog of the FoxO factors is named Abnormal Dauer Formation-16 (DAF-16) and in the D. melanogaster the homolog is named dFoxO. Four members of FoxO in mammals are existing, namely FoxO1, FoxO3, FoxO4 and FoxO6, all different regulated but they contain the same DNA binding motif. FoxO1, FoxO3 and FoxO4 are present in various tissues and organs, whereas FoxO6 can be found during the development from the brain and central nervous system (Wang, Yu, & Huang, 2016). Expression levels of FoxO1, FoxO3 and FoxO4 are dependent on the type of tissue. Because the expression levels and activity of FoxO6 is limited, in this thesis FoxO factors/proteins include FoxO1, 3 and 4.

The class of Forkhead proteins contains a highly conserved DNA-binding domain (DBD) (Carter & Brunet, 2007). The DBD of FoxO proteins contains three alpha-helices with two loops that binds to the consensus motif 5’-TTGTTTAC-3’ (Van Der Horst & Burgering, 2007).

The regulation of the FoxO factors is complex. They can mainly be regulated by post-translational modifications (PTMs), such as phosphorylation, acetylation and ubiquitylation. This thesis will concentrate on the phosphorylation of FoxO, while it is the most important regulator pathway of FoxO in cancer. It is carried out by the phosphoinositide 3-kinase (PI3K)-protein kinase B (PKB)/Akt pathway. On the other hand, FoxO can be phosphorylated by c-jun terminal kinase (Jnk) or Mst1. The effect of the two pathways on FoxO factors is opposite.

In this thesis I will discuss how FoxO transcription factors are regulated by these two different phosphorylation pathways and what the correlation is between FoxO proteins and cancer.

Function of FoxO

What can FoxO factors do?

The Forkhead box O (FoxO) proteins are transcription factors. They control cellular development, metabolism and survival. An overexpression of FoxO can trigger apoptosis (Tzivion, Dobson, & Ramakrishnan, 2011). Mainly, they play an important role in cell cycle arrest at the G1/S and the G2/M boundaries. Next to these functions, FoxO factors can decrease cell proliferation, can maintain redox homeostasis by reacting on reactive oxygen species (ROS) and nutrient deprivation and they play a role in the metabolism of fatty acids and glucose (gluconeogenesis).
The factors can perform all of these function by switching target gene transcription on or off.

Structure of FoxO

Each FoxO protein consists of a DNA-binding domain (DBD), a nuclear localization signal (NLS), a nuclear export sequence (NES) and a C-terminal transactivation domain (TA) (figure 1).

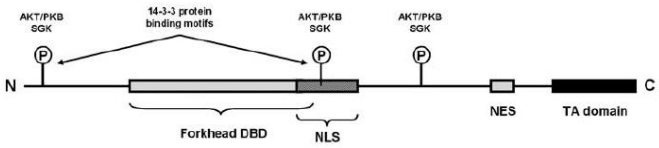

Figure 1: Structure of a primary FoxO protein. The DBD, NLS, NES and TA are shown. Also the Akt/PKB phosphorylation sites are present (Obsil & Obsilova, 2008).

The DBD consists three alpha-helices and two wing-like loops that binds to a consensus motif 5'-TTGTTTAC-3' on DNA of the target gene (Tia et al., 2018). This domain is the known 110 amino-acid Forkhead box, consisting of a N-terminal part and a C-terminal part or W2 wing (figure 2). While the N-terminal part is formed by three alpha-helices, the C-terminal part contains three beta-strands and two wing-like loops. Both parts of the DBD are essential in DNA binding stability (Boura et al., 2007).
The name of the protein is originated from the structure, because the whole structure of the protein looks like a fork.

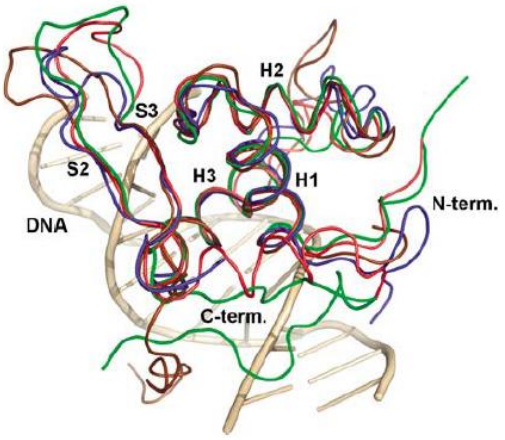

Figure 4 Superimposition of the FOXO4 DBD with FOXO3a DBD (Tsai *et al.*, 2007), FOXK1a DBD (Tsai *et al.*, 2006) and FoxA3 DBD (Clark *et al.*, 1993). For clarity, only the DNA in FOXO3a DBD–DNA complex is shown. The FOXO4 is shown in blue, FOXO3a in brown, FOXK1a in red and FoxA3 in green. This figure was created using PyMOL (http://www.pymol.org).

Figure 2: Structure of the DBD of FoxO. FoxO1 is shown in red, FoxO3 in brown and green and FoxO4 in blue (Obsil & Obsilova, 2008).

How are FoxO factors regulated?

FoxO factors are regulated via post-translational modifications (PTMs). Recently, over 400 protein PTMs are discovered. These modifications are especially phosphorylation, acetylation, ubiquitylation and methylation. FoxO proteins are mainly regulated by the phosphoinositide 3-kinase (PI3K)-protein kinase B (PKB)/Akt phosphorylation pathway that inhibits the function of FoxO factors (Wang et al., 2016). The known c-jun terminal kinase (Jnk) or Mst1 phosphorylation pathway has the opposite effect on FoxO. FoxO proteins can also be activated by extern stimuli such as cytokines and growth factors.

Nuclear to cytoplasm localization

FoxO factors can shuttle between the nucleus and the cytoplasm of a cell. In this regulation, 14-3-3 proteins play an important role through interaction with FoxO factors (Tzivion et al., 2011). They can interfere with their nuclear localisation sequence. This causes FoxO to be retained in the cytoplasm. The binding with 14-3-3 proteins makes it difficult to translocate back into the nucleus. Under stress conditions, the inhibition of FoxO by 14-3-3 proteins is overridden. In this case, the 14-3-3 proteins disconnect from FoxO factors, resulting in relocalization of FoxO factors into the nucleus (Rupp et al., 2017).

The target genes of FoxO

In the nucleus of the cell FoxO recognises DNA by a 5'-TTGTTTAC-3' motif on target genes (Wang et al., 2016). All of the genes regulated by FoxO have this DNA recognition specificity. FoxO makes a binding with DNA using hydrogen bonds, side chains-base interactions (water mediated) and van der Waals interactions (Obsil & Obsilova, 2008).

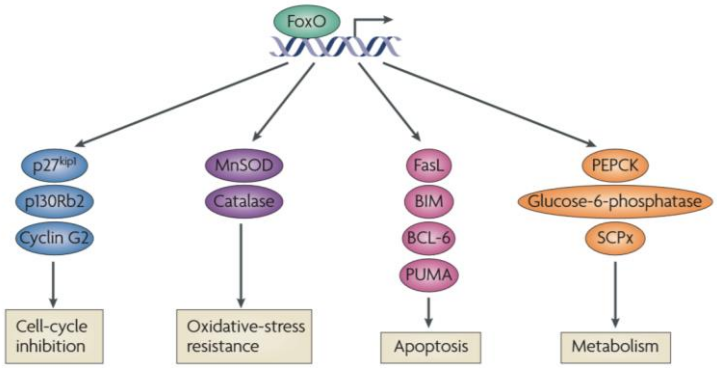

Figure 3: Target genes of FoxO transcription factors (Van Der Horst & Burgering, 2007).

For inducing apoptosis, FoxO factors can activate the expression of the pro-apoptotic genes FasL, BIM, BCL-6, TRAIL and PUMA (figure 3). FasL or Fas ligand is the necessary ligand for the Fas cell death pathway (Carter & Brunet, 2007). BIM is a BH3-only protein, which contains one binding site for FoxO (Van Der Horst & Burgering, 2007). BCL-6 induction can cause transcriptional inhibition (Greer & Brunet, 2008). TRAIL is member of the TNF family and FoxO factors can activate the TRAIL gene directly (Modur, Nagarajan, Evers, & Milbrandt, 2002b). PUMA is next to FoxO associated with p53, a suggested tumour suppressor which in this thesis is not further discussed (Van Der Horst & Burgering, 2007).

For cell cycle arrest, FoxO factors can induce $p27^{kip1}$, p21, p130Rb2, GADD45, Cyclin G2 gene expression. In this row of genes, especially $p27^{kip1}$ is regulator of cell cycle arrest (Van Der Horst & Burgering, 2007). $p27^{kip1}$ is a member of CDK inhibitor family Cip/Kip (Vos & Coffer, 2011). CDKs in complex with cyclins are essential for the progression of the cell cycle, so inhibition of these proteins establishes cell cycle arrest. The $p27^{kip1}$ gene can induce G1 arrest, by causing the inhibition of E-CDK2 (Modur et al., 2002b). FoxO factors can also regulate the p21 gene, another Cip/Kip family

member, by making a connection with SMAD3 and SMAD4, causing a block in cell cycle progression (Calnan & Brunet, 2008). P130 is a retinoblastoma protein family member that decides if genes will be expressed that are necessary for the entry of the S phase of the cell cycle (Vos & Coffer, 2011). GADD45 can induce G2 arrest and upregulation of this gene can repair damaged DNA (Carter & Brunet, 2007). Last but not least, the overexpression of the cyclin G2 can generate a inhibition in the cell cycle (Vos & Coffer, 2011).

FoxO factors also play a role in the glucose metabolism. Genes important in this regulation are PEPCK, Glucose-6-phosphatase and SCPx. In energy homeostasis the genes AgRP and NPY are induced.

Besides, under stress stimuli MnSOD and catalase are expressed. Both are enzymes involved in the detoxification of reactive oxygen species (Carter & Brunet, 2007).The expression of these genes might be a key in the oxidative-stress resistance.

There are more functions of FoxO factors and genes involved, but the genes described above are the most relevant with respect to FoxO factors in cancer.

The PI3K-PKB/Akt pathway

The function of PKB/Akt

Protein Kinase B (PKB), also called Akt, is involved in the regulation of almost every organ in our body. Akt is a serine threonine kinase, that can cause cellular survival due to inhibition of apoptosis. Akt functions through phosphorylation of target proteins, in this thesis I focus on FoxO factors. The minimum substrate motif that is needed for Akt to recognize and phosphorylate substrates is RXRXXS/T (R = arginin, X = a random amino acid, S/T = serin/threonin) (Alessi, Caudwell, Andjelkovic, Hemmings, & Cohen, 1996).
Akt is stimulated by insulin and insulin growth factor (IGF), but also by other growth factors and neurotrophic factors (Calnan & Brunet, 2008).

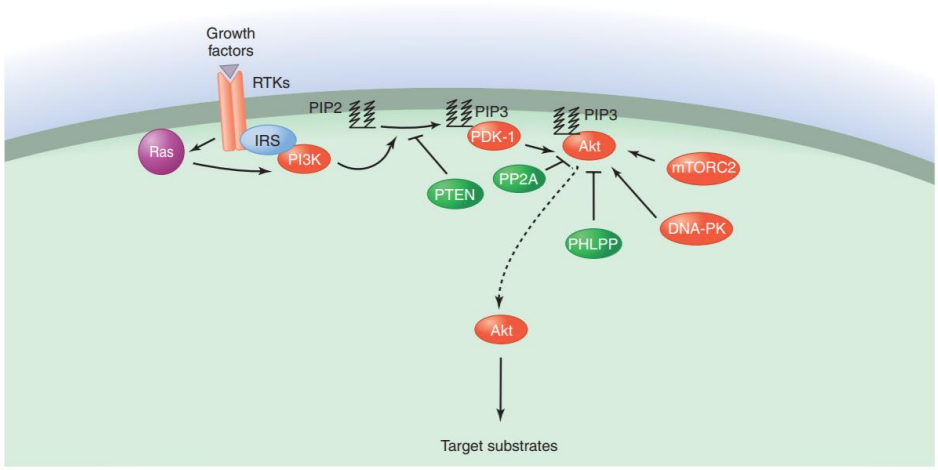

Figure 4: The regulation pathway of PI3K and Akt (Hemmings & Restuccia, 2012).

Reciprocity PI3K and Akt

Akt is a downstream target of PI3K (an agonist of IGF-1) and is activated by this pathway (figure 4). Phosphoinositide 3-kinase (PI3K) is involved in lipid metabolism and produces Ptdlns-3,4-P_2 (PIP_2) by insulin induction (Leibiger et al., 2010). When PIP_2 is phosphorylated by PI3K, it becomes Ptdlns-3,4,5-P_3 (PIP_3).
Both PIP_2 and PIP_3 bind to Akts pleckstrin homology (PH) domain, providing partial Akt activation but they are also rate limiting (Manning & Toker, 2017). In this case, Akt and phosphoinositide-dependent protein kinase 1 (PDK1) relocalize toward PIP2 or PIP3. This causes the conformational change and activation of the PH domain of Akt, after which phosphoinositide-dependent protein kinase 1 (PDK1) can phosphorylate T308 (catalytic protein kinase core).

Maximum activation of Akt is induced by the phosphorylation of both T308 (catalytic protein kinase core) and S473 (hydrophobic motif) Akt residues by PI3K (Yang et al., 2002). The phosphorylation of S473 is established by mTORC2 and can stabilize the phosphorylation of T308. It is not known if activation of PI3K is essential for phosphorylation by mTORC2, but (Manning & Toker, 2017) proposes that Akt itself bind to the involved membranes with its PH domain, causing mTOR2 to phosphorylate S473.

Phosphorylation of FoxO factors by Akt

When Akt is activated, it can recognize a phosphorylation consensus motif on FoxO proteins. FoxO1, 3 and 4 all have three conserved binding sites for Akt that are all necessary to be phosphorylated by Akt to have an effect on FoxO (Papers & Doi, 2007). Each of the three binding

sites contains the substrate motif (RXRXXS/T), two are located at the N- and C-termini and one at the DBD. FoxO can bind with its DBD to DNA and Akt prevents FoxO from binding to its target DNA.

First of all, FoxO1 can be phosphorylated on Thr 24, Ser 256 and Ser 319 (figure 5). Ser 256 is located in the Nuclear Localization Signal (NLS) (Manning & Toker, 2017). Phosphorylation of Ser 256 will cause a change in charge, from positive to negative. (Calnan & Brunet, 2008) argues this process takes place in the NLS, causing the inhibition of the re-entry of FoxO factors into the nucleus and (Wang et al., 2016) says it takes place in the DNA-binding domain (DBD). Both will have the same effect, namely the reduction in FoxO1 activity. In this case it seems logical that the charge changes in the NLS, because Ser 256 lays in the NLS. The phosphorylation of FoxO3 happens on Thr 32, Ser 253 and Ser 315. Ser 315 and Ser 253 have a synergistic effect. FoxO4 is phosphorylated on residues Thr 28, Ser 193 and Ser 258. This event will decrease the DNA-binding capacity of FoxO4 and its transcriptional activity. FoxO6 can be phosphorylated on Thr 26 and Ser 184 (Calnan & Brunet, 2008; Wang et al., 2016).

There are more kinases that can phosphorylate substrates at the RXRXXS/T motif. The Serum and glucocorticoid-regulated kinase (SGK) is also a serine threonine kinase that can phosphorylate the FoxO factors on the Akt sites, but it prefers different sites then Akt does. For example, on FoxO3 SGK rather phosphorylates S315 and Akt has a preference for Ser 253 (Brunet et al., 2001).

FoxO6 is an exception in the FoxO class, it is phosphorylated at two sites. The FoxO member does not contain the carboxy terminal site (Tzivion et al., 2011). Therefore, FoxO6 will be inactivated by phosphorylation of the two sites, but will not shuttle between the nucleus and cytoplasm of a cell.

Akt directly phosphorylates FoxO factors. However, phosphorylation by Akt itself does not have a direct effect on the function of FoxO proteins. Still it can change the conformation of FoxO and enhances a possibility for other enzymes to create a more efficient binding with FoxO, but it has not an effect on DNA binding. The 14-3-3 are required to affect the function and DNA binding of FoxO factors (Tzivion et al., 2011).

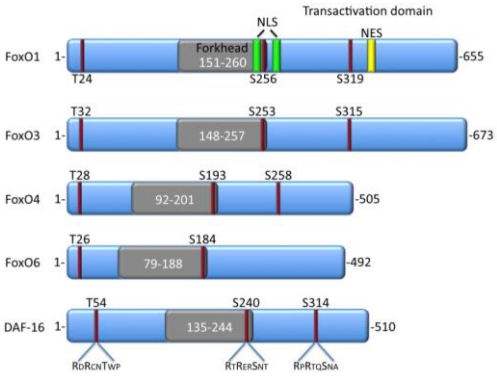

Figure 5: The binding site for Akt phosphorylation of FoxO proteins (Tzivion et al., 2011).

Nuclear exclusion by 14-3-3 proteins

Each phosphorylation at the first and second site of the FoxO factors (FoxO1: Thr 24 and Ser 256, FoxO3: Thr 32 and Ser 253, FoxO4: Thr 28 and Ser 193), generates two binding sites for the 14-3-3 protein. The recognition motif in the binding sites for 14-3-3 proteins is RSXpS/TXP.
The 14-3-3 has in fact two functions in regulating FoxO factors. They detach FoxO factors from their target genes, withholding FoxO from executing its functions. Second, the 14-3-3 have an effect on the NLS of FoxO proteins (figure 6).

Evidence is obtained with fluorescence spectroscopy that 14-3-3 proteins binding to FoxO, decreases the flexibility of the NLS by a conformational change of this region (Obsilova et al., 2005). In addition, the study suggests that the DBD of FoxO is not affected by 14-3-3 proteins.
Overall, the nuclear exclusion of FoxO factors and cytoplasmic sequestration is induced (Tzivion et al., 2011).

All taken together, the Akt pathway, stimulated by insulin and PI3K, inhibits the function of FoxO proteins. Thus, FoxO is a major downstream target of Akt.

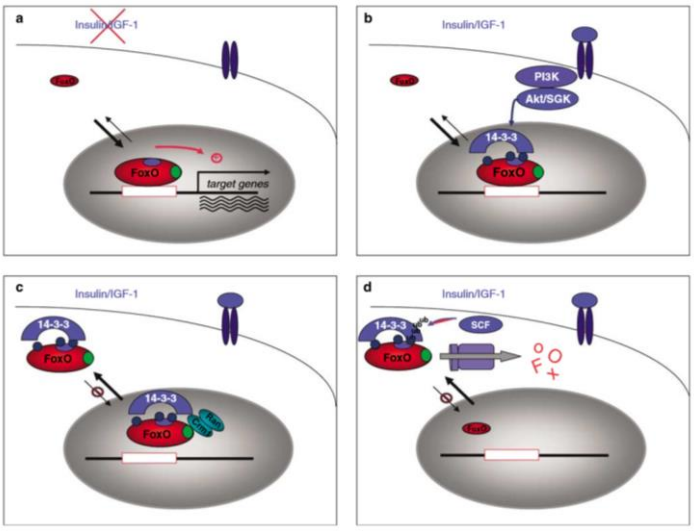

Figure 6: The mechanism of FoxOs nuclear exclusion by 14-3-3 proteins (Calnan & Brunet, 2008).

The Mst1 and Jnk pathway

Mst1 and Jnk activation

Under stress-stimuli, FoxO factors can re-entry into the nucleus, without dephosphorylation of the phosphorylated sites by the Akt (figure 7). Insulin, IGF and SGK are all inhibited when FoxO proteins entry the nucleus (Kim, et al., 2013). An example of a stress-stimulus is the presence of reactive oxygen species (ROS) or oxidative stress. In response to the oxidative stress, Mammalian Ste20-like kinase 1 (MST1) is stimulated (Calnan & Brunet, 2008). Mst1 belongs to the class II protein serine threonine kinases (Papers & Doi, 2007). Under oxidative stress, Mst1 activation is induced by phosphorylation of the sites Thr 183 and Thr 187, probably established by autophosphorylation (Bi et al., 2010). The serine threonine kinase is involved in regulation of cellular proliferation and apoptosis.

Another kinase, Jun N-terminal kinase (Jnk), is stimulated under oxidative stress (Calnan & Brunet, 2008). Mst1 is a upstream kinase of the Jnk pathway, what means that Mst1 can activate the Jnk pathway (Yu, Ji, & Guo, 2013). Interestingly, Jnk can phosphorylate Mst1 at Ser 82, which increases the kinase activity of Mst1 and its nuclear localization. Jnk can induce the cleavage of Mst1 by caspase-3, which is a necessary process for Mst1 to achieve its kinase activity (Bi et al., 2010).

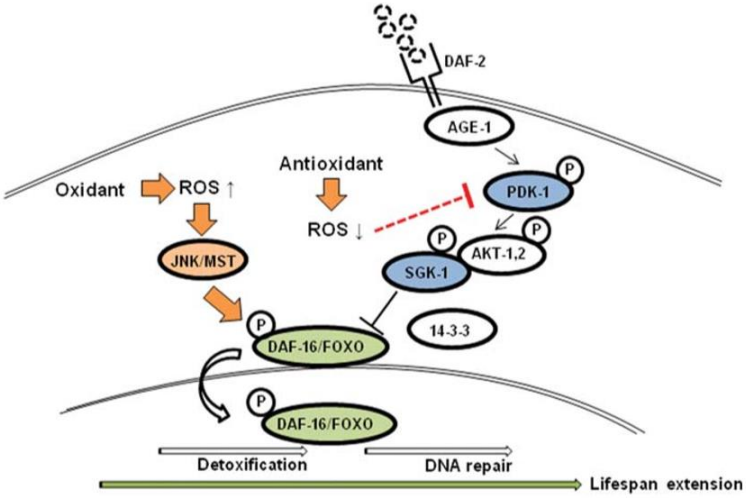

Figure 7: The regulation pathway of Mst1 and Jnk (Kim et al., 2013).

Phosphorylation of FoxO factors by Jnk and Mst1

Once activated, the Jnk/Mst1 pathway phosphorylates FoxO factors, just like the Akt/PKB pathway, however, it antagonized the effect of the Akt/PKB pathway. FoxO will be activated by Jnk and Mst1 and will relocalize to the nucleus. But how does this mechanism work? Both Jnk and Mst1 can phosphorylate FoxO proteins separately (Greer & Brunet, 2008). The difference between the two kinases is that Mst1 phosphorylates FoxO1 and FoxO3, whereas Jnk phosphorylates FoxO4 (figure 8). Jnk can probably phosphorylate FoxO1 and FoxO3 to, but the sites are not conserved. Suggested is that the FoxO proteins all have specific functions.

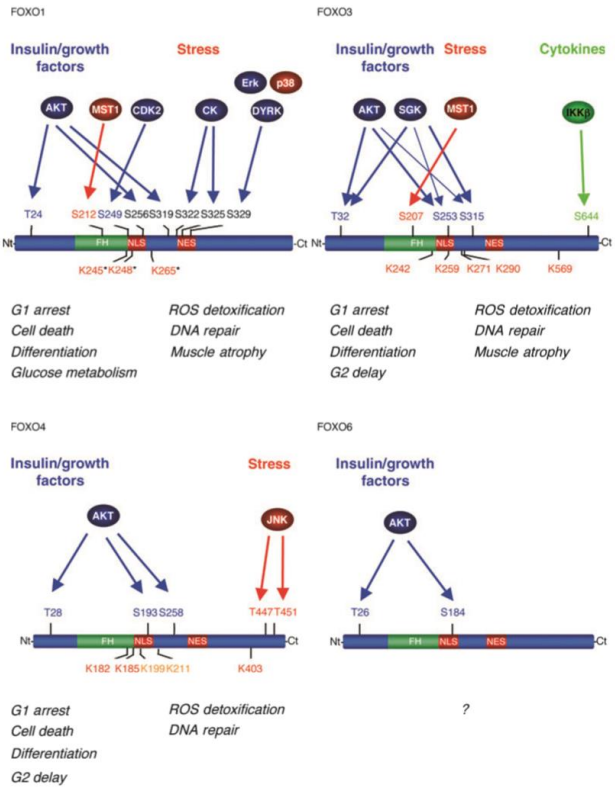

Figure 8: The phosphorylation sites of Mst1 and Jnk on FoxO proteins (Greer & Brunet, 2008).

The phosphorylation sites of this pathway are distinct to the Akt/PKB pathway sites. For FoxO1 the site is Ser 212, for FoxO3 it is Ser 207 and for FoxO4 the sites are Thr 447 and Thr 451. The Mst1 target sites are laying in the DBD.
As previously told, 14-3-3 proteins also bind within the DBD and provide cytoplasmic sequestering. Mst1 interferes with this binding and releases FoxO proteins from 14-3-3 proteins. FoxO factors can then in return localize back into the nucleus. Jnk has the same effect on FoxO4. Jnk has the possibility to phosphorylate the 14-3-3 proteins and to dissolve them from FoxO factors (Calnan & Brunet, 2008). This shows that Mst1 and Jnk antagonize the effect of the Akt/PKB pathway on FoxO proteins (Greer & Brunet, 2008; Calnan & Brunet, 2008).

Relocalization FoxO factors into the nucleus

When FoxO factors are stimulated by this pathway, they can relocalize into the nucleus. Once arrived in the nucleus, the transcription of cell death target genes can switch 'on'. The target genes of FoxO factors are previously discussed. One study argues that the FoxO phosphorylation by Mst1 causes steric hinder, whereby FoxO is unable to bind to DNA (Brent, Anand, & Marmorstein, 2008). There are probably more phosphatases involved in the mechanism, an example could be protein phosphatase 2 (PP2A). PP2A has a potential Akt dephosphorylation function (Calnan & Brunet, 2008).

Many studies describe the apoptotic function of Mst1 (Valis et al, 2011; Papers & Doi, 2007); Bi et al., 2010; Yuan et al., 2011). The pro-apoptotic genes that are expressed under stress conditions are BIM, FasL and Trail (Valis et al., 2011). But how this exactly works is unknown. When FoxO proteins are transported back into the nucleus, expected is that all of the FoxO target genes will be expressed. However, there is probably some Mst1 mechanism that regulates the pro-apoptotic target genes. Possible is that the intensity of the stress stimulus plays an important role and that FoxO proteins regulate other genes in different types of cells (Carter & Brunet, 2007). Thus, when the stress stimulus is low, Mst1 can induce stress resistance. Mst1 itself can localize in the nucleus and it might be that an overexpression of Mst1 or a high stress stimulus causes apoptosis (Bi et al., 2010).

The activation of FoxO and the killing of damaged cells or cells that act abnormal, is involved in extension of lifespan by oxidative stress resistance in D. melanogaster and C. elegans through Mst1. In the C. elegans Jnk also play a role in the extension of lifespan (Calnan & Brunet, 2008; Matsumoto & Accili, 2005; Kim, et al., 2013).

Correlation between Akt and Mst1 or Jnk pathways

Akt can inhibit the pro-apoptotic function of Mst1 by phosphorylating it on the Thr 387 site (Papers & Doi, 2007). The kinase activity of Mst1 is diminished and the FoxO transcriptional activity with it. FoxO1 and FoxO3 will sequester back into the cytoplasm. This process will promote cells to survive.

FoxO in cancer

FoxO as a tumour suppressor

In humans, the FoxO transcription factors were first identified in tumours at chromosomal breakpoints (Anderson, Viars, Czekay, Cavenee, & Arden, 1998). This finding suggest that the proteins might be tumour suppressors in cancer. Over the years the FoxO proteins are many times described as tumour suppressors (Hornsveld et al., 2018; Arden, 2007; Kim et al., 2018).
The function of FoxO factors, as described earlier, are diverse. They can regulate cell cycle arrest, DNA repair and apoptosis, all involved in the regulation of cancer. In addition, arrest of the cell cycle is a key feature of a tumour suppressor (Greer & Brunet, 2008).

The FoxO proteins can cause apoptosis in multiple types of cancer. The loss of both FoxO1, FoxO3 and FoxO4 can generate hematopoietic lineage-specific cancer (Hornsveld et al., 2018; Arden, 2007). The consequences can be severe. The lack of DNA repair by FoxO factors may lead to genomic instability and the cell cycle arrest can make cells grow extensively. Besides, the loss of apoptosis creates an opportunity for cancer cells to survive.

When one or two of the FoxO factors are disrupted, then a less severe tumour phenotype will be expressed (Arden, 2007). In addition, FoxO3 can act as an tumour suppressor by delaying metastasis, but they are not involved in the growth of a primary tumour (Hornsveld et al., 2018).

Examples of mutations in different cancer types

Incorrectly regulated FoxO proteins are correlated with multiple cancer types, such as prostate, brain, stomach, breast cancer, leukemia and lung cancer (Arden, 2007; Kim et al., 2018). Several mutations are present in FoxO genes in each type of cancer. The somatic alterations that can appear are somatic point mutations and chromosomal translocations (Coomans & Demoulin, 2016).

Various studies present evidence for a mutant of the phosphatase and tensin homolog (PTEN) gene in human tumours (Modur, Nagarajan, Evers, & Milbrandt, 2002; Cairns et al., 1997; Georgescu, 2010). Normally, PTEN in humans and other species is thought to be an inhibitor of the PI3K-PKB/Akt pathway (Modur, Nagarajan, Evers, & Milbrandt, 2002; Molina et al., 2012). An overexpression of the protein causes a dephosphorylation of FoxO factors and the translocation into the nucleus (Modur, Nagarajan, Evers, & Milbrandt, 2002a). Thus, the protein can lead to the stimulation of FoxO to exert its functions, such as cell cycle arrest and apoptosis. Evidence is showed for FoxO transcriptional activity as downstream target of PTEN. In contradiction, a mutation in the PTEN gene in prostate cancer leads to the loss of PTEN activity and with this an increase in phosphorylation of FoxO by Akt. This will in addition lead to a decrease in FoxO function and return of FoxO factors to the cytoplasm, which will cause a decrease in proliferation and apoptosis of cells. Because PTEN can inhibit the PI3K-PKB/Akt pathway and a mutation in the gene can cause uncontrolled cell growth, PTEN is suggested to be a tumour suppressor (Cairns et al., 1997; Kim et al., 2018). Along with this, FoxO is also a tumour suppressor, working downstream of PTEN. Besides, it is reported that with the interruption of the normal function of PTEN and FoxO factors, the TRAIL gene is not expressed in the tumours, leading to an inhibition of apoptosis (Modur et al., 2002a).

A different mutation that can occur in cancer is a Mst1 mutation of a Ser 82 residue (Bi et al., 2010). As described previously, Mst1 needs Jnk to phosphorylate its Ser 82 to enhance Mst1 kinase activity. When a mutation occurs on the Ser 82 residue to an alanine amino acid, Jnk is unable to phosphorylate Mst1 at this site. In this case the phosphorylation of Ser 207 on FoxO3 is inhibited. This leads to a not fully phosphorylated FoxO3 and to a loss of function of this FoxO factor.

In addition, one study reported evidence for FoxO proteins to play a role in tumour regulation in human breast. The presence of FoxO3 in the cytoplasm, mediated by Akt expression, would lead to a decreased survival of breast cancer patients, pointing out the tumour suppressive function of FoxO proteins (Hu et al., 2004).

A paradox?

There is a possibility that FoxO factors can control different genes in different kind of cells (Carter & Brunet, 2007; Greer & Brunet, 2008). In addition, it could be that the tumour phenotype is dependent on the tissue in which FoxO is or is not present. For example, when FoxO is absent in endothelial cells in the liver (thymocytes), these cells can proliferate and survive. Indeed, that is exactly what you would expect, if FoxO is a tumour suppressor. However, looking at endothelial cells originating from the lung, the opposite effect concerning FoxO deficiency is observed (Arden, 2007). Also in hematopoietic stem cells an increase in apoptosis was showed (Greer & Brunet, 2008). According to these findings the function of FoxO factors as tumour suppressors in cancer is challenging.

In addition, the question that raises is why 'normal' cells could benefit from the regulating functions of FoxO and cancer cells cannot? Indeed, tumour cells can also take advantage of FoxO factors, by using them for tumour growth, homeostasis and especially for metastasis (Hornsveld et al., 2018). Thus, FoxO factors can play a role in tumorigenesis. Suggested is a complex role of FoxO factors in cancer. For example, it can help breast cancer metastasis development.

A mice model with four to six deleted FoxO alleles showed in a late stage in life a tumour suppressive role of FoxO factors. In contrast, the loss of all three FoxO proteins, needed for a severe tumour phenotype, is unlikely to happen in human cancers and has not been reported yet (Hornsveld et al., 2018; Coomans & Demoulin, 2016).

For instance, FoxO1 mutations can be present at the start codon and at the Thr 24 residue, disrupting the phosphorylation of Akt. Especially in B-cell lymphomas this kind of mutations can occur. Cancer patients with a mutation in FoxO1 have lower survival levels then the ones with wild type FoxO1. This suggests an inhibition of the tumour suppressive role of FoxO1. Still, the tumour can keep the FoxO1 effects that are beneficial for its growth (Coomans & Demoulin, 2016).

In the same study the role of FoxO3 in leukamia is described, as a key player in the maintenance of this type of cancer. Probably, TGF-beta will activate FoxO3 by inhibiting Akt, FoxO3 is active in the nucleus of leukamia initiating cells (LICs) and BCL-6 can increase the self-renewal of LICs in leukamia. Taken together, this process will lead to survival of leukamia cells (Coomans & Demoulin, 2016).

Something else important to take in account is that FoxO factors can be present at different levels in different cell types and thus at which level of FoxO proteins are tumour suppressive or promote tumour survival must still be determined.

Discussion

The FoxO transcription factors fulfil many functions, but their association with cancer is unknown. The regulation of the FoxO proteins by post-translational modifications has been studied extensively, especially by phosphorylation. FoxO proteins are downregulated through phosphorylation by the PI3K-PKB/Akt pathway. It is clear that Akt phosphorylates FoxO proteins on three different sites (FoxO1: Thr 24, Ser 256, Ser 319; FoxO3: Thr 32, Ser 253, Ser 315; Thr 28, Ser 193, Ser 258), except for FoxO6 (Thr 26, Ser 184). The phosphorylation of FoxO by Akt creates binding sites for 14-3-3 proteins, which binding results in the nuclear export of FoxO factors. Mst1 and Jnk have a positive effect on FoxO factors by phosphorylating the FoxO factors on different binding sites (FoxO1: Ser 212; FoxO3 Ser 207; FoxO4 Thr 447 and Thr 451). The phosphorylation releases FoxO factors from 14-3-3 proteins and causes the re-entry of FoxO factors into the nucleus, resulting in the transcriptional expression of their target genes. Phosphorylation by Akt can override the phosphorylation by Mst1 kinase.

However, the FoxO factors are also regulated by acetylation and ubiquitylation. Acetylation is mainly regulated by the calcium response element-binding (CREB)-binding protein (CPB) and/or p300 pathway (Van Der Heide & Smidt, 2005). The CPB and p300 proteins have a transcriptional activating effect on FoxO, however in this pathway it is essential to discriminate between the acetylation of FoxO itself and the histone DNA acetylation. The exact outcome of the acetylation pathway is unclear. Two forms of ubiquitylation exist, namely monoubiquitylation and polyubiquitylation. Monoubiquitylation stimulate FoxOs nuclear translocation, whereas polyubiquitylation inhibits and degrades the FoxO factors (Eijkelenboom & Burgering, 2013). This regulatory pathways could meanwhile also have an impact on FoxO factors in cancer.

What remains unknown is, are FoxO proteins tumour suppressive or do they support tumour survival or both? It is import to take in consideration that the acetylation, ubiquitylation or other protein-protein interactions could have an effect on FoxO transcription factors. Added to the given, it might effect FoxO factors in different types of cancer. Thus, FoxO factors are regulated by numerous post-translational modifications that result in interaction with specific binding partners to regulate the factors differentially.

Furthermore, the date of the published articles have to be taken in account. Studies from about 10 years ago and older report that FoxO proteins are tumour suppressors by inhibition of for instance the cell cycle. Recent studies proposed FoxO factors as tumour supportive, because tumours can also benefit from the numerous cell maintaining functions of FoxO. At the same time, the more recent studies are doubting the role of FoxO in cancer. The evidence is not decisive about the cancer supportive or suppressive role of the factors. Taken in consideration, there are different types of FoxO, different levels of accumulating FoxO in different types of cancer, there is not an ascribed function for FoxO in cancer. Each case of cancer has to be looked at separately.
The role of FoxO factors in cancer remains to be established.
In the future, FoxO proteins may be a target for drug treatment against cancer. A couple of studies proposed a therapy with regarding to FoxO proteins in several types of cancer. For example, the effect of matrine in prostate cancer has been analysed by using a number of techniques. Matrine upregulates the expression of FoxO1, FoxO3, FoxO4 and FoxO6, trough downregulation of the PI3K-PKB/Akt pathway. This inhibits the development and progression of prostate cancer cells (Li et al., 2017). Besides, Paclitaxel is found to activate the expression of BIM via FoxO3 to induce apoptosis in breast cancer (Coomans & Demoulin, 2016).

However, targeting FoxO factors in cancer might lead to unwanted side effects. In general, each transcription factors exerts its function via the DNA-binding domain that can activate, silence or inhibit the target gene. So, looking at other transcription factors, might give more insight in the targeting of FoxO factors. For example, the transcription factor SMAD7 plays a role in the regulation of apoptosis in gastric cancer cells. SMAD7 was directly targeted at the 3'UTR, resulting in the inhibition of apoptosis, suggesting a better survival of gastric cancer cells (Yao, Pan, Du, Zhang, & Li,

2018). This is one example of targeting a transcription factor, generaaly it is very difficult to inhibit transcription factors. The role of, in this case, FoxO factors is unknown, thus it might be safer to target for example the target genes of the factors. The inhibition of genes as an epigenetic marker can be established by methylation (Wilson & Filipp, 2018). Other results showed evidence for dysregulated target genes of transcription factors by DNA methylation in mammary gland carcinomas (Daino et al., 2018).

I think it a good thing that the literature is doubtful about the exact role of FoxO factors in cancer. First it was thought that FoxO factors are important tumour suppressors, but looking more closely into the regulation of these proteins, they may also act as tumour supporters. Hence, if more becomes clear about the role of FoxO in cancer, the factors could be a part in the puzzle of solving a big problem, called cancer.

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