FOXO forwards

novel targets and feedback regulation

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FOXO forwards
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FOXO voorwaarts

nieuwe doelen en terugkoppel regulatie

(met een samenvatting in het Nederlands)

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Cogito ergo sum
Descartes

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CHAPTER 1

General introduction: The PKB/FOXO switch in ageing and cancer

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Abstract

Aging is characterised by a general decline in tissue and body function and increased susceptibility to age-related pathologies, such as cancer. To maintain optimal tissue and body function, organisms have developed complex mechanisms for tissue homeostasis. Importantly, it is becoming apparent that these same mechanisms when deregulated also result in the development of age-related diseases. The build-in failsafe mechanisms of homeostasis, which prevent skewing towards disease, themselves contribute to aspects of ageing. Thus, longevity is limited by an intrinsic trade-off between optimal tissue function and disease. Consequently, aging and age-related diseases, such as cancer and diabetes are driven by the same genetic determinants. Illustrative in this respect is the insulin/IGF1 signalling pathway acting through phosphoinositide 3-kinase (PI3K)-protein kinase B (PKB)/Akt and Forkhead box-O class of transcription factors. Loss of PKB signalling contributes to diabetes, whereas gain of function of PKB drives cancer. Enhanced FOXO-activity, at least in model organisms, contributes to extended lifespan and acts as a tumour suppressive mechanism. Here, we focus on the linkage between PKB and FOXO proteins as a central switch in contributing to tissue homeostasis and age-related diseases in particular cancer.

Introduction

Aging is considered the result of deterioration of the body due to progressive loss of tissue function and integrity. Tissue homeostasis entails renewal as well as maintenance of cells within the tissues, the importance of which of these particular processes is dependent on the particular tissue. While renewal is the result of both proliferation of (stem) cells, as well as eradication of differentiated cells, maintenance involves the turnover of cellular constituents themselves. It is estimated that the human body yearly eradicates and generates a number of cells that has a mass that is almost equal to that of its own [1]. To maintain tissue and body integrity, the processes of eradication and generation must be tightly controlled. While these processes have been studied intensively, their crosstalk – the actual homeostasis – is poorly understood. In addition, cell renewal is thought to occur in many more tissues than previously appreciated but the extent of cell turnover varies greatly among different tissues [2]. One of the prominent pathways involved in turnover of cellular constituents is autophagy. In particular, macro-autophagy and chaperone-mediated autophagy are responsible for regular removal of cellular constituents and organelles [3]. Aging is accompanied by the accumulation damaged constituents and organelles, as well as reduced regenerative capacity and renewal of tissues. Several cell intrinsic mechanisms have been proposed to underlie the decline in tissue homeostasis. A long standing hypothesis is based on the observations of Hayflick [4]. Due to the nature of DNA replicating enzymes, telomeres become shorter with each successive round of replication. After a number of replications, telomeres become critically short and this results in permanent withdrawal from the cell cycle. In some cells such as the germ line cells, this is counteracted by the activity of telomerase, but this enzyme is inactive in most somatic cells. While telomere-related signalling is considered a main barrier to malignant transformation, it has become apparent that telomere dysfunction can fuel cancer development through the induction of genome instability [5]. In addition, accumulating DNA damage due to both endogenous and exogenous sources has been proposed to be the origin of aging and cancer. Stem cells are no exception with regard to DNA damage, albeit possibly at a slower rate [6]. An important endogenous source of damage is thought to be reactive oxygen species (ROS), which are a normal consequence of metabolism. ROS can react with diverse components of the cell, including lipids, proteins and DNA. Such reactions might yield difficult-to-degrade products and give rise to a wide range of DNA lesions [7]. However, if and how aging of adult stem cells is related to this is currently controversial and unclear [8]. For example, while the abundance and ability of hematopoietic stem cells (HSCs) to form colonies does not seem to become impaired with aging, skewing towards certain lineages is observed, pointing to cell-intrinsic aging of these stem cells. On the other hand, the ability of satellite cells to regenerate muscle upon insult seems to be dependent on the age of the niche [8]. Aging is also accompanied by increased accumulation of macromolecular and organelle damage, and impairment of autophagic processes that normally counteract this hasten aging. Age-related impairment of lysosomal function, possibly as the result of increased ROS levels within lysosomes and the accumulation of lipofuscin, seems to be a main cause of reduced cellular turnover [3]. It is appreciated nowadays that the function of many genes influences ageing. Here, we will discuss involvement of the insulin pathway and its genes, which have set the paradigm for much of our current understanding of ageing.

Establishment of the PKB/FOXO switch

Ample evidence suggests that insulin and insulin-like growth factor (IGF) signalling (IIS) is

important in determining lifespan in various organisms, including mammals. Initially, the life-extending effects of dampening IIS were observed in the worm *Caenorhabditis elegans*. Worms that harbour mutations in the DAF-2 gene live twice as long compared to wild-type worms [9]. DAF-2 encodes an orthologue of the mammalian insulin and IGF1 receptors and at that time biochemical studies already provided possible mechanisms of signal propagation, which could be involved in mediating DAF-2 influence on lifespan. Binding of insulin to the insulin receptor (IR) results in activation of the intrinsic tyrosine kinase activity of the IR and consequent autophosphorylation and phosphorylation of cytoplasmic substrate(s) on tyrosine residues [10]. Members of the insulin receptor substrate family (IRS1/2/3/4) are the predominant IR substrate and IRS1 tyrosine phosphorylation provides access towards two major downstream signalling cascades: the phosphoinositide 3-kinase (PI3K) pathway and the Ras pathway [11]. SH2 domain mediated binding of the p85 regulatory subunit activates the catalytic p110 subunit of PI3K, resulting in increased level of 3' phosphorylated phosphoinositides (PtdIns(3,4,5)P_3, PtdIns(3,4)P_2 and PtdIns(3,5)P_2 hereafter referred to as PI3P lipids). Indeed, DAF-2 effects on lifespan were shown to involve AGE-2 (the PI3K orthologue) and importantly also the transcription factor DAF-16 [12, 13].

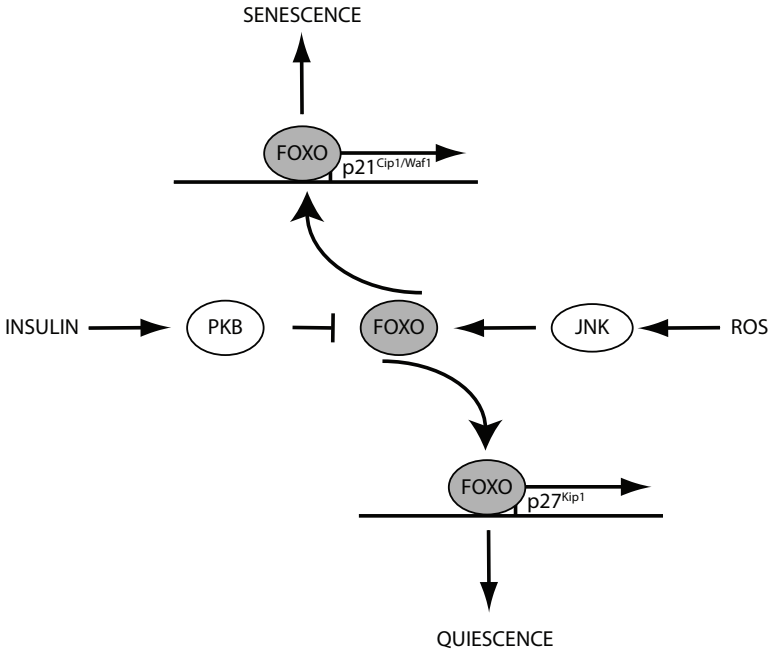

Figure 1: the PKB/FOXO switch. FOXO transcription factors are predominantly controlled by two signalling pathways. First, the insulin pathway acting through PI3K-PKB-mediated phosphorylation of FOXO proteins, resulting in cytoplasmic retention and inhibition of FOXO transcriptional activity. Second, a pathway activated by cellular stress acting through increased ROS and JNK-mediated phosphorylation of FOXO proteins resulting in increased nuclear localization and activation of transcriptional activity. The opposing forces of PKB versus JNK signalling will determine whether FOXO proteins will direct a transcriptional response regulating entry into quiescence or senescence. $p27^{Kip1}$ and $p21^{Cip1/Waf1}$ are provided as example of genes regulated by FOXO proteins to mediate quiescence or senescence onset respectively. However, other FOXO-regulated genes are in addition involved in imposing these cell fates (for discussion see text).
Abbreviations: protein kinase B (PKB); c-Jun amino-terminal kinase (JNK); reactive oxygen species (ROS); Forkhead box-O (FOXO).

Members of the Forkhead box-O class of transcription factors (FOXO1, 3, 4 and 6 hereafter collectively referred to as FOXO proteins), are the mammalian orthologues of DAF-16. By means of biochemistry the signalling gap between PI3K and FOXO proteins has been closed. PI3P lipids provide docking sites for proteins containing PI3P binding domains, such as the pleckstrin homology (PH) domain. Importantly PI3P increase results in activation PKB which harbours, next to the kinase domain, a PH domain (reviewed in [14]). PKB activation is complex and besides membrane recruitment requires phosphorylation at Thr308 by phosphoinositide-dependent protein kinase 1 (PDPK1), another PH domain containing kinase. Full activation of PKB also requires phosphorylation at the carboxyl-terminal Ser473 residue and several kinases have been proposed to mediate Ser473 phosphorylation, including PKB itself. At present genetic evidence suggest that, depending the context, either DNA-dependent protein kinase (DNA-PK) and/or the mammalian target of rapamycin (mTOR) complex 2 (mTORC2) mediate Ser473 phosphorylation (reviewed in [14]).

The notion that PKB mediates PI3K signalling prompted the possibility that FOXO proteins are direct substrates of PKB. Indeed, DAF-16 and FOXO proteins are phosphorylated by PKB on several conserved residues. PKB-mediated phosphorylation of FOXO proteins results in 14-3-3 binding, and this correlates with nuclear export, cytoplasmic retention, and inhibition of transcriptional activity of FOXO proteins (reviewed in [15]). The precise involvement of 14-3-3 in these processes is still unclear, but PKB phosphorylation and 14-3-3 binding occur in the nucleus and 14-3-3 binding reduces the affinity of FOXO proteins for DNA and shields the nuclear localisation signal (NLS), thereby affecting nuclear import (reviewed in [15]).

Besides PKB, PDPK1 can also activate other kinases of the AGC family of kinases including the PKB related serum and glucocorticoid inducible kinase (SGK) [16]. SGK can phosphorylate the same residues as PKB on FOXO proteins, albeit with different efficiency. In agreement, SGK is similarly implicated in *C. elegans* lifespan extension [17].

In addition to PKB and SGK, a number of other kinases have been shown to phosphorylate FOXO proteins and to contribute to FOXO-inactivation (reviewed in e.g. [15]). However, ROS activated signalling involving c-Jun N-terminal Kinase (JNK) leads to nuclear localisation and activation of FOXO proteins, even in the context of active PKB (reviewed in [18]). Activated JNK phosphorylates FOXO4 on a number of sites and this coincides with mouse double minute 2 homolog (MDM2)-dependent mono-ubiquination, nuclear localisation and activation of FOXO4 transcriptional activity [19]. Such JNK-mediated activation of DAF-16/dFOXO has also been reported in *C. elegans* and *Drosophila melanogaster* [20, 21]. However, whether JNK-dependent activation of FOXO4 applies to the other mammalian FOXO family members remains to be established. Interestingly, JNK opposing PKB signalling with respect to FOXO-regulation (figure 1) appears illustrative for a much broader network of antagonistic PKB/JNK interactions [22].

PKB peculiarities

The mammalian PKB/Akt family consists of three isoforms (PKBα/Akt1 PKBβ/Akt2 and PKBγ/Akt3). PKBβ is the predominant isoform in insulin sensitive tissues and cells. In agreement PKBβ$^{-/-}$, but not PKBα$^{-/-}$ mice display defective insulin-stimulated glucose uptake in muscle and adipocytes. In adipocytes cultured from PKBβ$^{-/-}$ mice this defect is relieved upon ectopic expression of PKBβ but not PKBα. Thus PKBβ appears to be the main mediator of insulin-induced glucose uptake [23-25]. The inducible glucose transporter GLUT4 is mainly responsible for the actual uptake of glucose after insulin increase. Numerous components regulating

GLUT4 dynamics have been identified and several of these are directly phosphorylated and regulated by PKB [26].
In addition to regulation of glucose uptake insulin also regulates storage of glucose into glycogen. Glycogen levels are controlled through synthesis catalyzed by glycogen synthase (GS) and lysis by glycogen phosphorylase. GS activity is repressed through phosphorylation by glycogen synthase kinase 3 (GSK3). Under basal conditions, GSK3 activity is high, but insulin-induced PKB activation results in PKB-mediated phosphorylation of GSK3 on its regulatory sites Ser21 and Ser9 (numbering GSK3α and GSK3β respectively). This results in inhibition of GSK3 activity and therefore increased GS activity [27].
Initially, it was shown that insulin through PKB could regulate PI3K-dependent p70 S6kinase activity [28]. However, this remained controversial until a series of genetic and biochemical studies outlined the pathway in detail. In brief, it was shown that PKB regulates the tuberous sclerosis 2 gene product (TSC2) by direct phosphorylation [29]. TSC2 functions in complex with TSC1 and the TSC1/2 complex has GTPase activating protein (GAP) activity. The small GTPase regulated by the TSC1/2 complex was identified as Rheb, identified previously as a component of the mTOR pathway by genetics in Drosophila. PKB phosphorylation inhibits GAP activity of the TSC1/2 complex and therefore a model was put forward in which PKB, through TSC1/2 and Rheb, regulates mTOR activity and hence p70 S6kinase. p70 S6kinase phosphorylates the 40S ribosomal subunit thereby affecting protein translation, but through the mTOR and the PI3K pathway, PKB regulates many other players involved in protein synthesis including eukaryotic translation initiation factor (eIF) 4G (eIF4G), eIF4B, eIF4E binding protein 1 (4E-BP1) and 4E-BP2 [30]. This results in a complex regulatory network, the details of which are still not fully understood. However the importance of this regulation is emphasised by the observation that deregulated expression of these components can cause (overexpression of eIF4E or eIF4G) or inhibit (overexpression of 4E-BP1 or 4E-BP2) malignant cell transformation [31, 32].
Besides insulin, nutrient availability is an important regulatory input towards mTOR. Nutrient sensing by cells is not fully understood but lack or surplus of nutrients may change the AMP/ATP balance within cells. Increased AMP/ATP ratio results in the activation of the AMP-dependent kinase (AMPK) and its upstream activator liver kinase B1 (LKB1). AMPK can directly phosphorylate two mTOR regulators i.e. regulatory-associated protein of mTOR (RAPTOR) and TSC1 and thereby regulates the mTOR pathway. Besides being regulated by multiple inputs, mTOR also regulates outputs other than protein synthesis, including regulation of autophagy, fat metabolism, and cell cycle progression [33, 34].
Whereas PKBβ is the isoform predominantly involved in regulating glucose homeostasis, PKBα appears to mainly regulate proliferation. Aberrant activation of PKB in human cancer is largely due to loss of phosphatase and tensin homolog (PTEN), but hyperactivity of PKB due to overexpression and gene mutation (E17K) have also been described [35]. PTEN heterozygous mice ($PTEN^{-/+}$) develop a wide range of tumours with high incidence and at early age. Strikingly, crossing these mice with mice nullizygous for PKBα markedly decreases tumour development in some but not all tissues [36, 37]. Thus PKB is involved in a variety of processes and we will further focus on the co-involvement of PKB and FOXO proteins in some of these processes.

The PKB/FOXO sandwich

Given its involvement in a large variety of cellular processes PKB has been shown to

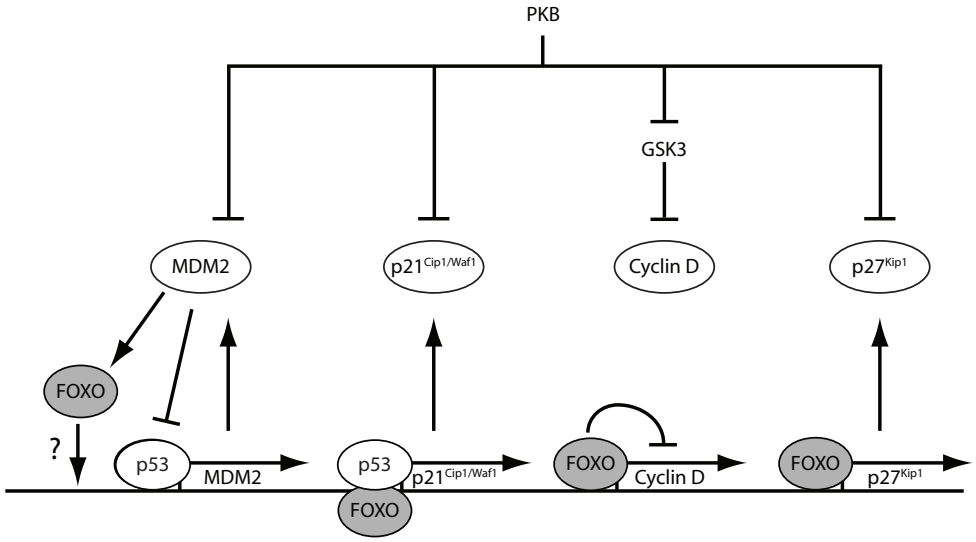

Figure 2: the PKB/FOXO sandwich. In addition to the direct control of FOXO proteins by PKB, a number of proteins are controlled separately by PKB and FOXO proteins, giving rise to antagonistic control. FOXO proteins control transcription of several PKB substrates especially genes involved in regulating cell cycle progression ($p27^{Kip1}$, $p21^{Cip1/Waf1}$, cyclin D1 and cyclin D2). MDM2 is involved in regulating p53, but also FOXO-ubiquitination and is transcriptionally controlled by p53, but possibly also by FOXO proteins (BB, unpublished observations). PKB phosphorylates MDM2 to increase its stability, which impairs p53-dependent $p21^{Cip1/Waf1}$ transcription, and possibly also induces MDM2-mediated polyubiquitination and degradation of FOXO proteins (for discussion on this see [19]).
Abbreviations: murine double minute 2 (MDM2), others see figure 1.

phosphorylate directly a large and diverse array of substrate proteins. Although the extent and rigor by which the various PKB substrates are defined, differs significantly, and probably not all will turn out genuine PKB substrates (reviewed in [38]) it is noteworthy that a number of these substrates as well as the events controlled hereby are also regulated by FOXO proteins. In keeping with PKB acting as a negative regulator of FOXO proteins, the effect of FOXO proteins on these substrates opposes that of PKB (figure 2).

Firstly, FOXO proteins activate transcription of the cell cycle inhibitors $p27^{Kip1}$ and $p21^{Cip1/Waf1}$ while they repress transcription of cyclin D1 and cyclin D2 (reviewed in [15]). PKB phosphorylates $p27^{Kip1}$ on Thr157 [39-41], resulting in 14-3-3 binding and cytosolic retention [42]. Interestingly, $p27^{Kip1}$ localised in the cytosol is ineffective in blocking cell cycle progression, but promotes cell migration and movement [43]. It should be noted however, that the PKB phosphorylation site Thr157 is not conserved in rodent versions of $p27^{Kip1}$. However, it has been suggested that in these species other sites of PKB phosphorylation may substitute for Thr157 phosphorylation, as otherwise similar to non-rodent cells and human $p27^{Kip1}$, relocalisation of $p27^{Kip1}$ following PI3K/PKB activation in rodent cells is observed [44]. PKB has also been found to phosphorylate the cyclin dependent kinase inhibitor $p21^{Cip1/Waf1}$ on Thr145, and, like $p27^{Kip1}$, this phosphorylation leads to $p21^{Cip1/Waf1}$ cytosolic localisation [45]. Interestingly, it has been suggested that PKBα, but not PKBβ, phosphorylates and inhibits $p21^{Cip1/Waf1}$, whereas PKBβ appears to bind and stabilise $p21^{Cip1/Waf1}$, thereby blocking cell cycle progression [46]. What these findings indicate in terms of the differential involvement of PKBα and PKBβ in the proliferative versus metabolic response to insulin is unclear. In addition, PKB can indirectly inhibit $p21^{Cip1/Waf1}$ expression through phosphorylation and activation of

MDM2 and subsequent downregulation of p53-mediated transcription of $p21^{Cip1/Waf1}$ [47, 48]. It is unknown whether this regulation of MDM2 involves a preferential PKB isozyme.
Secondly, active PKB mediates GSK3 phosphorylation and inhibition of GSK3 activity [14]. GSK3-mediated phosphorylation of cyclin D proteins, cyclin E proteins and c-MYC, targets them polyubiquitination and subsequent proteasomal degradation [49-51]. Consequently, PKB-mediated phosphorylation and inhibition of GSK3 activity will increase cyclin D proteins, cyclin E proteins and c-MYC expression. In agreement FOXO proteins repress expression of cyclin D proteins by a mechanism that is not fully understood [52, 53]. Furthermore, c-MYC opposes FOXO-function and vice versa (see further for discussion)
Lastly, besides acting in an antagonistic manner on critical cell cycle progression regulators, FOXO proteins also act to antagonise the activation of mTORC1 by PKB through Sestrin proteins. Sestrins were initially discovered as downstream transcriptional targets of p53 and potentially involved in antioxidant defences. Sestrins display cysteine sulfinyl-reductase activity *in vitro* and are therefore able to regenerate over-oxidised i.e. containing Cys-SO_2H, peroxiredoxins [54]. However, Sestrins display no sequence similarity to sulfiredoxin (Srx), the other known cysteine sulfinyl reductase, and Sestrin reductase activity could not be confirmed in another independent study [55]. Thus the mechanism by which Sestrins contribute to increased antioxidant capacity remains unclear. Sestrin expression is also regulated by FOXO proteins [56] and in Drosophila chronic mTOR activation results in increased dSestrin expression through increased ROS and dJNK-mediated dFOXO-activation [57]. How Sestrins inhibit mTORC1 is subject to some debate with regard to the requirements for the TSC1/2 complex (compare [54, 57] and [58]), but activation of AMPK is always observed [54, 57, 58]. In addition, dFOXO has been shown to regulate the expression of 4E-BP, reducing downstream signalling of mTORC1 [59, 60]. Thus FOXO proteins, indirectly via Sestrin regulation, counteract again PKB in this case in regulating mTOR.

Supersizing the sandwich: feedback signalling directly from FOXO proteins to PKB
Whereas FOXO proteins appear to generally inhibit signalling downstream of PKB, a number of upstream regulators of PKB are positively regulated by FOXO-mediated transcription. While IRS2 [61], PI3K (p110α) [62] and the insulin receptor [60, 63] seem to be direct transcriptional targets of FOXO proteins, increased rapamycin-insensitive companion of mTOR (RICTOR) expression is independent of DNA binding [58]. However, and importantly, mere increased expression of either of these components will not be sufficient to activate PKB and activation still requires an input signal, such as insulin. In this respect it is of interest that in Drosophila activation of JNK results in the downregulation of DILP2 expression (insulin resembling peptide) in a dFOXO-dependent manner [21].
The general picture that emerges from these observations – e.g. FOXO proteins inhibiting downstream signalling of PKB while at the same time enhancing expression of upstream regulators – is a model that favours fast switching (figure 3). From an evolutionary viewpoint, such rapid responses could have significant advantages. For example, during development of *C. elegans* larvae DAF-16 is activated due to stresses such as food shortage. DAF-16 activation causes a developmental arrest called dauer and exit of *dauer* occurs when stress is alleviated e.g. upon increased food availability. To be able to take advantage of the newly available nutrients, individuals should respond fast. Given regular occurrence of nutrient shortage, a shorter response speed would provide individuals with an advantage over those that respond slower. By narrowing the signalling system during periods of stress to the regulation of a single

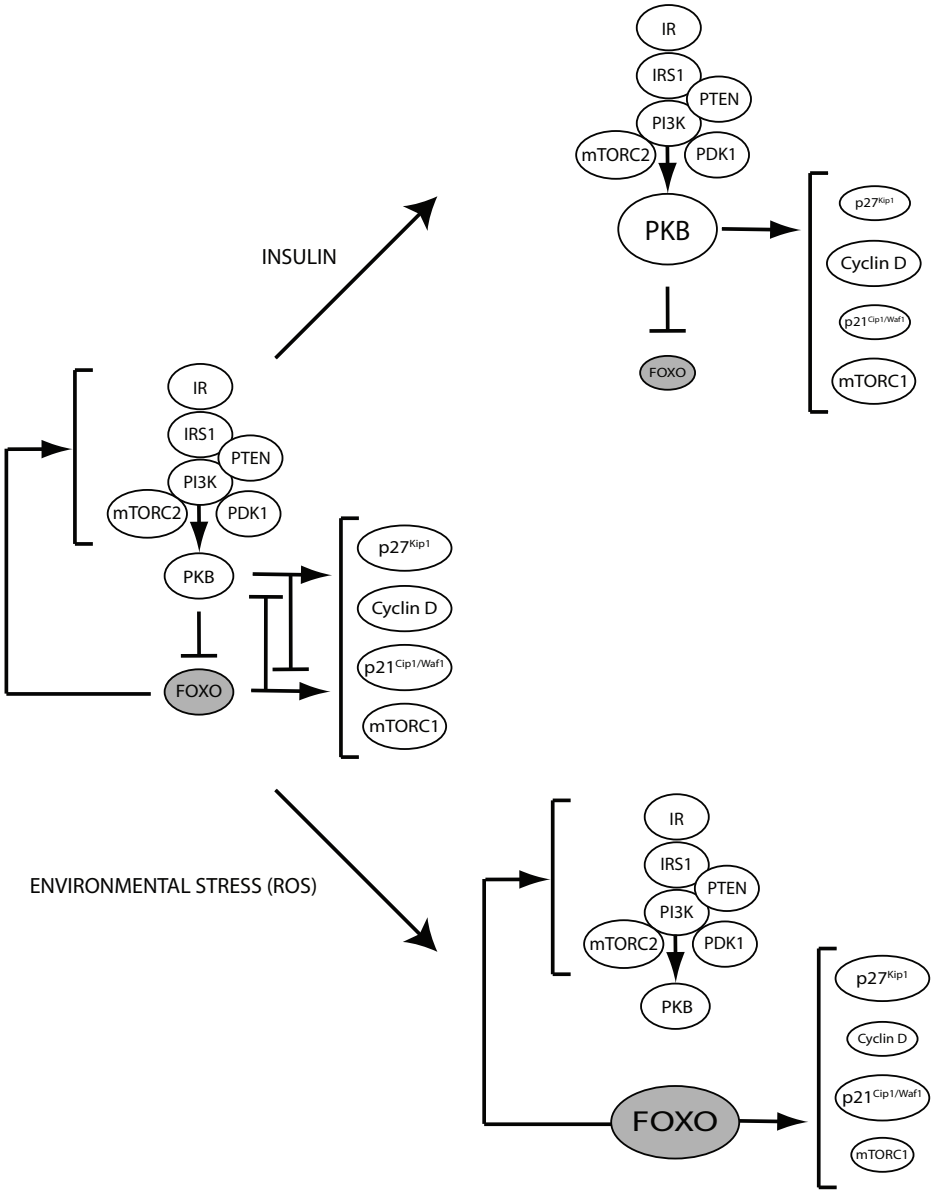

Figure 3: the PKB/FOXO super-size sandwich. Besides antagonistic interactions between FOXO proteins and PKB, FOXO-activity also increases expression of a number of PKB regulators that are involved in mediating PKB activity upon exposure of cells to extracellular ligands such as insulin. Under adverse conditions such as environmental stress, due to amongst others ROS-dependent signalling, the PKB/FOXO switch is set to active FOXO proteins (indicated by enlarged FOXO icon) and cells cease to proliferate, switch to FOXO-dependent metabolism and control of ROS. In addition upstream PKB signalling is set to optimal response in case conditions change to favourable. In case of favourable conditions insulin will drive PKB activation (indicated by enlarged PKB icon), followed by rapid shutdown of FOXO proteins (indicated by reduced FOXO icon) and loss of proliferation inhibition and accompanying changes in metabolism and ROS control.
Abbreviations: insulin receptor (IR), insulin receptor substrate 1 (IRS1), phosphatase and tensin homolog (PTEN), phosphoinositide-dependent protein kinase1 (PDPK1), mammalian target of rapamycin complex 1 (mTORC1), mammalian target of rapamycin complex 2 (mTORC2).

switch (PKB/FOXO) the organism enhances its speed of response when stress is alleviated. In addition, the reciprocal relationship between PKB and FOXO proteins ensures robust, binary switching. Possibly, this type of stress response has been conserved in mammalian cells where FOXO proteins also maintain the possibility of rapid and strong PKB activation by upregulating upstream activators, whilst inhibiting downstream PKB signalling. When stress is relieved and signals driving PKB are again present, FOXO proteins will be robustly switched off, as no feedback or interference will come from other downstream PKB signalling. Because all other PKB signalling is temporarily shut down by FOXO proteins, this will also allow for a rapid reactivation response of these other PKB regulated events.

Interestingly, the robust switching ensured by FOXO proteins and performed by DAF-16 in a whole organism, may also operate in a cell-autonomous manner and it may thus be concluded that cells compete with each other for rapid PKB activation and loss of FOXO-function, a model of cell competition that will be interesting to investigate. In addition this would predict/rationalise how cancer cells that have lost FOXO-function due to PI3K/PKB activation compete with normal cells during for example nutrient stress.

As discussed the PKB/FOXO switch is involved in lifespan and linked to at least two major cell regulatory events namely cell cycle progression regulation and mTOR-dependent signalling. This implies a role for the PKB/FOXO switch in two mechanisms of tumour suppression that are also directly linked to ageing, namely autophagy and senescence which will be discussed briefly.

Mechanisms of tumour suppression affecting ageing: autophagy

Aging is accompanied by increased accumulation of macromolecular and organelle damage and impairment of autophagic processes that normally counteract these aspects of aging. Age-related impairment of lysosomal function, possibly as the result of increased ROS levels within lysosomes and the accumulation of lipofuscin, seems to be a cause of reduced cellular turnover [3].

The lysosomal network is the major system that degrades long-lived proteins and larger structures, resulting in the release of raw building blocks to the cell [3]. Autophagy – to eat oneself – is a highly conserved pathway that is responsible for bulk degradation of cytoplasmic components through the lysosomal pathway. Among the several types of autophagy that are distinguished, macroautophagy and chaperone-mediated autophagy (CMA) are the best studied. Macroautophagy – which we and others refer to when speaking about autophagy – involves engulfing part of the cytoplasm inside double-membrane vesicles termed autophagosomes. These then fuse with lysosomes, which aids the degradation of the cargo [3]. The family of autophagy (ATG)-genes was first discovered in yeast to be essential for macroautophagy. At least 30 genes are nowadays recognised to be actively involved in this process and another 50 are implicated in the regulation of the process [65]. Macroautophagy is regulated downstream of mTORC1 by the *C. elegans* unc-51 like autophagy activating kinase 1 (ULK1)-complex, that contains ULK1, mATG13 and focal adhesion kinase (FAK)-family interacting protein of 200kDa (FIP200) [66]. ULK1 and mATG13 both bind directly to mTORC1 and ULK1 does so through interaction with the mTORC1-specific subunit RAPTOR. Both are thought to be phosphorylated by mTORC1, reducing their binding to one another and the kinase activity of ULK1. Formation of the complex is a critical step in proper localisation of the ULK1-complex, and activation of autophagy. In yeast, ATG17 is the most upstream protein responsible for proper localisation and initiation of autophagy and FIP200 is thought

to be the functional homologue of ATG17 [65]. Autophagosome maturation and lysosome fusion requires a number of additional steps, which have been extensively described (see for example [3]). Interestingly, a number of feedback loops seem to regulate these processes, in particular in autophagy-initiation involving mTORC1 and the ULK1-complex. For example, it has been shown that ULK1 itself can phosphorylate mATG13, which is predicted to cause inactivation of the complex, although not all data are consistent with such a model [65]. In addition, in Drosophila and mammals, ULK1 ablation or knockdown results in mTORC1 activation, pointing to a (negative) feedback [66].

As mentioned, autophagy has been implicated in both aging and disease [3, 67]. The most frequently reported characteristics of cells defective in autophagy are the progressive accumulation of protein aggregates and mitochondria with aberrant morphology. While mice that lack ATG5, ATG7 or ATG3 fail to survive the neonatal starvation period [67], tissue specific knockouts and pharmacological intervention have shed light on the consequences of loss of autophagy. For example, loss of autophagy in the nervous system results in the formation of inclusion bodies and neural degeneration with age [68, 69], while mice exposed to the Class III PI3K inhibitor 3-methyladenine show excessive accumulation of triglycerides and lipid drops, especially in the liver [70]. Furthermore, clearance of mitochondria from reticulocytes is an essential step in their development and as such impairment of this process results in anaemia [71]. Autophagy probably has a dual role in cancer development, being suggested to both prevent and facilitate cancer development. On the one hand, autophagy is thought to be essential for tumour cell survival, providing a mechanism to sustain viability under growth limiting conditions that are thought to be imposed on cancer cells frequently. On the other hand however, cancer development can be enhanced in settings of sustained inflammatory response, for example due to defective clearance of apoptotic cells. Thus, choosing between inhibiting or activating autophagy in cancer therapy is not a straightforward decision [67].

Given the above described, it is not surprising that autophagy is linked to aging and age-related deterioration of tissues [3]. In model organisms, autophagy has been shown to be important in aging and lifespan. Interestingly, both *dauer*-formation and lifespan extension observed in *C. elegans* mutant for DAF-2 are dependent on the Beclin 1 homologue of *C. elegans* bec-1 [72]. Furthermore, autophagy itself seems to be subject to aging. One of the hallmarks for this is the age-dependent accumulation of lipofuscin in lysosomes. The exact origin of lipofuscin is unknown, but it is thought to be constituted of oxidised, non-degradable molecules that may contain iron, as deduced from their yellow/brown colouring. Lipofuscin accumulation is thought to contribute to declining autophagy, possibly through reducing the ability of autophagosomes to fuse with lysosomes [3].

Decreased/loss-of-autophagy does not affect all tissues to the same extent, which can be explained by the fact that some tissues contain more long lived or stressed cells than others [67]. For example, skeletal muscle are believed to have low cell turnover [8] and the maintenance of muscle mass is dependent on functional autophagy [73, 74]. PI3K-PKB and FOXO signalling have been shown to be critically important for muscle hypertrophy and atrophy respectively [75-77]. Muscle atrophy is regulated by both ubiquitin-ligase-dependent degradation as well as autophagy [78]. The ability of FOXO proteins to cause atrophy was initially attributed to its ability to regulate the expression of two muscle-specific E3-ubiquitin ligases, namely Atrogin-1/muscle atrophy F-box protein (MAFbx) and muscle really-interesting-new-gene (RING)-finger protein-1 (MuRF1) [75, 79]. More recently, FOXO proteins were also shown to directly regulate autophagy-related genes in muscles, and other cell types, both *in*

vitro and *in vivo* [76, 78, 80]. Autophagy is negatively regulated by mTOR signalling, which is in turn positively regulated by PI3K-PKB. In agreement with the requirements of autophagy in lifespan extension of DAF-2 worms, FOXO proteins have been shown to regulate autophagy both directly and indirectly. Firstly, FOXO3 was shown to regulate a number of genes that are directly involved in autophagy, such as γ-aminobutyric acid (GABA) A receptor-associated protein-like 1 (Gabarapl1/ATG8), Microtubule-associated protein 1A/1B-light chain 3b (LC3b) and ATG12L [76]. Secondly, FOXO proteins were described to regulate one of the family members of the Sestrins, Sestrin3 [56-58]. Sestrins can inactivate mTORC1 in an AMPK-dependent manner [54] and have been implicated in ROS scavenging [56] (see also above). In Drosophila, constitutive activation of the mTOR pathway causes upregulation of dSestrin, which is dependent on ROS-activated dJNK and dFOXO. In the absence of dSestrin, these flies display lipid accumulation, decreased cardiac function, skeletal muscle degeneration and mitochondrial dysfunction [57]. These hallmarks of aging are also observed in flies that lack one of the constituents of autophagy (ATG1) [57], although this formally does not show that dFOXO exerts its protective function through autophagy. Another study showing the importance of FOXO proteins in age-related protein homeostasis – called proteostasis by the authors – implicates another dFOXO target, 4E-BP, in this [81]. This study confirms that the protective effects of dFOXO/4E-BP indeed are in part through upregulation of genes involved in autophagy, as reduction of ATG7 partly attenuates the beneficial effects of dFOXO/constitutively active 4E-BP expression [81]. Although the above described data seem to be conflicting – FOXO proteins causing muscle atrophy through autophagy, while loss of autophagy also results in atrophy – dynamic regulation of muscle size in response to exercise and maintenance of muscle cell integrity are clearly different aspects of muscle-biology.

Mechanisms of tumour suppression affecting ageing: senescence

When primary normal diploid cells are taken into culture these cells arrest after about 50 cell divisions *in vitro* and lose the ability to divide. This arrest is termed senescence and initially characterised as being permanent i.e. cells are no longer able to re-enter the cell cycle. This, trait distinguishes senescence from quiescence, the latter being characterised as reversible. However, studies have shown that p53-induced senescence is potentially reversible [82]. Consequently, it has proven difficult to establish a conclusive cellular marker of the senescent condition and hence difficult to establish whether senescent cells are present in living organisms or whether they are a cell culture artefact. Nevertheless, cells showing classical markers of senescence e.g. senescence-associated (SA)-β-galactosidase activity, are observed *in vivo* and currently the presence of senescent cells *in vivo* is accepted. Because senescence is under most conditions a permanent withdrawal of the cell cycle, it is considered a potential mechanism of tumour suppression. Indeed, oncogene expression was found to induce premature senescence in primary cells and senescence bypass is therefore a requirement of tumourigenesis.

Senescence is considered a stress response and various types of cellular stress, e.g. DNA damage and increased ROS can cause senescence onset. Human primary cells lack telomerase expression and progressive telomere shortening due to replication is thought to result in a chronic DNA damage response and induces senescence termed 'replicative senescence'. Mouse primary cells when taken into culture at ambient oxygen, suffer from hyperoxia and consequent oxidative stress triggers senescence onset, as reducing oxygen levels to apparent normoxia (approximately 3% O_2) greatly enhances the number of cell doublings before

senescence onset [83]. Compared to human primary cells, mouse primary cells express high telomerase activity and this may reveal a relative large contribution of ROS to senescence onset in mouse cells. However, also for human primary cells cellular ROS is important in senescence onset [84].

Because of the involvement of FOXO proteins in regulating cellular ROS, a role for FOXO proteins in ROS-mediated senescence can be invoked. In agreement with the role of FOXO proteins in ROS homeostasis, PKBα/β$^{-/-}$ MEFs (active FOXO proteins) are relatively resistant to signalling induced by ROS. Conversely, cells expressing active PKB (inactive FOXO proteins) are relatively sensitive to ROS signalling, including senescence induction [56]. In agreement with ROS contributing to oncogene-induced senescence, Ras-induced senescence was impaired in PKBα/β$^{-/-}$ MEFs [56]. However, it has also been suggested that a Ras-dependent negative feedback loop that represses PI3K-PKB activity [85] is responsible for induction of Ras-induced senescence. We showed that ectopic expression of FOXO4 and ROS-mediated activation of FOXO4 in BRAF^{V600E} melanoma cells induces senescence [86]. Moreover, recent genetic evidence shows that loss of PTEN inactivation strongly cooperates with BRAF^{V600E} to drive melanoma progression [87]. This would suggest that FOXO-inactivation resulting from loss of PTEN at least in melanoma development can mediate a senescence bypass.

Can these contradictory results regarding the involvement of FOXO proteins in senescence induction in the aforementioned studies be reconciled? Most certainly, oncogene induced senescence is only a partly understood phenomenon. At present several pathways by which oncogene expression induces senescence have been described and it is unclear whether these are general or oncogene specific. For example, whereas Ras proteins are upstream regulator of RAF kinases, oncogenic HRas^{G12V} expression in primary melanocytes induces senescence through the endoplasmic reticulum-associated unfolded protein response, whereas oncogenic (B)RAF does not [88]. Furthermore, these differences between Ras and RAF are also reflected in mice models in which BRAF^{V600E} can induce melanoma and hence a senescence bypass [89], whereas HRas^{G12V} and NRas^{Q61K} can induce melanoma only if combined with loss of tumour suppressors such as p16^{INK4a} or p19^{ARF} [90, 91]. Besides oncogene-specific differences in senescence induction, differences between cell types may also play a role. At least in cell culture systems, fibroblast senescence differs from melanocyte senescence (discussed in [92]). Summarising, p16^{INK4a}-deficient mouse or human fibroblasts senesce normally, but mouse or human melanocytes deficient for p16^{INK4a} show an impaired senescence response and extended lifespan (number of passages before they become senescent) [92]. Although clearly oncogene and cell type specific mechanisms of senescence induction are relevant and may explain differential involvement of FOXO proteins in senescence, it remains that senescence in general, including melanocyte senescence [93] frequently correlates with elevated levels of ROS and oncogene induced senescence can also be bypassed by ROS scavenging compounds such as N-acetyl cysteine (NAC) [94, 95]. How increased ROS exactly contributes to senescence is unclear at present but activation of p53 and/or induction of DNA damage are observed after increased cellular ROS and in agreement both can induce senescence.

ROS-regulation of FOXO proteins may also provide an explanation for the differential involvement of FOXO proteins in senescence. Decreased growth factor signalling results in activation of FOXO proteins through loss of inhibition by PKB, whereas increased cellular ROS activates FOXO proteins through a distinct mechanism involving JNK. These separate modes of FOXO-activation do not necessarily result in the same activation of downstream signalling.

Indeed, we observe a ROS-induced FOXO4-dependent increase in $p21^{Cip1/Waf1}$ expression, whereas growth factor deprivation-induced FOXO4 activity resulted in induction of $p27^{Kip1}$ expression. The first is associated with senescence induction, whereas the latter is involved in the induction of quiescence [96]. Thus distinction needs to be made between these two modes of FOXO-activation. In addition, the sensitivity of various signalling molecules such as FOXO proteins and PKB towards ROS enables differential regulation. For example, when comparing FOXO proteins and PKB, FOXO-regulation by ROS, as induced by hydrogen peroxide treatment, occurs already at low concentration (10-25μM H_2O_2) whereas PKB activation requires a relative high concentration (200-500μM H_2O_2) ([97] and unpublished results). Thus, FOXO proteins will respond different to changes in ROS levels and in general be activated by relative small changes in cellular ROS levels and inactivated at high ROS levels. Sensitisation of cells or biological systems will occur by depletion of PKB or FOXO proteins and will shift these systems to increased or decreased sensitivity towards changes in the levels of cellular ROS. Although not investigated in detail, such differences will probably occur in most studies employing genetically modified mice (e.g. [98]). Finally, the different and context-dependent involvement of FOXO proteins in senescence as outlined above is further indicated by more general observations. Senescence is considered a mechanism of tumour suppression and FOXO proteins are genuine tumour suppressors [99], hence senescence induction would be a means to achieve this function. However, and in contrast, cellular senescence contributes to aging [100, 101] and active FOXO proteins increase lifespan so could therefore be expected to reduce senescence. Importantly these considerations may be taken to suggest that the different and context-dependent functions of FOXO proteins to regulate senescence and tumour suppression provide a basis for the idea that FOXO proteins mediate the trade-off between tumour suppression and lifespan.

Interestingly, c-MYC overexpression is reported to suppress $BRAF^{V600E}$-induced senescence in melanoma cells [102] and this is independent of $p16^{INK4a}$ and p53. As mentioned previously, FOXO proteins can induce senescence and this involves $p21^{Cip1/Waf1}$ regulation, but is independent of $p16^{INK4a}$ [86]. Furthermore, c-MYC suppression of Ras-induced senescence requires CDK2 phosphorylation of c-MYC [103]. CDK2 also phosphorylates FOXO1 resulting in cytoplasmic retention of FOXO proteins and hence inhibition of its transcriptional activity [104]. These observations suggest that c-MYC requires FOXO-inhibition to prevent senescence onset in melanoma and are illustrative for the reciprocal relation between FOXO proteins and c-MYC as discussed below.

FOXO proteins clash with c-MYC

The classical experiment demonstrating the necessity for cooperation between oncogenes in driving tumorigenic transformation of primary cells, employed a combination of mutant HRas and overexpression of c-MYC [105]. Initially, we observed a block in cell cycle progression of HRas transformed cells following ectopic expression of FOXO4 and this suggested FOXO proteins to act as tumour suppressors, at least in the context of oncogenic Ras driven tumourigenesis [106]. To drive oncogenic transformation Ras employs several downstream signalling pathways most importantly PI3K, Ral and mitogen-activated protein kinase (MAPK)-signalling (reviewed in [107]). Thus, oncogenic Ras expression results in increased PKB activation and in agreement with Ras and c-MYC as cooperating oncogenes, constitutive PKB activity synergises with c-MYC in the Eμ-MYC mouse lymphoma model [108]. Combining these observations suggested that loss of FOXO-activity could possibly also cooperate with c-MYC

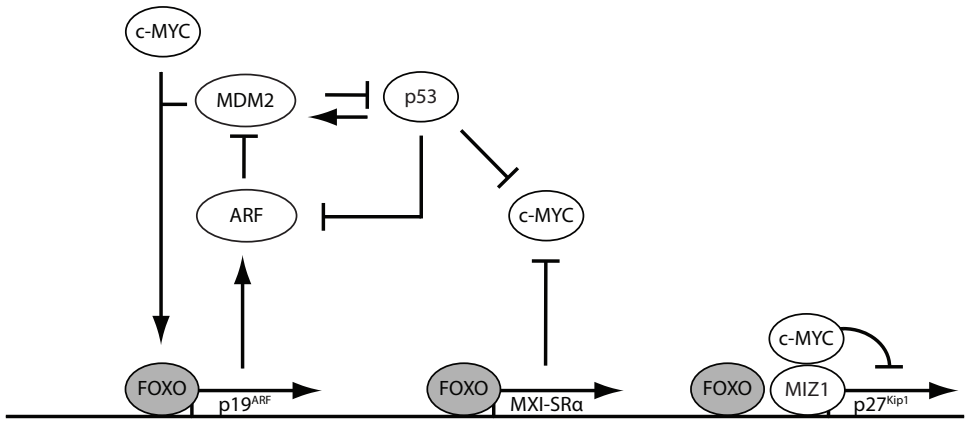

Figure 4: the c-MYC/FOXO clash. Analysis of available literature indicates an extensive network of antagonistic c-MYC/FOXO interactions. First, FOXO proteins and c-MYC regulate shared target genes in an opposing manner and this is illustrated by the $p27^{Kip1}$ gene. Here, MIZ1 binds to specific sequence elements present in the promoter of certain genes repressed by c-MYC, including the $p27^{Kip1}$ gene. MIZ1 binding is required for c-MYC to repress $p27^{Kip1}$ gene expression. In contrast, FOXO proteins bind to the $p27^{Kip1}$ promoter directly and activate $p27^{Kip1}$ gene transcription. Second, FOXO proteins regulate gene expression of a number of negative regulators of c-MYC, most importantly MXI1-SRα. This results in a general attenuation of c-MYC activity. Third, FOXO proteins can regulate $p19^{ARF}$ expression and as such participate in a c-MYC negative feedback loop to increase p53 activity and to repress c-MYC induced apoptosis. Abbreviations: alternative reading frame (ARF) of the human p19 (also known as cyclin dependent kinase inhibitor 2A) gene. MAX-interacting protein 1-string repressor α (MXI1-SRα); c-MYC-interacting zinc-finger protein 1 (MIZ1).

to drive cellular transformation. Indeed, expression of a dominant-negative form of FOXO proteins enables c-MYC to induce foci formation of primary cells in the absence of oncogenic Ras [109]. The mechanism underlying the observed cooperation between c-MYC and loss of FOXO proteins appears to be part of what we like to refer to as a general "clash between FOXO proteins and c-MYC" resulting in a reciprocal inhibition of transcriptional activity between FOXO proteins and c-MYC (figure 4). Repression of c-MYC transcriptional activity occurs through FOXO-dependent induction of expression of several members of the mothers against decapentaplegic (MAD)/c-MYC-associated factor X (MAX)-dimerization protein (MXD) family of transcriptional repressors, most notably the MAX interacting protein 1 (MXI1) splice variant strong repressor α (MXI1-SRα) [110]. Importantly, this results in a partial inhibition of the c-MYC transcriptional program as many but not all c-MYC target genes are repressed by FOXO proteins. Nevertheless, FOXO-repression of c-MYC results in reduced cell cycle entry following c-MYC activation [111]. Importantly, repression of c-MYC in return contributes to the cell cycle arrest imposed by active FOXO proteins as silencing of MXI1 alleviates this [110]. Furthermore, FOXO proteins impose a cell cycle arrest by induction of cell cycle inhibitors such as $p27^{Kip1}$ and $p21^{Cip1/Waf1}$ and repression of cyclin D1 and cyclin D2. Early studies already showed that c-MYC represses FOXO-induced $p27^{Kip1}$ [112]. How c-MYC represses $p27^{Kip1}$ expression is not fully understood but likely involves c-MYC-interacting zinc-finger protein 1 (MIZ1) [113]. c-MYC also represses $p21^{Cip1/Waf1}$ expression and induces cyclin D1, cyclin D2 and cyclin D3 expression [114, 115]. However, as yet, only direct binding of c-MYC to the promoter of cyclin D2 has been reported [116]. Importantly, c-MYC and its repression of $p21^{Cip1/Waf1}$, and cyclin D1 are suggested to be important mediators of canonical Wnt signalling downstream of T-cell factor (TCF)/β-catenin activation [117]. Interestingly, FOXO proteins bind to β-catenin and this partakes in FOXO-mediated repression of TCF signalling (reviewed

in [118]). Although this suggests the FOXO/c-MYC antagonism to be important in tuning Wnt and PI3K/PKB signalling, it is important to note that FOXO proteins only repress TCF/β-catenin under conditions of increased ROS [119, 120]. Also, we observed $p21^{Cip1/Waf1}$, rather than $p27^{Kip1}$ the preferred FOXO target gene under increased ROS [86]. Thus, an interesting suggestion that can be derived from these studies is that antagonistic regulation of $p21^{Cip1/Waf1}$ by c-MYC and FOXO proteins occurs under increased ROS whereas during growth factor PI3K/PKB signalling the clash occurs through $p27^{Kip1}$ and cyclin D expression. The importance of this distinction between conditions of enhanced ROS versus growth factor signalling in the antagonism between c-MYC and FOXO proteins may be illustrated further by the observation that loss of c-MYC impairs Ras-driven tumourigenesis, but the subsequent loss of $p21^{Cip1/Waf1}$ alleviates this [121]. c-MYC activity can induce both proliferation and apoptosis, but normally these two responses cancel each other out. Therefore, it is believed that for efficient tumourigenesis driven by c-MYC, apoptosis-induction needs to be repressed relative to the proliferative response. c-MYC activates the $p19^{ARF}$-MDM2-p53 pathway to suppress tumourigenesis by p53-dependent apoptosis [122] and in agreement with the above spontaneous inactivation of the $p19^{ARF}$-MDM2-p53 pathway occurs at high frequency in Eμ-MYC driven lymphomagenesis [123]. Recently, it was shown that impairing FOXO-activity by expression of a dominant-negative form of FOXO alleviates the need to loose p53 function in Eμ-MYC lymphomagenesis. This was attributed to the ability of FOXO proteins to regulate $p19^{ARF}$ transcription, which was impaired by expression of a dominant-negative FOXO. Thus, under normal circumstances, FOXO proteins counteract c-MYC induced proliferation by regulating the $p19^{ARF}$-MDM2-p53 pathway [111]. Although the mechanism by which c-MYC would increase FOXO-mediated $p19^{ARF}$ transcription has not been elucidated, it is noteworthy that c-MYC is reported to increase cellular ROS [95] and that increased ROS activates FOXO proteins [18]. Dependence on ROS signalling would agree with the observed importance of $p21^{Cip1/Waf1}$ deregulation in tumourigenesis following c-MYC activation [121] as, similar to p53, $p21^{Cip1/Waf1}$ is targeted by FOXO proteins after cellular stress [86]. Loss of $p21^{Cip1/Waf1}$ enhances p53-dependent apoptosis due to the loss of p53-dependent cell cycle arrest [124] suggesting that in tumourigenesis, repression of $p21^{Cip1/Waf1}$ by c-MYC also requires loss of FOXO/p53-dependent signalling as otherwise increased proliferation will be cancelled out by increased apoptosis. Thus, besides inhibition of the apoptosis arm also loss of repression on proliferation is important for c-MYC induced tumourigenesis. The possible importance of this feedback loop and the involvement of FOXO-repression in c-MYC induced tumourigenesis are further illustrated by the observation that concomitant loss of p53 and PTEN within the mouse central nervous system resulted in the onset of malignant glioma resembling human glioblastoma characterised by strong c-MYC activation. Thus, it may be concluded that loss of c-MYC-induced control of FOXO-dependent $p19^{ARF}$ transcription and hence p53 activation is insufficient to induce malignant glioma, and that further inhibition of FOXO-function by PTEN deletion is required. Finally, a recent study showed that in mice models FOXO proteins block progression from benign polycystic kidneys to renal tumours via suppression of c-MYC through upregulation of the c-MYC antagonists MXI1-SRα and miR-145 [125]. This provides further proof for the functional relevance of the c-MYC/FOXO-antagonism in tumourigenesis as well as an additional perspective on the role of FOXO proteins as tumour suppressors.

Stem cells and the c-MYC/FOXO antagonism

Embryonic stem cells are rapidly cycling cells whereas adult stem cells can be divided into

a relative quiescent dormant adult stem cell population and a population of stem cells that have exited quiescence and become rapidly proliferating yet fully competent stem cells (for discussion see [126]). Although this distinction may not fully apply to adult stem cells of all tissues, a number of adult stem cells such as those of the hematopoietic system have been shown to switch between a quiescent and proliferative self-renewing state [127]. Loss of FOXO proteins in haematopoietic stem cells (HSCs) results in uncontrolled switching from quiescence to proliferation, increased proliferation and (premature) exhaustion of the stem cell pool [128]. These observations are in line with observations in PTEN$^{-/-}$ [129] and PKB overexpressing mice [130], strongly suggesting the PKB/FOXO switch to be important in determining stem cell fate and/or stem cell renewal. Accordingly with the above described FOXO/c-MYC antagonism, c-MYC deficiency in the haematopoietic system resulted in an increased number of quiescent HSCs and conversely c-MYC activation resulted in loss of HSCs [131]. However, it has also been suggested that c-MYC is important in the regulation of differentiation of HSCs (reviewed in [132])

Premature stem cell differentiation in FOXO-deficient mice has also been reported for both neuronal stem cells [133, 134] and follicle cells [135]. These results further confirm an important role of FOXO proteins in stem cells, where they may be important to establish the quiescent state. This is in line with our observation that in some cells ligand-independent activation of FOXO proteins results in a reversible cell cycle arrest indicated as quiescence as it was characterised by hallmarks of quiescence i.e. reduced ^{35}S-methione incorporation, p130 Rb2 activation and a consequent shift in activity of the E2F family of transcription factors [136]. Interestingly, others showed that one of the target genes of FOXO proteins involved in ROS scavenging – manganese super oxide dismutase (MnSOD) [137] – is also required for acquiring the quiescent fate [138]. Thus, quiescence appears to depend on, as well as coincide with FOXO-induced changes in ROS control. In agreement, stem cell depletion due to loss of FOXO proteins correlates with an increase in cellular ROS, at least in HSCs and neural stem cells [128, 133].

During ageing, stem cell function is essential for replenishing damaged tissue with new cells and adult stem cell dysfunction is therefore considered a major cause for loss of tissue homeostasis in ageing (reviewed in [6]). However, the mechanism underlying age-dependent decline in stem cell function is still not understood. In general it appears that the mechanisms that restrict cancer cells are also limiting stem cell function, hence the strong functional similarities between cancer cells and stem cells. Therefore, telomere shortening, DNA damage and increased ROS may all contribute to gradually disabling stem cell function. As discussed, the PKB/FOXO switch appears relevant to regulate the reversible transition of stem cells between proliferation and quiescence. However, proliferative capacity per se does not necessarily decline during ageing [6] suggesting that the switch may function equally well at old age. However, the setup of this switch is strongly linked to external cues and surprisingly several transplantation experiments, in which cells derived from young animals are transplanted to aged animals, have shown signs of “rejuvenation” [139]. Apparently, external cues are relevant to stem cell ageing and hence may impinge on the PKB/FOXO switch. In addition, as argued above regulation of FOXO proteins by PKB differs from regulation of FOXO proteins by ROS, especially in the context of shifting from quiescence to senescence. Thus, increased ROS and ROS-induced damage accumulating during ageing may force FOXO proteins to tilt towards senescence rather than quiescence and thereby reduce stem cell fitness, without an apparent reduction of stem cell numbers. In agreement

with an important role for senescence mechanisms in the functional decline of stem cells are observations that important regulators of senescence e.g. $p16^{INK4a}$ and $p21^{Cip1/Waf1}$ affect stem cell function. Expression of $p16^{INK4a}$ increases during ageing and mice overexpressing $p16^{INK4a}$ show $p16^{INK4a}$ not only to be a marker of ageing, but also to contribute to age-induced regenerative failure of tissues [140-143]. Likewise, $p16^{INK4a}$-deficiency partly rescued age-induced decline in stem cell function. In mice with dysfunctional telomeres loss of $p21^{Cip1/Waf1}$ partially extends longevity of these mice and attenuates the proliferative defects, importantly without increased tumourigenesis [144]. Thus, current knowledge on extrinsic as well as intrinsic mechanisms of stem cell dysfunction during ageing suggests senescence as an important mechanism to limit stem cell function.

Stem cell dysfunction could also be a major contributor to cancer. Cancer initiating cells, or cancer stem cells, are believed to represent the resilient population of cancer cells that 'regenerate' tumours following stress insults, including chemotherapy. At present the cancer initiating cells are mostly studied based on their ability to cause tumour formation in a recipient mice following transplantation. Interestingly, FOXO3-deficiency disables cancer-initiating cells in chronic myeloid leukaemia to cause tumour formation [145]. As FOXO-deficiency also impairs the ability of normal HSCs to repopulate the bone marrow of recipient mice in serial transplantation experiments, this indicates a similar defect to underlie the role of FOXO proteins both in normal as well as in cancer stem cells. As argued the PKB/FOXO switch is primarily directed to regulate transition between quiescence and proliferation. Following this line of reasoning cancer initiating cells should express markers of quiescence. Indeed, a subpopulation of melanoma cells are slow cycling and they can maintain tumour growth, suggesting quiescence to be involved [146]. These observations suggest that an important role for FOXO proteins is maintaining viability of cancer initiating cells similar to that of normal stem cells. How this can be reconciled with FOXO proteins function as tumour suppressor is an important question that needs to be addressed.

Future perspectives

PI3K signalling remains one of the most intensely studied fields in biology and here we have provided only a snap-shot of a few of the many aspects of PI3K signalling, the PKB/FOXO switch. Initially, many studies focussed on this switch from the perspective of a linear PI3K-PKB pathway dedicated to switch off FOXO-function. However, it is becoming clear that the PKB/FOXO switch is also regulated by cellular ROS levels in a manner that may oppose PI3K/PKB. Understanding how ROS-regulation tunes the PKB/FOXO switch is complicated by the observation that FOXO proteins themselves also regulate cellular ROS levels. Therefore, the initial level and likely also the site of ROS formation becomes relevant for the outcome. As ROS is also considered a driving force of ageing and the PKB/FOXO switch is important to the ageing process, understanding the details of FOXO-regulation by ROS in the context of PI3K signalling will become a task for the future. One possible role in ageing for the opposing actions of the ROS/JNK pathway versus the insulin/PKB pathway, is to shift FOXO proteins from driving quiescence to driving senescence, especially as the demand for tumour suppression increases with age (see also figure 1). An important notion for comparing FOXO- and c-MYC-function is that FOXO proteins and c-MYC functionally and mechanistically antagonise each other at various levels. In disease, especially cancer, recent results clearly show this antagonism to be functionally relevant, but with respect to ageing the consequence or relevance of this antagonism remains to be investigated.

Thesis outline

The cells that make up our body need to behave in a coordinated manner to harness their unity against inevitable fluctuations in their environment. Besides cell specialisation, multicellular organisms have adopted strategies for individual cells to communicate with one another to achieve such coordination. These strategies come in the form of extracellular signalling molecules and intracellular signalling cascades that translate the various messages into cellular behaviour. Signal transduction is the study of which intracellular events are triggered by both intra- and extracellular signals and how these events result in altered cellular behaviour. We focus in this thesis on the regulation of Forkhead box-O (FOXO) transcription factors, which were originally identified as important downstream components of the growth factor-phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB; also known as Akt) signalling pathway. More importantly, nearly two decades of FOXO research have shown that FOXO proteins are important hubs that integrate numerous cues, including environmental nutrient status and intracellular redox status, and translate these into an equally large plethora of changes in cellular behaviour. Although recent advances in signal transduction research have underscored the importance and complexity of feedback loops, cross-talk and spatio-temporal regulation of signal transduction cascades, it remains unclear when and how the diverse roles of FOXO proteins are encoded in the topology of the signalling network. We have made a small step towards developing an integrative model of FOXO proteins and present our findings in this thesis.

In Chapter 1, we propose that FOXO proteins have important roles in maintaining homeostasis and present a functional model and rationale for this. The homeostatic function of FOXO proteins has become a focus of our research (see for example also [1]) and we characterised homeostatic functions of FOXO proteins in various cellular processes such as DNA damage [Charitou et al., under revision]. In Chapter 2 we have undertaken steps to understand the dynamic behaviour of PKB-FOXO signalling and uncovered a simple and robust mechanism whereby both PKB and FOXO proteins maintain steady state activity of one another. Furthermore, in Chapter 3 we have explored gene regulation of FOXO proteins in order to elucidate a common signature that can explain the organism wide slowing of aging attributed to FOXO-activity. We define a small set of genes that are regulated by FOXO3 or FOXO4 in both human and mouse cell lines and have extensively studied the function of one of these genes, CTDSP2, in Chapter 3 and Chapter 4. Reminiscent of FOXO proteins, ectopic expression of CTDSP2 regulates cell cycle progression through $p21^{Cip1/Waf1}$. Our results indicate that this is the result of Ras activation, pointing to previously unidentified cross-talk between FOXO proteins and Ras signalling. In Chapter 5 we have explored FOXO3 regulation of microRNAs, as FOXO proteins can regulate expression of these non-protein coding transcripts [2], which themselves regulate expression of protein expression post-transcriptionally. We have identified 11 miRNAs that are potentially regulated by FOXO3 and have more extensively studied the targets and functions of miR-26a, which is co-transcribed with CTDSP2. We identified a set of miR-26a-regulated proteins and validated their regulation. We have yet to clarify the exact role of miR-26a regulation in the context of FOXO-activity, but we suspect a potential contribution to the negative feedback regulation of FOXO proteins. In Chapter 6 we discuss opportunities and pitfalls of FOXO research in light of our data and speculate on how these data fit a general role of FOXO proteins in organismal homeostasis.

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CHAPTER 2

Reciprocal regulation of PKB and FOXO proteins through feed-back signalling

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Abstract

Protein kinase B (PKB)/Akt and Forkhead box-O (FOXO) transcription factors play important roles in cell cycle regulation, cell growth and apoptosis. PKB promotes cell cycle progression and cell growth, while it inhibits apoptosis, whereas FOXO proteins have opposite effects on these processes. Interestingly, PKB and FOXO proteins display reciprocal regulation: PKB inhibits FOXO proteins by direct phosphorylation whereas FOXO-activation through gene regulation results in increased phosphorylation of PKB, thereby also negatively regulating its own activity. These observations suggest that FOXO proteins induce a feedback mechanism to limit the duration for which they are active. In agreement with limited duration of FOXO-activity, the first authors to describe PKB regulation of FOXO3 noted that, in the presence of growth factors and active PI3K-PKB signalling, FOXO3 is predominantly cytoplasmic and presumably inactive. A number of FOXO target genes have been reported, which individually or combined can explain FOXO-induced activation of PKB. However, their role in limiting duration of FOXO-activity has not been studied.

Here, we have elaborated on the FOXO3-mediated regulation of PKB-activity and its impact on the dynamics of FOXO3 activity. We show that elevated FOXO3 expression results in elevated PKB phosphorylation, but that FOXO3 remains partially active, suggesting that PKB and FOXO-activity have reached a new equilibrium. We show that a large number of genes regulated in response FOXO3-activation can participate in regulation of PKB activity and this is conserved in multiple human cell lines of different origin. Moreover, elevated expression of these genes persists during prolonged expression of FOXO3, indicating that they are involved in maintaining the equilibrium of PKB and FOXO-activity. Lastly, we show that reduction of FOXO1 and FOXO3 results in reduced PKB phosphorylation, suggesting that endogenous FOXO proteins participate in regulation of PKB-activity. Based on this, we propose that, in contrast to previous reports suggesting PKB regulation of FOXO proteins to represent an on-off switch in controlling cellular processes, the reciprocal regulation of FOXO3 and PKB activity is an active, continues process aiming to maintain steady state PKB and FOXO-activity.

Introduction

The serine/threonine kinase protein kinase B (PKB), also known as Akt, plays a vital role in eukaryotic cells (reviewed in [1]). The three isoforms PKBα, PKBβ and PKBγ are members of the PKA/PKC/PKG (AGC) family of kinases (reviewed in [2]) and their activation involves phosphatidylinositol signalling [3]. More precisely, PKB-activation requires phosphatidylinositol-4,5-biphosphate ($PI(4,5)P_2$) or PI(4)P to be phosphorylated at the 3' location to generate $PI(3,4,5)P_3$ and $PI(3,4)P_2$, which in mammalian cells is carried out by members of the phosphatidylinositol 3-kinase (PI3K) family. PI3K lipid kinases are divided in three classes of which only class I lipid kinases have been shown to be relevant for PKB-activation to date. Class I PI3Ks are further divided in class IA and class IB. Class IA contains three catalytic and five regulatory subunits, while class IB contains only has one catalytic and two regulatory subunits, which are activated by G-coupled protein receptors (GPCRs) (reviewed in [4]). In contract, class IA catalytic/regulatory heterodimers can be activated by a large number of receptor tyrosine kinases (RTKs), including growth factor receptors for insulin, insulin-like growth factor (IGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), nerve growth factor (NGF) and platelet-derived growth factor (PDGF), soluble tyrosine kinases Scr, focal adhesion kinase (FAK) and members of the Janus kinase (JAK) family (reviewed in [5]), GTPases such as Ras, and GPCRs (reviewed in [4]). Of note, the major insulin receptor substrates are adaptor proteins of the insulin receptor substrate (IRS) family, which can mediate activation of PI3K [6]. The activity of PI3K family members is counteracted by lipid phosphatases, the most relevant for PI3K-PKB signalling being phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which is one of the most frequently mutated tumour suppressors [7]. Upon activation of PI3K, both PKB and its activator 3-phosphoinositide-dependent protein kinase 1 (PDPK1) are recruited to $PI(3,4,5)P_3$ or $PI(3,4)P_2$ containing membranes through their plekstrin homology domains. The constitutively active and general regulator of AGC kinases PDPK1 phosphorylates PKB in the activation segment or T-loop (Thr308 in PKBα), which results in partial activation [2]. Full activation requires PKB phosphorylation by mammalian target of rapamycin complex 2 (mTORC2) in the hydrophobic motif region (Ser473 in PKBα) [2], which also changes its substrate specificity [1]. Regulation of mTORC2 activity is poorly understood to date, but is increased in response to growth factors (reviewed in [8]). Lastly, PKB is constitutively phosphorylated in its turn motif (Thr450 in PKBα), which depends on mTORC2 activity and is important for the stability and activity of PKB [2]. Several proteins directly interacting with PKB can regulate its activity, including protein phosphatase 2A (PP2A) and PH domain leucinerich repeat phosphatase (PHLPP), heat shock protein 90 (HSP90) and carboxyl-terminal modulator protein (CTMP) (reviewed in [9]). PKB has been shown to directly phosphorylate a large number of proteins. For many of these described potential substrates it is unclear whether or not PKB-mediated phosphorylation is biologically relevant, but for a limited number of substrates there is independent, genetic evidence to substantiate a role for these substrates in PI3K-PKB signalling. Important PKB substrates include B-cell lymphoma 2 (Bcl-2)-associated death promoter (BAD), glycogen synthase kinase 3 (GSK3), tuberous sclerosis 2 (TSC2) and members of the Forkhead box-O (FOXO) transcription factor family FOXO1, FOXO3, FOXO4 and FOXO6. BAD, GSK3, TSC2 and FOXO-phosphorylation by PKB results in the inactivation of these proteins, which strikingly appears to be the case for most described PKB targets (reviewed in [10]). BAD is a pro-apoptotic BH-3 only protein that binds to and inhibits anti-apoptotic proteins, such as Bcl-2. GSK3 has been shown to regulate glycogen synthesis and GSK3-mediated phosphorylation decreases

the stability of c-MYC and cyclin D proteins. The TSC1/2 complex is a negative regulator of the mammalian target of rapamycin complex 1 (mTORC1) activating protein Rheb (reviewed in [1]). FOXO proteins are transcription factors that control gene expression of numerous genes and their activity is generally considered to oppose cell cycle progression, reactive oxygen species formation and promote apoptosis (reviewed in [11]). Thus, by its combined action towards downstream targets, PKB promotes cell cycle progression and cell growth whereas it inhibits apoptosis. Not surprisingly, it has become apparent that PI3K-PKB signalling plays a central role in cancer (reviewed in [12]) but also diabetes (reviewed in [13]). Interestingly, while many tumours display hyperactivation of PI3K-PKB signalling, diabetes is associated with reduced PI3K-PKB activity either through desensitisation or lack of stimulation by insulin [13]. Thus, precise and balanced regulation of PKB activity is of critical importance for cellular and organismal function.

Feedback mechanisms are a common feature of biological systems and are crucial for dynamic control of biological processes. PKB signalling is no exception to this and two important feedback mechanisms have been described to date. The first example of feedback to be described was the negative effect of mTORC1 and its direct target S6 kinase (S6K) on IRS1/2 stability (reviewed in [14]). More dispersed over the last decade, several papers have reported FOXO target genes that potentially result in increased PKB-activation, including IRS2 [15], the insulin receptor [16, 17], phosphoinositide 3-kinase, catalytic subunit α (PI3KCA) ([18], mTORC2 component rapamycin-insensitive companion of mTOR (RICTOR) [19, 20], human EGF receptor 3 (HER3)/v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 3 (ERBB3) [17, 21, 22] and IGF receptor 1 (IGFR1) [17, 23]. Indeed, PKB-activation in response to overexpression of FOXO family members has been documented previously [18, 19, 24]. Recently this has received extra attention because mTORC1-FOXO feedback regulation of PKB has major implications for targeting PI3K-mTORC signalling in cancer (reviewed in [25]). In brief, while inhibition of PI3K or mTORC1/2 by small molecule inhibitors initially results in PKB-inactivation, it was shown that PKB is rapidly re-activated, although our own unpublished observations suggest that this ability to reactivate PKB strongly depends on the dose of the drug. Both feedback signalling cascades have been shown to be important for this: relief of the negative regulation of PI3K by mTORC1-S6K [26] and FOXO-dependent regulation of several RTKs, including ERBB3 and the insulin receptor [17, 21, 22] or RICTOR [20].

Prior work has shown that FOXO proteins can inhibit c-MYC a downstream target of PKB-GSK3, through increasing c-MYC-associated factor X (MAX)-interacting protein 1 (MXI1) splice variant strong repressor α (MXI1-SRα) [Chapter 1], and mTORC1 through regulation of TSC1, Sestrin 3 and RICTOR (reviewed in [27]). At the same time FOXO proteins increase the expression of several pro-apoptotic genes, including Fas ligand (FASL), Bcl-2-interacting mediator (BIM)/BCL2-like 11 (BCL2L11) and B-cell CLL/lymphoma 6 (BCL6) (reviewed in [11]). Together, these studies reveal an intimate relationship between PKB and FOXO proteins, wherein they exactly oppose each other's functions, with the exception that FOXO proteins increase PKB-activity (reviewed in [Chapter 1]). Furthermore, it has been suggested that FOXO-activity is brief due to induction of negative feedback through regulation of PKB-activity, unless FOXO proteins are activated by e.g. oxidative stress [27]. However, most studies of FOXO proteins use constitutively active mutants of FOXO proteins in which the three PKB phosphorylation sites (Thr32, Ser253 and Ser315 in FOXO3) are mutated to alanine [28]. These mutants are referred to as the TM or A3 mutants, the latter annotation we will use in this manuscript. The use of constitutively active FOXO mutants has allowed analysis of

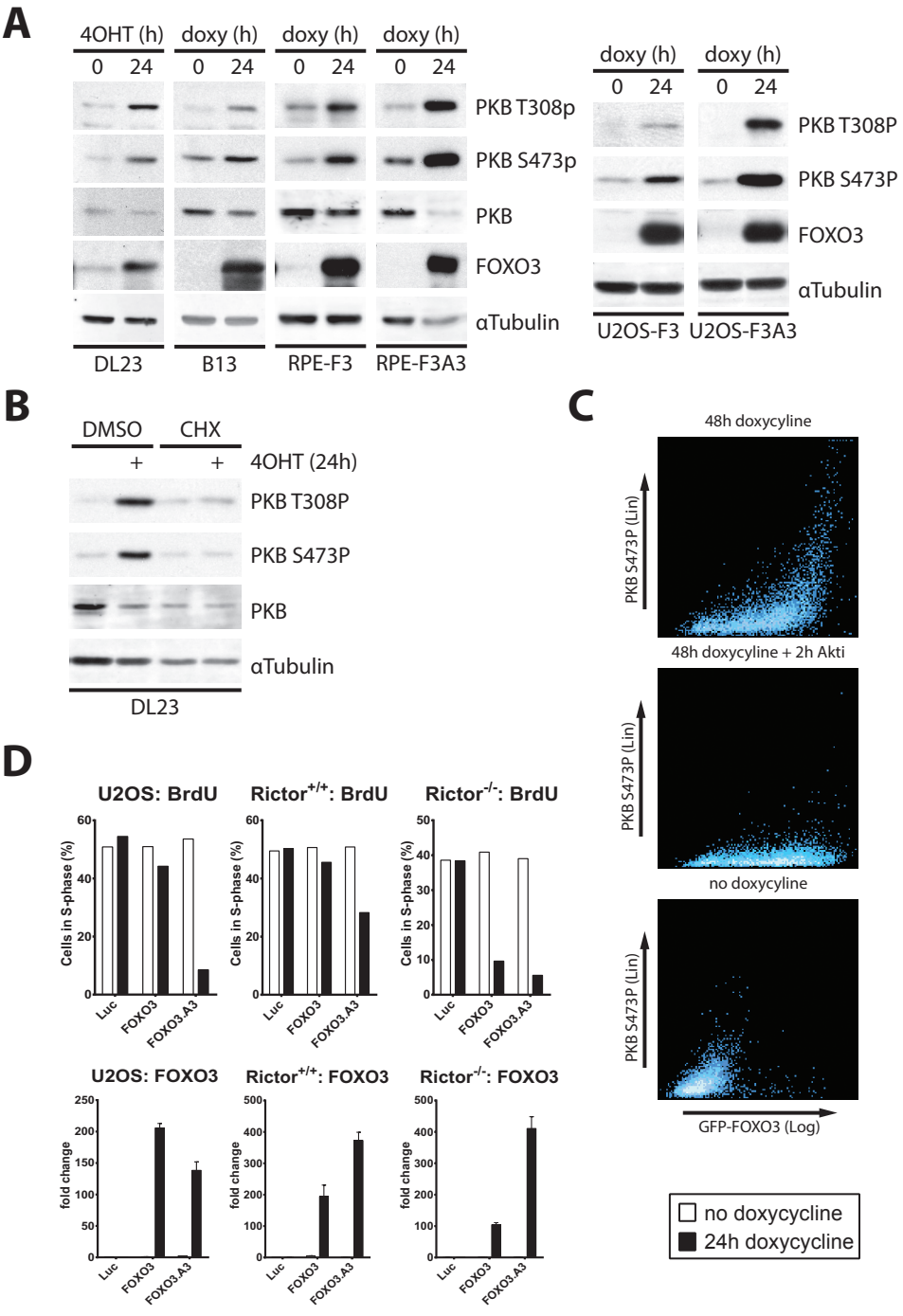
the effects of FOXO proteins in isolation, albeit usually in the context of sufficient nutrients and growth factors, which may affect results. Importantly, the dynamics of FOXO-activation and inactivation in unstressed conditions cannot be studied using the A3 mutant, because it does not permit PKB-mediated negative feedback.

Here we aimed to elaborate on the mechanisms that mediate FOXO3-induced PKB-activation and its impact on the activity of FOXO3 itself. In agreement with previously proposed feedback-mediated inactivation of FOXO3, we find that the effects of FOXO3 expression are generally mild and that, upon prolonged expression of FOXO3, expression of $p27^{Kip1}$ and CTDSP2 is only affected briefly. However, elevated expression of FOXO3 continues to affect cell cycle progression, expression of SOD2 and GADD45B, as well as PKB-activation, suggesting that a new balance between PKB and FOXO3 activity has been established, which may involve FOXO-directed gene regulation. We show that FOXO3 activates a large number of potential PKB regulating proteins, predominantly signal transducers that require ligand-mediated activation but also ligands that would enhance activation of these transducers. Interestingly, both focal adhesion (FA) and JAK-STAT signalling may play a larger role in this than previously appreciated. Most of the tested targets are regulated in response to expression of constitutively active or wild-type FOXO3 in human cell lines of different origin, suggesting that these are part of a common set of FOXO3 target genes, at least in humans. Of note, discrepancies seem to reflect the promoter status in the particular cell type, rather than the ability of FOXO3 to promote transcription of these genes. Increased expression of part of these genes persists during prolonged expression of FOXO3, indicating that they participate in balancing PKB and FOXO3-activity. Finally, we show that endogenous FOXO1 and FOXO3 are required for maintaining PKB-activity and proliferation/survival of cells. Also FOXO1 and FOXO3 are shown to link to cell size control, although we have yet to clarify the mechanisms underlying this process. In summary, we propose that reciprocal regulation of FOXO3 and PKB-activity is an active, continues process aiming to maintain steady state PKB and FOXO-activity, thereby dampening the effects of fluctuations in growth factor levels, as well as shortening the time of strong FOXO3-activation.

Results

Elevated FOXO3-activity increases PKB phosphorylation

Several publications have reported FOXO target genes that are involved in activation of PKB (reviewed in [Chapter 1]). Indeed ectopic expression of FOXO3/FOXO3.A3 in cardiomyocytes [24], expression of FOXO3.A3 in K562 cells [18] or expression of FOXO1.A3 in several cell lines [19] results in increased PKB phosphorylation. Furthermore, ectopic expression of wild-type FOXO3 has little or no effect on eye size unless PKB-activity is blocked [39]. Thus, it has been proposed that FOXO-activation can only be brief, unless FOXO proteins are activated by e.g. oxidative stress [27]. We wanted to characterise these dynamics and determine how they affect transcriptional output of FOXO proteins, also with respect to FOXO-induced changes in for example cell cycle progression. First, we determined if ectopic expression of wild-type FOXO3 or FOXO3.A3 results in increased phosphorylation of PKB in our experimental setup. To this end, we established several cell lines with doxycycline inducible wild-type FOXO3 or constitutively active mutant FOXO3.A3 for comparison. For reference, we also included DL23 cells, which are derived from colon carcinoma cell line DLD1 and express FOXO3.A3 fused to the ligand binding domain of the estrogen receptor [30] that allows activation of FOXO3.A3 by 4OHT. Similar to activation of FOXO3.A3 in DL23 cells, induced expression of FOXO3.A3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PKB S473P (Lin)
no doxycyline
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Rictor+/+: BrdU
Rictor-/-: BrdU
Cells in S-phase (%)
Luc
FOXO3
FOXO3.A3
U2OS: FOXO3
Rictor+/+: FOXO3
Rictor-/-: FOXO3
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← Figure 1: expression of FOXO3 increases PKB phosphorylation and PKB is required for the reduced effects of wild-type FOXO3
A) DLD1 derived DL23 cells expressing FOXO3.A3-ER, U2OS derived B13 cells expressing tet-inducible GFP-FOXO3, RPE and U2OS cells expressing tet-inducible FOXO3 or FOXO3.A3 were treated with EtOH/4OHT or doxycycline for 24 hours to activate FOXO3.A3-ER or induce expression of FOXO3 or FOXO3.A3, respectively. All cell lines show increased PKB phosphorylation at Thr308 and Ser473 upon activation/induction of FOXO3. FOXO3 induced increase in PKB phosphorylation is mild compared to FOXO3.A3 induced increase in PKB phosphorylation. B) DL23 cells were treated for 24 hours with EtOH or 4OHT together with DMSO or cycloheximide (CHX). FOXO3.A3-ER-activation induced PKB phosphorylation is inhibited by CHX. C) B13 cells were left untreated or treated with doxycycline for 48 hours, the last two in the presence of DMSO or Akti, fixed and stained with phospho-PKB Ser473 antibody. Phospho-PKB Ser473 antibody staining increases with GFP-FOXO3 signal and this can be inhibited by Akti treatment. D) U2OS, RICTOR$^{+/+}$ or RICTOR$^{-/-}$ cells expressing tet-inducible Luciferase (Luc), FOXO3 or FOXO3.A3 were treated with doxycycline for 24 hours. Neither U2OS nor RICTOR$^{+/+}$ cells expressing FOXO3 show strong reduction in BrdU-positive cells, in contrast to RICTOR$^{-/-}$ cells, indicating that inactivation of overexpressed FOXO3 requires PKB activity towards FOXO3 (see main text for references).
Data presented are representative experiment with technical S.D. T-tests: * $p < 0.05$, ** $p < 0.005$; indication absent or n.s. means $p > 0.05$.

or FOXO3 in U2OS or RPE cells results in elevated PKB phosphorylation at both Thr308 and Ser473 (figure 1A). In agreement with PKB-mediated inhibition, wild-type FOXO3 induced PKB phosphorylation to a lesser extent compared to FOXO3.A3 with similar protein expression level (figure 1A). Furthermore, the FOXO3 induced increase in PKB phosphorylation depends on protein synthesis, as activation of FOXO3.A3 in DL23 cells does not result in elevated PKB phosphorylation in the presence of translation inhibitor cycloheximide (figure 1B). Lastly, at the level of individual cells, PKB Ser473 phosphorylation correlates with FOXO3 expression level, being highest in cells with most FOXO3 (figure 1C). FOXO3.A3 expression has been shown to arrest cells through regulation of p27^{Kip1}, p130 Rb2 and cyclin D proteins (reviewed in [11]). In line with the moderate effects of wild-type FOXO3 on PKB phosphorylation, cell cycle progression of U2OS or wild-type mouse embryonic fibroblasts (MEFs; RICTOR$^{+/+}$ in figure 1D) is only mildly affected by FOXO3 overexpression, in contrast to FOXO3.A3 expression (figure 1D). However, MEFs deficient for mTORC2 due to loss of RICTOR, which are strongly impaired in FOXO phosphorylation by PKB [29], respond strongly to expression of wild-type FOXO3 expression (figure 1D), further suggesting that PKB-mediated feedback signalling compensates for increased FOXO3 levels.

PKB-activation results in FOXO3-inactivation

The effects of wild-type FOXO3 expression are much less pronounced than those of FOXO3.A3, but still visible. To establish the time frame required for complete inactivation of overexpressed wild-type FOXO3, we extended the duration of expression of FOXO3 or FOXO3.A3 for comparison. We again measured cell cycle progression and also expression of well-known target genes p27^{Kip1} [40], SOD2 [30], GADD45 [41] and CTDSP2 [Chapter 3]. Interestingly, FOXO3.A3 expression is not tolerated well by U2OS cells and decreases rapidly from 48 hours of induction onwards (figure 2A). This is reflected in decreased expression of exogenous FOXO3 mRNA (figure 2A – graphs), although we have not excluded additional mechanisms such as FOXO3-mediated increased transcription of enzymes regulating FOXO3 stability. Regardless, wild-type FOXO3 expression is not diminished and this allows us to study the long-term effects of elevated FOXO3 expression. In agreement with PKB-mediated FOXO3-inactivation, prolonged expression of wild-type FOXO3 has little effect on cell cycle progression; after an initial dip in S-phase entry, cells resume division, albeit at slower rate than without FOXO3 expression (figure 2B). This is consistent with upregulation of p27^{Kip1}, of which the expression levels return to basal (without FOXO3 expression) expression after 72 hours of FOXO3 expression (figure 2C). Expression of SOD2, CTDSP2 and GADD45B are

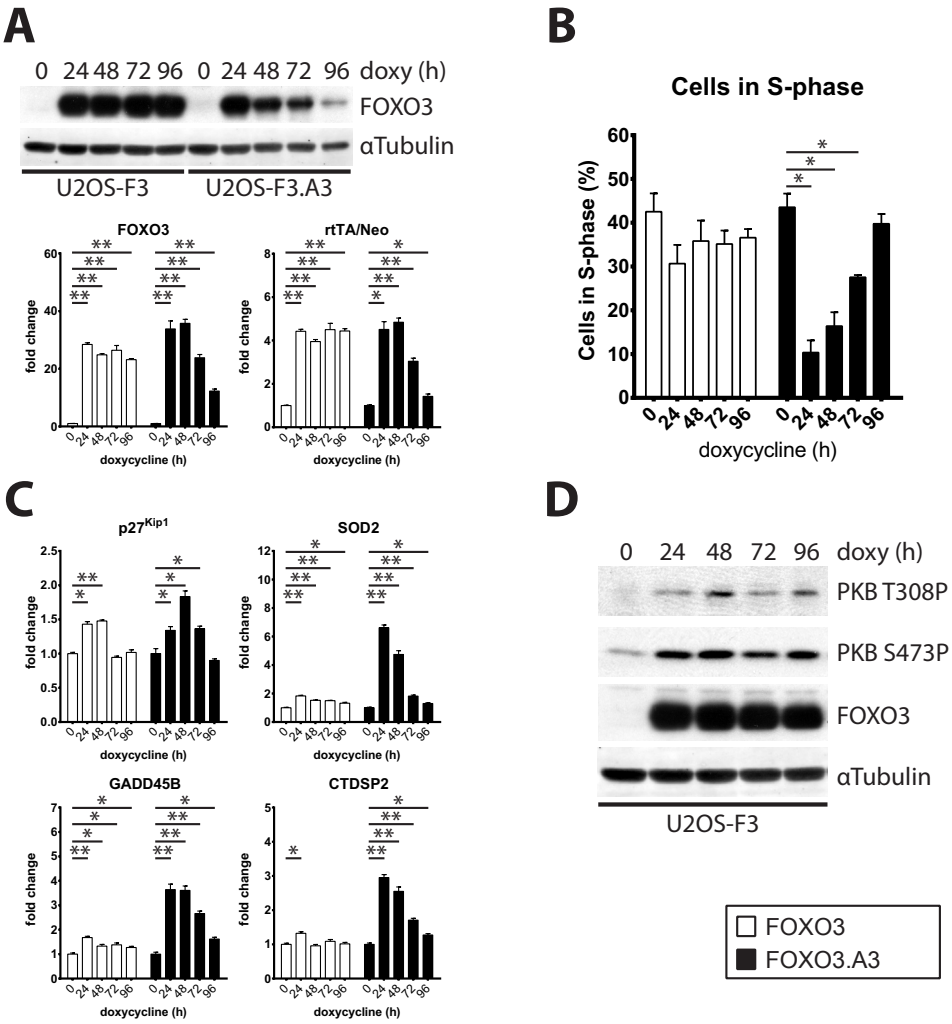

Figure 2: expression of wild-type FOXO3 has mild but sustained effects on cell cycle progression and target gene expression

A) U2OS expressing FOXO3 or FOXO3.A3 were treated with doxycycline for indicated time. U2OS cells tolerate prolonged expression of wild-type FOXO3 but not constitutively active FOXO3.A3, which involves decreased expression of the exogenous construct elements. Left graph shows (endogenous and) induced FOXO3 expression. Right graph shows expression of constitutive tet-activator and resistance gene, which are also induced due to lack of terminator sequence in between FOXO3 CDS and rtTA/Neo. B) Prolonged expression of FOXO3 in U2OS cells has mild effects on cell cycle progression, compared to FOXO3.A3. C) FOXO3 expression results in smaller induction of target genes $p27^{Kip1}$, SOD2, CTDSP2 and GADD45B, compared to FOXO3.A3. However, in contrast to $p27^{Kip1}$ and CTDSP2, GADD45B and SOD2 remain higher at later time points, than without FOXO3 expression. D) FOXO3 induced increase in PKB phosphorylation is sustained during prolonged expression of FOXO3.

Graphical data presented are representative experiment with technical S.D. T-tests: * $p < 0.05$, ** $p < 0.005$; indication absent or n.s. means $p > 0.05$.

increased after 24 hours of FOXO3 expression and declined thereafter (figure 2C). However, expression of SOD2 and GADD45B, but not CTDSP2, remains higher than without FOXO3 expression (figure 2B and 2C). In line with above described effects on PKB phosphorylation, the effect of elevated wild-type FOXO3 expression on expression of these genes is very mild

compared to those observed in response to expression of constitutively active FOXO3.A3, at least for the first 48 hours (figure 2B and 2C). These striking difference in effect between the wild-type and A3 mutant suggests that feedback regulated activation of PKB is rapid and strong enough to compensate almost completely for the increase in FOXO3-activity. However, the small but maintained effect of FOXO3 expression on cell cycle progression, SOD2 and GADD45B expression suggests that FOXO3 is still partially active, although this is not reflected by the expression of $p27^{Kip1}$ and CTDSP2. Indeed, FOXO3 expression induced PKB phosphorylation is not dramatically changed after prolonged expression, suggesting that a new balance between PKB and FOXO-activity has been established, which may involve FOXO3-dependent gene regulation or at least requires synthesis of new proteins (figure 1B).

PKB-activation involves multiple pathways

We wondered which genes are important for the maintained increase in PKB phosphorylation. A number of genes involved in PKB-activity has been reported to be regulated by FOXO proteins, both in mammalian systems and *Drosophila melanogaster* (reviewed in [Chapter 1]). However, none of these genes are in our recently published short list of genes that are consistently regulated by FOXO3 and FOXO4 in human and mouse cell lines [Chapter 3]. This may be the result of cell type specific usage of different FOXO3 target genes that potentially regulate the activity of PKB, thus we wanted to determine the possible candidates and explore possible differential usage in DL23, RPE and U2OS cells. As a starting point, we used DL23 cells for which we have previously described ChIP-seq data of both exogenous and endogenous FOXO3 [42] and expression data of gene changes after 8 and 24 hour FOXO3. A3-activation [Chapter 3]. In addition, we collected proteome data for these cells after 24 hour activation of FOXO3.A3, which covered approximately 35% of all proteins expressed in these cells. We combined these datasets and analysed expression changes of genes that are directly or indirectly upstream of PKB according to the Kyoto encyclopaedia of genes and genomes (KEGG) [43]. Clusters that contain at least one gene linked to PKB include the PI3K-AKT signalling pathway (PKB), the B-cell receptor signalling pathway (B cell), the chemokine signalling pathway (Chemokine), the ErbB signalling pathway (ErbB), the focal adhesion signalling pathway (FA), the insulin signalling pathway (Insulin), the JAK-STAT signalling pathway (JAK-STAT), the Toll-like receptor signalling pathway (TLR) and the mTOR signalling pathway (TOR). Different clusters often contain the same genes, which complicates the analysis. Therefore, we assigned each gene to a single cluster. The B cell, Chemokine, ErbB, FA, Insulin, JAK-STAT and TLR signalling clusters now include only pathway specific ligands, receptors and adaptor proteins as well as some signalling mediators that are not included in any of the other clusters. The TOR cluster includes all components, regulators and relevant effectors of the two mTOR complexes. Lastly, the PKB cluster includes several growth factors, their receptors, PI3K signalling components and genes that directly influence PKB-activity. In the PKB cluster, we have removed the reference to TORC2, which in KEGG refers to cAMP response element-binding protein (CREB)-regulated transcription co-activator 2 (CRTC2), assuming that it was meant to be mTOR complex 2 (which is included in the TOR cluster).
Collectively, these clusters contain a total of 434 genes in our dataset, of which 64 are statistically significantly (ANOVA; $p < 0.05$) changed after 24 hour FOXO3.A3-activation in DL23 cells. We have graphically summarised these genes, depicting the number of genes per cluster and the number of in- or decreased genes along the arrows or flanking the total number of genes, in the case of the PKB cluster (figure 3A). Quantitatively, most expression

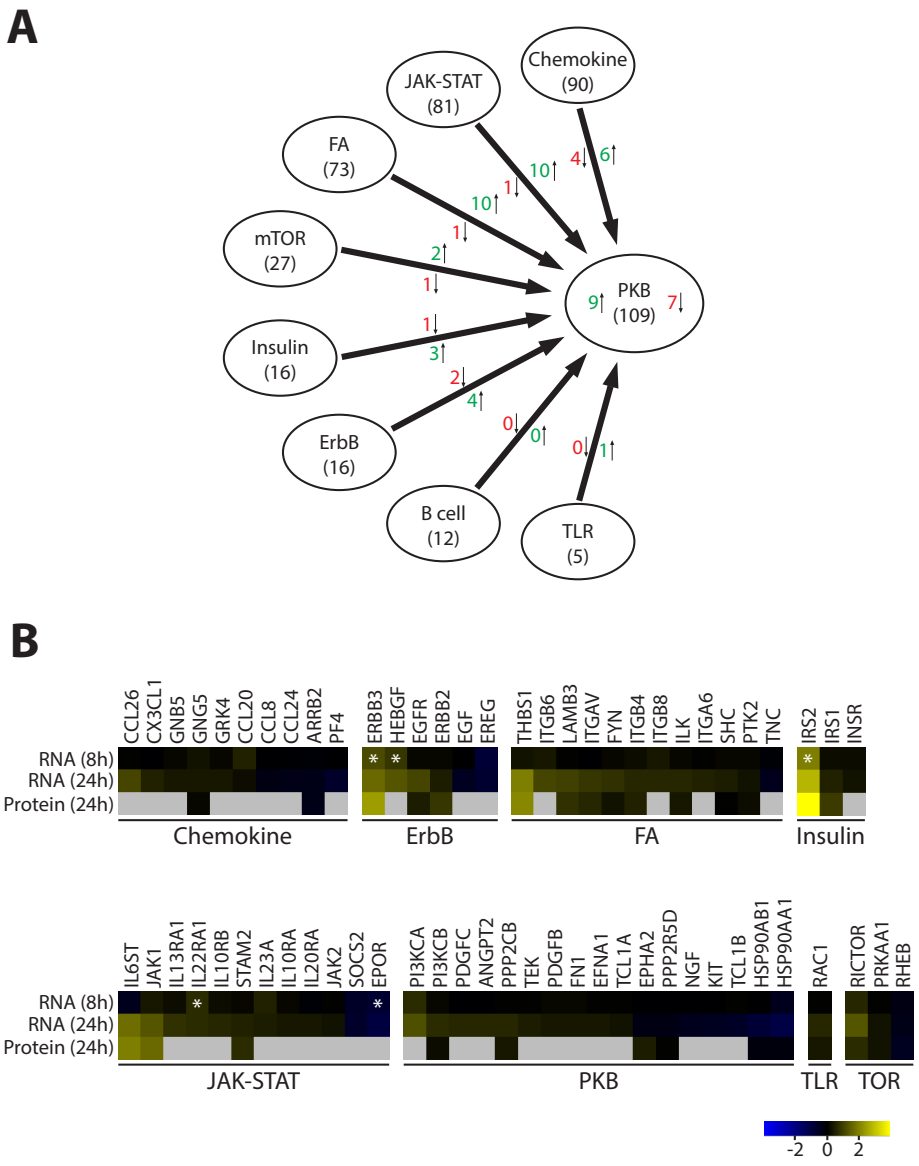

Figure 3: FOXO3 activates many genes that potentially regulate PKB activity
A) Graphical overview of statistically significantly (ANOVA; $p < 0.05$) regulated genes in DL23 cells treated with 4OHT for 24 hours, that directly or indirectly regulate PKB activity according to KEGG (see main text for details). Number of genes in each cluster are in black within the circles. Clusters affecting PI3K-PKB signalling are placed around the central PKB cluster. Regulated genes per cluster are in green (up) or red (down), either along arrows or inside the PKB-cluster. FA: focal adhesion cluster B) Heatmap of changed genes per cluster, ordered by cluster and fold change and separated by cluster for convenience. First row from top contains mRNA expression data of DL23 cells treated with 4OHT for 8 hours, second and third row respectively contain mRNA and protein expression data of DL23 cells treated with 4OHT for 24 hours. Asterisks in first row denote that this gene is statistically significantly (ANOVA; $p < 0.05$) regulated. Grey squares in third row denote that there is no protein data for this gene.

changes are moderate or low (fold change < 1.5 up/down for 42 genes; figure 3B), although interpretation of visual data is complicated by the strong regulation of IRS2 (7.8 fold up). The extensive number of changed genes that potentially regulate PKB-activity indicates that FOXO3 induced gene expression changes can affect PKB-activity in a wide range of contexts. Furthermore, these data suggest that activation of JAK-STAT signalling and FA signalling may have a larger contribution to activation of PKB in response to FOXO3.A3-activation than previously appreciated, although FOXO-regulation of JAK-STAT signalling has been reported [44]. Surprisingly, only five genes are statistically significantly (ANOVA, $p < 0.05$) regulated after 8 hours of FOXO3.A3-activation (figure 3B). These include previously reported IRS2 [15], ERBB3 [17, 21, 22], but also interleukin 22 receptor alpha 1 (IL22RA1), heparin binding epidermal growth factor (HBEGF) and erythropoietin receptor (EPOR), of which expression is decreased.

A

Gene	Cluster	DL23 (8h)	DL23 (24h)	HUVECs	Protein	Peak no.	Distance
IRS2	Insulin	1.94*	2.76	1.17	3.94	7	-4848
ERBB3	ErbB	1.13*	1.62	0.41	2.38	1	1916
RICTOR	TOR	0.59	1.31	0.52	0.66	1	935
HBEGF	ErbB	0.80*	1.30	1.75	NA	3	52030
JAK1	JAK/STAT	0.38	1.28	0.81	1.64	4	-14625
PIK3CA	Akt	0.70	1.25	1.62	NA	1	-918
INSR	Insulin	0.19	0.31	0.22	NA	0	NA

B

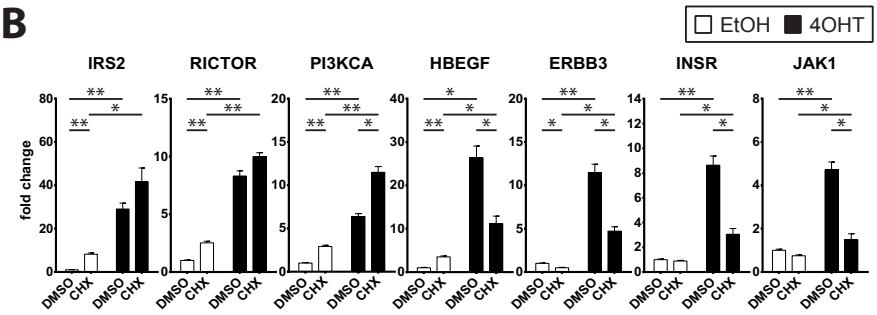

Figure 4: FOXO3 regulates PKB activating genes through direct and indirect mechanisms

A) Table with numerical data of figure 3 and more, for 7 selected genes (see main text), sorted from high to low fold change in DL23 cells treated with 4OHT for 24 hours. Column *Cluster* contains names for clusters as described in main text and depicted in figure 3A. Columns *DL23 (8h) and DL23 (24h)* contain mRNA level fold change for each gene in DL23 cells treated with 4OHT for 8 and 24 hours respectively. Asterisks behind fold change in *DL23 (8h)* indicate that this gene is statistically significantly (ANOVA; $p < 0.05$) regulated in this set; all changes in *DL23 (24h)* are statistically significant (ANOVA; $p < 0.05$). Column *HUVECs* contains mRNA level fold change for each gene in publicly available dataset of HUVECs expressing FOXO3.A3-ER, treated with 4OHT for 12 hours (see main text for details and references), which are all statistically significant (LIMMA [45] with Benjamini & Hochberg correction; $p < 0.05$). Column *Protein* contains protein level fold change for each gene in DL23 cells treated with 4OHT for 24 hours. Columns *Peak no.* and *Distance* summarise previously published ChIP-seq data for DL23 cells. *Peak no.* indicates the total number of peaks within 200kb of the gene and *Distance* indicates the distance of the closest peak to the transcriptional start site in base pairs, unless not applicable (NA). B) DL23 cells were treated with 4OHT for 24 hours in the presence of DMSO or protein translation inhibitor cycloheximide (CHX) and expression changes were measured for the genes in A). FOXO3 induced expression of IRS2, RICTOR and PI3KCA is not dependent on protein translation as their increased expression is maintained or even higher in the presence of CHX. Increased expression of JAK1, INSR, HBEGF and ERBB3 is partially or completely dependent on protein translation, indicating that their regulation may not be direct. Of note, CHX decreases the expression of ERBB3 without 4OHT, indicating that ERBB3 requires additional factors for its sustained expression, which have lower stability than FOXO3.A3-ER.
Data presented are representative experiment with technical S.D. T-tests: * $p < 0.05$, ** $p < 0.005$; indication absent or n.s. means $p > 0.05$.

FOXO3 regulates PKB activating genes through direct and indirect mechanisms

Regulation of most targets is relatively late, which leaves room for secondary effects i.e. FOXO3 target genes that themselves alter expression of genes. To further validate these findings, we measured expression changes of seven genes after 24 hours FOXO3.A3-activation in DL23, either in the presence of vehicle DMSO or cycloheximide. We selected genes that are strongly regulated after 24 hours 4OHT treatment and/or have been described to be involved in FOXO3-mediated regulation of PI3K-PKB [15-22]. Interestingly, except INSR, all genes have FOXO3 binding elements in proximity (Geo dataset GSE35486 of [42]) and all are statistically significantly (LIMMA [45] with Benjamini & Hochberg correction; $p < 0.05$) regulated in the publicly available dataset for HUVECs expressing FOXO3.A3-ER (Geo dataset GSE16573 of [46]) (figure 4A). Moreover, although not statistically significantly different, expression of RICTOR, PI3KCA and, to a lesser extent, JAK1 are increased after 8 hours FOXO3.A3-activation (figure 4A), further suggesting that these are also direct FOXO3 targets. Indeed, increased transcription of IRS2, PI3KCA and RICTOR does not depend on protein translation (figure 4B). FOXO3 induced expression of INSR, HBEGF and JAK1 is partially or completely dependent on translation (figure 4B) and thus their regulation may not be direct. Lastly, ERBB3 expression follows the pattern observed for INSR and HBEGF, suggesting that its increase in expression does not directly involve FOXO3. However, ERBB3 expression is decreased upon cycloheximide, indicating that ERBB3 regulation may require other additional (transcription) factors with high turnover. In general, interpretation of these data is somewhat complicated because many genes are increased by the cycloheximide treatment, possibly due to activation of endogenous FOXO proteins. Furthermore, in the absence of cycloheximide, gene regulation by FOXO3 is exacerbated by regulation of FOXO1 expression (2.07 and 3.92 fold up after 8 and 24 hours 4OHT respectively), as has been described [47]. Lastly, FOXO3 may require other proteins for regulation of certain targets such as e.g. SMADs for regulation of $p21^{Cip1/Waf1}$, which may have higher turnover and lower abundance than ectopically expressed FOXO3.A3-ER. In conclusion, we have identified several genes of which the expression is strongly influenced by FOXO3-activity, probably largely due to direct regulation of transcription of these genes.

FOXO3 regulation of selected PKB activating genes is observed in different human cell types

As argued, data from the DL23 cells expressing the A3 variant of FOXO3, can be misleading as it does not permit feedback signalling and we have observed that FOXO3 target genes are differently affected by the feedback regulation (see above). To assess if regulation of these genes is an effect that can be solely attributed to the A3 mutant or in particular to DL23 cells, we also measured expression changes of these seven genes in RPE cells expressing FOXO3 or FOXO3.A3. Similarly to DL23 cells, IRS2, PI3KCA, RICTOR and JAK1 are regulated by FOXO3 and FOXO3.A3 in RPE cells (figure 5A). Furthermore, expression of HBEGF and INSR are increased by FOXO3.A3, but not persistently by wild-type FOXO3 (figure 5A, but see also 5B). Lastly, ERBB3 is not included because it is expressed at very low levels in RPE cells (roughly 600 fold lower than in DL23 cells, assuming that HNRNPA1 expression is equal), impeding proper analysis of its expression. However, we do observe ERBB3 levels to increase from barely detectable to well detectable levels in response to FOXO3.A3 expression, suggesting that this gene is also regulated in RPE cells (data not shown). Next we assessed the regulation of these genes during extended expression of FOXO3, both in RPE cells and U2OS cells. Unfortunately, expression of FOXO3 in RPE cells is not maintained throughout the experiment

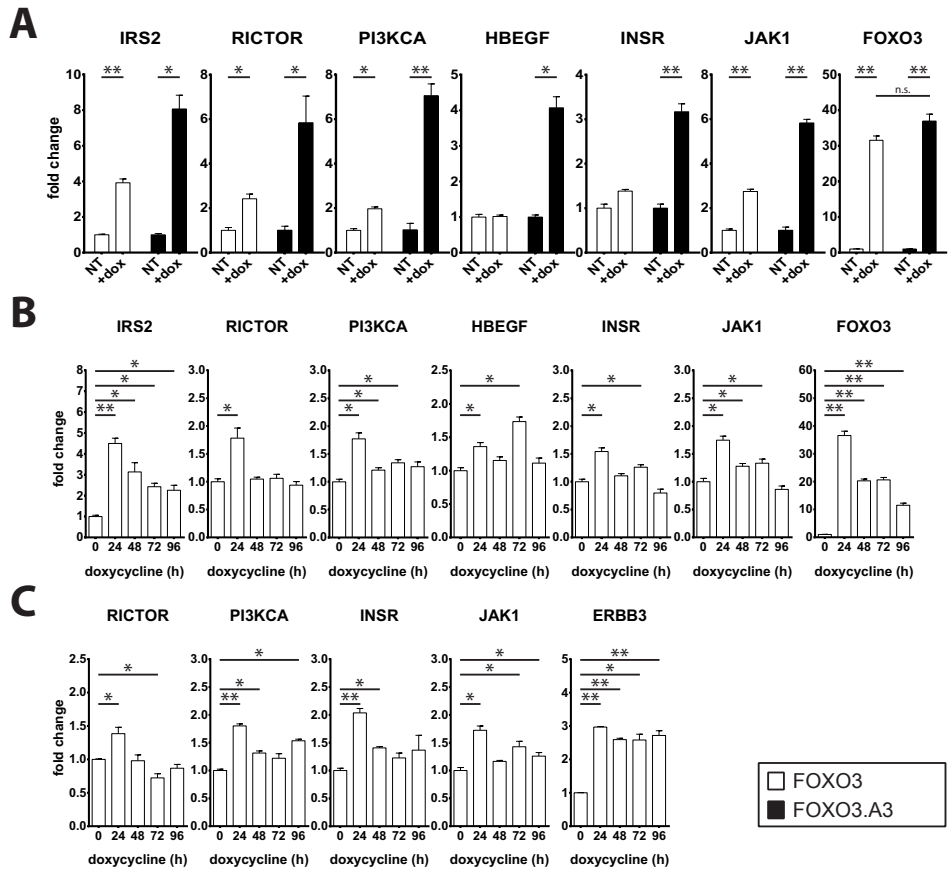

Figure 5: FOXO3 regulates the same PKB activating genes in different cell lines
A) FOXO3 or FOXO3.A3 expression was induced in RPE cells for 24 hours and expression changes of PKB activating genes from figure 4 were measured. ERBB3 expression is low in RPE cells and therefore not included. FOXO3.A3 induces expression of all six genes. FOXO3 induces all but HBEGF, showing that their regulation is not cell type specific or particular for constitutively active FOXO3.A3. B) FOXO3 expression was induced in RPE cells for indicated duration and expression data from PKB regulating genes was collected. FOXO3 expression decreases with increasing time, similar to observed for FOXO3.A3 in U2OS cells (see figure 2A). Elevated expression of IRS2 and PI3KCA clearly persists after initial peak in expression. Expression changes of HBEGF, INSR and JAK1 fluctuate, possibly resulting from the changes in FOXO3 expression levels. Increased RICTOR expression is only observed after 24 hours of doxycycline treatment and returns to unstimulated levels thereafter, indicating additional regulatory mechanisms. C) FOXO3 expression was induced in U2OS cells for indicated duration and expression data from PKB regulating genes was collected. Elevated expression of ERBB3 clearly persists upon prolonged expression of FOXO3. PI3KCA, INSR and JAK1 expression decreases after initial peak, but remain above basal (no doxycycline) level. RICTOR expression dips after initial peak and even below basal expression after 72 hours, again indicating additional regulatory mechanisms. IRS2 expression is low in U2OS cells and therefore not included.
Data presented are representative experiment with technical S.D. T-tests: * $p < 0.05$, ** $p < 0.005$; indication absent or n.s. means $p > 0.05$.

and decreased to almost 30% after 96 hours, compared to expression at 24 hours (figure 5B – rightmost graph). Nonetheless, expression of IRS2, PI3KCA and JAK1 is clearly maintained from 48 hours onwards, although JAK1 expression is decreased below the level of untreated cells after 96 hours, possibly due to feedback regulation and reduced FOXO3 expression. Maintained expression of INSR is much less clear, probably due to very mild regulation by FOXO3 in general (figure 5A and 5B). RICTOR expression returns to initial levels from 48 hours

expression on, which may either reflect feedback signalling or the expression level of FOXO3 (figure 5B – second graph). In U2OS cells, FOXO3 expression is maintained throughout the time course (figure 2A). Again, expression of PI3KCA, INSR and JAK1 is maintained from 48 hours to a similar extent as in RPE cells (figure 5C). Furthermore, RICTOR expression returns to initial expression (or below) after 24 hours (figure 5C – leftmost graph), thus further suggesting that expression of RICTOR is controlled by additional feedback signalling. Lastly, IRS2 is not depicted because it is expressed much lower in U2OS compared to DL23 or RPE cells (64 and 142 fold lower respectively, assuming that expression of HNRNPA1 is equal), impeding proper analysis of its expression. Taken together, expression of PI3KCA, JAK1 and INSR is consistently regulated in response to wild-type or constitutively active FOXO3 in DL23, RPE and U2OS cells, INSR regulation being the least clear thus far. Expression regulation of ERBB3 and IRS2 is clear whenever they are expressed at sufficient levels for detection, which may depend on cell line specific promoter status of these genes.

FOXO1/3 expression maintains PKB-activity and regulates cell division and size

It is often assumed that in the presence of growth factors, FOXO proteins are completely inactive. However, if elevated levels of FOXO3 increase PKB-activity, resulting in a new balance through maintained activity, they could well be engaged in balancing PKB-activity constantly. Indeed, it has previously been reported that FOXO3 knockout MEFs have lower phosphorylation of PKB [19], although this could be cell line specific differences. Thus, we tested if reduction of FOXO1 and FOXO3 would also decrease PKB phosphorylation in RPE cells. Indeed we observe decreased PKB phosphorylation after we reduce the expression of FOXO1 and FOXO3 using a doxycycline inducible short hairpin (figure 6A). Furthermore, expression of IRS2, RICTOR, INSR and JAK1 is decreased upon FOXO3 knockdown (figure 6B), although these differences are small and due to variation not statistically significant. Surprisingly, PI3KCA expression is elevated upon FOXO1/3 knockdown, suggesting additional regulatory mechanism controlling PI3KCA expression (figure 6B – third graph). We wondered if decreased PKB-activity is reflected in cell survival as has been described (reviewed in [1]). Indeed, FOXO1/3 depleted cell count is reduced by 78±3% after 96 hours of doxycycline treatment, which is not observed in cells expressing a short hairpin targeting luciferase (5±10% reduction) (figure 6C – leftmost graph). In line with progressive reduction of FOXO1/3 expression (figure 6A), cell count gradually decreases with duration of doxycycline induced FOXO1/3 short hairpin expression (data not shown). Surprisingly, the reduction is cell number is not reflected in protein amount (data not shown) or α-Tubulin expression (figure 6A). Indeed, in contrast to the reduction in cell number, both mean and peak (shown) diameter and volume are increased upon FOXO1/3 depletion (figure 6C – second and third graph). In summary, FOXO1 and FOXO3 are involved in maintaining PKB-activity in RPE cells, at least in part through continues regulation of IRS2, RICTOR, INSR and JAK1. In addition, their presence is required for cell survival and/or proliferation and affects cell size through yet to be dissected mechanisms.

Conclusions and discussion

Here we show that expression of wild-type FOXO3 or constitutively active FOXO3.A3 have different effects on gene regulation and cell cycle progression, and this depends on the ability of PKB to phosphorylate FOXO3. However, elevated expression of wild-type FOXO3 still does significantly affect cell cycle progression and expression of many genes, best visible at

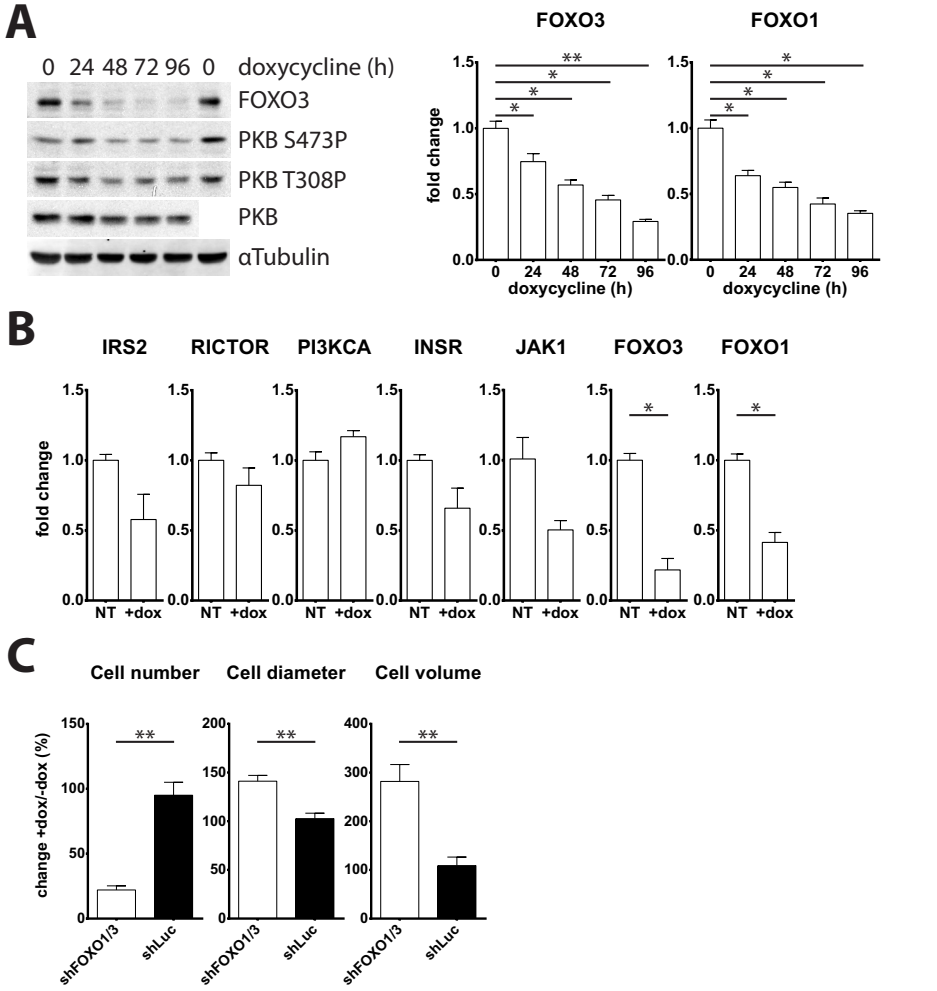

Figure 6: FOXO1 and FOXO3 are required for maintaining PKB activity, cell proliferation and/or survival and possibly involved in cell size regulation

A) Expression of a FOXO1 and FOXO3 targeting short hairpin was induced in RPE cells for indicated duration. Graphs show gradual decrease of FOXO1 and FOXO3 mRNA with increasing length of treatment, in agreement with gradual FOXO3 protein level reduction. Protein samples from cells not treated with doxycycline are loaded twice, at the beginning and at the end, to facilitate visualisation of differences. PKB phosphorylation at both Thr308 and Ser473, FOXO3 expression and to a lesser extent PKB expression, gradually decrease with time of short hairpin induction, indicating that FOXO1 and FOXO3 are required for maintaining PKB activity. B) FOXO1/3 short hairpin was induced for 0 or 96 hours in RPE cells. Except for PI3KCA, expression of FOXO3 target genes which may be relevant for maintaining PKB activity in RPE cells are decreased, indicating that under these conditions, FOXO1 and FOXO3 could be responsible for continued expression of these genes, thereby maintaining steady state PKB activity. C) A luciferase targeting short hairpin (control) or FOXO1/3 short hairpin were induced for 0 or 96 hours in RPE cells. Cells were counted with a CASY cell counter (MERCK/Millpore) and normalised cell count (treated/non-treated) are depicted (first graph). Expression of the FOXO1/3 short hairpin, but not the luciferase short hairpin, decreases the number of cells, indicating that FOXO1 and FOXO3 are required for survival and/or proliferation of these cells. In addition, peak diameter and peak volume ratios (treated/non-treated) are depicted (second and third graph), which are both increased upon expression of the FOXO1/3 short hairpin, indicating that FOXO1 and FOXO3 may be involved in regulation of cell size.

Data presented are representative experiment with technical S.D., or a summary of three experiments in the case of cell counting. T-tests: * $p < 0.05$, ** $p < 0.005$; indication absent or n.s. means $p > 0.05$.

early time points. These effects are largely or completely diminished upon prolonged expression of FOXO3, suggesting a time-dependent compensatory mechanism that, given the afore-mentioned, probably involves increasing the activity of PKB. Based on reported FOXO-regulation of several genes involved in regulation of PKB-activity [15-23], we and others [27] have proposed that this compensatory mechanism involves FOXO-mediated changes in transcription, resulting in PKB-mediated negative feedback regulation of FOXO proteins (reviewed in [Chapter 1]). In line with this and supporting our data, increased PKB phosphorylation in response to FOXO3 expression has been observed [18, 19, 24]. We have interrogated the dynamics of FOXO3-activity by elevating the expression of FOXO3 and measuring well-known effects of activated FOXO3 during prolonged expression of FOXO3. In our experimental setup, FOXO3-activity measured by target gene expression and cell cycle regulation, is highest at 24 hours of doxycycline induced expression and declines thereafter. PKB phosphorylation is increased by FOXO3 expression and both PKB phosphorylation and FOXO3 effects remain relatively stable in cells that have had increased expression of FOXO3 for 48 hours or more, suggesting PKB and FOXO-activities have reached a new equilibrium. Importantly, our data show that FOXO3 is not completely inactivated as reflected by sustained elevated expression of SOD2 and GADD45B, but not $p27^{Kip1}$ and CTDSP2 (discussed below). These observations fit the proposed model in which FOXO-induced transcription regulates PKB-activity, their mutual influence on one another's activity reaching a new equilibrium that requires both their activities. We show that increased PKB phosphorylation requires translation of new proteins suggesting that indeed changes in gene expression induced by DNA binding of FOXO3 mediate this feedback. In addition, it will be informative to test if FOXO3 lacking its DNA binding domain or DNA binding impaired $FOXO3^{H215R}$ can elevate expression of PKB, which is not expected if FOXO3 target gene regulation is responsible for elevated PKB phosphorylation. Furthermore, we have not provided direct evidence that FOXO3-inactivation is the result of increased PKB-activity or that this results from active PKB already present without FOXO3 overexpression. However, our data on extended FOXO3 expression suggest that at least part of the increased FOXO3-activity is compensated for by increased PKB-activity, because it is not expected that exceeding PKB-activity would require as much time to inactivate the newly added FOXO3. Nonetheless, a final experiment wherein wild-type FOXO3-ER is activated in the presence of cycloheximide will allow us to estimate the contribution of FOXO3 induced PKB-activity to inactivation of surplus FOXO3.

Based on the frequently observed regulation of PKB phosphorylation by FOXO proteins and the number of reported FOXO target genes that are involved in regulation of PKB-activity, it can be anticipated that this function of FOXO proteins is important in physiological context and this is substantiated by persistent target gene regulation in a range of cell types. However, none of the previously published target genes is in our shortlist of FOXO3/4 regulated genes in human and cell mouse lines [Chapter 3]. In an effort to determine which genes can be responsible for the observed increase in PKB-activation, we created a list of genes that can alter the activity of PKB according to KEGG and looked which of these genes are regulated in response to FOXO3-activation in DL23 cells. We observe 64 out of 434 selected genes statistically significantly (ANOVA; $p < 0.05$) regulated in response to 24 hour FOXO3. A3-activation in DL23 cells. For approximately two thirds of these genes, changes are small (less than 1.5 fold change) and their individual contribution to PKB-activity might therefore be negligible. However, although we have no means to test this, it is conceivable that their combined action can affect PKB-activity significantly. In addition, the remaining one third

contains several strongly regulated genes, some of which we have investigated in more detail (see below). Lastly, these data suggest that activation of JAK-STAT signalling and FA signalling may have a larger contribution to activation of PKB in response to FOXO3.A3-activation than previously appreciated, although we have not specifically focused on these two pathways in this study.

The KEGG analysis is a useful starting point for tracing back PKB regulating proteins that are regulated by FOXO3. However, it is also limited because this database does not contain all homology-based putative and/or published PKB regulating proteins. For example, Sestrin 3 and/or BCL2/adenovirus E1B 19kDa protein-interacting protein 3 (BNIP3) are not assigned to any pathway and have been shown to mediate FOXO-control of mTORC1-activity [19, 48], which is expected to influence PKB-activity (reviewed in [14]), and which we have not elaborated on in this study. Furthermore, several receptor tyrosine kinases including discoidin domain receptor tyrosine kinase 1 (DDR1) and Axl, are not assigned to any pathway in KEGG, while overexpressed DDR1 and activation of endogenous Axl have been shown to active PKB [49, 50]. These two receptors are of particular interest because their promoters contain FOXO3 binding sites, are regulated in DL23 cells after FOXO3-activation and at least DDR1 is abundantly expressed (data not shown). Also not included in any pathway in KEGG is adaptor protein growth factor receptor-bound protein 7 (GRB7), which is increased in response to FOXO3-activation in DL23 cells. Interestingly in light of ERBB3/HBEGF regulation by FOXO3, GRB7 overexpression activates PKB in HER2 overexpressing cells [51, 52], suggesting that GRB7 can couple ERBB signalling to PI3K-PKB signalling. However, although PKB inhibition results in increased GRB7 mRNA level, FOXO1.A3 expression does not alter GRB7 expression [53] in line with no detectable FOXO3 binding within 200kb of the TSS of GRB7 [42]. Also, GRB7 expression is not increased after FOXO3.A3-activation in HUVECs [46]. Lastly, KEGG does not include CTDSP2 or miR-26a in any pathway, which we show can both increase PKB phosphorylation when overexpressed [Chapter 4; Chapter 5].

Of 64 regulated genes we selected ERBB3, HBEGF, IRS2, INSR, JAK1, PIK3CA and RICTOR because their expression is affected strongly by FOXO3-activity, they have FOXO3 peaks in proximity and/or they have been reported FOXO target genes previously [15-22]. We show that FOXO3 regulates these targets by direct and indirect mechanisms and that most targets are increased to varying extent upon elevated expression of wild-type FOXO3 in cell lines of different origin, although expression levels of IRS2 and ERBB3 varies between cell lines (discussed below). Importantly, increased expression of PI3KCA, INSR, JAK1, ERBB3 (in U2OS cells only) and IRS2 (in RPE cells only) persists during prolonged expression of FOXO3 in U2OS and RPE cells, indicating that FOXO3 regulation of these genes is important for the FOXO-induced PKB-activation. Taken together, elevated expression of at least PI3KCA, INSR, JAK1, ERBB3 and IRS2 is likely to play a role in elevating PKB-activity to re-establish a new equilibrium upon increased expression of FOXO3. Surprisingly, increased RICTOR expression does not persist during extended expression of FOXO3, although it has been reported that increased RICTOR expression resulting from mTOR-inhibition requires FOXO proteins [20]. However, we have not assessed protein levels of RICTOR, which may be subject to additional regulation.

Increased expression of FOXO3 for more than 24 hours has persistent but mild effects on target gene expression and cell cycle progression. Contrary to this, increased PKB phosphorylation remains more similar throughout the time course, although this requires more extensive analysis of the data. Nonetheless, this may reflect the small contributions of

many FOXO3 regulated genes, rather than strong regulation of a single gene. Such flexibility of this network may be required since we observe that, in different cell lines, other FOXO target genes have larger contribution to PKB-activation (i.e. IRS2 in RPE cells versus ERBB3 in U2OS cells). Connected to this and notwithstanding its cell type specific basal expression level, we observed that ERBB3 expression is regulated by FOXO3.A3 in RPE cells (data not shown). Thus, FOXO3 appears to regulate these genes in all tissues, but their identification by expression profiling may be occluded by initial the status of their promoter, i.e. genes with low expression levels are less likely to be considered statistically significantly regulated. In this respect, promoter binding studied would be more informative for identification of a common FOXO target gene signature, although it is unknown if promoter recruitment of FOXO proteins is affected by the basal promoter-activity, as is the case for enhancers [54]. Lastly, the apparent discrepancies between non-persisted $p27^{Kip1}$ or CTDSP2 regulation and persisted regulation of e.g. GADD45B and SOD2 probably results from PKB-activation induced stabilisation of c-MYC (reviewed in [10]). c-MYC has been shown to decrease the expression of $p27^{Kip1}$ [55], miR-26a and CTDSP2 [56, 57]. Thus, these two genes are examples of the antagonistic relationship between FOXO proteins and c-MYC (reviewed in [Chapter 1]).
We speculated that FOXO proteins may well be continuously involved in regulation of PKB-activity. Indeed, preliminary data show that PKB phosphorylation is markedly decreased upon FOXO1/3 depletion in RPE cells, which coincides with reduced expression levels of IRS2, JAK1 and INSR, further substantiating that these genes are involved in the reciprocal relationship between FOXO proteins and PKB. In addition to the effects on gene expression, we observed a strong decrease in cell number, indicating that FOXO1 and FOXO3 are required for proliferation and/or survival of RPE cells, possibly resulting from decreased PKB-activity, although additional experiments are required to substantiate this claim. Lastly, we show that FOXO1/3 depletion results in increased cell size of RPE cells, possibly resulting from reduced inhibition of mTORC1, which can be regulated by FOXO proteins [19, 48]. However, to date we have not yet determined in influence of (prolonged) FOXO3 expression on the activity of mTORC1 and/or contribution of inhibition of mTORC1 to PKB-activation.
In conclusion, we have studied the dynamics of FOXO-activity and conclude that FOXO proteins continuously participate in regulation of PKB-activity, resulting in maintained and restricted activity of both FOXO proteins and PKB. These findings have major implications for our current understanding of FOXO-regulation and its effects.

Materials and methods

Tissue culture, reagents, BrdU staining

HEK293T (ATCC CRL-11268), U2OS (ATCC HTB-96), B13 [Chapter 3] cells, $RICTOR^{+/+}$ and $RICTOR^{-/-}$ [29] and all derived lines were maintained in DMEM with 10% FBS, L-Glutamine and Pen/Strep. RPE (ATCC CRL-4000), RPE-shFOXO1/3 and RPE-shLUC [Charitou et al., under revision] cells were maintained in DF-12 with 10% FBS, L-Glutamine and Pen/Strep. DL23 [30] cells were maintained in RPMI-1640 with 10% FBS, L-Glutamine and Pen/Strep. Doxycycline (final concentration 1µg/mL; Sigma-Aldrich) and G418 (Invitrogen) were dissolved in water. 4-hydroxy Tamoxifen (4OHT; final concentration 500nM; Sigma-Aldrich) was dissolved in 96% EtOH. S-phase cells were detected using FITC-BrdU or APC-BrdU flow kit (BD Biosciences) according to the manufacturer's instructions. For all cell lines used, BrdU was incubated for 30 minutes at 37°C.

Plasmids, lentivirus particle production and infection

Full length human FOXO3 (NM_001455.3) was amplified with flanking attB sites and transferred into pDONOR (Invitrogen) using Gateway BP clonase (Invitrogen). The FOXO3$^{T32A-S253A-S315A}$ (FOXO3.A3) mutant was generated by subsequent rounds of mutagenisis PCR following the Strategene Quickchange protocol. All construct were sequence verified before further cloning. pCNDA3-HA-FOXO3 was a kind gift from prof. dr. Paul Coffer and HA-FOXO3 was cloned HindIII-BamHI into pENTR4-GFP-C3 (Addgene plasmid 17397) [31]. Plasmid pENTR4-V5-Luc has been previously described (Addgene plasmid 19135) [31]. Using Gateway LR clonase (Invitrogen), V5-LUC, FOXO3, FOXO3.A3 and GFP-HA-FOXO3 were transferred into pInducer20 (Addgene plasmid 44012) [32]. Protein expression was induced using doxycycline for indicated duration.

Lentiviral particles were generated in HEK293T as previously described [33]. Transient transfection of HEK293T cells was mediated by MaxPEI (Polysciences Inc.) with DNA-PEI ratio 1:3µg. Polyclonal cell lines were selected for three weeks using 400-600µg/mL G418 (Invitrogen).

Mass spectrometry sample preparation and analysis

For proteome analysis, DL23 cells were grown in RPMI without arginine and lysine (PAA), supplemented with L-Glutamine, Penicillin/Streptomycin, 10% dialysed FBS (Gibco), 73µg/mL light or heavy L-Lysine ($^{+8}$K; Sigma/Silantes) and 58.8µg/mL light or heavy L-arginine ($^{+10}$R; Sigma/Silantes) to obtain SILAC medium. Cells were allowed 10 doublings in SILAC medium after which labelling efficiency was confirmed >95%. Approximately 40% confluent DL23 cells were treated with vehicle or 4OHT for 24 hours. Per experiment, one light and one heavy labelled plate was treated with 4OHT to obtain label swapped experiments (light untreated vs. heavy treated and light treated vs. heavy untreated). After 24 hours, cells were washed twice in PBS and lysed in 100mM Tris-HCl pH 7.5, 4% SDS and 100mM DTT. Approximately 100mg of protein of each condition was mixed in indicated combinations and washed, alkylated and digested as described before [34]. Peptides were basified to pH 11 using Britton buffer pH 11 and NaOH to pH 11, and bound to Strong anion-exchange (SAX) matrix (Dr. Maisch Gmbh HPLC). Eight fractions, including a flow though fraction, were obtained using Britton buffers pH 11, 8, 6, 5, 4, 3 and 2 in this order and as described [34]. Peptides were desalted on C18 matrix (Dr. Maisch Gmbh HPLC) and separated in 4 hour gradients (0-80% acetonitrile) using an EASY-nLC (Proxeon) connected online to an LTQ-Orbitrap Velos mass spectrometer (Thermo) as described [35]. Spectra were analysed using MaxQuant V1.2.2.5 [36] and mapped using protein database IPI human V3.87. Identified protein groups were log2 transformed and contaminants, reverse peptides, single peptide identifications and protein groups identified in only one of two experiments were discarded using Perseus V1.2.0.16 [37]. Using Excel (Microsoft), protein groups were selected that have ratios with opposite signs in the forward and reverse experiment.

Western blotting and antibodies

Western blotting was performed according to standard laboratory protocols. Briefly, cells were directly lysed in Leammli buffer, boiled for 3 minutes and separated on denaturing SDS-PAGE gels. Proteins were transferred to PVDF membrane, blocked with 2% BSA in TBS and strained with desired antibodies. The following antibodies were used: anti-FOXO3 (H144; Santa Cruz), anti-αTubulin (Calbiochem), anti-PKB (in house), anti-PKB Ser473P (D9E; Cell

Signalling) and anti-PKB T308P (D25E6; Cell Signalling). Secondary HRP conjugated antibodies (BioRad) and Alexa680/Alexa800 conjugated antibodies (Invitrogen) were used for ECL and Odessey respectively, according to manufacturers' instructions.

RNA extraction, cDNA synthesis, qPCR and statistical analysis

RNA was extracted using RNA easy kit (Qiagen). cDNA was synthesised using iScript kit (BioRad) with 0.5µg RNA input. qPCR was performed using FastStart SYBRgreen mix (Roche) in CFX96 Real-Time Detection System (BioRad) and analysed using the software provided by the manufacturer. Expression levels were normalised to hTUBA1A, hHNRNPA1 or mTUBA1A unless stated otherwise. Where applicable, qPCR data presented are mean and S.D. (technical and biological) of three biological replicates. For each primer set, samples were measured in triplicate average C(t) value of the control sample triplicate was used to calculate fold change for all data points. Average and technical standard deviation were calculated per sample measured and S.D. propagated from normalisation gene measurements to target gene measurements. S.D. calculations for biological replicates was performed according to [38], equation 5.38.

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CHAPTER 3

FOXO target gene CTDSP2 inhibits cell cycle progression through regulation of p21$^{Cip1/Waf1}$

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Abstract

Activity of Forkhead box O (FOXO) transcription factors is inhibited by growth factor-phosphoinositide 3-kinase (PI3K)-protein kinase B (PKB)/Akt signalling to control a variety of cellular processes including cell cycle progression. Through comparative analysis of a number of microarray datasets we identified a set of genes commonly regulated by FOXO proteins and PI3K-PKB, which includes carboxyl-terminal domain small phosphatase 2 (CTDSP2). We validated CTDSP2 as a genuine FOXO target gene and show that ectopic CTDSP2 can induce cell cycle arrest. We analysed transcriptional regulation after CTDSP2 expression and identified extensive regulation of genes involved in cell cycle progression, which depends on the phosphatase-activity of CTDSP2. Most notably regulated genes are CDK inhibitor $p21^{Cip1/Waf1}$ and transcription factor E2F1, both implicated in S-phase entry. We show that $p21^{Cip1/Waf1}$ is regulated by CTDSP2 in a p53-independent manner and that $p21^{Cip1/Waf1}$ upregulation results in decreased cyclin/CDK2 and cyclin/CDK6-activity. Thus we identify FOXO-dependent CTDSP2 regulation as a novel regulatory mechanism for inhibiting proliferation in the absence of growth factor-PI3K-PKB signalling.

Introduction

The Forkhead box transcription factors are a large family of transcription factors characterized by a conserved DNA binding domain – the Forkhead box. The O-group of Forkhead box-factors consists of four members in mammals; FOXO1, FOXO3, FOXO4 and FOXO6. FOXO6 expression appears less ubiquitous than the other members and FOXO6 is suggested to be regulated different compared to the other family members (reviewed in [1]), thus with FOXO proteins we only refer to FOXO1, FOXO3 and FOXO4. FOXO proteins shuttle between the cytoplasm and the nucleus, which regulates their activity as transcription factors. The growth factor-phosphoinositide 3-kinase (PI3K)-protein kinase B (PKB)/Akt is a major regulator of FOXO-activity. FOXO proteins are direct substrates of PKB and phosphorylated on three conserved residues. PKB-phosphorylated FOXO proteins bind to members of the 14-3-3 family, which correlates with nuclear export and cytoplasmic localisation (reviewed in [2]). On the other hand, under conditions of cellular stress i.e. following an increase in reactive oxygen species, FOXO proteins are localised to the nucleus, which involves the activation of c-Jun N-terminal Kinase (JNK) and ubiquitination of FOXO proteins (reviewed in [3]). FOXO proteins have been implicated in the regulation of a large number of processes (reviewed in [4]). Initially FOXO proteins were shown to control cell cycle progression by inhibiting S-phase entry [5]. Depending on cell type specific or additional signalling cues FOXO-arrested cells can enter quiescence, senescence or apoptosis (reviewed in [3]). Cell cycle deregulation appears the most prominent phenotype of individual FOXO knockout and FOXO1/3/4 triple knockout mice [6]. Relevant to this, FOXO proteins have been shown to upregulate both the cyclin dependant kinase (CDK) inhibitor $p27^{Kip1}$ [5] and the pocket protein p130 Rb2 [7] as well as to downregulate cyclin D proteins [8]. During S-phase, the genomic DNA is replicated and the onset of S-phase is marked the initiation of DNA synthesis. DNA replication starts at the first group of origins of replication (ORIs) and initiation of replication is usually referred to as ORI firing (reviewed in [9]). Onset of S-phase is dependent on a number of factors, including active (Cyclin D bound) CDK4/6, (Cyclin E bound) CDK2 and E2F1/2/3a which we will refer to as E2F1. E2F-members E2F3b-8 and the pocket proteins Rb, p130 Rb2 and p107 negatively regulate E2F1-activity and must therefore be inactivated. The transition to S-phase consists of two steps: during early-mid G1 Cyclin D proteins accumulate, provided mitogenic factors are present. Active CDK4/6 phosphorylates and partially inactivates the pocket proteins, resulting in increased activation of E2F1 and inactivation of E2F4/5. E2F1 is an important regulator of expression of Cyclin E proteins and thus CDK2-activity will rise while more E2F1 becomes active. Active CDK2 also phosphorylates the pocket proteins, thus establishing a feed-forward loop. When active E2F1 levels reach a critical threshold, its activity becomes independent of CDK4/6-activity due to the Cyclin E/CDK2-dependent feed-forward loop; the so-called restriction point has been passed (reviewed in [10]). Full activation of E2F1 is required for two important aspects: firstly, transcription of several genes required for maturation of the ORIs is regulated by E2Fs, as well as transcription of other genes involved in e.g. synthesis of nucleotides. Secondly, Cyclin E-CDK2-activity is required not only for E2F1-activation, but also to phosphorylate a number of proteins involved in replication, such as CDC6. These and other phosphorylations are required for DNA replication initiation and thus S-phase onset (reviewed in [9]).

FOXO signalling is evolutionary conserved and in the nematode *Caenorhabditis elegans* growth factor-PI3K-PKB signalling regulates the FOXO orthologue DAF-16. Enhanced DAF-16-activity due to reduced insulin signalling increases lifespan of *C. elegans* and similarly

in *Drosophila melanogaster* dFOXO-activity affects lifespan. In humans, small nucleotide polymorphisms (SNPs) have been identified in FOXO3, which associate with longevity. These and other results suggest that a FOXO-induced gene expression program affects lifespan. To identify genes transcriptionally controlled by FOXO proteins, which are critical in mediating FOXO proteins effect on lifespan, a number of labs have used microarrays to explore mRNA changes after FOXO-activation (this manuscript and [11-14]). These studies show that a large part of FOXO transcriptional output is highly context-dependent and FOXO-regulation of most genes is only observed in a limited number of settings or cell types. However, here we show that regulation by FOXO proteins of some genes is consistent when analysing a limited data set. We combined several selected datasets from our own lab and others that are publicly available through NCBI. Considering only statistically significantly changed genes, we generated a list of FOXO3/FOXO4 regulated genes that are consistently regulated throughout these datasets.

Carboxyl-terminal domain small phosphatase 2 (CTDSP2, also referred to as SCP2 or OS4) is regulated in all datasets we analysed but has not been described as a FOXO target gene previously. CTDSP1, CTDSP2, CTDSPL and CTDSPL2 are phosphatases and related to CTDP1 (FCP1 in yeast) because of their characteristic phosphatase domain [15]. Similar to CTDP1, CTDSP family members have been shown to dephosphorylate the carboxyl-terminal domain (CTD) of RNA Polymerase II (RNAPII) core-subunit RBP1 [15, 16] and thereby inhibit gene expression [16-18]. Other studies have highlighted different roles of CTDSP1, CTDSP2 and CTDSPL, including regulation of TGFβ signalling [19-22], Snail protein stability [23] and cell cycle progression [24, 25]. In conclusion, members of the CTDSP family of phosphatases are involved in regulation of both signalling and transcription.

Here we show that CTDSP2 is consistently regulated in a range of microarray datasets generated from cell lines overexpressing FOXO3 or FOXO4. Furthermore, CTDSP2 is regulated in response to activation of endogenous FOXO proteins as well as in the absence of protein translation. We find that CTDSP2 is a direct target gene of FOXO proteins with FOXO proteins binding directly adjacent to the transcriptional start site (TSS) of CTDSP2. One of the consequences of elevated expression of CTDSP2 is a strong reduction of the number of S-phase cells. However, unlike previous suggestions, we do not confirm the requirement of the pocket protein retinoblastoma (Rb) for this [24, 25]. Instead, microarray analysis of cells expressing CTDSP2 reveals several genes regulated, which in turn are potentially involved in regulating S-phase onset. Of these, we show that the CDK inhibitor $p21^{Cip1/Waf1}$ is largely responsible for the decreased cell cycle progression of CTDSP2 overexpressing cells.

Results

Defining a limited FOXO target gene signature

Active FOXO proteins affect a number of cellular processes. To identify genes transcriptionally controlled by FOXO proteins that convey regulation of these processes, different studies including the present one have generated microarray datasets [11-14]. These studies identified numerous FOXO target genes which, when analysed under different conditions, are shown to be regulated only in a particular context or cell type. To identify a set of FOXO3 and FOXO4 regulated genes that is common to at least a limited number of cell types, we merged our datasets including E-MTAB-4 [11] with datasets GSE16573, GSE23926 and GSE35705 from NCBI [12-14]. The datasets included in the analysis were generated from cells stably expressing estrogen receptor-ligand binding domain (ER) fusions of FOXO3 or FOXO4 that

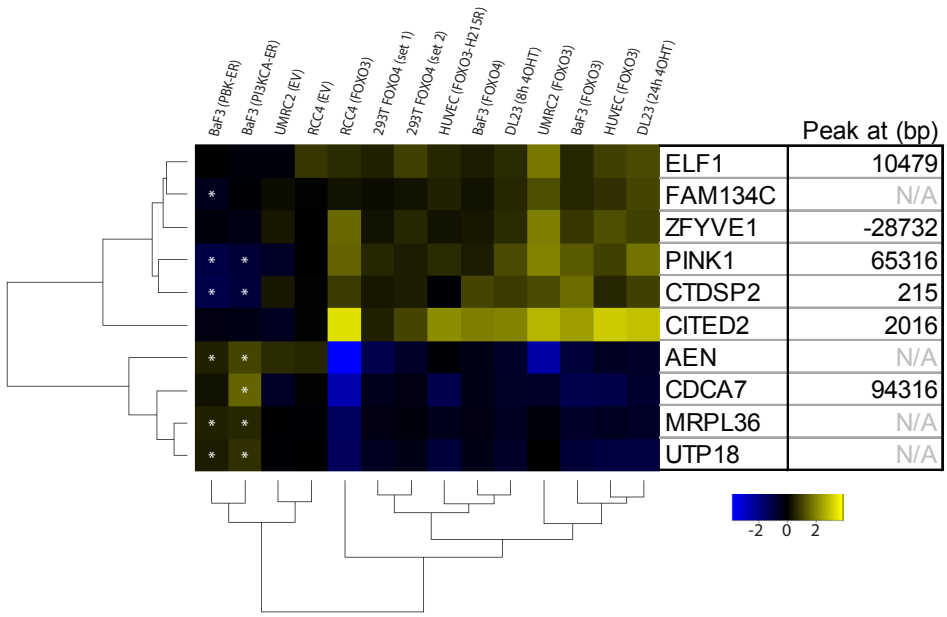

Figure 1: A common set of FOXO-regulated genes in human and mouse cell lines overexpressing FOXO3 or FOXO4
In house and publicly available datasets (see main text for references) of cell lines overexpressing FOXO3 or FOXO4 were merged as described in results and materials and methods section. Genes depicted are statistically significantly ($p < 0.05$) regulated in all datasets, excluding HUVECs expressing DNA binding defective FOXO3^{H215R} and BaF3 cells expressing PKB or PI3K. Asterisks indicate genes that are statistically significantly (ANOVA, $p < 0.05$) regulated by PI3K or PKB-activation, in opposing direction compared to FOXO3/4-activation. Right table column shows distance of closest FOXO3 peak in DL23 cells.

are insensitive to PKB inactivation due to mutation of the PKB phosphorylation sites [38] and can be activated using 4-hydroxy Tamoxifen (4OHT). In addition we included two datasets generated from HEK293T cells that transiently overexpressed wild-type FOXO4. In this way, we combined data from HUVEC, UMRC2, RCC4, BaF3, DLD1 derived DL23 [7] and HEK293T cells. We found ten genes that are regulated in all datasets, excluding HUVECs expressing DNA binding defective FOXO3^{H215R} and BaF3 cells expressing PKB or PI3K (figure 1). Datasets generated in our institute (DL23, HEK293T and BaF3) contain four samples per experiment, which are dye-swapped, dye-corrected and for which the differences are tested for significance using ANOVA-testing (included genes have $p < 0.05$). The HUVEC dataset has three replicates that were tested for significance using LIMMA [39] with Benjamini & Hochberg correction (included genes have $p < 0.05$ in the HUVEC-FOXO3-ER set). The datasets for UMRC2 and RCC4 have only a single replicate per cell line and construct which can not been tested for significance. For reference, we included the cell lines that were infected with the empty vector. These two cell lines cluster furthest away from the FOXO3/4-overexpression datasets (figure 1).

We recently published that FOXO-activation correlates best with increased expression of genes [37] and hence we focused on genes that are upregulated in our combined datasets. Of the six genes upregulated in all datasets, CITED2 and PINK1 have been previously described to be FOXO target genes [40, 41]. Besides these, CTDSP2 is the most strongly regulated gene in these datasets but it has not been described as a FOXO target gene of to date. Interestingly, CTDSP2 expression is decreased significantly (ANOVA, $p < 0.05$) in response to activation of

PI3K or PKB (figure 1; two rightmost columns), suggesting that CTDSP2 expression is highly sensitive to FOXO-activity.

CTDSP2 is regulated by exogenous and endogenous FOXO proteins

To further confirm the regulation of CTDSP2 by FOXO proteins, we tested CTDSP2 mRNA regulation using qPCR in response to activation of exogenous and endogenous FOXO proteins. Indeed, CTDSP2 mRNA levels increase significantly over time, starting from one hour after FOXO3-activation in DL23 cells (figure 2A – left graph). Furthermore, the increase in CTDSP2 mRNA upon FOXO3-activation is also observed in the presence of the translation inhibitor cycloheximide, indicating that FOXO3 directly regulates the expression of CTDSP2 (figure 2A – right graph). To test CTDSP2 mRNA regulation by endogenous FOXO proteins, we treated several cell lines with PI3K or PKB inhibitors (LY294002 and Akti respectively). As shown in figure 2B, all tested cell lines show increased expression of CTDSP2 mRNA upon inhibition of PI3K/PKB.

FOXO proteins bind to the CTDSP2 promoter

Next we analysed our previously published Chip-seq data [37] and identified FOXO3 binding sites within the promoter of CTDSP2 (figure 3A). We confirmed these data by ChIP-qPCR, where we identify the CTDSP2 promoter upon precipitation of exogenous FOXO3 (figure 3B – left graph) or endogenous FOXO3 (figure 3B – right graph). In addition, we confirmed

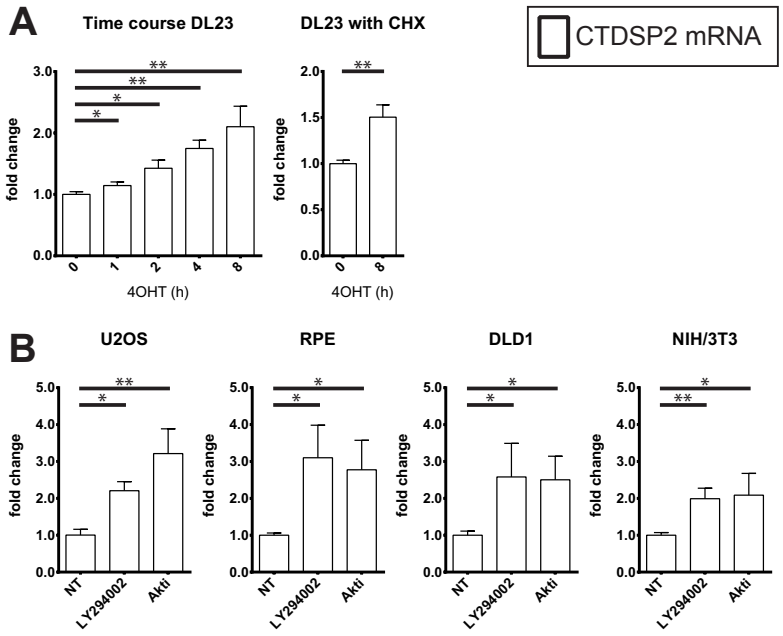

Figure 2: CTDSP2 mRNA is rapidly upregulated, independent of protein translation, after activation of exogenous FOXO3 and in multiple cell lines after inhibition of PI3K or PKB

A) CTDSP2 mRNA was measured by qPCR in DL23 cells after FOXO3 was activated by 4OHT for indicated times points (left) or for 8 hours in the presence of cycloheximide (right) B) DLD1, U2OS, RPE and NIH/3T3 cells were treated for 16 hours with LY294002 (LY) or Akti1/2 (VIII) to inhibit PI3K and PKB respectively, and CTDSP2 mRNA was measured by qPCR. Data presented are mean and S.D. of three biological replicates. T-tests: * $p < 0.05$, ** $p < 0.005$; indication absent or n.s. means $p > 0.05$.

that GFP-tagged FOXO1, FOXO3 and FOXO4 bind to the same region in U2OS cells (figure 3C), which is in line with the proposed general and direct regulation of CTDSP2 by FOXO proteins. Taken together, these results show that CTDSP2 is a genuine and direct target of FOXO proteins.

CTDSP2 expression decreases the number of cells in S-phase

To study the effects of elevated CTDSP2 expression in isolation, we used a doxycycline regulated expression system to ectopically express CTDSP2. Expression of either CTDSP1, CTDSP2 or CTDSPL has previously been described to induce cell cycle arrest [24, 25]. To negate overexpression artefacts, we compared expression of wild-type, phosphatase-proficient CTDSP2 to a previously described phosphatase-dead mutant CTDSP2^{pd} [15]. We measured BrdU incorporation of U2OS, RPE, DLD1 and NIH/3T3 cells of which U2OS and RPE cells indeed showed a phosphatase-activity-dependent reduction in number of cells in S-phase (data U2OS in figure 4A – left graph; data not shown for RPE). qPCR of CTDSP2 (figure 4B – left and right graph) and constitutive bicistronic Tet-Activator/APH mRNA (figure 4B – middle and right graph) as well as protein levels (figure 4C), all confirm that these differences

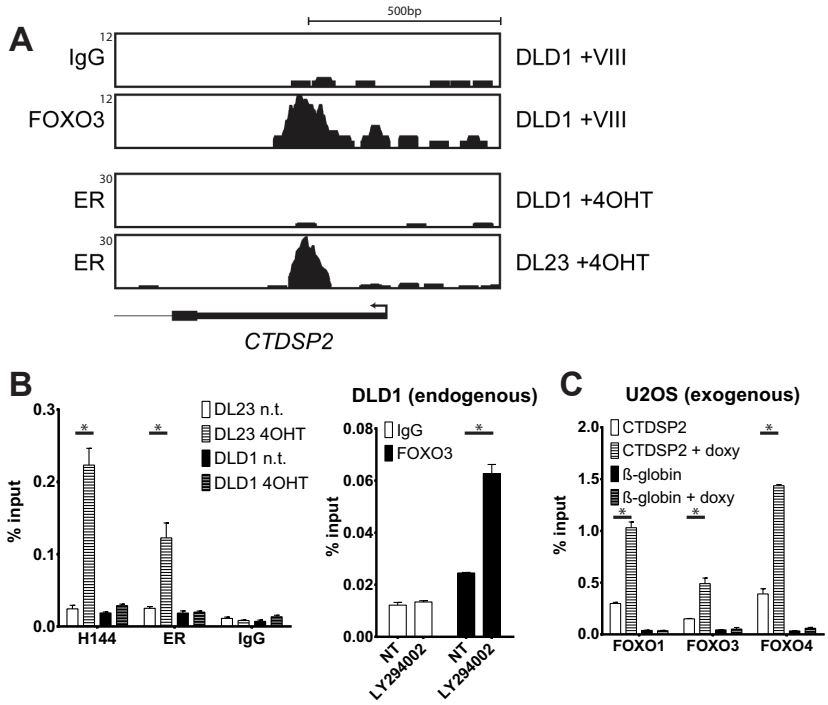

Figure 3: FOXO proteins bind to the promoter of CTDSP2 in DLD1 and U2OS cells

A) Graphical view of published endogenous (top) and exogenous (bottom) ChIP-seq read-density at the CTDSP2 promoter in DLD1/DL23 cells [37]. B) Left: ChIP-qPCR determined percentage input of the CTDSP2 promoter or non-specific βglobin locus precipitated by non-specific IgG or exogenous FOXO3 using FOXO3 or ER antibody. Right: ChIP-qPCR determined percentage input of the CTDSP2 promoter precipitated by endogenous FOXO3 or non-specific IgG. Nuclear localisation of endogenous FOXO3 was induced using LY294002 and precipitated using FOXO3 antibody. C) ChIP-qPCR determined percentage input of the CTDSP2 promoter or non-specific βglobin locus precipitated by FOXO1, FOXO3 or FOXO4. FOXO1, FOXO3 and FOXO4 expression was induced by doxycycline treatment where indicated and precipitated using GFP antibody. All conditions were treated with Akti for 30 minutes. Data presented is mean and S.D. of three technical replicates. The experiment is representative of three or more independent biological replicates. T-tests: * $p < 0.002$; indication absent means $p > 0.002$.

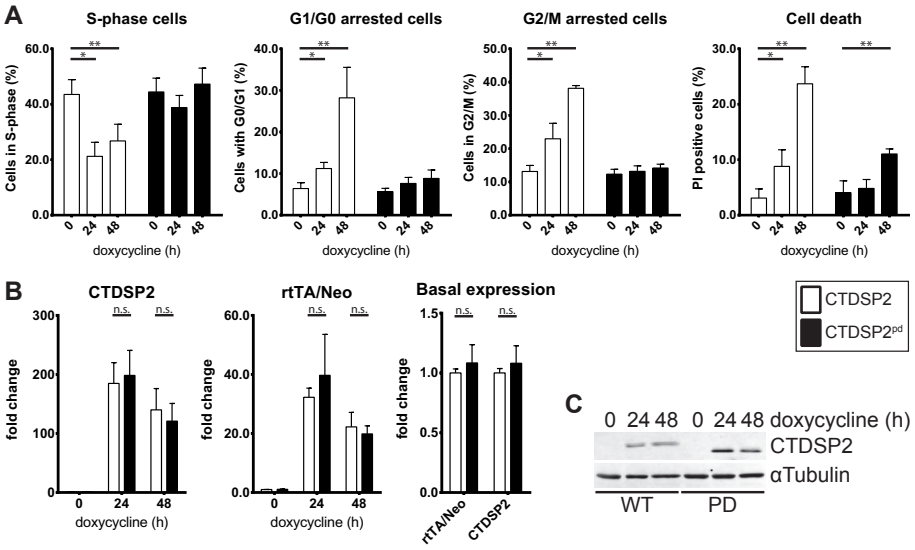

Figure 4: CTDSP2 expression causes phosphatase-dependent cell cycle arrest and cell death in U2OS cells.
A) Percentage of BrdU-positive cells is decreased after doxycycline-induced expression of CTDSP2 but not a phosphatase-deficient mutant phosphatase-dead CTDSP2^{pd} (left). Both G0/G1 cell population (second graph) and G2/M cell population (third graph) are increased in CTDSP2 expressing cells blocked with nocodazole or thymidine respectively. PI exclusion measured cell viability decreases after doxycycline-induced expression of CTDSP2. B) Induced expression of CTDSP2 and phosphatase-dead CTDSP2^{pd} mRNA (left) and bicistronic Tet-Activator/APH mRNA (middle). Using U2OS-CTDSP2 mRNA levels without doxycycline as a reference point for either transcript, unstimulated expression is identical, indicating expression potential is similar. C) Doxycycline stimulated protein expression of CTDSP2 (WT) and phosphatase-dead CTDSP2^{pd} (PD) are similar after 24 and 48 hours. αTubulin was used a loading control. Data presented are mean and S.D. of three biological replicates. T-tests: * $p < 0.05$, ** $p < 0.005$; indication absent or n.s. means $p > 0.05$.

are not due to differential expression of CTDSP2 compared to phosphatase-dead CTDSP2^{pd}. To dissect if this reduction in BrdU-positive cells is the result of an arrest in either G1 or G2 phase, we blocked cell cycle progression in either phase. In response to expression of CTDSP2, U2OS cells arrest in both G1/G0 and G2/M phases whereas RPE cells arrest only in G1/G0 (data U2OS in figure 4A – middle two graphs; RPE data not shown). Furthermore, both U2OS and RPE cells show increased cell death upon prolonged expression of CTDSP2 (U2OS data in figure 4A – right graph; RPE data not shown).

Pocket protein family members are dispensable for CTDSP2-induced cell cycle arrest

It has been observed that expression of CTDSPL alone [24] or in combination with CTDSP1 and CTDSP2 [25], results in decreased phosphorylation of Rb. Based on this, CTDSPL was initially suggested to be named Rb1 serine phosphatase from human chromosome 3 (RBSP3) [24]. However, decreased phosphorylation of Rb can be both cause and consequence of reduced cell cycle progression. To investigate the involvement of Rb and the other two pocket proteins p107 and p130, we analysed the effect of CTDSP2 or phosphatase-dead CTDSP2^{pd} expression on cell cycle progression in wild-type MEFs and MEFs that lack either of the three pocket proteins, further referred to as RbTKO MEFs [26]. Surprisingly, MEFs lacking pocket proteins still show a reduction in BrdU-positive cells in response to CTDSP2 expression (figure 5A), albeit to a lesser extent (29.2±12.4% reduction) compared to wild-type MEFs (40.0±6.7% reduction) or U2OS cells (46.8±11.4% reduction). Presumably, the weaker inhibition of cell

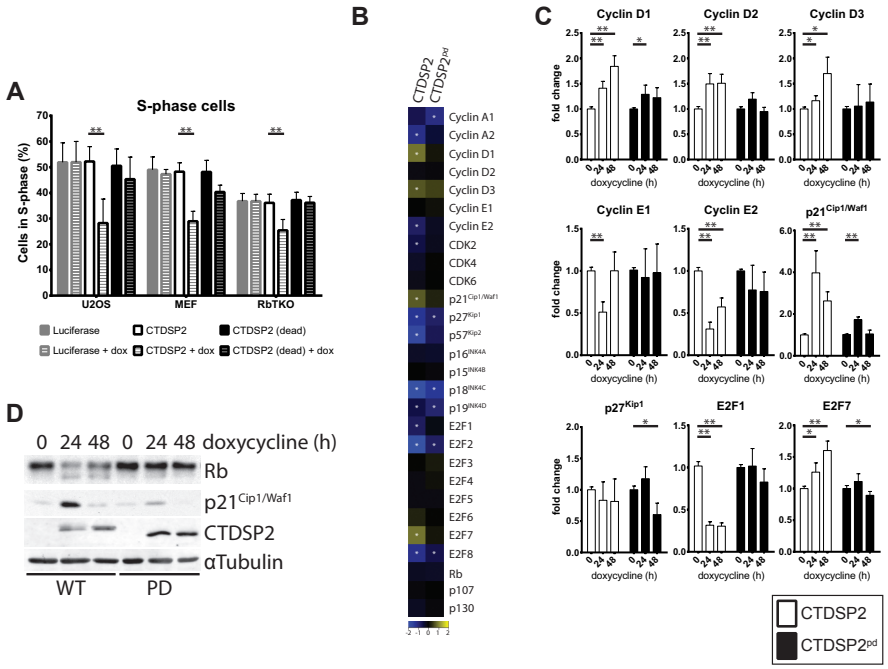

Figure 5: CTDSP2-induced cell cycle arrest does not require pocket protein family members
A) Expression of CTDSP2 reduces BrdU-positive cells in U2OS cells, wild-type MEFs and RbTKO MEFs lacking Rb, p107 and p130. All cell lines were established three times and BrdU-incorporating cells were measured after 24 hour doxycycline treatment. B) Microarray results show that several S-phase entry regulating genes are statistically significantly (ANOVA; $p < 0.05$) changed in CTDSP2, but not phosphatase-dead CTDSP2^{pd} expressing cells. In agreement with reduced S-phase entry in CTDSP2 expressing cells, Cyclin E2 (1.77 fold down), p21$^{Cip1/Waf1}$ (1.64 fold up), E2F1 (1.74 fold down) and E2F7 (1.88 fold up) are changed. C) qPCR results confirm regulation of p21$^{Cip1/Waf1}$, Cyclin E2, E2F1 and E2F7 after 24h and 48h CTDSP2 expression. D) Western blots confirm decreased phosphorylation of Rb and upregulation of p21$^{Cip1/Waf1}$. Dox indicates doxycycline treatment. Data presented are mean and S.D. of three biological replicates. T-tests: * $p < 0.05$, ** $p < 0.005$; indication absent or n.s. means $p > 0.05$.

cycle progression in RbTKO cells is due to Rb in part determines the threshold for cells to enter S-phase. Thus, CTDSP2 expression arrests cells, but this does not critically depend on Rb or any of the other pocket proteins.

Cell cycle regulators p21$^{Cip1/Waf1}$, E2F7, E2F1 and Cyclin E2 are regulated in response to CTDSP2 expression

To determine which genes are involved in the observed CTDSP2-induced cell cycle arrest, we collected expression profiles of CTDSP2 and phosphatase-dead CTDSP2^{pd} expressing U2OS cells. We focused on the main regulators of the G1-to-S transition (reviewed in [10]) and found several genes regulated (figure 5B). Interestingly we observed statistically significant upregulation of p21$^{Cip1/Waf1}$ and E2F7 in CTDSP2 expressing cells whereas these two genes are not statistically significantly regulated in phosphatase-dead CTDSP2^{pd} expressing cells (figure 5B). Furthermore, E2F1 and Cyclin E2 were downregulated in a phosphatase-activity-dependent manner. E2F2 is downregulated in both CTDSP2 and phosphatase-dead CTDSP2^{pd} expressing cells, albeit to a lesser extent (4 fold versus 1.8 fold down resp.). Surprisingly, Cyclin D1 was specifically upregulated (1.8 fold) in CTDSP2 expressing cells and Cyclin D3 was upregulated in both cell lines (1.6 fold and 1.4 fold resp.; figure 5B), indicating that mitogenic

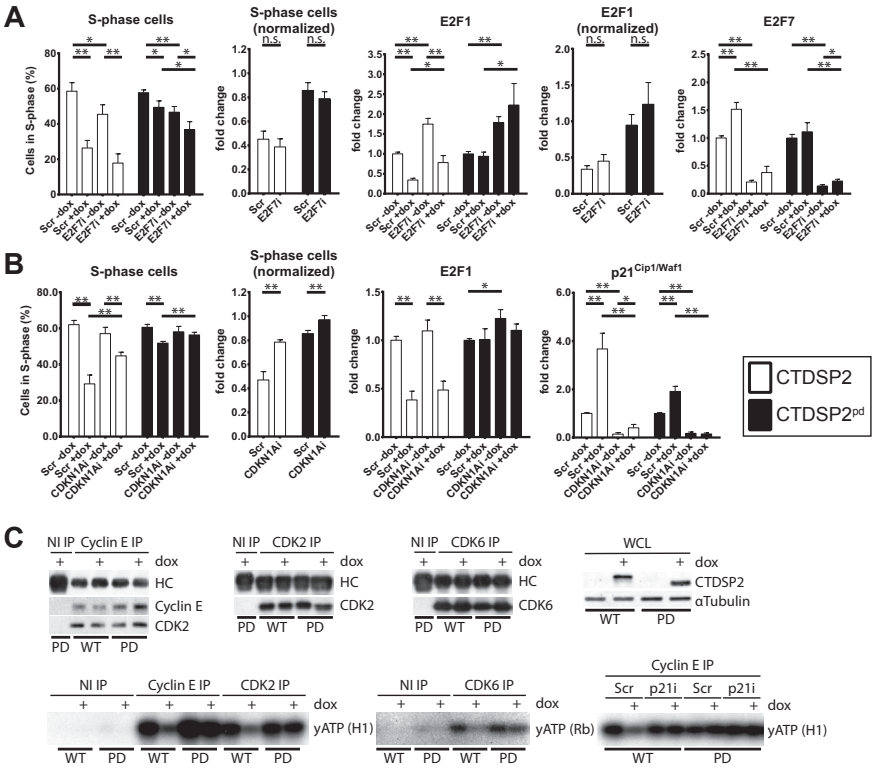

Figure 6: p21[Cip1/Waf1] RNAi, but not E2F7 RNAi, attenuates reduced cell cycle progression resulting from CTDSP2 expression and restores Cyclin E bound CDK2-activity.
A) Knockdown of E2F7 (fifth graph) does not reduce absolute (first) or relative (second graph), CTDSP2-induced decrease in BrdU-positive cells. E2F7 knockdown does result in absolute increase of E2F1 levels (third graph), but not the relative fold decrease in E2F1 mRNA in response to CTDSP2 (fourth graph). B) p21[Cip1/Waf1] knockdown (fifth graph) does attenuate absolute (first) and relative (second graph) decrease in BrdU-positive cells upon CTDSP2 expression. However, CTDSP2 expression-induced decreased E2F1 expression is only moderately restored in response to p21[Cip1/Waf1] RNAi (fourth graph). C) Cyclin E-bound and total CDK2 and CDK6 *in vitro* kinase activities are reduced (bottom left and middle γATP detection) upon CTDSP2 but not phosphatase-dead CTDSP2[pd] expression (top fourth western blot showing CTDSP2 and phosphatase-dead CTDSP2[pd] expression in whole cell lysates). Input protein levels per cell line and precipitated protein are similar (top first, second and third western blots of precipitated cyclin E1/CDK2, CDK2 or CDK6 resp.). p21[Cip1/Waf1] RNAi (p21i) restores Cyclin E1-bound CDK2-activity (bottom right γATP detection). Dox indicates 24 hour doxycycline treatment. HC: heavy chain. Data presented are mean and S.D. of three biological replicates. T-tests: * $p < 0.05$, ** $p < 0.005$; indication absent or n.s. means $p > 0.05$.

signalling per se is not impaired in these cells. We confirmed regulation of several of these genes using qPCR, including upregulation of p21[Cip1/Waf1] and E2F7 as well as downregulation of E2F1 and Cyclin E2 (figure 5C). Western blots confirm reduced phosphorylation of Rb as well as increased expression of p21[Cip1/Waf1] (figure 5D).

p21[Cip1/Waf1], but not E2F7, knockdown attenuates the effects of CTDSP2 on cell cycle progression

E2F1 and Cyclin E2 are major constituents of the feed-forward loop important for cells to commit to S-phase (reviewed in [10]). Hence, reduced Rb phosphorylation and downregulation of E2F1 potentially result in the observed reduction in Cyclin E2 expression. Rb is phosphorylated by Cyclin D-CDK4/6 and Cyclin E-CDK2, while E2F7 regulates E2F1 expression [42]. We wanted to determine if either or both E2F7 and p21[Cip1/Waf1] play a role

in the observed reduction in cell cycle progression after CTDSP2 expression. To test if the cells arrest due to increased expression of E2F7 upon CTDSP2 expression, we tested the ability of CTDSP2 to induce cell cycle arrest in cells depleted of E2F7 using RNAi. While E2F7 levels were decreased significantly (figure 6A – fifth graph) and resulted in increased absolute E2F1 expression (figure 6A – middle graph), neither the relative decrease in E2F1 expression levels (figure 6A – fourth graph) nor the absolute or relative decrease in BrdU-incorporating cells was affected by E2F7 RNAi (figure 6A – first and second graph resp.). Unexpectedly, we observed a statistically significant decrease in number of BrdU-incorporating cells that have E2F7 depleted, compared to the control (figure 6A – first graph). Contrary to E2F7 knockdown, cells that have been depleted for $p21^{Cip1/Waf1}$ using RNAi (figure 6B – fourth graph) do not arrest to the same extent as the controls, indicating that $p21^{Cip1/Waf1}$ is in part responsible for the effects of CTDSP2 on cell cycle progression. Knockdown of $p21^{Cip1/Waf1}$ statistically significantly increased the absolute (figure 6B – first graph) and relative numbers (figure 6B – second graph) of cells in S-phase. However, $p21^{Cip1/Waf1}$ knockdown does not restore E2F1 expression to its original level (figure 6B – third graph).

The effect of $p21^{Cip1/Waf1}$ on cell cycle progression is generally attributed to its ability to inhibit CDKs, although this is complicated in the case of cyclin D-bound CDKs (reviewed in [43]). CDK2-activity depends on binding to either cyclin E or cyclin A, the latter cyclin being part of a negative feedback loop activated during S-phase (reviewed in [10]). Thus, most relevant to S-phase entry is cyclin E-bound CDK2. We measured the activity of CDK2 and CDK6 after CTDSP2 and phosphatase-dead $CTDSP2^{pd}$ expression using *in vitro* kinase assays. In line with decreased G1-to-S signalling upon CTDSP2 expression, we find that activity of CDK6, total

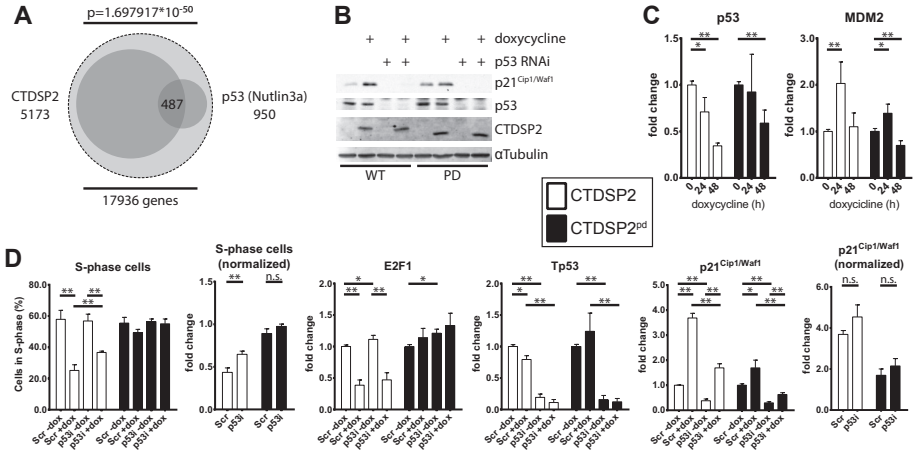

Figure 7: p53 is activated by CTDSP2 expression, but not responsible for $p21^{Cip1/Waf1}$ upregulation upon CTDSP2 expression

A) A statistically significant overlap exists between regulated genes in response to CTDSP2 expression and genes regulated in response to p53 stabilisation using Nultin3a (GSE46493 from [44]). Overlap only includes genes that are regulated in the same direction and p-value indicates probability of overlap estimated by hypergeometric test. B) Western blot analysis of p53 and $p21^{Cip1/Waf1}$ levels in response to CTDSP2 expression shows p53 is largely responsible for $p21^{Cip1/Waf1}$ expression. However, p53 is not stabilised in response to CTDSP2 expression. C) MDM2 is upregulated after 24 of CTDSP2 expression while p53 mRNA gradually decreases, indicating brief activation of p53 and activation of negative feedback through MDM2. D) p53 knockdown (fourth graph) does reduce basal but not CTDSP2 increased expression of $p21^{Cip1/Waf1}$ (fifth and sixth graph). p53 depletion does alleviate part of the CTDSP2-induced decrease in cell cycle progression, but to a lesser extent than $p21^{Cip1/Waf1}$ depletion (first and second graph). E2F1 levels are only moderately restored (third graph). Data presented are mean and S.D. of three biological replicates. T-tests: * $p < 0.05$, ** $p < 0.005$; indication absent or n.s. means $p > 0.05$.

CDK2 and cyclin E-bound CDK2 are decreased in CTDSP2 expressing cells (figure 6C – bottom left and middle films). This decrease in activity is not the result of reduced kinase input in either of the assays (figure 6C – top western blots). Instead, $p21^{Cip1/Waf1}$ RNAi alleviated the CTDSP2-induced inhibition of cyclin E-bound CDK2 (figure 6C – bottom right film).
Taken together, CTDSP2 expression results in elevated expression of $p21^{Cip1/Waf1}$, which inhibits cyclin/CDK-activity thereby reducing cell cycle progression.

p53 is activated by CTDSP2 expression but is not responsible for elevated $p21^{Cip1/Waf1}$ levels
CTDSP2 does not harbour a DNA binding domain and gene regulation therefore likely involves DNA binding factors, such as transcription factors. p53 is the best known transcriptional regulator of $p21^{Cip1/Waf1}$ expression. Therefore, we tested the involvement of p53 in CTDSP2 regulation of $p21^{Cip1/Waf1}$. To explore this possibility, we first compared CTDSP2-induced gene changes to a recently published dataset (GSE46493) of genes changed upon treatment of p53 stabilising compound Nutlin3a [44], only counting genes that are upregulated in both datasets or downregulated in both datasets. Indeed, a statistically significant overlap exists between genes regulated in response to CTDSP2 expression and genes changed after a 24 hour treatment with Nutlin3a (figure 7A). Unexpectedly however, we do not observe stabilisation of p53 (figure 7B). It is generally believed that p53-activity is predominantly regulated at the level of protein stability through the E3 ubiquitin ligase MDM2 (reviewed in [45]), which itself is a target gene of p53 thereby creating a negative feedback loop [46]. Our microarray data show upregulation of MDM2 following CTDSP2 expression (1.88 fold up, $p < 0.05$). Thus, we explored the expression levels of p53 and MDM2 with respect to duration of CTDSP2 expression. In line with initial activation and concomitant negative feedback regulation of p53, we find that MDM2 is upregulated after 24 hours of CTDSP2 expression (figure 7C). Surprisingly, p53 expression is also gradually downregulated with time (figure 7C), which is also observed in our microarray data (p53 is 2.26 fold down, $p < 0.05$). Thus, it is possible that $p21^{Cip1/Waf1}$ is upregulated due to initial p53-activation by CTDSP2. To test if $p21^{Cip1/Waf1}$ upregulation depends on the presence of p53, we expressed CTDSP2 in cells depleted of p53 using RNAi (figure 7D – fourth graph). Unexpectedly, even though knockdown of p53 was sufficient to decrease basal $p21^{Cip1/Waf1}$ expression to a large extent (2.70±0.13 fold down), $p21^{Cip1/Waf1}$ upregulation still occurs upon expression of CTDSP2 (figure 7D – fifth and sixth graph). Furthermore, p53 RNAi does alleviate the S-phase entry inhibition (figure 7D – first and graph), but not to the same extent as $p21^{Cip1/Waf1}$ RNAi (compared to control + doxycycline: 17.89±6.86% and 30.97±8.63% more cells in S-phase in p53 and $p21^{Cip1/Waf1}$ depleted cells resp.). Lastly, similar to $p21^{Cip1/Waf1}$ depletion, E2F1 expression levels are not restored to their original levels by p53 knockdown. In summary, we conclude that p53 is not the main mediator of increased $p21^{Cip1/Waf1}$ expression in response to CTDSP2 expression.

Conclusions and discussion
FOXO3 and FOXO4 target gene expression has been extensively studied over the years, but the datasets have not been combined before. Here we present a short list of genes regulated in all included datasets included, thus representing a set of genes regulated by FOXO3 and FOXO4 in different cell types. In line with the notion that FOXO3 binding correlates predominantly with increased expression of genes [37], most upregulated genes have one or more FOXO3 binding sites within 100kb of the TSS (figure 1). Only two out of six upregulated genes were previously reported to be regulated by FOXO proteins, i.e. CITED2 and PINK1

[40, 41]. The remaining genes might be of interest for further investigation. For example, ZFYVE1 is a phosphatidylinositol 3-phosphate binding protein that has been proposed to be involved in autophagy [47], a process that has also been linked to FOXO proteins [14]. About FAM134C relatively little is known. Interestingly, it has been shown a target gene of Nrf1 [48], a transcription factor that is important for stress resistance and linked to FOXO proteins [49]. Lastly, Elf1 is an Ets-related transcription factor important in embryonic haematopoiesis and angio-genesis, its expression in adults being restriction to the lymphoid system (reviewed in [50]). Interestingly, FOXO1 knockout mice are embryonic lethal due to defects in vascular development [51, 52] and induced loss of individual or multiple FOXO proteins at later age results in vascular abnormalities [6].

In this study, we have focused on the effects of CTDSP2, which has not been described as a FOXO target gene before. We confirmed that CTDSP2 is regulated in response to FOXO-activation in all cell lines we tested (figure 2), underscoring the power of this approach. Also, we show that FOXO1, FOXO3 and FOXO4 bind to the proximal promoter of CTDSP2 using ChIP-qPCR (figure 3), confirming and extending previous results from our lab [37]. Thus, CTDSP2 is a novel, ubiquitously regulated and direct target of FOXO proteins. CTDSP2 mRNA is ubiquitously expressed and relatively abundant (GSE50243: 45.01 ± 8.34 KRPM compared to average 25.9223 KRPM with 0.9 KRPM cut off) [53]. However, despite the abundance and persistent regulation of CTDSP2 mRNA, we were unable to detect endogenous CTDSP2 protein using different commercially available antibodies that all recognise ectopically expressed CTDSP2. In agreement, available proteomics datasets of different cell lines such as [54] almost never report CTDSP2 expression (data not shown). Two datasets do contain a single modified peptides of CTDSP2, either phosphorylated on Ser5 [55] or ubiquitinated at Lys20 after DNA damage [56]. Interestingly, it has been shown that translation of zebra fish CTDSP2 is inhibited by miR-26b, which in early embryogenesis is inhibited in its maturation [57]. Members of the miR-26 family are co-transcribed from the same loci as CTDSP1, CTDSP2 and CTDSPL [58]. However, when we co-expressed ectopic miR-26a together with a luciferase gene fused to the 3'UTR of human CTDSP2, it was equally expressed compared to a luciferase gene lacking this 3'UTR (data not shown).

Members of the CTDSP family have been shown to be involved in regulation of RNAPII CTD phosphorylation [15, 16], as well as a number of other processes, including the regulation of BMP/TGFβ signalling [19-22] and the expression of Snail [23]. It was also shown that they can regulate cell cycle progression [24, 25] and that their expression is downregulated upon re-entry of the cell cycle [25]. Because FOXO proteins have been extensively studied for their ability to regulate cell cycle progression, we chose to investigate cell cycle regulation of CTDSP2. We show that expression of CTDSP2 results in a decreased number of cells in S-phase and that this is dependent on its phosphatase-activity. In U2OS cells, expression causes accumulation of cells in both the G0/G1 and G2/M phase. Prolonged expression induces cell death through mechanisms that we did not further elaborate on (figure 4). These findings are remarkably reminiscent for those described for FOXO proteins [5, 59]. The ability of CTDSPs to induce cell cycle arrest was attributed to the reduced phosphorylation of Rb [24, 25]. However, evidence that Rb is a direct substrate of CTDSPs is lacking. We show that MEFs devoid of the pocket proteins p107, p110 (Rb) and p130 arrest upon expression of CTDSP2, indicating that Rb is not strictly required for the inhibition of cell cycle progression. Microarray analysis of CTDSP2 expression-induced gene changes showed that expression of E2F7 and $p21^{Cip1/Waf1}$ are increased (figure 5), both of which are able to reduce S-phase entry

(reviewed in [10]). We have investigated the relative contribution of both of these genes and determined that $p21^{Cip1/Waf1}$ has the largest contribution to the effects we observe. Depletion of $p21^{Cip1/Waf1}$ attenuated the CTDSP2 inhibition of cell cycle progression and restored Cyclin E-bound CDK2-activity (figure 6). The upregulation of $p21^{Cip1/Waf1}$ does not seem to depend on p53, although p53 is transiently activated in response to CTDSP2 expression (figure 7). It is plausible that $p21^{Cip1/Waf1}$ is upregulated due to activation of SMAD2/3, which has been reported for CTDSP2 [19-22]. However, our microarray data reveals SMAD2 and SMAD3 are strongly downregulated, as well as several TGFβ-SMAD non-cell specific target genes including SMAD7 and SKIL (reviewed in [60]). Preliminary data confirm that EGF/TPA-induced phosphorylation of the SMAD2 linker region, but not TGFβ-induced SXS motif phosphorylation, are inhibited by CTDSP2. However, basal phosphorylation of both sites is inhibited by CTDSP2 expression and SMAD7 mRNA levels decrease strongly within 4 hours after doxycycline-induced expression of CTDSP2 (data not shown).
In summary, we provide evidence for a new target gene of FOXO proteins, regulated in a broad set of cellular contexts. Furthermore, we confirm and refine previous findings with respect to the regulation of cell cycle progression by CTDSP2 and point to $p21^{Cip1/Waf1}$ as a specific mediator of these effects.

Materials and methods

Tissue culture

HEK293T (ATCC CRL-11268), NIH/3T3 (ATCC CRL-1658), U2OS (ATCC HTB-96) cells, wild-type MEFs and p107/p110/p130 deleted MEFs [26] and all derived lines were maintained in DMEM with 10% FBS, L-Glutamine and Penicillin/Streptomycin. RPE (ATCC CRL-4000) cells were maintained in DF12 with 10% FBS, L-Glutamine and Penicillin/Streptomycin. DLD1 (ATCC CCL-221) and derived DL23 [7] cells were maintained in RPMI-1640 with 10% FBS, L-Glutamine and Penicillin/Streptomycin.

Antibodies and reagents

Antibodies for CTDSP2 were purchased from Abcam (mAb 2230C1a and pAb ab97463). TUBA was purchased from Calbiochem. $p21^{Cip1/Waf1}$ was purchased from BD Biosciences. p53 (DO-1), Cyclin E (C-19), Cyclin E (HE-12), CDK2 (M-2), CDK6 (M-21) from Santa Cruz Biotechnology. Secondary HRP conjugated antibodies (BioRad) and Alexa680/Alexa800 conjugated antibodies (Invitrogen) were used for ECL and Odyssey respectively, according to manufacturers' instructions. LY294002 (final concentration 10μM; SelleckChem), Akt1/2 inhibitor VIII (Akti; final concentration 10μM; Santa Cruz Biotechnology), nocodazole (final concentration 250ng/mL), thymidine (final concentration 2.5mM; Sigma-Aldrich) were dissolved in DMSO. 4-hydroxy Tamoxifen (4OHT; final concentration 500nM; Sigma-Aldrich) was dissolved in 96% EtOH.

Plasmids, transfections and lentiviral transductions

Full length human CTDSP2 (NM_005730.3) was amplified from HEK293T cDNA, BamHI-EcoRI-cloned into pFakeEntry2Dummy (derived from vector pAZ1; containing extended MCS) and sequence verified. The D98E/D101N mutant of CTDSP2 ($CTDSP2^{pd}$), which lacks phosphatase-activity [15], was generated following the Strategene Quickchange protocol. Plasmid pENTR4-V5-LUC has been previously described (Addgene plasmid 19135) [27]. Using Gateway LR clonase (Invitrogen), V5-Luc, CTDSP2 and $CTDSP2^{pd}$ were transferred into

pInducer20 (Addgene plasmid 44012) [28]. Protein expression was induced using sterile filtered doxycycline (Sigma-Aldrich) at a final concentration of 1µg/mL for 24 hours unless stated otherwise.
siRNA SmartPOOLs against hE2F7, hTp53 and hCDKN1A ($p21^{Cip1/Waf1}$) were all purchased from Fisher-Dharmacon and reverse transfected using Hyperfect (Qiagen) with RNA-Hyperfect ratio 40pmol:5µL.
Cell lines expressing different GFP-tagged FOXO isoforms were established by transfecting UTR [29] cells with pBIOPSF-DEST (modified from [30] to Gateway destination vector by F. Zwartkruis) containing full length hFOXO1 (NM_002015.3), hFOXO3 (NM_001455.3) or hFOXO4 (NM_005938.3) using ExtremeGene (Roche). DNA:ExtremeGene ratio 1µg:3µL. Polyclonal lines were selected using 200µg/mL Zeocin (Invitrogen) and monoclonal lines A13, B13 and C13 expressing comparable levels of Tet-inducible GFP-hFOXO1, GFP-hFOXO3 and GFP-hFOXO4 respectively, were established from these.
Lentiviral particles were generated in HEK293T as previously described [31]. Transient transfection of HEK293T cells was mediated by MaxPEI (Polysciences Inc.) with DNA-PEI ratio 1:3µg. Established polyclonal cell lines were selected for three weeks using 400-600µg/mL G418 (Invitrogen).

Cell cycle profiling and viability assays

S-phase cells were detected using FITC-BrdU staining kit (BD Biosciences) according to the manufacturer's instructions. For all cell lines used, BrdU was incubated for 30 min at 37°C. Alternatively, DNA content was measured using propidium iodide (PI; Sigma-Aldrich) staining. For any FACS analysis, medium was collected, cells were washed once with PBS (which was also collected) and then trypsinised. Using the collected medium, cells were resuspended, collected by centrifugation at 300g after which the supernatant was discarded. If used for BrdU-staining, cells were washed with 1mL PBS, collected by centrifugation, the PBS discarded and further processed according to the manufacturers' instructions. If used for PI DNA content measurement, cells were resuspended by flicking cells and immediately fixed using 70% EtOH while vortexing. Before FACS analysis, cells were incubated with 20µg/mL PI and 0.2µg/mL RNAse A (Sigma) for 30 minutes at 37°C.
Cell viability was measured by the ability of live cells to exclude PI. Cells were collected as described above, but kept on ice after first centrifugation. Collected cells were resuspended in PBS with 20µg/mL PI and immediately analysed for PI staining with the FACS. For all samples, 10000 cells were counted on a BD Calibur (BD Biosciences) and analysed using the provided software. For both BrdU-stained as well as PI-staining for DNA content measurements, multi-cell-aggregates as well as sub-G1 cells were discarded for the analysis and shown percentages G0/G1, S (when applicable) and G2/M populations were expressed as percentage of total live cells (without sub-G1). FACS data presented are mean and S.D. of three biological experiments.

RNA extraction, qPCR and western blotting

RNA was extracted using RNA easy kit (Qiagen). cDNA was synthesised using iScript kit (BioRad) with 0.5µg RNA input. qPCR was performed using FastStart SYBRgreen mix (Roche) in CFX96 Real-Time Detection System (BioRad) and analysed using the software provided by the manufacturer. Expression levels were normalised to hTUBA1A or mHMBS (PBGD) unless stated otherwise. qPCR data presented are mean and S.D. (technical and biological)

of three biological replicates. For each primer set, average C(t) value of the control samples was used to calculate fold change for all wells. Average and technical standard deviation were calculated per sample measured and S.D. propagated from normalisation gene measurements to target gene measurements. S.D. calculations for biological replicates was performed according to [32], equation 5.38. Western blotting was performed according to standard laboratory protocols. Briefly, cells were directly lysed in Leammli buffer, boiled for 3 minutes and separated on denaturing SDS-PAGE gels. Proteins were transferred to PVDF membrane, blocked with 2% BSA in TBS and strained with desired antibodies.

Microarrays

DL23 cells were mock (EtOH) or 4OHT stimulated for 8 or 24 hours before RNA extraction. DLD1 RNA was used as common reference for 24 hour stimulated DL23 samples (all vs. DLD1), whereas 8 hour stimulated DL23 samples were directly compared (mock vs. 4OHT). Fold changed were calculated for DL23+mock vs. DL23+4OHT. HEK293T (set 2) cells were transiently transfected with empty (EV) vector or Flag-FOXO4 using Fugene (Promega) with DNA:Fugene ratio 1:2.5µL. After 48 hours, cells were collected for RNA extraction. RNA of non-transfected HEK293T cells was used as common reference (all vs. HEK293T non-transfected). Fold change was calculated for HEK293T+EV vs. HEK293T+Flag-FOXO4. Parental U2OS, U2OS-CTDSP2 and U2OS- CTDSP2^{pd} cells were mock or doxycycline stimulated for 48 hours before RNA extraction. For hybridisations, mock treated parental U2OS RNA was used as common reference (all vs. U2OS w/o doxycycline). Fold change was calculated for U2OS+doxycycline vs. U2OS-CTDSP2+doxycycline or U2OS- CTDSP2^{pd}+doxycycline.

RNA integrity was evaluated using 2100 Bioanalyzer (Agilent) and all samples used had scores higher than 9. Microarray experiments were performed 4 times. Of each sample group two samples were labelled with Cy5 and co-hybridised with reference RNA labelled with Cy3, and two samples were labelled and hybridised in the opposite way (dye-swapped). DL23 and HEK293T samples were hybridised to Operon V2 collection spotted onto Codelink slides (Codelink), representing 21329 H. sapiens 70-mer probes. U2OS samples were hybridised on human whole genome gene expression microarrays V2 (Agilent) representing 34127 H. sapiens 60-mer probes in a 4x44K layout. cDNA synthesis, cRNA amplification, labelling, quantification, quality control and fragmentation were performed with an automated system (Caliper Life Sciences NV/SA), starting with 3µg total RNA from each sample, all as previously described in detail [33]. Microarray hybridisation and washing were performed with a HS4800PRO system (Tecan, Benelux) using 1000 ng, 1-2% Cy5/Cy3 labelled cRNA per channel as described [33]. Slides were scanned on an Agilent G2565BA scanner (Agilent) at 100% laser power, 30% PMT. After automated data extraction using Imagene 8.0 (BioDiscovery), Loess normalisation was performed on mean spot-intensities according to [34]. Gene-specific dye bias was corrected by a within-set estimate as described in [35]. Data were further analysed using described MAANOVA [36], modelling sample, array and dye effects in a fixed effect analysis. P-values were determined by a permutation F2-test, in which residuals were shuffled 5000 times globally. Gene probes with $p < 0.05$ after family wise error correction (FWER) were considered significantly changed. In cases of multiple probes per gene, the values from the most 3' probe were used. All data are deposited in GEO/ArrayExpress. References are in main text or above (to be added for the sets presented in this paper; presently submitted).

ChIP

ERα (M-20) and FOXO3 (H-144) were purchased from Santa Cruz Biotechnology. Rabbit anti-GFP was a generous gift from Prof.dr. G. Kops (UMC Utrecht, The Netherlands). The ChIP protocol has been described previously for DLD1/DL23 cells [37]. A13/B13/C13 cells treated for 16 hours with doxycycline and for 30 min with Akti prior to crosslinking, after which we followed the above described protocol [37].

In vitro kinase assays

Active CDK complexes were precipitated from cells were treated with doxycycline for 24 hours, washed twice with cold PBS and lysed in 50mM Tris-HCl pH 7.5, 1% NP-40, 10% glycerol, 100mM NaCl and protease/phosphatase inhibitors. Lysates were collected and cleared by centrifugation. Supernatants were incubated for two hours with antibody pre-coupled ProtA beads (Sigma-Aldrich), washed three times with lysis buffer and to times with kinase buffer 25mM Tris-HCl pH 7.5, 10mM $MgCl_2$ and 1mM DTT. Beads were incubated for 30 minutes at 30°C in kinase buffer supplemented with 100μM ATP, 10μCi γATP and 1μg/reaction recombinant H1 (Millipore) or 0.5μg/reaction recombinant GST-Rb (Sigma-Aldrich). Protein were separated on SDS Page and gels were briefly fixed in 30% methanol/10% acetic acid and wrapped in Saran-wrap before X-film exposure.

Dataset mapping and heat map

DL23 and HEK293T of our institute were mapped on microarray Probe ID. Other datasets were mapped on Ensembl ID; BaF3 datasets were provided with human Ensembl ID and HUVEC, RCC4 and UMCR2 dataset Entrez ID was converted to Ensembl ID using a Biomart generated mapping table. Data clustering and heat maps were generated using R 3.0.3 using gplots (CRAN).

Author contribution

DK and BB designed the experiments. DK and PP carried out most of the experiments. AE, LS and MT carried out ChIP experiments. MB and AE cloned the GFP-FOXO1/3/4 constructs, established and characterised A13/B13/C13 cell lines. FH supervises microarray facility where MGK and DL carried out microarray hybridisations and analysis. Microarray analysis pipeline was designed by PL, who also helped with statistical analysis of the data. DK and BB wrote the manuscript.

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CHAPTER 4

CTDSP2 interacting proteins and gene regulation

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Abstract

We have described Forkhead box-O 3 (FOXO3) regulation of carboxyl-terminal domain small phosphatase 2 (CTDSP2) expression and have shown that expression of CTDSP2 causes cell cycle arrest, partially through the regulation of cyclin-dependent kinase (CDK)-inhibitor $p21^{Cip1/Waf1}$ expression [Chapter 3]. However, it is unclear how CTDSP2 precisely regulates gene expression. CTDSP2 has been shown to dephosphorylate, and thereby inhibit, RNA polymerase II (RNAPII) [1]. However, for lack of any identified structural domain(s) other than its phosphatase domain, it is unlikely that CTDSP2 binds to DNA or to phosphorylated RNAPII directly. Indeed, it was shown that CTDSP1 requires the RE1-silencing transcription factor (REST)/neuron-restrictive silencer factor (NRSF) complex for it to regulate neuronal gene expression. To gain further insight into possible mechanism(s) whereby CTDSP2 regulates gene expression and to delineate the events that cause cell cycle arrest and $p21^{Cip1/Waf1}$ upregulation, we have employed a proteomic approach to identify novel interacting proteins of CTDSP2. We identified a significant number of novel potentially interacting proteins for CTDSP2 and of these we selected candidates for further analysis. E2F family member E2F7-binding to CTDSP2 was confirmed by co-immunoprecipitation assays, yet the interaction between CTDSP2 and E2F7 appeared not to mediate the effects of CTDSP2 on cell cycle progression and/or $p21^{Cip1/Waf1}$ expression. In addition to E2F7, neither insulin receptor substrate 4 (IRS4), cell division cycle associated 3 (CDCA3) nor small body size/mothers against decapentaplegic 2/3 (SMAD2/3) appear to be required for CTDSP2-induced upregulation of $p21^{Cip1/Waf1}$ expression and/or cell cycle arrest. However, during the course of our study we noted a contribution of the Ras-Raf-MEK-ERK signalling pathway, to CTDSP2 signalling and regulation of $p21^{Cip1/Waf1}$ expression, but not cell cycle regulation. Further experiments are required to confirm the direct involvement of Ras and to understand how the (Ras-Raf-)MEK-ERK pathway is activated in response to CTDSP2 expression.

Introduction

The four members of the Forkhead box-O (FOXO) family of transcription factors (FOXO1, FOXO3, FOXO4 and FOXO6) have been shown to regulate gene expression and thereby to regulate a number of processes, including control of cell cycle progression, apoptosis and life span. Extensive documentation exists of particular genes regulated directly or indirectly by FOXO family members attributing by themselves for phenotypes observed after FOXO-activation (reviewed in [2]). However, a common gene signature that can explain the diverse effects of FOXO-activity remains enigmatic, although we have recently published a short list of genes that are consistently regulated by FOXO3 and FOXO4, at least in a restricted number of cell lines [Chapter 3]. One of relatively few target genes we identified is carboxyl-terminal domain small phosphatase 2 (CTDSP2). CTDSP2 has been implicated in a number of processes including regulation of phosphorylation of the carboxyl-terminal domain (CTD) of RNA Polymerase II (RNAPII) core-subunit RBP1 [1, 3]. Phosphorylation of the RBP1 CTD, which in mammals consists of 52 repeats of a heptapeptide with consensus sequence $Y_1S_2P_3T_4S_5P_6S_7$, changes co-ordinately during gene transcription by RNAPII. Generally, the CTD of RPB1 has high levels of Ser5 phosphorylation when RNAPII is at 5' end of a gene, while gradually being replaced for phosphorylation of Ser2 while RNAPII progresses towards the 3' end of the gene. Both phosphorylated Ser5 and Ser2 can attract complexes that are relevant to the position of RNAPII, i.e. Ser5 was shown to be important for recruitment of the capping machinery and Ser2 for recruitment of the poly-A ligating machinery (reviewed in [4]). Phosphorylation of Ser5 precedes initiation of transcription and indeed CTDSP1 was shown to inhibit gene expression [3, 5]. However, CTDSP1 and CTDSP2 do not have harbour any obvious structure or domain that can mediate an interaction with RNAPII or DNA for that matter. This is in contrast to the functionally related CTDP1, which harbours a breast cancer associated 1 (BRCA1) carboxyl-terminal (BRCT) domain [6], a known phospho-serine/threonine-binding domain. Instead, for localisation, CTDSP1 was shown to bind to the repressive, DNA-binding RE1-silencing transcription factor (REST)/neuron-restrictive silencer factor (NRSF) complex [5] and CTDSP2 was shown to bind to the androgen receptor (AR) [3]. We and others have shown that ectopic expression of CTDSP2 results in reduced cell cycle progression [7, 8] and our recent work has implicated $p21^{Cip1/Waf1}$ regulation in this [Chapter 3]. AR has also been reported to increase $p21^{Cip1/Waf1}$ expression (reviewed in [9]), making it a candidate to mediate CTDSP2 regulation of $p21^{Cip1/Waf1}$. However, AR expression in U2OS cells, for which we observe CTDSP2-dependent cell cycle regulation, is low and even further decreased upon expression of CTDSP2 (2.10 fold down [Chapter 3]). Furthermore, expression of AR target gene kallikrein-related peptidase 3 (KLK3, also known as PSA), which was used as primary readout by Thompson et al. [3], is not affected by expression of CTDSP2 or phosphatase-dead $CTDSP2^{pd}$ (data not shown). On the other hand, gene suppression by REST/NRSF is continuous in non-neuronal tissues, thus it is expected that effects mediated by CTDSP2 binding to REST/NRSF are observed upon depletion of CTDSP2 or overexpression of a (dominant negative) phosphatase-dead $CTDSP2^{pd}$ as described [5], but not with wild-type CTDSP2 expression. However, our microarray data [Chapter 3] revealed no CTDSP2 or phosphatase-dead $CTDSP2^{pd}$-induced changes in expression of previously reported CTDSP2-REST/NRSF repressed genes [5], including *Drosophila melanogaster* embryonic lethal, abnormal vision-like (ELAVL) family members, sodium channel, voltage gated, type II alpha or beta (SCN2A/B) and glutamate receptor or ionotropic, N-methyl D-aspartate 1 (GRIN1) (data not shown). Taken together, CTDSP2 binding to either REST or AR does not appear relevant for mediating

CTDSP2-induced gene expression changes in our experimental setting, and thus are not likely to be responsible for increased $p21^{Cip1/Waf1}$ expression and/or cell cycle arrest in U2OS cells. Therefore, we wished to identify novel interacting and DNA-localising proteins for CTDSP2, which may provide further insight into the regulation of cell cycle progression and/or $p21^{Cip1/Waf1}$ in response to CTDSP2 expression. Using mass spectrometry, we identified a number of novel CTDSP2 interacting proteins. Of these we analysed in further detail adenovirus E2 promoter binding factor 7 (E2F7), insulin receptor substrate 4 (IRS4) and cell division cycle-associated protein 3 (CDCA3) as potential binding partners of CTDSP2 to mediate regulation of $p21^{Cip1/Waf1}$ and cell cycle progression.

In the case of negative regulator of E2F signalling E2F7 (reviewed in [10]), we confirm binding between CTDSP2 and E2F7 but have not been able to uncover the function of this, neither in gene expression regulation by E2F7 combined with CTDSP2 nor in cell cycle regulation [Chapter 3]. Secondly, as a possible consequence of IRS4-binding and/or regulation (reviewed in [11]), CTDSP2 expression results in increased PKB phosphorylation. However, this does not involve changes in feedback-mediated IRS stability (reviewed in [12]) and occurs also in the absence of growth factors. Furthermore, the activation of PKB contributes to CTDSP2-induced increase in $p21^{Cip1/Waf1}$ protein level, but not to elevated mRNA expression, and is probably not required for the cell cycle arrest, as E2F1 mRNA levels are decreased in response to CTDSP2 expression, regardless of PKB inhibition. The last potential CTDSP2 interacting protein that we have studied is the G2-M transition regulating F-box protein CDCA3 [13]. However, in our experimental setup, CDCA3 does not have an overt function in cell cycle progression, nor does it play a role in CTDSP2-induced cell cycle arrest or ubiquitination of CTDSP2 itself. In addition to our own data on CTDSP2 interacting proteins, we have explored a possible role for transforming growth factor β (TGFβ) signalling in CTDSP2-induced upregulation of $p21^{Cip1/Waf1}$, since inhibitory phosphorylation of small body size/mothers against decapentaplegic 2/3 (SMAD2/3) has been shown to be decreased by CTDSP2 [14, 15]. However, we conclude that basal activity of the TGFβ signalling network is decreased upon expression of CTDSP2 and therefore unlikely to be responsible for $p21^{Cip1/Waf1}$ regulation in our experimental setup. Interestingly, several controls indicated that CTDSP2 expression results in strong activation of Ras. For example, CTDSP2 expression results in increased phosphorylation of extracellular signal regulated kinase (ERK), which is commonly used as a readout of activity of the Ras-Raf-MEK-ERK pathway. In addition to ERK phosphorylation, strong Ras-activation can result in PKB-activation and increased $p21^{Cip1/Waf1}$ expression (reviewed in [16]). Indeed, inhibition of MEK inhibits CTDSP2-induced upregulation of $p21^{Cip1/Waf1}$, but surprisingly did not influence CTDSP2-induced cell cycle arrest. Taken together, we have explored several proteins that may directly influenced by CTDSP2 and could potentially play a role in CTDSP2 regulation of cell cycle progression and $p21^{Cip1/Waf1}$ expression. Thus far, direct or indirect activation of Ras seems to most likely mechanism for changes in $p21^{Cip1/Waf1}$ expression, but further experiments are required to substantiate this conclusion.

Results and discussion

Novel binding partners of CTDSP2

CTDSP family members have been shown to directly dephosphorylate the CTD of RBP1 [1, 3]. However, unlike related CTDP1, CTDSP family members do not have any obvious structure to localise them to RBP1. Instead, CTDSP1 has been shown to interact with the REST/NRSF DNA-binding complex, which represses neuronal gene expression in non-neuronal

Gene	Total (fwd)	Total (rev)	Nuclear (fwd)	Nuclear (rev)
CTDSP2	3.51	-3.44	2.91	-7.38
CDCA3	3.22	-3.59	ND	-3.14
ZNF281	ND	ND	1.49	-1.06
E2F7	ND	ND	0.51	-0.94
TARDBP	0.49	-0.17	0.92	-0.46
PPIH	ND	ND	0.68	-0.35
IRS4	1.98	-1.99	1.42	-0.43
NCBP1	0.47	-0.62	0.49	-0.66
SKP1	0.47	-0.12	-0.28	0.35

Table 1: list of putative CTDSP2 interacting proteins
Ectopically expressed CTDSP2 was immunoprecipitated and co-enriched proteins were identified as described in main text. Depicted are fold enrichment over median value of each experiment. *Total* columns contain enrichment values of proteins co-precipitated with CTDSP2 from whole cell lysates; *Nuclear* columns contain enrichment values of proteins co-precipitated with CTDSP2 from nuclear extract. Ratios are always calculated by dividing heavy label intensity over light label intensity and log2 transformed. *Fwd* and *rev* indicate the labelling as follows: fwd has heavy labelled CTDSP2 with light labelled empty vector (enrichment values > 0), rev has light labelled CTDSP2 and heavy labelled empty vector (enrichment values < 0). ND indicates 'no data'. CCT1 and CYP20 are enriched with CTDSP2 precipitated from of nuclear extract and fulfil our criteria (see materials and methods), but were excluded from this table for clarity.

tissues [5]. Furthermore, CTDSP2 has been shown to bind to the androgen receptor and repress androgen-dependent transcription [3]. CTDSP1, CTDSP2, CTDSPL and CTDSPL2 share only their carboxyl-terminal phosphatase domain whereas the amino-terminal part is unstructured and divergent between isoforms. Taken together, the amino-terminus could serve to target different isoforms to different gene sets dependent on the interacting DNA-binding protein. To identify DNA interacting proteins binding to CTDSP2, we transfected empty vector or Flag-tagged CTDSP2 in SILAC labelled HEK293T (two experiments with label swap), precipitated Flag-CTDSP2 with interacting proteins and quantified relative abundance of precipitated proteins using mass spectrometry. For our initial experiment, we precipitated CTDSP2 from whole cell lysates. In a second experiment, we separated the nucleoplasm from cytoplasm to allow for better identification of low abundant DNA-binding proteins. Using our criteria (see materials and methods) we found 139 proteins enriched together with CTDSP2 when precipitated from total cell lysates and 7 proteins enriched with CTDSP2 when precipitated from nuclear extract. Several potentially interesting putative CTDSP2-binding proteins, including E2F7, IRS4 and CDCA3 are listed in table 1. We chose to investigate these three potential interacting proteins further, because E2F7 is a repressive transcription factor involved in cell cycle regulation (reviewed in [10]), IRS4 is involved in PKB signalling (reviewed in [23]) and CDCA3 in mitotic entry [13].

E2F7 and CTDSP2 function

The E2F7 transcription factor belongs to the E2F family of transcription factors, which are important in regulation of S-phase entry. E2F target genes, many of which are involved in DNA replication, typically have an E2F box in their promoter and can be activated or repressed, dependent on the E2F family member bound. Whereas E2F1, E2F2 and E2F3a promote transcription of E2F target genes, E2F3b, E2F4, E2F5, E2F6, E2F7 and E2F8 have been shown to repress transcription from these genes in various ways (reviewed in [10]). In contrast to other

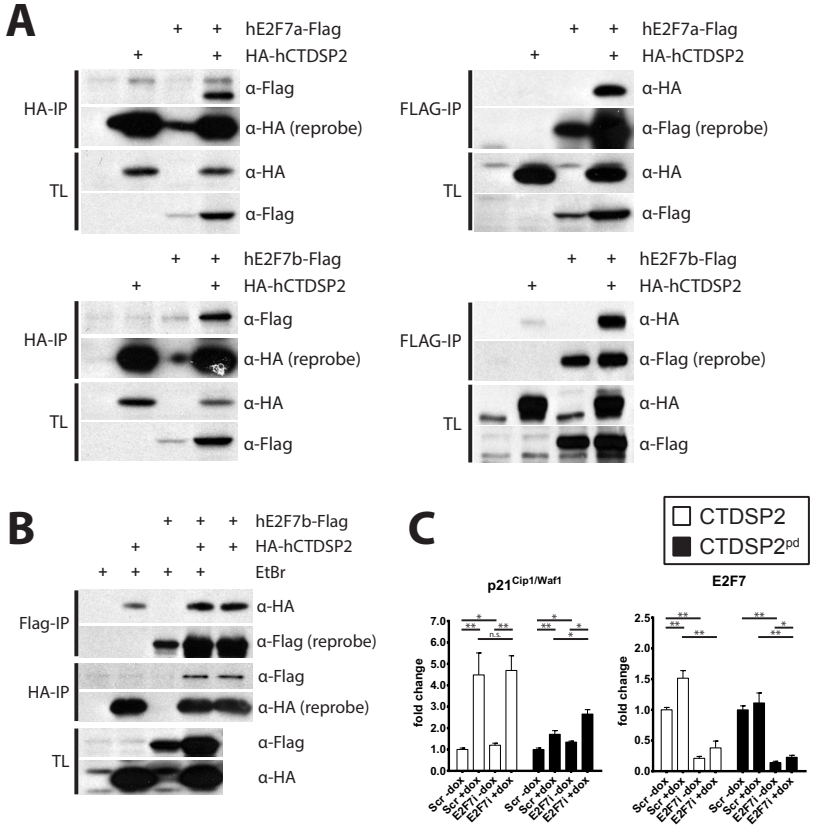


Figure 1: CTDSP2 interacts with E2F7, but does not collaborate to induce p21$^{Cip1/Waf1}$
A) HEK293T cells were transfected with empty vector and either or both HA-CTDSP2 and E2F7a/b-Flag and lysates were used in Flag or HA immunoprecipitations. Both E2F7a-Flag and E2F7b-Flag co-precipitate with HA-CTDSP2 and similarly HA-CTDSP2 co-precipitates with both E2F7a-Flag and E2F7b-Flag. B) Cells were transfected and used for immunoprecipitations as in A) but in the presence of EtBr, which inhibits DNA-mediated interactions, indicating that CTDSP2-E2F7 binding is not DNA-mediated. C) Extended analysis samples Chapter 3 – figure 6: knockdown of E2F7 in U2OS cells expressing either wild-type of phosphatase-dead CTDSP2 (left graph) does not inhibit CTDSP2-induced upregulation of p21$^{Cip1/Waf1}$ mRNA (right graph). TL: total lysate. T-tests: * $p < 0.05$, ** $p < 0.005$; indication absent or n.s. means $p > 0.05$.

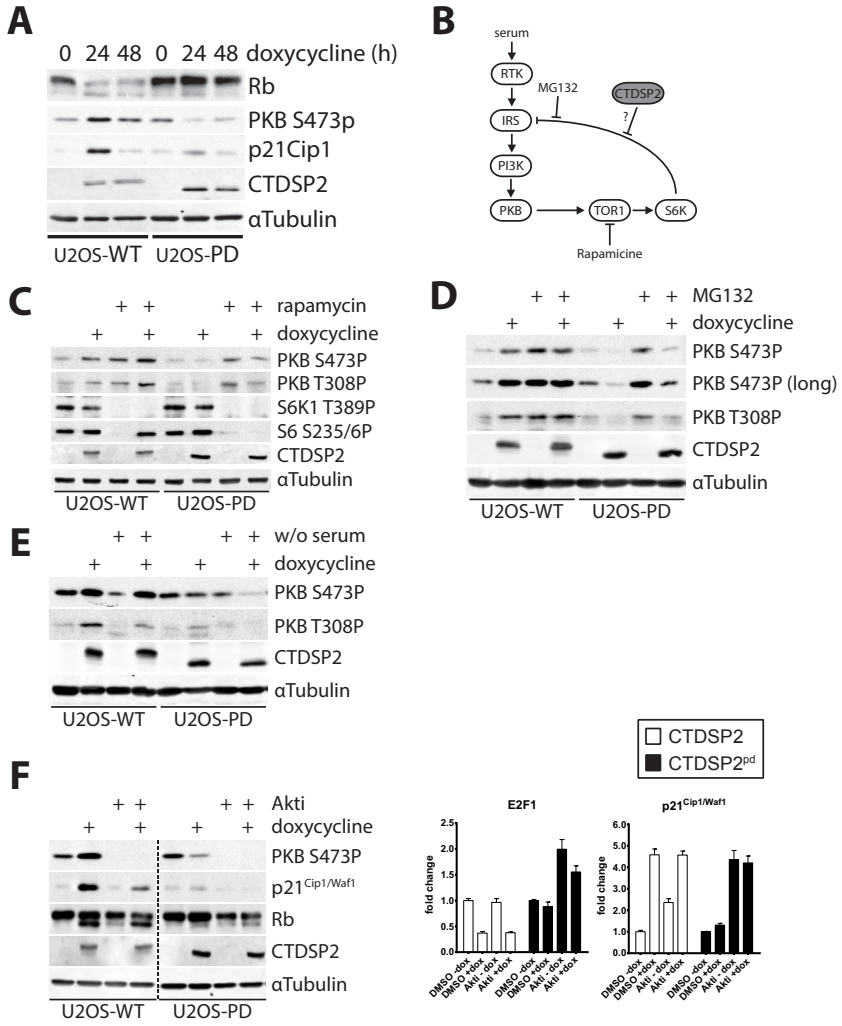
E2F family members the closely related E2F7 and E2F8 do not require additional proteins for DNA-binding per se [24, 25]. E2F7 has two major splice variants termed E2F7a and E2F7b, the latter being the shortest [17]. Furthermore, E2F7 has been described to be a p53 target gene and was proposed to be involved in DNA damage-induced G1 arrest in this context [26, 27]. Interestingly, we observe E2F7 upregulation upon doxycycline-induced CTDSP2 expression in U2OS cells [Chapter 3]. Thus, CTDSP2 might play a role in E2F7-induced gene repression, being recruited to E2F promoters by E2F7 and decreasing CTD Ser5 phosphorylation of RBP1 at these locations.

To confirm binding between E2F7 and CTDSP2, we expressed Flag-E2F7a or Flag-E2F7b together with HA-CTDSP2 and immunoprecipitated these proteins though either the Flag or HA epitope, in separate experiments. As shown in figure 1A, both E2F7a and E2F7b co-precipitate with CTDSP2 and vice versa. Furthermore, this interaction seems not to depend

on DNA as it is maintained in the presence of ethidium bromide (figure 1B), which inhibits DNA-mediated interactions [28]. However, luciferase, qPCR and RNAPII-ChIP experiments did not show consistent changes in E2F target gene expression when either E2F7 alone or E2F7 together with CTDSP2 was overexpressed or expression of either gene was reduced using siRNA (data not shown). Furthermore, we have shown that E2F7 does not play a major role in CTDSP2-mediated reduction in cell cycle progression [Chapter 3]. Lastly, we investigated the role of E2F7 in the observed increase of $p21^{Cip1/Waf1}$ expression observed in response to CTDSP2 expression. E2F7 binds to the $p21^{Cip1/Waf1}$ promoter [29] and has been described to suppress $p21^{Cip1/Waf1}$ expression [30], although this could be the result of increased E2F1-activity [31], which we observe upon E2F7 knockdown [Chapter 3]. Nonetheless, it remains possible that CTDSP2 represses E2F7-activity, rather than collaborate with it to suppress gene expression, and by that increases $p21^{Cip1/Waf1}$ expression. However, we do not observe differences in CTDSP2-induced increase in $p21^{Cip1/Waf1}$ mRNA levels when comparing control and E2F7 depleted cells (figure 1C). In summary, E2F7 and CTDSP2 can bind to one another but the physiological relevance remains to be identified. In addition, E2F7 appears not required for CTDSP2-induced upregulation of $p21^{Cip1/Waf1}$ expression.
It is possible that E2F7 and CTDSP2 collaborate to repress gene expression, but interpretation of these data is complicated by the observed regulation of $p21^{Cip1/Waf1}$ by CTDSP2 [Chapter 3], which in return also affects E2F signalling. On the other hand, CTDSP2 could modulate phosphorylation of E2F7, as it has been described for SMAD2 proteins [14, 15, 32, 33] and Snail [34]. Interestingly, the latter study showed that CTDSP2 and Snail co-expression greatly increased the stability of Snail and we observe a similar effect on E2F7 when it is co-expressed with CTDSP2 (figure 1: Flag total lysates) but have not further elaborated on this.

IRS4; CTDSP2 regulates PKB-activity

Another CTDSP2 interacting protein identified was IRS4, which was found in both experiments performed (table 1). To investigate a possible link between CTDSP2 and IRS4, we have interrogated the influence of CTDSP2 on PKB-activation as this in principle should reflect CTDSP2-induced changes in IRS4-mediated PI3K-activation (reviewed in [23]). Interestingly, CTDSP2 expression in U2OS cells results in increased phosphorylation of PKB Ser473 and expression of phosphatase-dead $CTDSP2^{pd}$ represses PKB phosphorylation, suggesting the phosphatase-dead $CTDSP2^{pd}$ variant to act in a dominant-negative manner in this context (figure 2A). These observations fit a model in which CTDSP2 counters inhibitory phosphorylation on IRS family members, such as those resulting from mammalian target of rapamycin complex 1 (mTORC1) and ribosomal protein S6 kinase (S6K)-activity (reviewed in [12]), although this has not been described for IRS4 specifically. S6K is a direct target of mTORC1, the activity of which is indirectly increased by PKB (reviewed in [23]). Both S6K and mTORC1 have been shown to phosphorylate IRS1 and IRS2 on multiple residues and this results in increased proteasome-dependent degradation of IRS1 (reviewed in [11]). Consequently, mTORC1 and S6K are part of a negative feedback mechanism operating to control PKB-activity. To test this model (figure 2B) in which CTDSP2 inhibits the IRS degradation-induced by active mTORC1-S6K, we blocked mTORC1-activity by rapamycin or (IRS) degradation by the proteasome-inhibitor MG132 treatment. Both rapamycin and MG132 elevate PKB Thr308 and Ser473 phosphorylation as expected (figure 2C and 2D). However, in disagreement with the proposed model, only MG132 treated cells fail to further increase PKB phosphorylation upon CTDSP2 expression and expression of phosphatase-dead $CTDSP2^{pd}$ still results in decreased phosphorylation



(figure 2D). In addition, if CTDSP2 affects IRS4 stability and thereby PKB-activity status, the CTDSP2 effect on PKB-activity would require active growth factor signalling, but the presence of serum contained growth factors is not strictly required for CTDSP2 regulated PKB-activation (figure 2E). Concluding, CTDSP2 enhances PKB phosphorylation, but this does probably not involve changes in IRS stability. Of note, although IRS family members are often considered redundant (e.g. [35]) it was recently published that IRS4 can activate PKB, even in the absence of growth factors [36]. Thus, further experiments using IRS4 depletion would be required to rule out IRS4 involvement in PKB-activation in response to CTDSP2 expression.

We have investigated the role of PKB-activation in the increased expression of $p21^{Cip1/Waf1}$. In agreement with previous observations [37], we have previously observed that $p21^{Cip1/Waf1}$ levels are regulated by PKB [Chapter 5], although it has been shown that PKB phosphorylation of $p21^{Cip1/Waf1}$ results in cytoplasmic localisation and reduced CDK inhibition (reviewed in Chapter 1). In agreement with these observations, treatment with PKB-inhibitor Akti reduced

← Figure 2: CTDSP2-mediated increased PKB phosphorylation does not involve regulation by CTDSP2 of mTORC1-IRS-mediated negative feedback on PKB
A) Expressing of CTDSP2 (WT) or phosphatase-dead CTDSP2^{pd} (PD) in U2OS cells was induced with doxycycline for indicated time. CTDSP2 expression induces PKB Ser473 phosphorylation while phosphatase-dead CTDSP2^{pd} decreases PKB Ser473 phosphorylation, best visible after 24 hours. B) Tested model for CTDSP2-induced increase in PKB Ser473 phosphorylation. IRS family members are relocated and degraded in response to mTORC1-S6K phosphorylation of multiple serine residues of IRS1/2 (see main text for references), which may be removed by CTDSP2. RTK: receptor tyrosine kinase. C) Expression of CTDSP2 or phosphatase-dead CTDSP2^{pd} was induced for 24 hours in U2OS cells, in the presence of vehicle DMSO or mTORC1-inhibitor rapamycin. PKB Ser473 phosphorylation is increased in rapamycin treated cells, as expected. However, PKB Ser473 phosphorylation is still (further) increased by CTDSP2 expression and decreased by phosphatase-dead CTDSP2^{pd} expression in both DMSO and rapamycin treated cells. D) Expression of CTDSP2 or phosphatase-dead CTDSP2^{pd} was induced for 24 hours in U2OS cells, the last 6 hours in the presence of vehicle DMSO or proteasome-inhibitor MG132. PKB Ser473 phosphorylation is increased by treatment with MG132, as expected. Expression of CTDSP2 does not further increase PKB Ser473 phosphorylation in MG132 treated cells, in agreement with the proposed model. However, PKB Ser473 phosphorylation is still decreased by phosphatase-dead CTDSP2^{pd} in both DMSO and MG132 treated cells. E) U2OS medium was replaced with fresh medium containing 10% FBS or 0% FBS, either without or with doxycycline to induce expression of CTDSP2 or phosphatase-dead CTDSP2^{pd}, and cells were harvested 24 hours later. PKB Ser473 phosphorylation is decreased in growth factor deprived cells, as expected. However, PKB Ser473 phosphorylation is still increased by CTDSP2 expression and (further) decreased by phosphatase-dead CTDSP2^{pd} expression regardless of the presence of exogenous growth factors. F) Expression of CTDSP2 or phosphatase-dead CTDSP2^{pd} was induced for 24 hours in U2OS cells, in the presence of vehicle DMSO or PKB-inhibitor Akti. PKB-activity influences the abundance of p21$^{Cip1/Waf1}$ (presumably by direct phosphorylation – see main text), but is not responsible for increased expression of p21$^{Cip1/Waf1}$ mRNA (right graph) and PKB inhibition does not rescue CTDSP2-induced reduction of E2F1 mRNA (left graph).

the abundance of CTDSP2-induced p21$^{Cip1/Waf1}$ (figure 2F). However, PKB inhibition does not abrogate p21$^{Cip1/Waf1}$ mRNA upregulation upon CTDSP2 expression (figure 2F – left graph). Furthermore, the CTDSP2-induced decrease in E2F1 expression is not affected by PKB inhibition, suggesting that the cell cycle arrest is not dependent on PKB-activation per se (figure 2F – right graph), although we have not determined cell cycle progression in BrdU-incorporation assays. Concluding, CTDSP2-induced p21$^{Cip1/Waf1}$ expression most likely involves other mechanisms than PKB-activation, although PKB-activation contributes to p21$^{Cip1/Waf1}$ protein abundance.

CDCA3 involvement in CTDSP2 regulation or its effects

Next, we investigated the F-box containing protein CDCA3, also known as trigger of mitotic entry 1 (TOME1), which has also been shown to interact with CTDSP1 in a large scale protein-protein interaction mapping effort [38]. CDCA3 is part of an S-phase-associated kinase 1 (SKP1)/cullin 1 (CUL1)/F-box (SCF) complex involved in mitotic entry at the end of G2 [13]. SCF complexes are E3 ubiquitin ligase complexes in which the variable F-box containing adaptor protein confers substrate specificity (reviewed in [39]). CDCA3 has been reported to have similar functions as β-transducin repeat-containing protein 1/2 (βTrCP1/2) [13], which targets Wee1 for degradation in G2, an essential step in mitotic entry (reviewed in [40]). However, unlike βTrCP, CDCA3 does not bind Wee1 directly [41], although shRNA-mediated knockdown of CDCA3 reduces mitotic entry to a similar extent as βTrCP in Hela cells [42]. Indeed, we also observe SKP1, but not CUL1, enriched with CTDSP2 extracted from total lysates but depleted from nuclear CTDSP2 and in both at much lower abundance (table 1). In addition, we observe accumulation of U2OS cells in G2/M upon expression of CTDSP2 [Chapter 3]. However, upon siRNA-mediated depletion of CDCA3 (figure 3A – lower, most left graph), we do not observe large changes in cell cycle progression of U2OS cells and no effect on CTDSP2-induced cell cycle arrest or changes in distribution among the different phases (figure 3A – upper three graphs). In addition, neither CTDSP2 expression-induced changes in E2F1 nor p21$^{Cip1/Waf1}$ mRNA levels are altered by CDCA3 depletion, although depletion itself increases the abundance of both p21$^{Cip1/Waf1}$ mRNA (figure 3A – lower, right and middle graphs) and protein (figure

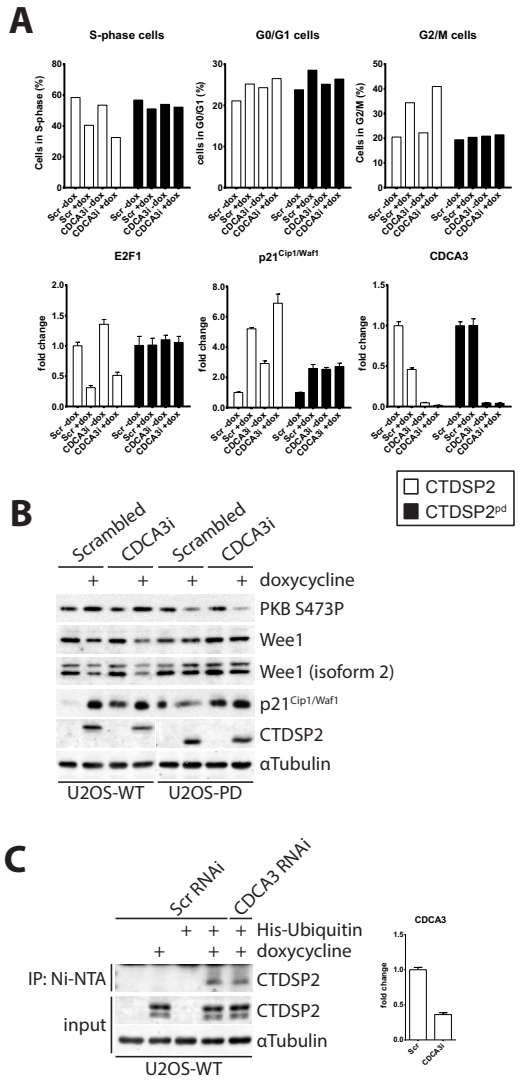


Figure 3: CDCA3 knockdown does not influence cell cycle distribution, CTDSP2-induced cell cycle arrest, CTDSP2-induced increase in $p21^{Cip1/Waf1}$ expression or CTDSP2 ubiquitination in U2OS cells
A) CDCA3 was depleted using siRNA and CTDSP2-induced as described for E2F7, $p21^{Cip1/Waf1}$ and p53 [Chapter 3]. Knockdown of CDCA3 (lower left graph) does not strongly influence cell cycle distribution without or with CTDSP2 expression (upper three graphs). CDCA3 knockdown increases both E2F1 (lower right graph) and $p21^{Cip1/Waf1}$ mRNA levels (lower middle graph), either without or with expression of CTDSP2. CDCA3 itself is decreased upon CTDSP2 expression. B) Western blot analysis to determine CDCA3 contribution to some of the other CTDSP2 expression-induced effects. In disagreement with previously published results (see main text), CDCA3 depletion does not have a strong effect on Wee1 abundance. CDCA3 depletion does not influence PKB phosphorylation and does increase basal, but not CTDSP2-induced, $p21^{Cip1/Waf1}$ expression. C) U2OS cells with inducible CTDSP2 expression were transfected with His-tagged ubiquitin and treated with doxycycline as indicated. Ubiquitinated proteins were precipitated and CTDSP2 detected using a specific antibody. CDCA3 depletion does not affect CTDSP2 ubiquitination, although the knockdown is incomplete (graph).

3B). Western blotting analysis showed that CDCA3 is not required for increased PKB phosphorylation and has only very little effect on Wee1 expression levels in our experimental setup. Nonetheless, CTDSP2 could be a target of SCF^{CDCA3} in G2 cells, thus we compared ubiquitination of CTDSP2 in control or CDCA3 depleted cells. However, despite largely reduced mRNA levels of CDCA3 (figure 3C – graph), we do not observe obvious changes in CTDSP2 ubiquitination (figure 3C – blots), although the experimental setup may require improvements. In conclusion, CDCA3 does not seem to play a significant role in either CTDSP2-induced cell cycle arrest, $p21^{Cip1/Waf1}$ mRNA upregulation or CTDSP2 ubiquitination.

Involvement of p53 in CTDSP2-induced $p21^{Cip1/Waf1}$ regulation and cell cycle arrest

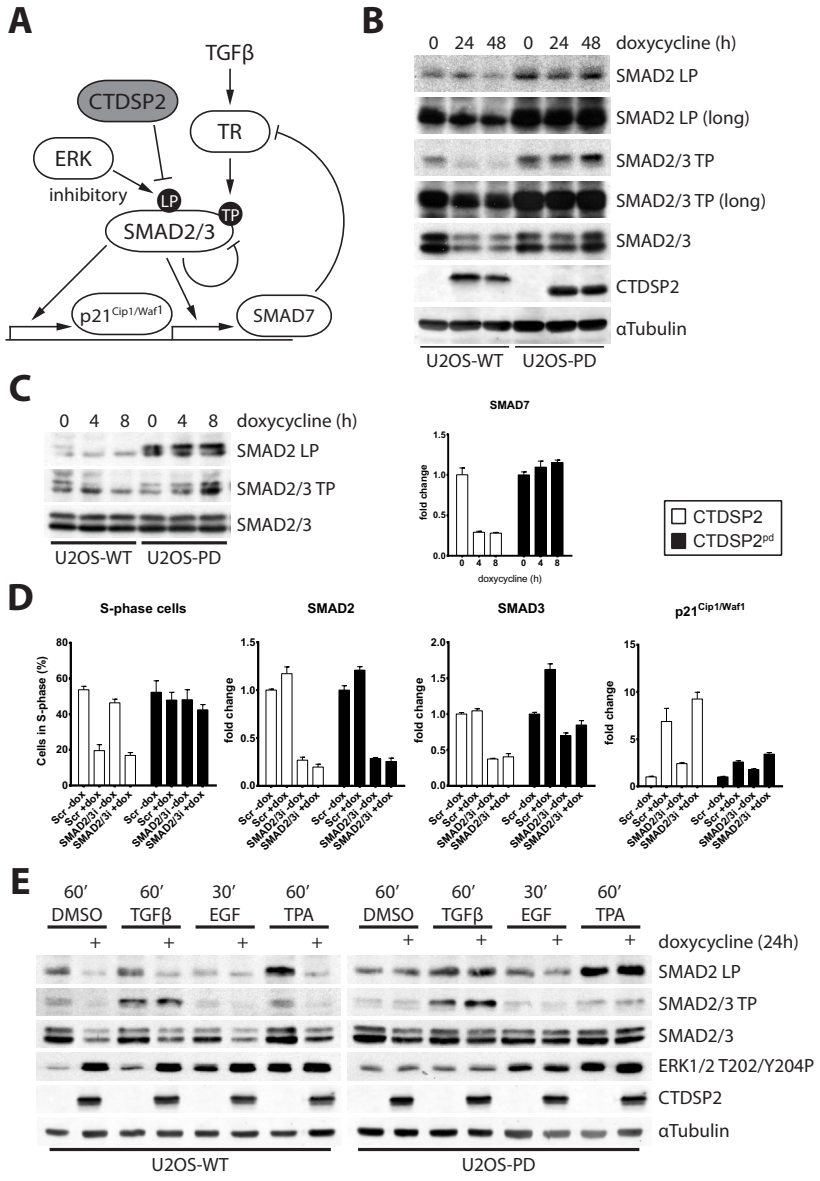
Besides the novel interacting proteins of CTDSP2 we also investigated whether any of the known regulators of $p21^{Cip1/Waf1}$ are required for CTDSP2-dependent $p21^{Cip1/Waf1}$ regulation. The best understood regulator of $p21^{Cip1/Waf1}$ expression is p53 and we have explored if p53 is responsible for CTDSP2-induced upregulation of $p21^{Cip1/Waf1}$ mRNA [Chapter 3]. However, although p53 is largely responsible for basal expression of $p21^{Cip1/Waf1}$, it does not seem to be required for the CTDSP2-induced increase in $p21^{Cip1/Waf1}$ expression [Chapter 3]. Notwithstanding, active p53 possibly contributes to the observed $p21^{Cip1/Waf1}$ increase.

CTDSP2 decreases basal TGFβ signalling

TGFβ-mediated activation of SMAD2/3 containing complexes can also regulate $p21^{Cip1/Waf1}$ expression, which requires cooperation with FOXO family members (reviewed in [43]). Interestingly, CTDSP1 and CTDSP2 have been shown to prolong TGFβ-induced activation of SMAD2/3 through inhibition of the inhibitory linker phosphorylation (LP), but not the activating phosphorylation of the carboxyl-terminal SXS motif, referred to as tail phosphorylation (TP) [14, 15]. Both members of the extracellular signal-regulated kinase (ERK) family – e.g. ERK1 and ERK2 – and cyclin-dependent kinase (CDK) family – e.g. CDK8 and CDK9 – can phosphorylate SMAD2/3 in the linker region, which primes SMAD2/3 for glycogen-synthase kinase (GSK) phosphorylation and consequent degradation (reviewed in [43]). Thus, as schematically presented in figure 4A, CTDSP2 could activate SMAD2/3-dependent transcription, possibly activated by low levels of TGFβ ligands present in tissue culture serum or low intrinsic activity of the various upstream receptors. Indeed, we observe basal LP and TP (figure 4B). However, unlike reported for TGFβ stimulated TP, basal TP is decreased by CTDSP2 expression, as well as LP and protein levels of SMAD2/3 (figure 4B). SMAD2/3-activity is tightly regulated by feedback signalling (summarised in figure 4A), which involves target gene SMAD7 stimulated proteolysis of TGFβ receptors, LP and GSK3-dependent SMAD2/3 degradation as well as dephosphorylation of the SXS motif (reviewed in [43]). Thus, decreased activity and abundance of SMAD2/3 after 24 to 48 hours of CTDSP2 expression could be the result of feedback signalling. However, already after 4 hours of doxycycline-induced CTDSP2 expression, TP and LP are decreased, as well as the expression of target gene SMAD7 (figure 4C). In addition, siRNA-mediated reduction of SMAD2/3 (figure 4D – rightmost two graphs) expression cannot prevent the CTDSP2-induced cell cycle arrest (figure 4D – left graph) or increase in $p21^{Cip1/Waf1}$ mRNA expression (figure 4D – second graph). To confirm that these effects are not specific to our experimental setup or cell lines, we tested the effects of CTDSP2 expression in U2OS cells in a similar setup as described [14, 15]. Indeed, we observe that TGFβ stimulated TP is not blocked by CTDSP2 whereas 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced LP is reduced by CTDSP2 expression (figure 4E). In conclusion, we confirmed previously reported regulation of TGFβ signalling by CTDSP2 but show that these effects depend strongly on the context e.g. TGFβ ligand concentration.

CTDSP2 expression results in ERK-activation

We noted that S6 phosphorylation is greatly increased upon CTDSP2 expression (figure 2C) and that this does not depend on the activity of mTORC1 (figure 2C) or PKB-activity (figure 5A). In addition, PKB-activation after CTDSP2 expression does not depend on the presence of exogenous growth factor (figure 2E). Lastly, we observed increased phosphorylation of ERK upon CTDSP2 expression, when we confirmed TPA or EGF stimulation of ERK phosphorylation (figure 4E). Thus, it is possible that CTDSP2 activates Ras which can, when activated sufficiently strong, activate PI3K and consequently PKB (e.g. figure 2A) (reviewed in [44]), result in ERK-mediated increased activation of ribosomal S6 kinase (RSK) family members with concomitant increased S6 phosphorylation (figure 2B) (reviewed in [45]) and lastly, indirectly increase $p21^{Cip1/Waf1}$ expression [Chapter 3], which depends on p38 mitogen activated protein kinase (MAPK) and p53 (reviewed in [16]). Furthermore, our microarray data [Chapter 3] show strong upregulation of several ErbB components, including the two ligands amphiregulin and epiregulin (respectively 51.85 and 32.22 fold up with CTDSP2 expression and 2.62 and 1.14



fold up with phosphatase-dead CTDSP2 expression), although the expression of epiregulin is very low (intensity 5.22). Indeed, when we induce expression of CTDSP2, MEK and ERK, but also c-Jun amino-terminal kinase (JNK), family members are activated (figure 5B). Thus, we tested the involvement of MEK-ERK or JNK in $p21^{Cip1/Waf1}$ and cell cycle regulation by CTDSP2, using specific inhibitors PD325901 (MEKi) and SP600125 (JNKi) for ERK and JNK respectively. In line with our hypothesis, the presence of MEKi prevents increased expression of $p21^{Cip1/Waf1}$ mRNA and, to a lesser extent, increased protein expression resulting from CTDSP2 expression (figure 5C – lower left graph). Furthermore, MEK-ERK-mediated $p21^{Cip1/Waf1}$ expression may

← Figure 4: CTDSP2 expression reduces unstimulated SMAD2/3 phosphorylation and activity, which cannot account for CTDSP2-induced increase in $p21^{Cip1/Waf1}$ expression
A) Summarising model of TGFβ signalling and its feedback regulation. TGFβ receptor family members activate SMAD2/3 by carboxyl-terminal SXS phosphorylation (TP). ERK (and CDK; see main text) family members prime SMAD2/3 for degradation by linker phosphorylation (LP). B) Expressing of CTDSP2 (WT) or phosphatase-dead $CTDSP2^{pd}$ (PD) in U2OS cells was induced with doxycycline for indicated time. CTDSP2 expression decreases both TP and LP while phosphatase-dead $CTDSP2^{pd}$ has little or no effect. C) Four or eight hour induction of CTDSP2 expression reduces LP and TP to undetectable levels and decreases expression of target gene SMAD7 by 75% (graph), indicating that the results in B) are not due to feedback signalling. D) SMAD2 and SMAD3 were depleted using siRNA and CTDSP2-induced as described for E2F7, $p21^{Cip1/Waf1}$, p53 and CDCA3 ([Chapter 3] and see above). Combined knockdown of SMAD2 and SMAD3 (middle two graph) does not abrogate CTDSP2 inhibition of cell cycle progression (right graph) or induction of $p21^{Cip1/Waf1}$ expression (left graph). E) Expression of CTDSP2 or phosphatase-dead $CTDSP2^{pd}$ was induced for 24 hours in U2OS cells and cells were left untreated, treated with TGFβ for 30 minutes, treated epidermal growth factor (EGF) for 60 minutes or treated with TPA for 60 minutes. In contrast to basal TP, TGFβ-induced TP is not inhibited by CTDSP2 expression, whereas both basal and TPA-induced LP are decreased to the same level, all in agreement with previously published results (see main text for references). EGF stimulation does not have a strong effect on LP, although ERK phosphorylation is increased in response to EGF treatment.

involve activation of JNK because JNK is also activated and JNK inhibition alone also results in mild inhibition of CTDSP2-induced upregulation of $p21^{Cip1/Waf1}$.

In contrast to attenuated $p21^{Cip1/Waf1}$ regulation, E2F1 expression levels are decreased in response to CTDSP2 expression, regardless of MEK-ERK or JNK inhibition (figure 5C – lower right graph) and indeed, we do not observe that cell cycle progression is restored in MEKi treated cells expressing CTDSP2 (figure 5C – upper right graph). Although we have previously seen that siRNA-mediated $p21^{Cip1/Waf1}$ depletion partially restores cell cycle progression in CTDSP2 expressing cells [Chapter 3], MEKi treatment does not decrease basal expression of $p21^{Cip1/Waf1}$. Possibly, the remaining CTDSP2-induced increase in $p21^{Cip1/Waf1}$ protein in the presence of MEKi, which may result from PKB-activation (see above), is sufficient for inhibition of cell cycle progression. However, it seems likely that CTDSP2 has additional effects on cell cycle progression, besides $p21^{Cip1/Waf1}$ regulation. Remarkably, MEKi and JNKi have very different effects on cell cycle phase distribution both before and after induction of CTDSP2 expression. Whereas MEKi treatment increases the length of G0/G1, JNKi treatment increases the duration cells remain in G2/M, the latter which is exacerbated by CTDSP2 expression (figure 5C – upper middle and left graph).

Activation of Ras may be responsible for the increased PKB phosphorylation, which we have discussed above. However, this cannot explain the decrease in PKB phosphorylation in response to expression of phosphatase-dead $CTDSP2^{pd}$, because also phosphatase-dead $CTDSP2^{pd}$ expression increases ERK phosphorylation, albeit to much lower levels (figure 5B). Mild activation or inactivation of Ras in response to expression of phosphatase-dead $CTDSP2^{pd}$ would provide an explanation for our observations that cancer cell lines with oncogenic endogenous Ras (e.g. DLD1) or U2OS cells expressing with $HRas^{G12V}$, respond strongly to expression of phosphatase-dead $CTDSP2^{pd}$ (unpublished observations). However, actual measurements of changes in Ras-activity in response to either CTDSP2 or phosphatase-dead $CTDSP2^{pd}$, are required substantiate these claims. Taken together, CTDSP2-induced expression of $p21^{Cip1/Waf1}$ mRNA requires activation of ERK, while changes in $p21^{Cip1/Waf1}$ protein level are subject to additional regulation. It will be very interesting to determine changes in GTP loading if Ras upon expression of CTDSP2 and phosphatase-dead $CTDSP2^{pd}$. Furthermore, we will determine CTDSP2-induced changes in $p21^{Cip1/Waf1}$ expression when blocking Ras with ectopic expression of its endogenous inhibitor Ras GTPase activating protein 1 (RasGAP1), which should also result in attenuation of PKB-activation.

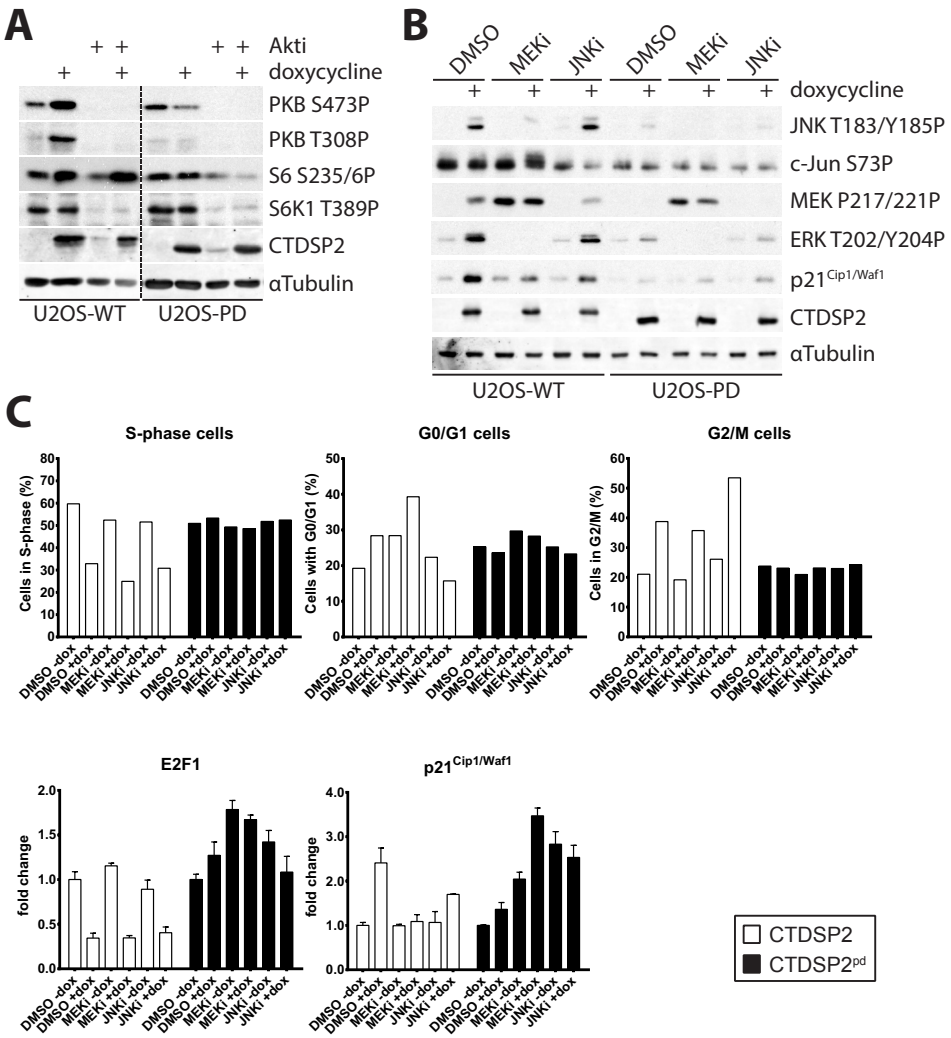


Figure 5: CTDSP2 expression results in increased JNK, MEK and ERK phosphorylation and inhibition of the latter abrogates CTDSP2 expression-induced upregulation of $p21^{Cip1/Waf1}$

A) Expression of CTDSP2 (WT) or phosphatase-dead $CTDSP2^{pd}$ (PD) was induced for 24 hours in U2OS cells, in the presence of vehicle DMSO or PKB-inhibitor Akti. Activity of mTORC1 is decreased by PKB inhibition, as determined by phosphorylation of S6K. However, phosphorylation of S6K target S6, despite reduced S6K-activity. Expression of phosphatase-dead $CTDSP2^{pd}$ does not affect either S6K or S6 phosphorylation. B) Expression of CTDSP2 (WT) or phosphatase-dead $CTDSP2^{pd}$ was induced for 24 hours in U2OS cells, in the presence of vehicle DMSO or MEK1/2-inhibitor PD325901 (MEKi) or JNK1/2/3-inhibitor SP600125 (JNKi). Effectiveness of inhibition was confirmed by decreased ERK phosphorylation in MEKi treated cells and decreased phosphorylation of JNK target c-Jun. MEKi treatment abrogates p46, but not p54, JNK phosphorylation and attenuates $p21^{Cip1/Waf1}$ upregulation resulting from CTDSP2 expression. In contrast to JNKi, MEKi does not influence c-Jun phosphorylation, possibly due to the remaining activity of p54 JNK. JNKi attenuates c-Jun phosphorylation, ERK phosphorylation and $p21^{Cip1/Waf1}$ upregulation resulting from CTDSP2 expression. C) Cells were treated as in B). MEKi or JNKi treatment do not dramatically alter cell cycle progression or CTDSP2-induced cell cycle arrest (upper right graph), although MEKi treatment increased the number of cells in G0/G1 (upper middle graph) and JNKi treatment increased the number of cell in G2/M (upper left graph). Neither MEKi nor JNKi treatment can rescue CTDSP2-induced decrease in E2F1 expression (lower right graph), but MEKi and JNKi treatments respectively abrogate and attenuate CTDSP2-induced upregulation of $p21^{Cip1/Waf1}$ mRNA (lower left graph).

Concluding remarks

Here we presented a number of exploratory experiments, which we have conducted in the course of our investigation into the mechanisms by which CTDSP2 affects cell cycle progression and $p21^{Cip1/Waf1}$ expression. In many cases where we observe changes in expression upon expression of CTDSP2, phosphatase-deficient $CTDSP2^{pd}$ has similar effects, albeit much smaller than those of wild-type CTDSP2. However, phosphatase-dead $CTDSP2^{pd}$ does not have phosphatase-activity in *in vitro* phosphatase assays (data now shown), which makes it unlikely that this is the result of residual phosphatase-activity *in vivo*. Interestingly, CTDP1 homologues FCP1 in yeast has been shown to increase RNAPII elongation independent of its phosphatase-activity [46], which may explain our observations and challenge that phosphatase-dead $CTDSP2^{pd}$ must have dominant-negative effects on gene expression. To investigate how CTDSP2 regulates expression of (specific) genes, we have explored involvement of several (putative) CTDSP2 interacting proteins, focussing on their requirement for regulation of cell cycle progression and $p21^{Cip1/Waf1}$ expression. However, we have not found compelling evidence for the involvement of E2F7, IRS4, CDCA3 or SMAD2/3 in either cell cycle arrest or $p21^{Cip1/Waf1}$ upregulation-induced by CTDSP2 expression. Interestingly, we have uncovered a role for MEK-ERK-activation in the changes in of $p21^{Cip1/Waf1}$ expression and will elaborate further on this in the near future.

Materials and methods

Tissue culture, reagents, plasmids and siRNA

HEK293T (ATCC CRL-11268), U2OS (ATCC HTB-96) cells and derived lines were maintained in DMEM with 10% FBS, L-Glutamine and Penicillin/Streptomycin. Polyclonal U2OS derived U2OS-CTDSP2 and U2OS-$CTDSP2^{pd}$ have been described before [Chapter 3]. PKB-inhibitor VIII (Akti; final concentration 10µM; Santa Cruz), cycloheximide (CHX; final concentration 0.1µg/mL; Santa Cruz), MG132 (MG; final concentration 20µM; Sigma-Aldrich), rapamicine (Rapa; final concentration 5nM; SelleckChem), PD325901 (MEKi; final concentration 1µM; SelleckChem) and SP600125 (JNKi; final concentration 20µM; Sigma-Aldrich) were all dissolved in DMSO at 1000 times final concentration. Ethidium Bromide (EtBr; final concentration 0.16mg/mL; Sigma) was dissolved in water.

For pCDNA3-Flag-CTDSP2, full length CTDSP2 was amplified with Flag-peptide sequence containing forward primer from HEK293T cDNA, cloned EcoRI-NotI into pCDNA3 and sequence verified. For HA-CTDSP2, full length CTDSP2 was amplified from pCDNA3-Flag-CTDSP2 with original start codon containing forward primer, cloned EcoRI-NotI into pFakeEntry (a kind gift from dr. Holger Rehman) and sequence verified. Using Gateway LR clonase (Invitrogen), CTDSP2 was transferred into Gateway-compatible pMT2-HA (a kind gift from dr. Holger Rehman). pCDNA3.1-E2F7a-Flag and pCDNA3.1-E2F7b-Flag containing Flag-tagged human E2F7a and E2F7b were previously described [17]. siRNA SmartPOOLs against hCDCA3 was purchased from Fisher-Dharmacon and reverse transfected using Hyperfect (Qiagen) with RNA-Hyperfect ratio 40pmol:5µL.

Cellular fractionation, mass spectrometry sample preparation and analysis

For identification of CTDSP2 interacting proteins, HEK293T cells were grown in DMEM without arginine and lysine (PAA), supplemented with L-Glutamine, Penicillin/Streptomycin, 10% dialysed FBS (Gibco), 73µg/mL light/K^{+0} (Sigma) or heavy/K^{+8} (Sigma or Silantes) L-Lysine and 29.4µg/mL light/R^{+0} (Sigma) or heavy/R^{+10} (Sigma or Silantes) L-arginine, termed SILAC

medium. Cells were allowed ten doublings in SILAC medium after labelling efficiency was confirmed >95%. Approximately 30% confluent HEK293T were transfected with 2.5µg/10cm plate pCNDA3 or pCDNA3-Flag-CTDSP2 and harvested 48h post transfection. Per experiment, one plate of cells with light-labelled K/R and one plate of cells with heavy-labelled K/R were transfected with each construct, later to be combined into a forward experiment – i.e. light pCDNA3 with heavy pCDNA3-Flag-CTDSP2 – or reverse experiment – i.e. light pCDNA3-Flag-CTDSP2 with heavy pCDNA3. For whole cell immunoprecipitation, cells were lysed directly using lysis buffer containing 25mM Tris-HCl pH 7.5, 100mM NaCl, 5mM EDTA, 1% NP40, 1mM $NaVO_3$, 1mM NaF and EDTA-free protease-inhibitor cocktail (1 tablet/50mL buffer; Roche). Cellular fractionation was performed as described previously [18]. Briefly, cells were collected by trypsinisation, washed with PBS and resuspended in hypotonic buffer containing 10mM HEPES KOH pH 7.9, 1.5mM $MgCl_2$, 10mM KCl. Swollen cells were lysed by dounce homogenising in the presence of 0.15% NP40 and EDTA-free protease-inhibitor cocktail. Nuclei were collected by centrifugation, lysed in 20mM HEPES KOH pH 7.9, 420mM NaCl, 20% v/v glycerol, 2mM MgCl2, 200µM EDTA, 0.15% NP40, EDTA-free protease-inhibitor cocktail and 500µM DTT and cleared by centrifugation. Soluble nuclear extracts were used for subsequent immunoprecipitations.

Light and heavy labelled lysate pairs were mixed 1:1 and proteins were precipitated using 40µl cross-linked anti-Flag M2 agarose resin (Sigma). After 2 hours incubation at 4°C and four washes with lysis buffer, proteins were eluted from the beads using 100µL 100mM glycine pH 2 for 10 minutes at room temperature. Eluents were collected and neutralised using 10µL 1.5M Tris-HCl pH 8.8. Proteins were washed, reduced, alkylated and digested as described before [19]. Peptides were desalted on C18 matrix (Dr. Maisch Gmbh HPLC) and separated using an EASY-nLC (Proxeon) connected online to an LTQ-Orbitrap Velos mass spectrometer (Thermo) as described [20]. Spectra were analysed using MaxQuant V1.2.2.5 [21] and mapped using protein database IPI human V3.68. Identified protein groups were log2 transformed and contaminants, reverse peptides, single peptide identifications were discarded using Perseus V1.2.0.16 [22]. Using Excel (Microsoft), protein groups were selected that have ratios with opposite signs in the forward and reverse experiment. High abundance proteins with names containing HSP, RPS, RPL, RNP, TUB and ACT as well as proteins with average ratio of forward/reverse lower than 0.5 were not considered genuine interacting proteins.

Immuno-precipitations, ubiquitination-assessment, western blotting and antibodies

For immunoprecipitations, 30% confluent HEK293T cells were transfected with 1µg E2F7a-Flag or E2F7b-Flag and 0.5µg HA-CTDSP2 using ExtremeGene (Roche) and harvested 48 hours post transfection in lysis buffer containing 25mM Tris-HCl pH 7.5, 100mM NaCl, 5mM EDTA, 1% NP40 and phosphatase and protease inhibitors. Cell lysates were cleared by centrifugation, total lysate samples (approximately 15%) collected and remainder incubated with ProtA agarose pre-coupled anti-HA (12CA5; in house hybridoma) or anti-Flag (M2; Sigma) for 2 hours at 4°C. After 4 washes in lysis buffer, proteins were released using Leammli buffer and boiling for 3 minutes. For *in vivo* ubiquitination, U2OS-CTDSP2 cells were transfected with control or CDCA3 RNAi SMARTpool (Dharmacon) as indicated, using Hiperfect (Qiagen) according to the manufacturers' instructions. After 24 hours, medium was replaced and the cells were transfected with 0.5µg pBlueScript or His-Ubiquitin as indicated, using Fugene (Promega) according to the manufacturers' instructions. The next day, doxycycline was added

and after 24 hours cells were lysed in urea lysis buffer containing 8M Urea, 10mM Tris-HCl pH 8.0, 100mM Na_2HPO_4/NaH_2PO_4 pH 8.0, 0.2% TX-100, 5mM N-ethylmaleimide (NEM) and protease inhibitors. Lysates were incubated with solid nickel-nitrilotriacetic acid (Ni-NTA) beads (Sigma) for 2 hours at room temperature. After 4 washes with urea lysis buffer, proteins were released using Leammli buffer and boiling for 3 minutes.
Proteins were separated on denaturing SDS-PAGE gels, transferred to PVDF membrane, blocked with 2% BSA in TBS, incubated overnight with anti-Flag (M2; Sigma), anti-HA (in house 12CA5 or Y-11; Santa Cruz Biotechnology), anti-αTubulin (Calbiochem), anti-CTDSP2 (Abcam or in house), anti-Wee1 (Millipore), anti-$p21^{Cip1/Waf1}$ (BD Biosciences), anti-PKB S473P, anti-PKB T308P, anti-S6Kinase T389P, anti-S6 S235/6P, anti-SAPK/JNK T183/Y185P, anti-p44/42 MAPK (ERK1/2) T202/Y204P, anti-MEK1/2 S217/221P or anti-c-Jun S73P (all from Cell Signalling) antibody. Secondary HRP conjugated antibodies (BioRad) or Alexa680/Alexa800 conjugated antibodies (Invitrogen) were used for detection with ECL or Odessey respectively, according to manufacturers' instructions.

BrdU-incorporation, RNA extraction, cDNA synthesis and qPCR

BrdU-incorporation was detected using FITC-BrdU Flow kit (BD) according to the manufacturers' instructions. RNA was isolated using RNAeasy kit (Qiagen) and cDNA synthesis of 0.5µg RNA with iScript (BioRad) according to the manufacturers' instructions. qPCR was performed using FastStart SYBRgreen mix (Roche) in CFX96 Real-Time Detection System (BioRad) and analysed using the software provided by the manufacturer. Expression levels were normalised using hTUBA1A expression.

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CHAPTER 5

FOXO3 regulates miRNA 26a expression

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Abstract

The Forkhead box-O (FOXO) class of transcription factors controls a number of cellular processes through regulation of gene transcription. In addition to protein encoding genes, FOXO-activation also results in the transcription of non-coding genes including micro RNAs [1]. Here, we show regulation of 11 microRNAs, including miR-26a, in response to FOXO3-activation in DLD1 colon carcinoma cells. We validated regulation of miR-26a in both human and mouse cells. Importantly, miR-26a regulation is likely resulting from FOXO3-dependent regulation of its host gene carboxyl-terminal domain small phosphatase 2 (CTDSP2), which we have extensively characterised in [Chapter 3]. Using mass spectrometry, we observed decreased protein expression of 22 genes upon ectopic expression of miR-26a, of which 16 are TargetScan predicted miR-26a targets. We confirmed 3'UTR-mediated regulation of high-mobility group AT-hook 1 (HMGA1), prostaglandin-endoperoxide synthase 2 (PTGS2; also known as COX-2) and protein kinase C δ (PKCδ) by miR-26a. Surprisingly, despite the upregulation of miR-26a expression after FOXO3-activation, we observed little or even opposing regulation in protein changes by miR-26a upon FOXO3-activation. This leaves it unclear at present what the physiological consequences of elevated miR-26a are. This chapter also contains an exempt from the results of our collaborative work to elucidate the functions of age- and DNA damage regulated miRNAs, which is centred on miR-26a regulation of phosphatase and tensin homolog (PTEN).

Introduction

The Forkhead box-O (FOXO) transcription factor family members affect expression of genes in a diverse set of cellular processes, such as cell cycle regulation, cell survival and regulation of metabolic as well as redox status (reviewed in [2]). Of the four members FOXO1, FOXO3, FOXO4 and FOXO6 that have been characterised in mammals, FOXO6 is the least well studied and has been shown to have different shuttling dynamics (reviewed in [3]). Thus, when referring to FOXO proteins, we include only FOXO1, FOXO3 and FOXO4. The ability of FOXO proteins to regulate transcription is largely determined by shuttling between nucleus and cytoplasm. Two important signalling pathway regulate FOXO-shuttling and consequently its activity. Best studied is the regulation of FOXO proteins by growth factor-phosphoinositide 3-kinase (PI3K)-Protein Kinase B (PKB)/Akt signalling. Growth factor activated PKB phosphorylates FOXO proteins on multiple, conserved residues, which results in binding to members of the 14-3-3 family, nuclear exclusion and decreased DNA-binding affinity (reviewed in [4]). More recently, it has been shown that changes in redox potential, such as those resulting from acute increase in reactive oxygen species, result in nuclear accumulation and increased transcriptional activity, which involves c-Jun N-terminal Kinase (JNK) and ubiquitination of FOXO proteins (reviewed in [5]). A number of target genes have been described to date (reviewed in [6]), although not all of them may be direct targets [7]. The described target genes are mostly protein coding genes, but also include both microRNA miR-30d [8] and a cluster of microRNAs containing miR-506, miR-507, miR-508 and members of the miR-513 family [9].

MicroRNAs (miRNA) are small 21-24bp long RNA molecules. After their initial discovery in *Caenorhabditis elegans*, thousands of miRNAs have been identified in different species (reviewed in [10]). MiRNAs are part of the miRNA-induced silencing complex (miRISC), which contains members of the Argonaute (AGO) family, glycine-tryptophan (GW) repeat-containing protein of 182kDa (GW182) and others (reviewed in [11]). MiRNAs direct miRISC to specific target (m)RNA, thereby regulating the expression of these target RNAs in various ways, including repression of protein translation or by (m)RNA degradation (reviewed in [12]). Target recognition is complex but perfect pairing of the base pairs 2 to 7 of the 5' end of the miRNA is usually sufficient for recognition. Given the limited understanding of target recognition, *in silico* target prediction remains a major challenge. Nonetheless, several target prediction algorithms based on our current understanding of target recognition have been published. These algorithms generally predict that a single miRNA can have hundreds of targets and that half of the human genome is potentially under control of miRNAs (reviewed in [10]). MiRNAs originate from RNA polymerase II (RNAPII) transcribed loci. They can be under control of their own promoters or contained in introns of protein-coding genes and transcribed together with these genes, although these options are not mutually exclusive. Upon transcription, pri-miRNAs fold into a hairpin structure, which is processed by the RNAse DROSHA into a 70bp pre-miRNA that is exported from the nucleus. In the cytoplasm, the pre-miRNA is further processed into a duplex by the RNAse DICER and incorporated in the miRISC (reviewed in [13]).

Since FOXO proteins regulate the recruitment of RNAPII to promoters, we reasoned that they could regulate miRNA genes or miRNA containing genes. Here, we show that FOXO3 can regulate of 11 microRNAs in DL23 cells, which are derived from colon carcinoma cell line DLD1 and stably express a PKB-regulation insensitive (and consequently constitutively active) FOXO3-estrogen receptor (ER) ligand-binding domain fusion (FOXO3.A3-ER) that can be

activated by 4-hydroxy Tamoxifen (4OHT) [14]. We also show regulation of miR-26a by FOXO3 in mouse embryo fibroblasts (MEFs), indicating that this regulation is not cell type specific. Indeed, miR-26a is contained in the fifth intron of the transcript of carboxyl-terminal domain small phosphatase 2 (CTDSP2), which we have shown to be one of relatively few common FOXO3 target genes in many different cell lines [Chapter 3]. Using mass spectrometry, we observed decreased expression of 22 genes upon overexpression of miR-26a, of which 16 are TargetScan [15] predicted targets. We have confirmed 3'UTR-mediated regulation of regulation of high-mobility group AT-hook 1 (HMGA1), prostaglandin-endoperoxide synthase 2 (PTGS2; also known as COX-2) and protein kinase C δ (PKCδ), in addition to regulation of phosphatase and tensin homolog (PTEN), which we previously identified as a miR-26a in the context of a collaborative project [Pothof et al., under revision]. Surprisingly, most proteins regulated by miR-26a are increased upon FOXO3-activation in DL23. Thus, further experiments are required to elucidate the contribution of miR-26a regulation in protein changes upon FOXO-activation.

Results and discussion

FOXO3 regulates 11 miRNAs, including CTDSP2 contained miR-26

Most miRNAs are transcribed by RNAPII and thus their expression can be regulated by specific transcription factors (reviewed in [13]), such as those of the FOXO family. Thus, we determined changes in expression of 328 miRNAs after 4 and 8 hours 4OHT-mediated activation of FOXO3 in colon carcinoma cell line DLD1 derived DL23 cells that express a 4OHT inducible, constitutively active form of FOXO3, FOXO3.A3-ER [14]. Eleven miRNAs are statistically significantly (T-test; $p < 0.05$) differentially regulated at both time points and not in similarly treated parental cell line DLD1 (figure 1A). Except for miR-516, targets have been described for all these miRNAs to date. While miR-361, miR-431, miR-493, miR-506 and miR-510 are less extensively documented, a large body of work exists for miR-26a, miR-125a, miR-130a, miR-150 and miR-499. Generally, these miRNAs have been implicated in developmental processes such as cell type specification. For example, miR-499 plays important functions in muscle cell and cardiomyocite specification (reviewed in [25, 26]) but is also associated with cancer (reviewed in [27]). Furthermore, through regulation of e.g. c-Myb [28], miR-150 has been shown to play an important role in haematopoiesis and deregulation thereof (reviewed in [29]). Less clear is the consensus on the functions of miR-130a. However, it has been shown to regulate vascularogenesis (reviewed in [30]) and can act as a tumour suppressor [31]. Several studies into the role of miR-125a have revealed its roles in different diseases including cancer, being able to promote tumour initiation as well as inhibit cancer progression (reviewed in [32]). Interestingly, miR-125a which is homologous to lin-4 in *C. elegans* [33] is downregulated in our dataset. This is in agreement with FOXO orthologue DAF-16-dependent repression of lin-4 during starvation-induced L1 arrest referred to as *dauer* [34]. Lastly, miR-26a has been shown to be required for skeletal muscle cell differentiation [35]. However, miR-26a has gained most attention for its regulation of tumour suppressor PTEN [36], but has also been reported a tumour suppressor itself by regulation of cyclin D proteins [37] and EZH2 [38-40]. Many papers have confirmed these findings, showing that ectopic miR-26a expression can both promote (e.g. [34, 41, 42]) and inhibit cell cycle progression (e.g. [43-45]). In conclusion, miR-26a regulates cell cycle progression, but the outcome of miR-26a expression is determined by the contribution of its targets to cell cycle progression in the cell line or tissue under study (reviewed in [46]).

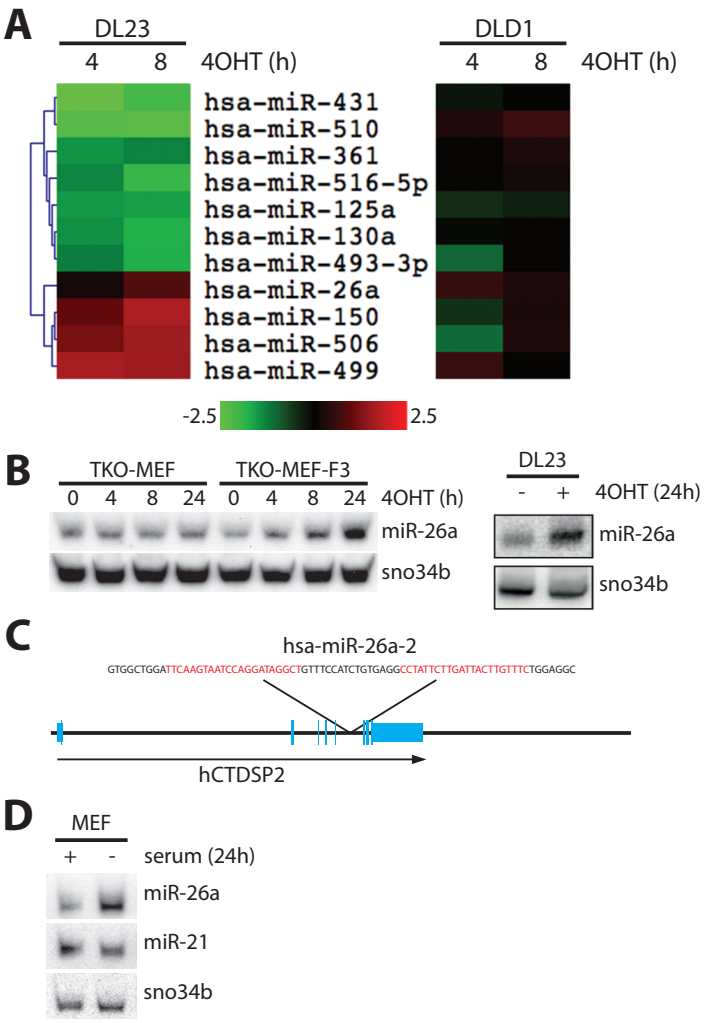


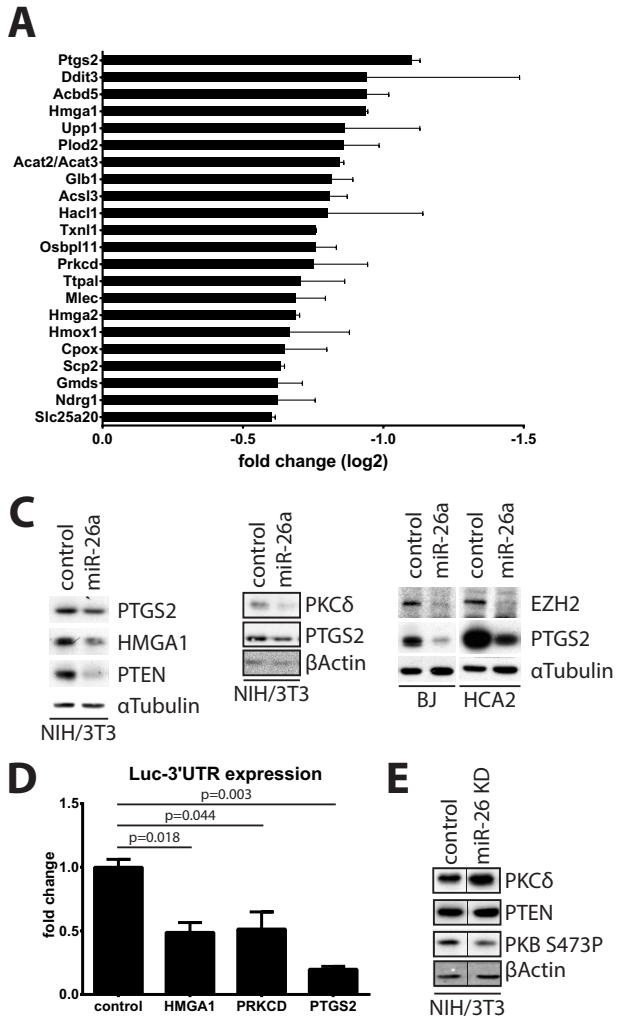
Figure 1: FOXO3 regulates 11 miRNAs, including miR-26a and its host gene CTDSP2 [Chapter 3]
A) DLD1/DL23 cells were treated with 4OHT for 4 or 8 hours and statistically significant (T-test; $p < 0.05$) differentially regulated miRNAs at both time points are depicted in the heatmap. B) Northern blot showing miR-26a upregulation in DL23 cells after 24h 4OHT treatment and TKO-MEFs without or with FOXO3.A3-ER treated with 4OHT for indicated times. C) Schematic representation of genomic organisation of CTDSP2/miR-26a-2 locus. D) Northern blot analysis of miR-21 and miR-26a expression changes upon 24h serum deprivation of wild-type MEFs. Expression of miR-26a is increased as expected. Expression of miR-21 is decreased upon growth factor withdrawal, in agreement with its increased expression in cells that re-enter cycling [73].

Using Northern blotting, we confirmed FOXO3-mediated regulation of miR-26a in DL23 and FOXO1/3/4 triple-knockout (TKO) MEF cells, the latter either without or with FOXO3. A3-ER (figure 1B). We were unable to validate expression changes observed for the other 10 miRNAs, possibly due to low expression or poor hybridisation of their probes (data not shown). Members of the miR-26 family are located within the transcribed regions of three out of four members of the carboxyl-terminal domain small phosphatase (CTDSP) family members, i.e. miR-26b is in the fourth intron of CTDSP1, while miR-26a-1 and miR-26a-2

are located in the fifth intron of CTDSPL and CTDSP2 respectively (figure 1C). Furthermore, relatively high correlation exists between expression of either miRNA and its host gene, although this is complicated for CTDSPL [47]. In agreement, we have shown that FOXO3 regulates the expression of CTDSP2 [Chapter 3], which most likely accounts for the observed increase in miR-26a. Furthermore, in agreement with previously published results [43], miR-26a is also upregulated during growth factor starvation (figure 1D), a condition which is known to activate FOXO proteins.
Others have shown that the expression of miR-26a can be regulated by p53 and that miR-26a regulates Wee1 and Chk1 in this context. In this case, p53 was shown to regulate miR-26a-1 from an internal promoter within the CDTSP1 gene [48], most likely independent of CTDSP1 expression regulation. Furthermore, miR-26a expression is repressed by c-MYC [38]. However, whether c-MYC-dependent regulation of miR-26a underlies the observation that miR-26a levels are higher in arrested/quiescent [43] and senescent cells [49], remains to be established. Alternatively, FOXO-activation also correlates with these cell fates, thus miR-26a regulation illustrates the antagonistic relationship between FOXO proteins and MYC proteins as we proposed (reviewed in [Chapter 1]) and by all means the CTDSP2 locus appears an excellent example of this.

Proteome profiling of miR-26a overexpressing cells reveals 22 downregulated genes

In silico prediction algorithms of miRNA-targets perform poorly in validation experiments, although new technologies have the potential to improve our understanding of targeting rules (reviewed in [10]). In order to directly assess miR-26a-induced changes in protein expression thereby circumventing the need for prediction to find leads, we measured protein expression changes upon overexpression of miR-26a using SILAC based proteomics. We identified 22 genes that are downregulated upon ectopic miR-26a expression in NIH/3T3 cells (figure 2A). Of these 22 genes, 16 genes are predicted miR-26a targets in TargetScan 6.2 [15] and for 12 out of these 16 targets the predicted seed sequences in their 3'UTR are broadly conserved among species (figure 2B). Indeed, in independent experiments, we show that newly identified PTGS2, HMGA1 and PKCδ but also PTEN and EZH2 are repressed by ectopic expression of miR-26a in NIH/3T3 cells but also other cell lines (figure 2C). To confirm that the observed repression is due to the 3'UTR as predicted by TargetScan, we fused the 3'UTR of SV40 (control), PTGS2, HMGA1 and PKCδ to a luciferase coding sequence and measured expression of luciferase with or without miR-26a overexpression. Indeed, we find that the 3'UTR sequences of either PTGS2, HMGA1 or PKCδ are sufficient for miR-26a-mediated repression of luciferase expression to various degrees (figure 2D). To test the extent of regulation of PTEN and PKCδ by endogenous miR-26a, we inhibited miR-26a by non-degradable complementary oligo's, which clearly reveals regulation of PKCδ but not PTEN, although PKB phosphorylation is affected by the treatment suggesting that PTEN levels may indeed have decreased (figure 2E). Thus, we identified many putative endogenous and direct targets of miR-26a and have confirmed miR-26a regulation of PTGS2, HMGA1 and PKCδ through their 3'UTR. In addition, endogenous PKCδ is readily suppressed by endogenous miR-26a in NIH/3T3 cells under basal conditions.
We have determined the expression status of miR-26a regulated proteins in the proteome dataset of FOXO3-induced protein expression changes in DL23 cells, which is presented in [Chapter 2]. Unfortunately there is relatively little overlap between these data sets (only approximately 50% of miR-26a regulated proteins is covered in the dataset of FOXO3-



B

Gene	Conserved	All	DL23 protein data
Ptgs2	1	2	nd
Ddit3	0	0	nd
Acbd5	1	1	+
Hmga1	1	1	+
Upp1	0	0	nd
Plod2	2	3	++
Acat2/Acat3	0	0	-
Glb1	0	1	+
Acsl3	1	1	nd
Hacl1	0	1	nd
Txnl1	0	0	nd
Osbpl11	2	2	-
Prkcd	1	1	nd
Ttpal	1	2	nd
Mlec	1	2	=
Hmga2	1	2	nd
Hmox1	0	0	+
Cpox	0	1	+
Scp2	0	0	+
Gmds	1	1	=
Ndrg1	0	1	++
Slc25a20	1	1	nd

Figure 2: miR-26a expression decreases expression 22 proteins in NIH/3T3 cells, a number of which were validated in independent experiments

A) Mass spectrometry determined fold change of >1.5 fold decreased genes in NIH/3T3 cells overexpressing synthetic miR-26a. B) Table showing number of conserved and total miR-26a binding sites as predicted by TargetScan 6.2. The last column shows status of the protein in proteome data of DL23 cells treated for 24 hours with 4OHT [Chapter 2]. Legend: nd means no or inconclusive data; - means downregulated; = means not changed; ++/+ means (> 2 fold) induced. C) miR-26a overexpression-induced decrease in PTGS2, HMGA1 and PKCδ and previously described PTEN and EZH2 (see main text for references) in both NIH/3T3 cells and primary non-transformed human fibroblast lines HCA2 and BJ. D) miR-26a-induced decrease of expression of luciferase bearing the 3'UTR of HMGA1, PKCδ and PTGS2, but not SV40 (control). E) PKCδ expression is increased when miR-26a/b are suppressed with complementary oligo's. In addition, PKB phosphorylation is downregulated by the same treatment, which is suggestive of increased PTEN expression, although this is not readily visible in the presented Western blot.

induced protein expression changes), partly because our proteome data sets cover only 4000 proteins, which still represents only an estimated 20-30% of all proteins that can be expressed in a given cell type. Surprisingly, the miR-26a targets that we have data for, are mostly upregulated by FOXO3-activation (figure 2B). Interestingly, it has been reported that

microRNAs can increase the expression of their targets in quiescent/arrested cells [50] and FOXO3-activation results in cell cycle arrest in G0/G1 [51]. However, we have not yet further investigated this. Thus the contribution of FOXO-induced miR-26a to FOXO-induced protein level changes remains elusive.

A number of miR-26a targets have been reported and several of these agree with our dataset. As mentioned, miR-26a regulation of PTEN has been extensively documented [36, 46, 52, 53], but also more recently regulation of HMGA1 [54], HMGA2 [55], PTGS2 [56, 57] and PLOD2 [41] has been shown. Bearing this in mind, it is of interest to note that a number of these targets have been implicated in cellular senescence, including PTGS2 [58], HMGA1, HMGA2 [59] and PKCδ [60]. We also observe decreased expression of the β-galactosidase GLB1 and increased activity of this gene is one of the earliest described markers for senescence cells [61]. GLB1 does not harbour a conserved miR-26a seed sequence in its 3'UTR, which may suggest indirect regulation of GLB1 by miR-26a, although both mouse and human GLB1 do bear a non-conserved miR-26a binding site. Lastly, we observe a 1.76±0.32 fold increase in histone 1 (H1.1) expression, and expression of H1.1 has been observed to decrease in senescent cells [62]. Based upon these notions, we have explored the possibility that miR-26a upregulation could inhibit H_2O_2 or oncogene-induced senescence. However, we did not observe a reduction in the induction of senescence as we did not observe a decreased in marker expression for those that we have looked at (SA-β-GAL and IL6; data not shown). In addition, miR-26a expression is increased in H_2O_2-induced or replicative senescence [49] and in response to DNA damage ([48] and see below).

Aging and DNA damage regulated miR-26a controls PTEN levels to increase PKB-activity and $p21^{Cip1/Waf1}$ stability

As part of a collaborative effort to elucidate the function of miRNAs regulated in aging, we studied the effects of aging and DNA damage regulated miRNAs on insulin signalling. Initially, Pothof and colleagues collected mRNA and miRNA expression profiles from four different tissues obtained from 13, 52 and 104 week old mice. By comparative analysis it was shown that expression of 14 miRNAs increases progressively with aging, in response to UV-induced DNA damage and in tissues of 17 weeks old mice defective in nucleotide excision repair ($ERCC1^{\delta/-}$; [63]). A compiled list of targets of these miRNAs shows that genes involved in insulin signalling are overrepresented [Pothof et al., under revision]. PTEN is a predicted target of miR-26a in TargetScan 6.2 and we show that overexpression of the aging regulated miR-26a inhibits the expression of PTEN, which results in enhanced PKB activation (figure 3A). PTEN is among the most frequently mutated tumour suppressors and in this respect important for regulating the activity of PI3K and PKB (reviewed in [64]) Thus, the increased basal and stimulated PKB-activity observed in miR-26a overexpressing cells is probably the result of reduced PTEN expression, although we cannot exclude other mechanisms. Pothof and colleagues extended these findings, showing that UV-induced repression of PTEN depends on miR-26a/b upregulation and that the 3'UTR of PTEN is sufficient for miR-26a/b repression of expression. Importantly, they also show that miR-26a/b-dependent regulation of PTEN is important for survival of UV treated cells [Pothof et al., under revision].

Interestingly, upon ectopic expression of miR-26a, we also observed increased expression of the cell cycle progression inhibitors $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ (figure 3B). Both $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ are phosphorylated by PKB, which results in their cytoplasmic localisation and inactivation as CDK inhibitors (reviewed in [Chapter 1]). Loss of PTEN as well as activation

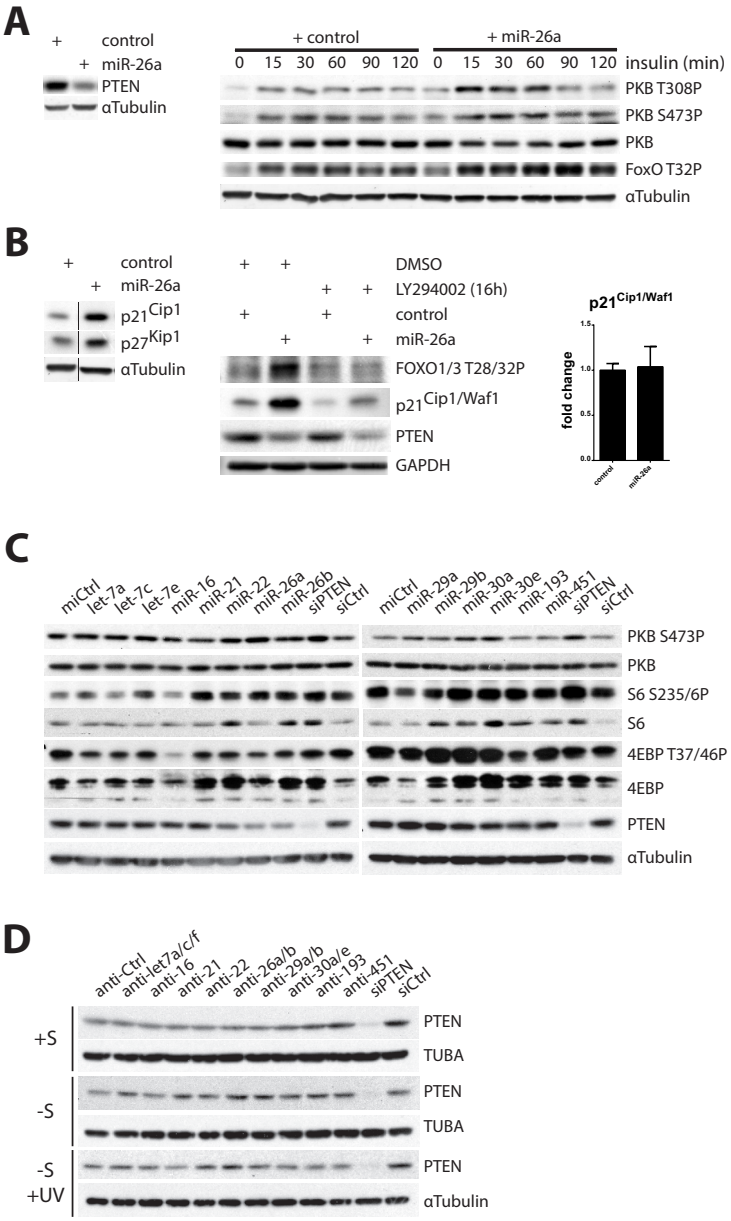


Figure 3: UV and aging-induced miR-26a regulates PTEN and p21Cip1/Waf1 expression levels
A) miR-26a regulates PTEN expression (left) and this results in enhanced basal and insulin stimulated PKB-activation of overnight serum deprived cells (right). B) miR-26a overexpression results in increased $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ levels (left), which is not the result of increased mRNA expression (right) and can be attenuated by PI3K inhibition using LY294002 (middle). C) Many of the 14 age-regulated miRNAs influence PKB-mTORC1 signalling, reflected in either increased PKB phosphorylation or decreased phosphorylation of direct and indirect mTORC1 targets 4EBP1 and S6. D) PTEN in mildly increased in response to miR-26 inhibitory oligo's combined with overnight serum deprivation or overnight serum deprivation combined with UVC treatment (20J/m^2). Also inhibition of other age-regulated miRNAs has similar effects when cells are serum deprived, including let7a/c/f, miR-29a/b and miR-193. The last also reduces PTEN expression upon ectopic expression (see C).

of PKB have been reported to result in stabilisation of $p21^{Cip1/Waf1}$ [65, 66], which can be attributed to the enhanced phosphorylation of $p21^{Cip1/Waf1}$ by PKB [65]. Indeed, increased $p21^{Cip1/Waf1}$ is not due to increased expression of $p21^{Cip1/Waf1}$ mRNA and can be attenuated by the PI3K inhibition (figure 3B). Interestingly, we have shown that expression of miR-26a-2 host gene and FOXO target gene CTDSP2 increases $p21^{Cip1/Waf1}$ mRNA levels [Chapter 3], tempting us to speculate that these two co-transcribed molecules collaborate to regulate $p21^{Cip1/Waf1}$ under physiological conditions. Pothof and colleagues showed that expression several FOXO target genes is increased with aging and after DNA damage. However, FOXO-activity itself is decreased as measured by the decreased activation of an artificial promoter in response to UVC [Pothof et al., under revision], in agreement with decreased expression of CTDSP2 after UVC treatment [67]. Interestingly, $p21^{Cip1/Waf1}$ has been described to bind to and stabilise NRF2 by competing for binding to its destruction complex [68] and in aged tissues as well as in response to DNA damage, expression of several NRF2 target genes is increased [Pothof et al., under revision]. Furthermore, Pothof and colleagues have shown that basal NRF2 nuclear localisation is increased by miR-26a overexpression or PTEN knockdown and this depends on the presence of $p21^{Cip1/Waf1}$. Most importantly, UVC treatment-induced NRF2 nuclear localisation in a miR-26a/b, PTEN and $p21^{Cip1/Waf1}$-dependent manner. Interestingly, the increased resistance to 100μM H_2O_2-induced cell death, resulting from 8J/m^2 UVC pre-treatment also depends on miR-26a/b, PTEN, $p21^{Cip1/Waf1}$ and NRF2, illustrating that endogenous levels of all these components are sufficient to operate in the model that we propose [Pothof et al., under revision].

In a broader context, we propose that age- and DNA damage-regulated miRNAs are responsible for part of gene expression changes observed during aging, thus suggesting that aging itself is the result of (accumulated) DNA damage. Furthermore, increased expression of miR-26a results in enhanced survival cells by increased NRF2-mediated protection, which depends on miR-26a regulation of PTEN and $p21^{Cip1/Waf1}$ and we predict this to occur *in vivo* and in aging. In agreement, insult-induced apoptosis decreases with age possibly due to decreasing regenerative capacity [69-72]. In such scenario, prolonged life of individual cells resulting from increased miR-26a expression can serve to maintain tissue and body integrity.

In response to a first round of review, we have collected data on the influence of all 14 age- and DNA damage regulated miRNAs on the PI3K-PKB-mTORC1 pathway. We show that ectopic expression of many of these miRNAs can influence PKB-activity and/or mTORC1-activity (figure 3C). In addition, we investigated if inhibition of either miRNA family would influence the expression of PTEN, either in the presence or absence of growth factors and with or without UV-induced DNA damage. Although these effects are difficult to visualise, expression of PTEN is most influenced by miR-26a/b inhibitions and possibly by let7a/c/f, miR-29a/b and miR-193 inhibition (figure 3D).

Taken together, we have made important contributions to elucidating the consequences of age and DNA damage increased miRNA expression. Of particular interest to us is the regulation of miR-26, although this is likely the result of (p53-dependent) expression of miR-26a-1 and not miR-26a-2/CTDSP2. Regardless of its origin, the link to NRF2 provided by miR-26a may be interesting to explore in the context of FOXO-activity.

Materials and methods

Tissue culture, reagents, transfection, luciferase reporter assays and UVC treatments

NIH/3T3 (ATCC CRL-1658), U2OS (ATCC HTB-96) cells, FOXO1/FOXO3/FOXO4 deleted MEFs

(TKO-MEF) and isogenic wild-type MEFs (WT-MEF) [16], and derived lines were maintained in DMEM with 10% FBS, L-Glutamine and Penicillin/Streptomycin. DLD1 (ATCC CCL-221) and derived DL23 [14] cells were maintained in RPMI-1640 with 10% FBS, L-Glutamine and Penicillin/Streptomycin. BJ (ATCC CRL2522) and HCA2 [17] were maintained in DF12 with 10% FBS, L-Glutamine and Penicillin/Streptomycin. Monoclonal cell line TKO-MEF-F3 derived from TKO-MEF transduced with pBabe-puro-hFOXO3.A3-ER was a kind gift from M. Putker and T. Dansen. 4-hydroxy Tamoxifen (4OHT; final concentration 500nM; Sigma-Aldrich) was dissolved in 96% EtOH. LY294002 (final concentration 10µM; Sigma-Aldrich) was dissolved in DMSO. Insulin (5µg/mL final concentration; Sigma-Aldrich) was dissolved in 10mM acetic acid.
NIH/3T3 cells were transfected at approximately 25% density with control #2 or miR-26a duplexes (Ambion) using Lullaby (OZBIOSCIENCES) according to the manufacturers' instructions. For luciferase reporter assays, U2OS cells were reverse transfected at approximately 12.5% confluence with control #2 or miR-26a duplexes using Hiperfect (Qiagen). After 24 hours, cells were again transfected with LightSwitch GoClone 3'UTR Reporter Constructs (SwitchGear Genomics) of SV40 (control), HMGA1, PRKCD or PTGS2 using ExtremeGene (Roche). Renilla expression was measured 48 hours after the second transfection using Dual-Luciferase Reporter Assay System (Promega) in a MicroLumat Plus LB 96V (Berthold Technologies), according to the manufacturers' instructions (only Renilla values were used). For miRNA knockdown experiments, NIH/3T3 cells were transfected at approximately 25% density with miRCURY LNA microRNA inhibitor (Exiqon) control or anti-miR-26a/b oligo's and harvested 48 hours post-transfection.
For UVC treatments, 50% confluent NIH/3T3 cells were washed twice in PBS, aspirated dry and left untreated or exposed to indicated amount of UVC in Stratalinker 2400 (Stratagene).

Lentiviral plasmids, particles and transductions

To generate pWPXLd V2, the BamHI site in pWPXLd (deposited by Didier Trono as Addgene plasmid 12258) was removed with mutagenesis according to the Strategene Quickchange protocol. The 5' and 3' splice sites of intron 5 of CTDSP2 were amplified from genomic DNA, fused to create a 415bp fragment that contains a 277bp intron with adjacent BamHI and EcoRI site for future cloning, and cloned BstBI – SpeI behind GFP into pWPXLd V2. The IRES-Puro fragment was amplified from pLV-CMV-IRES-Puro [18] and cloned into the SpeI site to obtain pWPXLd V2-GFP-Intron-IRES-Puro (pDK-GFP). A 391bp fragment containing miR-26a-1 was amplified from genomic DNA and cloned BamHI – EcoRI into pDK-GFP to obtain pDK-GFP-26a. Inserted elements were all sequence verified. Lentiviral particles of pDK-GFP or pDK-GFP-26a were generated and concentrated as described [19]. HCA2 and BJ cells were transduced using 50µL concentrated virus in a 10cm plate and selected for 2 weeks using 1.5µg/mL puromycin.

Microarrays, qPCR and analysis

For miRNA microarrays, 70% confluent DLD1 or DL23 cells were treated with vehicle or 4OHT for 4 or 8 hours prior to total RNA extraction using RNAbee (Amsbio). RNA integrity was evaluated using 2100 Bioanalyzer (Agilent) and all samples used had scores higher than 8. Four replicates were collected and miRNA microarrays were performed and analysed as described previously [20].
For qPCR analysis, RNA was extracted using RNAeasy kit (Qiagen) and cDNA was synthesised

using iScript kit (BioRad) with 1ug RNA input. qPCR was performed using iQ SYBR green Supermix (BioRad) in a CFX96 Real-Time Detection System (BioRad) and analysed using the software provided by the manufacturer. Expression levels were normalised to mHMBS (PBGD).

Northern and Western blots and antibodies

For Northern blot analysis of miRNA expression, 50µg of total RNA was mixed with loading dye (95% deionised formamide, 5% 0.5M EDTA (pH = 8.0) and Phenol Broom Blue plus Xylene Cyanol) in >1:1 ratio, denatured at 95°C, chilled on ice and separated using 15% polyacrylamide (19:1 polyacrylamide:bisacrylamide) TBE buffered gels. RNA was transferred to BrightStar Plus nitrocellulose membrane (Ambion) using Trans-Blot SD Semi-dry (30 minutes 200mA) or Mini Trans-Blot (overnight 12V) and cross-linked with 2400J/m2 using UV Stratalinker (Stratagene). Membranes were pre-hybridised at 37°C in 50mL Falcon tubes with 7mL 68°C pre-heated UltraHyb Oligo solution (Ambion) inside glass incubator tubes in a hybridisation incubator model 400 (SciGene) for 30 minutes. Complementary DNA probes were end-labelled for 1 hour at 37°C using polynucleotide kinase (PNK; NEB) according to the manufacturers' instructions and in the presence of 50µCi γ-32P-ATP, after which PNK was heat inactivated for 2 minutes at 95°C. Membranes were incubated overnight with labelled oligo's in 2mL fresh UltraHyb Oligo solution, washed 3 times with 2xSSC, 0.2% SDS, wrapped in plastic and used for phosphoscreen or overnight X-ray film exposure. A probe recognising SNORD2 (snR39b) was used as a loading control.

Western blotting was performed according to standard laboratory practises. Cells were directly lysed in Leammli buffer, denatured by boiling, separated using SDS-PAGE gels and transferred to PVDF membrane. Membranes were blocked with TBS containing 2% BSA and stained with desired antibodies recognising PTEN (Cell Signalling), phospho-PKB S473 (Cell Signalling), phospho-PKB T308 (Cell Signalling), phospho-FOXO1/3 T28/32 (Cell Signalling), EZH2 (Cell Signalling), 4EBP (Cell Signalling), phospho-4EBP T37/46 (Cell Signalling), S6 (Cell Signalling), phospho-S6 S235/6 (Cell Signalling), PKB (in house), SCP2 (a kind gift from T. Dansen), α-Tubulin (Calbiochem), PKCδ (Abcam), PTGS2 (COX-2; Abcam), $p21^{Cip1/Waf1}$ (BD Biosciences), $p27^{Kip1}$ (BD Biosciences), GAPDH (Santa Cruz Biotechnology) or β-Actin (Sigma-Aldrich).

Mass spectrometry sample preparation and analysis

For stable isotope labelling by amino acids in cell culture (SILAC) based proteome analysis, NIH/3T3 cells were grown in DMEM without arginine and lysine (PAA), supplemented with L-Glutamine, Penicillin/Streptomycin, 10% dialyzed FBS (Gibco), 73µg/mL light ($^{+0}$K; Sigma) or heavy ($^{+8}$K; Sigma) L-lysine and 29.4µg/mL light ($^{+0}$R; Sigma) or heavy ($^{+10}$R; Sigma) L-arginine. Cells were allowed ten doublings in SILAC medium after which labelling efficiency was confirmed >95%. Cells were transfected with control #2 or miR-26a as described above. Per experiment, one light and one heavy labelled plate were transfected with control duplex and one light and one heavy labelled plate with miR-26a duplex to obtain label swapped experiments (light control vs. heavy miR-26a and light miR-26a vs. heavy control). After 48 hours, cells were washed twice in PBS and lysed in 100mM Tris-HCL pH 7.5, 4%SDS and 100mM DTT. Approximately 100mg of protein of each condition was mixed in indicated combinations and washed, alkylated and digested as described before [21]. Peptides were basified to pH 11 using Britton buffer pH 11 and NaOH, and bound to Strong anion-exchange

(SAX) matrix (Dr. Maisch Gmbh HPLC). Eight fractions, including a flow though fraction, were obtained using Britton buffers pH 11, 8, 6, 5, 4, 3 and 2 in this order [21]. Peptides were desalted on C18 matrix (Dr. Maisch Gmbh HPLC) and separated in 4 hour gradients (0-80% acetonitrile) using an EASY-nLC (Proxeon) connected online to an LTQ-Orbitrap Velos mass spectrometer (Thermo) as described [22]. Spectra were analysed using MaxQuant V1.4.3.0 [23] and mapped using protein database IPI mouse V3.65. Identified protein groups were log2 transformed and contaminants, reverse peptides, single peptide identifications and protein groups identified in only one of two experiments were discarded using Perseus V1.2.0.16 [24]. Using Excel (Microsoft), protein groups were selected that have ratios with opposite signs in the forward and reverse experiment and average change and standard deviation thereof was calculated.

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CHAPTER 6

General discussion

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Protein kinase B (also known as Akt) and Forkhead box-O (FOXO) transcription factors have been implicated in many processes including cell cycle regulation, cell survival and metabolism (reviewed in [1, 2]). As we have discusses in **Chapter 1**, proper control of these processes is important throughout organismal existence and imbalances result in diseases such as cancer and diabetes, the incidence of which increases with age. Work in model organisms has revealed that FOXO-activity can prolong lifespan in laboratory setup and single nucleotide polymorphisms in FOXO3 have been associated with longevity in humans (reviewed in [3]). Thus, understanding regulation of FOXO proteins and the consequences of their activity can help us understand the underlying mechanisms of the changes that we collectively refer to as aging. More than two decades of research have shed light on many aspects of PKB and FOXO-regulation and how these proteins are interconnected. PKB was discovered as the transforming gene of a viral oncogene and later its activation was shown to require activity of Class I phosphatidylinositol 3-kinase (PI3K) family members. Class I PI3K-activity is increased by growth factors and other extracellular stimuli, and controls PKB phosphorylation and activation by 3-phosphoinositide-dependent protein kinase 1 (PDPK1) and mammalian target of rapamycin complex 2 (mTORC2) (reviewed in [4]). FOXO family members have been discovered independently and were soon after implicated in lifespan regulation by work in *Caenorhabditis elegans*, under negative control of PI3K-PKB signalling (reviewed in [5]). Inhibitory regulation of FOXO proteins by PKB results from direct phosphorylation and involves localisation of FOXO proteins to the cytoplasm and other mechanisms, which inhibit its function as a transcription factor (reviewed in [6]). Although growth factor regulation of FOXO-activity is most extensively studied, it has been shown that the PI3K-PKB pathway is not the only signalling cascade that affects FOXO-activity. An important additional level of FOXO-regulation is induced by cellular stress, in particular changes in cellular redox state, which results in FOXO-activation (reviewed in [7]). Taken together, growth factor-PI3K-PKB-FOXO signalling plays vital roles in cells and organisms and understanding their regulation helps us unravel mechanisms underlying aging and age-related pathologies such as cancer and diabetes.

FOXO gene regulation: a common signature

FOXO proteins have been studied mostly for their role in regulation of gene transcription and have been shown to regulate expression of a large number of genes (reviewed in [2]), although not all of these may be regulated by FOXO proteins directly [8]. Throughout this thesis, we have further extended identification of FOXO target genes. Not only have we collected genome-wide gene expression data as presented in **Chapter 3** and **Chapter 5**, we also combined our datasets with those of others. In particular, in **Chapter 3** we aimed to determine a common signature of FOXO family members, which may explain the conserved and organism-wide regulation of disease and aging (reviewed in [5]). Such common signature of FOXO gene regulation has remained enigmatic thus far, which has led to the conclusion that FOXO gene regulation is tissue and/or context specific [9, 10]. However, in **Chapter 3** we present a small subset of genes that are consistently regulated by FOXO3 and FOXO4 in different cells lines of both human and mouse origin, although we have not included data for FOXO1 because no dataset of sufficient quality is available. Importantly, in **Chapter 2** we observed that, although absolute gene expression can differ greatly between cell lines, their regulation by FOXO proteins was observed regardless, suggesting that the ability to ***observe*** FOXO3-regulation of genes is context-dependent rather than the FOXO3-regulation

of these genes itself. In technical terms, expression changes are less likely to be statistically significant with decreasing signal intensity, because technical and biological noise becomes more and more likely to result in false positive results towards the detection limit of a given technology. In addition, if unstimulated gene expression is below or close to detection levels of the particular method, changes are automatically underestimated, further reducing the likelihood of them being considered to result from (technical and/or biological) variation. Thus the lack of a universal FOXO target gene signature may be the result of many factors besides promoter-binding.
Regardless of FOXO proteins binding to the same promoters in different cell types or not, the multitude of FOXO-regulated genes involved in PKB regulation that we identified in **Chapter 2** suggests an additional solution by which context-independent functional consequences of FOXO-activity can be achieved. Given the number FOXO3-regulated candidates regulating PKB-activity, FOXO-regulation of PKB may be present in all cell types, irrespective of their particular set of genes that is expressed or if these genes are regulated by FOXO proteins in that cell type. Combining these two approaches, not simply matching single genes, but comparing overlap of regulated functionally related genes in different cell types, may aid further characterisation of FOXO-function and can be a useful starting point to narrow down and individually test specific gene regulation by individual FOXO isoforms and/or in different tissues. If not for any of the above, function cluster aided matching of expression patterns will at least reduce mismatching resulting from specific gene family members that are expressed in different cell types but most likely have the same function; a problem that we frequently encounter when combining datasets.

FOXO effects: novel FOXO target genes and their functions
FOXO proteins affect many processes through regulation of gene expression and we have identified new target genes in **Chapter 3** and **Chapter 5**, two of which we have studied in more detail. As discussed above, we have compiled a list of commonly regulated genes in FOXO3/4 overexpressing cell lines. To substantiate these findings, we have explored the consequences FOXO-regulation of carboxyl-terminal domain small phosphatase 2 (CTDSP2) in **Chapter 3** and **Chapter 4**. We show that CTDSP2 expression is controlled by endogenous FOXO proteins and through direct promoter-binding of FOXO proteins. CTDSP2 has been shown to regulate gene expression through direct regulation of RNA polymerase II [11], the primary RNA polymerase responsible for regulated expression of protein and non-protein coding genes. In addition, ectopic expression of CTDSP2 has been reported to decrease cell cycle progression [12, 13], which is reminiscent of the observed effect of expression of (constitutively active) FOXO3 [14]. In **Chapter 3**, we confirmed CTDSP2 regulation of cell cycle progression and show that this involves increased expression of $p21^{Cip1/Waf1}$. In **Chapter 4** we have explored many possible mechanisms by which CTDSP2 may regulate cell cycle progression and $p21^{Cip1/Waf1}$ expression and conclude that CTDSP2 promotes Ras-activity, which may account for both these effects and will be the subject of additional experiments. In addition, the contribution of CTDSP2 to FOXO-induced changes in cell cycle progression and/or gene regulation remains to be investigated. Interestingly, both observed Ras activation and PKB activation by CTDSP2 may be part of negative feedback regulation of FOXO proteins, which is the main topic op **Chapter 2** and will be discussed below. In **Chapter 5** we have explored regulation of microRNA expression by FOXO3 and show that 11 miRNAs are differentially regulated upon its activation. We have validated FOXO3-mediated regulation of miR-26a, which is likely the result of FOXO

proteins binding to the promoter of the miR-26a host gene, CTDSP2. Ectopic expression of miR-26a showed regulation of a number of proteins, but expression of most of these miR-26a-downregulated proteins is increased upon activation of FOXO3 as observed determined from proteome data (presented in **Chapter 2**). Interestingly, microRNAs can increase expression of proteins under certain circumstances, most notably in cells that are arrested in G0/G1 [15], which is one of the best-characterised consequences of FOXO-activation [14]. Moreover, miR-26a expression increases with aging, which has become apparent in a collaborative effort to define a microRNA-signature of aging [Pothof et al., under revision], although this seems to be independent of FOXO-regulation. In this context, we identified phosphatase and tensin homolog deleted on chromosome 10 (PTEN) as a miR-26a target gene and indeed overexpression of miR-26a increases PKB-activity, which we show to result in increased $p21^{Cip1/Waf1}$ protein levels. Intriguingly, separate ectopic expression of normally co-transcribed CTDSP2 and miR-26a has similar effects on both PKB-activity and $p21^{Cip1/Waf1}$ protein levels and it is tempting to speculate that they collaborate under physiological conditions, as was shown for cell cycle regulation [13]. In addition, separate and/or combined action of CTDSP2 and miR-26a may be part of the negative feedback regulation described in **Chapter 2**.
In a broader perspective, these two FOXO target genes are part of the many FOXO target genes which themselves can regulate transcription and/or translation of other genes. This underscores the complexity of FOXO-induced expression changes and the need for proper controls to discriminate primary and secondary effects of FOXO-activation, like the experiments shown in figure 4 of **Chapter 2**. Interestingly, secondary expression regulation might be an important aspect of temporal control of FOXO-induced changes in cellular behaviour, a concept that has not yet been explored to date. Due to the delay that inevitably results from secondary expression changes, it is possible to elicit different responses from short or sustained activation of FOXO proteins (see also below), which may be key to explaining how FOXO-activation can have so many different effects. For example, FOXO-activation in response to redox-stress may immediately elicit expression of detoxification enzymes, while increasing the levels of pro-apoptotic proteins requires more time, thus allowing cells some time to restore redox-levels and only activate a cell death program if restoring stress-induced damage takes too long (i.e. when FOXO proteins remain active for too long).

FOXO-activity: from static endpoint to dynamic continuum

In **Chapter 1**, we summarised our current understanding of PKB and FOXO-regulated processes, and we conclude that there is striking overlap between PKB and FOXO-output, albeit with precisely opposite outcome. In brief, whereas PKB has been shown to promote cell cycle progression, cell growth and cell survival, FOXO proteins inhibit these processes, often through the very same mediators that are affected by PKB. Interestingly, this mutual relation is even extended to PKB and FOXO proteins themselves, as PKB inhibits FOXO gene regulation while FOXO proteins have been shown to promote expression of various genes that increase PKB-activity as discussed in **Chapter 1**. Although not pre-conceived initially, PKB regulation by FOXO proteins has become a recurrent theme in this thesis (see above). It is widely accepted that FOXO proteins induce negative feedback regulation and it is expected that this influences the outcome of FOXO-activation (reviewed in [16]), also suggesting that results obtained using constitutively active mutants of FOXO proteins must be treated with caution as they do not permit PKB-mediated negative feedback regulation. Determining the precise wiring and strength of feedback signalling is of critical importance, since these

network properties can have dramatic influence on signalling patterns and output in time (see figure 1 in [17]). In **Chapter 2** we provide evidence that PKB and FOXO proteins are part of a dynamic continuum, which influences either activity significantly. We have elaborated on the PKB-mediated negative feedback regulation of FOXO proteins by studying the changes in activity of elevated FOXO3 expression. In this setup, a number of FOXO3 target genes participate in its inactivation, although the relative contribution of individual genes differs dependent on the context (i.e. the cell type). Interestingly, even when a new equilibrium has been established, FOXO proteins remain partially active as is to be expected from its active role in feedback regulation, but challenges the dogma that FOXO proteins are inactive under favourable conditions (i.e. sufficient nutrients and growth factors). From this, we anticipated a continuous involvement of FOXO proteins in regulation of PKB-activity and indeed show that, under the experimental conditions we used, FOXO1/3 depletion results in decreased PKB-activity, although some critical experiments remain to be performed. From these data it seems that continuous balancing of activity of all components of the PI3K-PKB-FOXO network maintains relatively steady activity of each individual component, which depends on the pathway as a unity. Importantly, these findings are of particular interest because perturbations of PI3K-PKB-FOXO signalling have been linked to disease and aging. In addition, with this work, we have focussed on two highly important, but rarely addressed questions: *why* and *for how long* are FOXO proteins active. A large body of data exists for changes that occur *if* FOXO proteins are active, but very little is known about the precise physiological and local circumstances under which FOXO-activity becomes apparent, although studies in model organisms have shed some light on this (reviewed in [18]). We propose that FOXO proteins are continuously engaged in transcription to a lesser or larger extent, dependent on fluctuations of the environment, to maintain constant PI3K-PKB-FOXO signalling activity.

FOXO in health and disease: homeostasis

We have discussed the role of cell and tissue homeostasis in aging and cancer, and how PKB and FOXO proteins have been linked to these processes (reviewed in **Chapter 1**). The term 'homeostasis' refers to a mechanism that aims to keep a given parameter within a certain range. For example, blood glucose level homeostasis aims to maintain blood glucose levels between 4mM and 7mM [19], and mechanistically involves insulin-mediated communication between multiple organs (reviewed in [20]). PKB-activity needs to be maintained at a level high enough to sustain normal growth and metabolism, but low enough prevent hyperproliferation and cancer (reviewed in [21]) and our data in **Chapter 2** indicate that FOXO proteins are mechanistically involved in this. One of the important reasons why sufficient PKB-activity is required for normal metabolism, is illustrated by experiments in mice with reduced insulin signalling. Whereas in *C. elegans*, elevated activity of FOXO homologues DAF-16 resulting from impaired PKB-activation appears to have only lifespan extending properties [3], mice with reduced insulin signalling due to loss of IRS2 develop a diabetes-like syndrome (reviewed in [22]). This may be a particular challenge, although a very real one, in lifespan extension in mammals or it may reflect that *C. elegans* are largely post-mitotic and require PKB activity only during development, while mammals require PKB signalling throughout their life to maintain tissue and body integrity. On a speculative level, the continued renewal of tissues may be a major contributor to our approximately 1500 times longer life span than *C. elegans*, leaving out the question why it has been an evolutionary successful strategy to get as old as we do. Interestingly, the diabetic phenotype of IRS2 knockout mice can be alleviated

by removal of a single allele of FOXO1 (reviewed in [22]), whereas FOXO knockout mice develop cancer [23] and hematopoietic stem cell (HSC)-specific deletion of FOXO proteins results in increased proliferation and premature depletion of HSC pool [24]. Taken together, these observations further support the idea that PKB and FOXO proteins act in reciprocal homeostasis of one another, aiming to keep either activity within a certain range, which mechanistically involves the other.

In Eijkelenboom and Burgering [10] FOXO-regulation of protein homeostasis was highlighted, although 'proper protein folding' may not be a continuous but a discrete parameter and as such, it is hard to speak of homeostasis. Interestingly, we observe upregulation of heat shock 70kDa protein 5 (HSPA5), better known as glucose-regulated protein, 78kDa (GRP78), upon FOXO-depletion in Hela cells [Charitou et al., under revision]. GRP78 is upregulated during endoplasmic reticulum (ER) stress as part of the unfolded protein response (UPR), which can, among other things, be induced by glucose deprivation-induced ATP depletion (reviewed in [25]). Increased GRP78 expression during UPR is important for pro-survival signalling (reviewed in [25]), possibly in part by increasing PKB activity [26]. ATP depletion also activates AMP-activated protein kinase (AMPK) (reviewed in [27]) and eukaryotic translation initiation factor 2-alpha kinase 3 (EIF2AK3), better known as protein kinase RNA-like endoplasmic reticulum kinase (PERK) (reviewed in [25]), and both AMPK and PERK can phosphorylate and activate FOXO proteins [28, 29]. In addition, essential mTORC2 component SIN [30] is destabilised by ATP depletion due to decreased activity of mTORC2 caused by low ATP levels [31] and mTORC2 activity is required for efficient phosphorylation of FOXO proteins by PKB [30, 32] (see also **Chapter 2** – figure 1E). Thus, it seems plausible that FOXO proteins are activated upon ATP depletion, possibly to activate PKB resulting in increased glucose uptake (reviewed in [33]). Indeed, FOXO3-knockout mouse embryonic fibroblasts have been reported to have a lower ATP content, which could be the result of the observed decrease in activity of PKB [34] and we observe GLUT1 upregulation in DL23 cells upon FOXO3-activation, which depends on PKB activity (data not shown). Therefore, we measured glucose uptake in response to either FOXO3-activation in DL23 cells or FOXO1/3-depletion in RPE cells, as well as FOXO-dependence of observed IRS2 upregulation in response to glucose deprivation. Surprisingly, we did not observe dramatic changes in glucose uptake in either DL23 cells with activated FOXO3, or RPE cells depleted for FOXO1 and FOXO3, nor was increased IRS2 abrogated by FOXO-depletion in RPE cells deprived of glucose (data not shown). However, PKB-mediated regulation of glucose uptake is probably dependent on the experimental setup [35] and very high in cancer cells such as DLD1 [36], while IRS2 regulation could be subject to additional factors in RPE cells. Thus, this remains an attractive line of research to pursue.

To extent this line of thought, stresses that enhance the activity of FOXO proteins and consequently PKB activity, could function as signalling mediators to drive division and increase organ capacity in accordance with demand. Such a model may be supported by data in HSCs, which are stimulated to produce blood cells in response to ischemic stroke (reviewed in [37]), a process that requires the generation of reactive oxygen species (ROS), which are known to activate FOXO proteins (reviewed in [7]). With a delay, the same FOXO-activity could restrict proliferation of these HSCs, which explains the results obtained from triple FOXO-knockout HSCs in mice, where it was shown that FOXO-loss results in hyperproliferation of HSCs that can be blocked by antioxidants [24]. To further test such a model, it would be interesting to observe how triple FOXO-knockout mice respond to ischemic injury and address specifically if they are able to mount the same response in a similar time-frame as wild-type littermates.

If not, this would illustrate both the timing effects (see above) and reciprocal homeostasis of PKB and FOXO proteins. However, this particular question has not been addressed to date, which leaves us only with the model.
In conclusion, many leads point to the involvement of FOXO proteins in different homeostasis mechanisms, ultimately resulting in maintenance of tissue and body integrity, as we have argued in **Chapter 1** and a view that we also share with others (reviewed in [9, 10]). Perturbation studies of homeostatic mechanisms are complicated, because the mechanism aims to negate the perturbations efficiently. Thus, we need to adapt our experiments and broaden our view on the number of regulatory inputs that influence our results to further elucidate these exciting possibilities. Let's get to work.

David Kloet, September 2014

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APPENDICES

List of abbreviations
Nederlandse samenvatting
Curriculum vitae
List of publications
Dankwoord

Abbreviations used in this manuscript

FOXO	Forkhead box-O
PI3K	phosphatidylinositol 3-kinase
PKB/Akt	protein kinase B/Akt
IRS	insulin receptor substrate
mTORC1/2	mammalian target of rapamycin complex 1/2
TSC1/2	tuberous sclerosis 1/2
RICTOR	rapamycin-insensitive companion of mTOR
RAPTOR	regulatory-associated protein of mTOR
GSK3	glycogen synthase kinase 3
c-MYC	cellular myelocytomatosis oncogene
S6K	ribosomal protein S6 kinase
BCL2	B-cell lymphoma 2
BCL6	B-cell CLL/lymphoma 6
BIM	BCL2-interacting mediator
BAD	BCL2-associated death promoter
PTEN	phosphatase and tensin homolog deleted on chromosome 10
HER/ERBB	human EGF receptor /v-erb-b2 avian erythroblastic leukemia viral oncogene homolog
CDK	cyclin-dependent kinase
p53	53kDa tumour protein
$p21^{Cip1/Waf1}$	21kDa CDK-interacting protein 1/wild-type p53-activated fragment 1
$p27^{Kip1}$	27kDa CDK-inhibiting protein 1
E2F	adenovirus E2 promoter binding factor
Rb	retinoblastoma p107
CTD	carboxyl-terminal domain
SCP	small CTD phosphatase
OS4	amplified region 12q13-q15 clone 4
CTDSP1/2/L/L2	CTD small phosphatase 1/2/Like/Like 2
TGFβ	transforming growth factor β
PD	phosphatase-dead
DMEM	Dulbecco's modified Eagle's medium
HRP	horseradish peroxidase
MEF	mouse embryonic fibroblast
RPE	retinal pigment epithelium
U2OS	human bone osteosarcoma cells
HEK293T	human embryonic kidney 293 (with SV40 large T)
MEF	mouse embryonic fibroblasts
NIH/3T3	NIH Swiss mouse embryo fibroblasts (immortalized with the 3T3 protocol)
FBS	foetal bovine serum
PEI	polyethylenimine
EtOH	ethanol

Nederlandse samenvatting (voor de leek)

Ons lichaam bestaat uit cellen; kleine gesloten eenheden die op zichzelf staan, maar die samen ons lichaam vormen. Alle cellen in ons lichaam hebben in beginsel dezelfde capaciteiten, maar omdat ze in bijvoorbeeld organen in een bepaalde organisatie zitten, zijn ze van elkaar afhankelijk en moeten cellen met elkaar communiceren. Dat gebeurd met chemische verbindingen die zij uitscheiden, zoals hormonen. Deze chemische verbindingen – liganden – stimuleren bepaalde veranderingen in de cellen die de liganden waarnemen. Zo kunnen de liganden zorgen voor de opname of juist afgifte van glucose in ons bloed, maar ook voor celdeling of celdood. Deze processen worden geregeld door een opeenstapeling van heel veel kleine veranderingen in de van een cel. De cascades van deze veranderingen heten samengevat signaal transductie.

De cellen zijn georganiseerd in compartimenten die worden gescheiden door membranen. Deze membranen laten kleine moleculen zoals water passeren, maar houden grote moleculen zoals eiwitten tegen. De buitenste membraan heet plasmamembraan en deze vormt de scheiding met de omgeving. In de cel zijn ook compartimenten, waaronder de celkern, die wordt gescheiden door de kernmembraan. In de kern bevindt zich het DNA; de informatie voor de het leven (zie figuur 1).

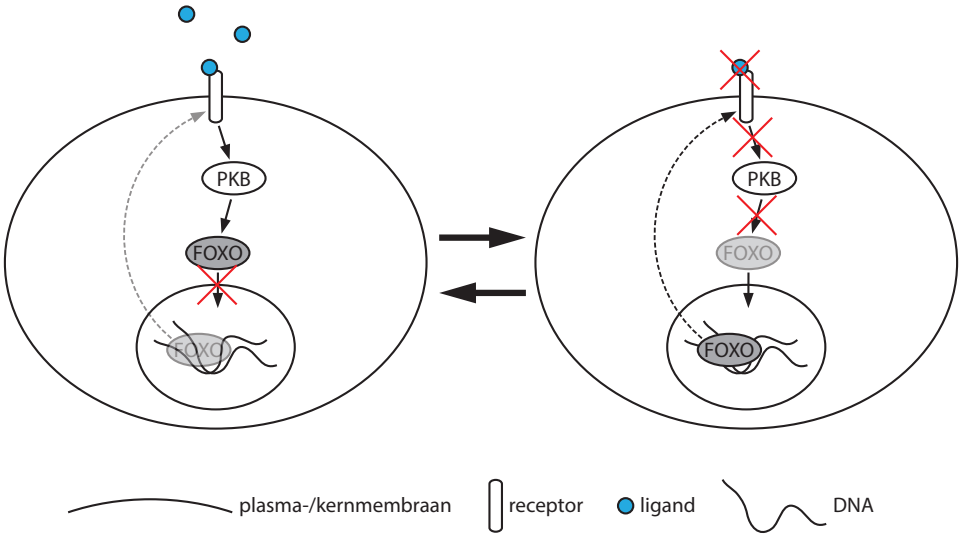
In ons laboratorium bestuderen we een specifieke familie van eiwitten die wordt gekarakteriseerd door het eiwitdomein dat we Forkhead box noemen. Dit domein zorgt ervoor zorgt ze aan het DNA kunnen binden en zo de aanmaak van eiwitten kunnen beïnvloeden. De eiwitten waar wij ons mee bezig houden heten de Forkhead box-O (FOXO) eiwitten.

Om aan het DNA te kunnen binden moeten FOXO eiwitten de kern in. Dit proces wordt geremd door eiwit kinase B (PKB). PKB is actief na stimulatie met liganden van buiten de cel. De liganden kunnen niet de cel in, maar binden aan speciale eiwitten die door de plasmamembraan heen steken: receptor eiwitten (zie figuur 1). Receptor eiwitten activeren PKB als de liganden aan hen binden.

Als PKB actief wordt kunnen de FOXO eiwitten dus niet naar het DNA, en zijn ze dus niet in staat hun functie uit te voeren (figuur 1- links). Als PKB minder of niet actief is kunnen de FOXO eiwitten de kern in gaan en zo de aanmaak van een heleboel eiwitten stimuleren (figuur 1- rechts). PKB stimuleert celdeling en remt celdood, terwijl FOXO eiwitten celdeling juist remmen en celdood bevorderen. Interessant maar tegenstrijdig is dat verhoogde activiteit van FOXO eiwitten ook zorgt voor een langer en gezonder leven.

Weefsels in ons lichaam worden steeds vernieuwd, onder andere door celdeling en cel sterfte Dit wordt beïnvloed door veel verschillende signalen, waaronder het activeren van PKB. In oude cellen of kankercellen raakt deze regulatie vaak verstoord: de cellen zetten PKB continu “aan” of “uit”. Dit leidt tot ziekten zoals kanker of diabtes, en uiteindelijk tot het ineenstorten van het systeem (ons lichaam). Het is dus belangrijk dat er niet te veel, maar ook niet te weinig stimulatie van PKB optreedt.

Wij hebben ons gericht op verhouding tussen de PKB en FOXO eiwitten. Ondanks dat PKB het tegenovergestelde doet van FOXO, kan FOXO ook de activiteit van PKB stimuleren (de



Figuur 1: schematische weergave van groeifactor-PKB-FOXO signalering met terugkoppelregulatie
Links: groeifactoren binden aan receptoren, welke PKB (indirect) activeren. Actief PKB zorgt dat FOXO eiwitten voornamelijk buiten de kern blijven, niet aan het DNA kunnen binden en dus niet de aanmaak van andere eiwitten kan beïnvloeden.
Rechts: als er geen groeifactoren zijn, wordt PKB minder actief en kunnen FOXO eiwitten makkelijker in de kern komen. Door aan het DNA te binden zorgen FOXO eiwitten voor meer receptoren en andere eiwitten die nodig zijn om PKB actief te maken. Op die manier stimuleren de FOXO eiwitten dat PKB weer actiever wordt en ze zelf weer minder de kern in kunnen (en ze dus ook minder kunnen helpen om PKB te activeren).
Dit terugkoppelmechanisme zorgt ervoor dat zowel PKB als FOXO eiwitten nooit te weinig of te veel actief worden.

gestippelde lijn in figuur 1). Daarmee doen FOXO eiwitten hun eigen effecten gedeeltelijk teniet en worden ze zelf weer inactief. Deze negatieve terugkoppeling is iets wat je veel vaker ziet in cellen. Het signaal gaat dus niet aan of uit, maar verandert zeer specifiek en gebalanceerd, al naar gelang wat de cel op dat moment nodig heeft. Uit de literatuur (samengevat in hoofdstuk 1) en ons onderzoek (samengevat in hoofdstuk 2) is gebleken dat de activiteit van FOXO eiwitten veel dynamischer zijn dan we dachten (figuur 1: de toestand van de cel wisselt steeds tussen links en rechts).

We hebben ons ook gericht op de gevolgen van activering van FOXO eiwitten (samengevat in hoofdstukken 2, 3, 4 en 5). We vermoeden FOXO eiwitten een heel algemene rol spelen in het verzorgen van levensduur en gezondheid. We hebben geprobeerd om te achterhalen hoe FOXO eiwitten dit teweeg brengt, door te kijken hoe verschillende celtypen op FOXO eiwitten reageren: van welke eiwitten ze meer en minder gaan produceren (samengevat in hoofdstuk 3). Er zijn maar weinig eiwitten die in alle celtypen veranderen. We hebben één van die eiwitten (CTDSP2) uitvoerig bestudeerd (samengevat in hoofdstukken 3 en 4). Van CTDSP2 is bekend dat het zelf ook de aanmaak van eiwitten regelt. Daarnaast kan het net als PKB de functie van andere eiwitten beïnvloeden. Meer CTDSP2 dan normaal is voldoende om celdeling te remmen en celdood te stimuleren. Dit heeft veel weg van de gevolgen van verhoogde activiteit van FOXO eiwitten. We hebben onderzocht hoe CTDSP2 dit doet(samengevat in hoofdstuk 3) en gevonden dat dit het gevolg is van stimulatie van een andere signaleringroute (samengevat in hoofdstuk 4). Als laatste hebben we ook gekeken naar de gevolgen van actief FOXO op de aanmaak van een speciale groep moleculen (microRNA's)

die de aanmaak van eiwitten kunnen remmen (samengevat in hoofdstuk 5). FOXO eiwitten beïnvloeden de aanmaak van een aantal van deze microRNA moleculen. We hebben van één microRNA bestudeerd welke eiwitten het remt. Het zal interessant zijn om hier nog verder onderzoek naar te doen, juist omdat FOXO eiwitten ook de verhoogde aanmaak van dit microRNA kan induceren.

Samengevat hebben we dus veel geleerd over de dynamiek van FOXO-activiteit, de gevolgen van deze activiteit en de invloed van deze activiteit op celdeling. Deze kennis kan in de toekomst bijdragen aan de behandeling van kanker, maar ook juist om te begrijpen waarom kanker ontstaat.

Curriculum vitae

David Ernst Adriaan Kloet werd geboren op 11 mei 1981 te Groningen. Van 1987 tot en met 1999 zat hij op de Vrije School Groningen en in 2000 behaalde hij zijn VWO diploma aan het Noorderpoort College Groningen. In 2001 begon hij zijn Bachelor Biologie in Wageningen en rondde in 2007 de Master Biologie – Cell Biology af. Tijdens deze Master heeft hij drie stages gelopen. De eerste stage was bij de groep Plant Cell Biology, Universiteit Wageningen onder leiding van prof. dr. Anne-Mie Emons en dr. Tijs Ketelaar. Daar heeft hij zich verdiept in mechanismen van cel strekking van epidermale cellen van plantenwortels. De tweede stage was bij het department Neuroscience, Erasmus MC onder leiding van prof. dr. Ype Elgersma en Mitja Vandeputte. Tijdens deze stage heeft hij calmodulin-dependent kinase II-mutante embryonale stamcellen gemaakt. De derde stage was bij Solvay Pharmaceuticals onder leiding van dr. Hiskias Keizer. Hier heeft hij geprobeerd een test-assay te ontwikkelen om de activiteit van een specifieke peptidase te meten. Op 1 oktober 2007 is David begonnen als onderzoeker in opleiding in de groep van prof. dr. Boudewijn Burgering, binnen de afdeling Molecular Cancer Research, UMC Utrecht. Het werk dat hij daar heeft verricht is beschreven in dit proefschrift.

List of publications

Kloet, D.E.A. and Burgering, B.M.T. (2011) The PKB/FOXO switch in aging and cancer. *Biochimica et Biophysica Acta* **1813**, 1926-1937

Dankwoord

Dit is het dan, het einde van een weg met veel kuilen en andere decepties. Het is toch gelukt en daar ben ik blij om. Het staat buiten kijf dat ik hier niet alleen verantwoordelijk voor ben, in tegendeel. Ik wil iedereen heel erg bedanken en ga nu een poging doen hen allen bij naam te noemen :-)

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Maria, we have met two times during my PhD. I believe the first time, I may have made a bad impression… Please forgive me for that. I think we both always felt we understand each naturally and I have always valued that very much. I was very happy to hear you came back, although I knew already that I'd be soon be leaving. I have enjoyed our workaholic-madness and never-ending science talk, but even more so to learn about what moves you and made you this way. Thank you so much for sharing your thoughts and ideas with me; I hope you have enjoyed mine equally well! We'll keep in touch this time, ok?

Lydia, altijd met twee benen in het lab en in je leven, en trouwe ridder in de garde van Boudewijn. Alhoewel ik de term 'lab-mamma' niet zelf heb bedacht en ook niet direct gekozen zou hebben, past hij wel bij je: 'Als je het niet weet, dan vraag je het even aan Lydia'. Het kletsen en kibbelen was voor mij altijd vertrouwd; misschien houden we allebei evenzeer niet van verandering. Ik had in ieder geval altijd het gevoel dat we elkaar begrepen, of in ieder geval dat je mijn mogelijkheden en beperkingen goed door had. Bedankt voor al je goede zorgen!

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jaren. Doordat je nu veel meer tijd kwijt bent met je eigen groep en verplichtingen, is dat logischerwijs minder geworden. Bedankt voor je steun en welwillendheid me te helpen met de FACS. Hopelijk kun je nog verder je onderzoek uitbouwen en op termijn een onafhankelijke groep vormen.

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Sabina, je werkt nog maar kort bij ons en ik weet dus niet veel van je, behalve een onvermijdelijke link naar mijn vroegere studiestad Wageningen. Ik vind het bijzonder dat je van Voeding en Gezondheid bent overgestapt naar biochemie en dat je zo goed je weg kunt vinden in deze nieuwe omgeving. Hopelijk gaan deze ontwikkelingen zich ook voortzetten bij je thuis en kunnen jullie samen proeven aan het leven van werken; dat bevalt mij althans heel goed! **Maaike** (Meerlo), je bent een vrolijke aanwinst voor het lab en ik weet zeker dat je hier veel kunt leren. Ik was in ieder geval blij met je aanwezigheid en bijdrage :-) Hopelijk blijf je nog een tijdje! **Sasha**, ook jij werkt nog maar kort in ons lab en we hebben nog niet echt de tijd gehad om elkaar beter te leren kennen. Ik hoop dat we daar nog een beetje verandering in kunnen brengen. **Robert**, onze meest recente aanwinst! Ik heb nog niet de kans gehad om meer met je uit te wisselen dan hallo. Hopelijk bevalt het werk je en kun je een goed team vormen met Harmjan!

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&

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