Complex regulation of Forkhead Box O transcription factors

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Complex regulation of Forkhead Box O transcription factors

Complexe regulatie van Forkhead Box O transcriptie factoren

(met een samenvatting in het Nederlands)

Proefschrift

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“You never fail until you stop trying”

- Albert Einstein -

Table of Contents

CHAPTER 1
General introduction

Integrating opposing signals towards Forkhead Box O

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Abstract

Transcription factors are the common convergence points of signal transduction pathways to affect gene transcription. Signal transduction activity results in posttranslational modification [PTM] of transcription factors and the sum of these modifications at any given time point will determine the action of the transcription factor. It has been suggested that these PTMs provide a transcription factor code analogous to the histone code. However, the number and variety of these modifications and the lack of knowledge in general of their dynamics precludes at present a concise view of how combinations of PTMs affect transcription factor function. Also, a single type of PTM such as phosphorylation can have opposing effects on transcription factor activity. Transcription factors of the Forkhead box O [FOXO] class are predominantly regulated through signaling, by phosphoinositide 3-kinase (PI-3K)/protein kinase B [PKB also known as AKT] pathway and a reactive oxygen species [ROS]/c-jun terminal kinase [JNK] pathway. Both pathways result in increased FOXO phosphorylation yet with opposing result. Whereas PKB-mediated phosphorylation inactivates FOXO, JNK-mediated phosphorylation results in activation of FOXO. Here we discuss regulation of FOXO transcription factors by phosphorylation as an example for understanding integration of signal transduction at the level of transcription activity.

Introduction

The transcription factor family of forkhead proteins participates in numerous cellular processes, such as development, differentiation, metabolism, proliferation, apoptosis and stress resistance. The family name forkhead is derived from its founding member the *Drosophila Melanogaster Forkhead (fkh)* gene product. A conserved DNA binding domain also known as the 'forkhead box' characterizes Forkhead proteins [1]. This domain is a variant of the helix-turn-helix motif and is made up of three alpha helices and two characteristic large loops or butterfly-like "wings"[2, 3]. There is a high degree of sequence homology within the DNA binding domain, but an almost complete lack of similarity in the transactivation domains.

In mammals, the class O of Fox transcription factors (FOXO) contains 4 members: FOXO1, FOXO3, FOXO4 and FOXO6. The DNA binding domain of FOXO, in particular helix 3, mediates binding to promoters that contain the FOXO consensus motif 5'-TTGTTTAC-3' [4]. In general it seems that expression of FOXO-regulated genes can be controlled by any of the FOXO transcription factors, and that specificity is obtained either by their specific expression pattern or by isoform-specific regulation. Therefore, throughout this review we will use the general term FOXO, if not specified otherwise.

FOXOs are subject to regulation by many signaling pathways but initially it was shown that FOXO activity is controlled by insulin/ insulin-like growth factor (IGF) signaling, in particular through phosphoinositide 3 kinase (PI3K)/ protein kinase B (PKB also known as AKT) signaling resulting in PKB-mediated phosphorylation (Fig. 1) [5-10]. As FOXOs therefore were the first identified downstream transcriptional regulators of this pathway, it became of interest to identify what PI3K/PKB dependent cellular processes are regulated through FOXO and what gene transcription would therefore be required.

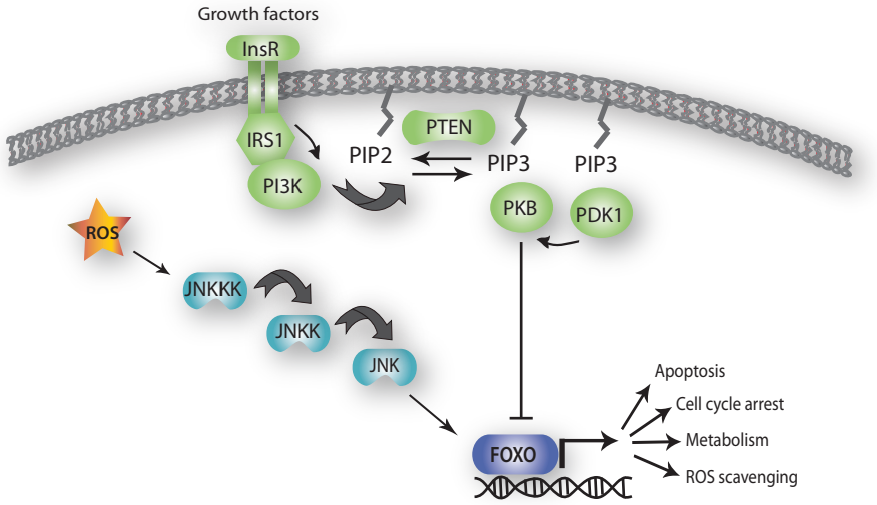


FIGURE 1: Opposing signals towards FOXO.A simplified model of the PBK-induced negative and JNK-induced positive signaling pathways toward FOXO. FOXO, Forkhead box O; InsR, insulin receptor; IRS, insulin receptor substrate; JNK, c-Jun N-terminal kinase; PDK1, 3-phosphoinositide- dependent protein kinase 1; PIP, phospho-inositide-phosphate; PKB, protein kinase B; PTEN, phosphatase and tensin homolog.

Control of cell fate by FOXO

Ectopic expression of FOXO factors in a large variety of mammalian cell types causes a strong inhibition of cell proliferation [11-14]. This anti-proliferative effect of FOXO expression is also observed in transformed cell types, such as Ras-transformed cells and cells deficient in PTEN [12, 13]. Thus, FOXO factors can oppose the growth stimulating effects of PKB/AKT when expressed at sufficiently high levels. In many cell types, FOXO-induced growth inhibition is associated with a block in cell cycle progression in the G1 phase. FOXOs regulate transcription of the cdk inhibitor $p27^{kip1}$ [12, 13] and FOXO-mediated upregulation of $p27^{kip1}$ controls G1 progression. Next to $p27^{kip1}$, FOXO-mediated transcriptional repression of D-type cyclins is crucial to the FOXO-induced cell cycle arrest [15, 16]. Under conditions of increased cellular stress, mostly oxidative stress FOXOs may also induce a cell cycle arrest at G2/M [17]. However what target genes mediate G2/M arrest is presently unclear. Cell cycle arrest in G1/S or G2/M cells oftentimes is a prerequisite to proceed to a specific cellular fate, including quiescence, senescence, differentiation or cell death. FOXOs are more or less implicated in all these cellular fates. For example FOXO-induced arrest in DLD-1 cells is timely and reversible, an important criterion for quiescent cells [14]. In contrast to quiescence, a number of cell types respond to FOXO activation by inducing apoptosis. Importantly, at least in BaF3 cells, induction of cell death follows a G1 arrest, showing that FOXOs first induce a cell cycle arrest followed by a switch to a 'desired' cell fate [18]. A number of FOXO gene targets that may mediate apoptosis have been identified, including Fas-ligand [9], Bim [19, 20], Bcl-6, negatively regulating BCL-XL expression [21], TRAIL (Tumor Necrosis Factor-related Apoptosis Inducing Ligand) [22] and TRADD (tumor necrosis factor receptor-associated death domain) [23].

In order to enable regulation of these complex and diverse cell fates FOXOs have to regulate other gene programs such as cell metabolism and stress response programs. In mammalian cell systems and in the nematode *Caenorhabditis elegans*, FOXO factors have been shown to mediate protection of cells against oxidative stress. FOXOs control the expression of numerous enzymes involved in anti-oxidant defenses including manganese superoxide dismutase (MnSOD) [24] catalase [25] sestrins [26] and selenoprotein P [27]. In addition, FOXO was shown to control the expression of GADD45, a protein involved in DNA repair mechanisms [17, 28]. Taken together, these data demonstrate that FOXO factors regulate the expression of a number of genes that are important in the protection of cells against oxidative stress. As many studies employ H_2O_2 addition to cells, the nature of this oxidative stress is not extensively specified, but at least metabolic stress (e.g. glucose starvation and caloric restriction) are thought to contribute to endogenous regulation of FOXO by cellular stress. FOXO activity has also an important impact on cell metabolism. For instance in situations where there is lack of, or impaired insulin signaling, such as during fasting or diabetes, FOXOs play an important role in regulating gene expression important for hepatic gluconeogenesis, like glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) [29, 30]. In addition FOXO1 plays an important role in lipid metabolism where it regulates microsomal triglyceride transfer protein (MTP) production [31], a membrane associated chaperone protein that catalyzes transfer of lipids to nascent apolipoprotein B (apoB). MTP is necessary for VLDL formation; as the liver specific $MTP^{-/-}$ mice are unable to produce VLDL [32] and genetic mutations in the gene encoding MTP (*MTTP),* cause abetalipoproteinemia (eliminated VLDL formation) in humans [33]. So in situations of impaired insulin signaling, FOXO1 is active and induces MTP production and thereby increased VLDL levels, resulting in hypertriglyceridemia [31]. Next to the regulation of MTP, FOXO1 also promotes the expression of apolipoprotein CIII (apoCIII) [34]. ApoCIII inhibits the clearance of triclycerides, so increased ApoCIII expression by FOXO1 can result in hypertriglyceridemia as well. Besides the liver, FOXOs also play an important metabolic function in muscle cells, where they are involved in muscle atrophy and myoblast differentiation. Muscle atrophy

is a process in which rapid loss of muscle proteins takes place. This process occurs systemically with diseases like diabetes and cancer and during fasting, circumstances in which FOXOs are activated. So not unexpectedly, during catabolic states, FOXOs translocate to the nucleus and induces antrogin-1 and MuRF-1 gene expression, atrophy-specific ubiquitin ligases, important in the proteosomal mediated degradation of muscle proteins [35, 36]. Recently, Zhao *et al.* and Mammucari *et al.* discovered an additional role for FOXO3 in the regulation of muscle atrophy, by regulation of autophagy through transcriptional regulation of a set of autophagy genes [37, 38]. Thus, muscle atrophy is regulated through two different pathways; ubiquitin/proteosomal pathway and autophagic/lysosomal pathway in which FOXO plays an essential role by transcriptional regulation of genes involved in both pathways.
Next to the above described metabolic functions of FOXO, they are important in regulating metabolic processes in a subset of other tissues/cell types as well; pancreatic β-cells, adipose tissue, hypothalamus and endothelial cells. (For more detailed information, see reviews [39, 40]).

FOXOs through evolution

All of the above studies are in fact guided by the initial observation that FOXO transcription factors are orthologous to the nematode *Caenorhabditis elegans* DAF-16 (Abnormal Dauer Formation-16) protein. DAF-16, like FOXO is also negatively regulated by the PI(3)K (*age-1*) / PKB (*akt-1/2*) pathway [41-43]. In *C. elegans*, DAF-16 promotes entry into the Dauer stage. The Dauer stage represents an alternative larval stage that is induced by starvation, a dauer pheromone or high temperature. This developmental arrest is associated with reduced metabolic activity and increased resistance to oxidative stress. Besides controlling dauer diapause, DAF-16 has also been shown to be a key component in controlling *C. elegans* lifespan. Thus, increased DAF-16 activity is responsible for enhanced *C. elegans* lifespan and further studies have shown that at least to some extend this DAF-16/FOXO function is preserved through evolution. This observation has turned out to be key of current thinking with respect to ageing. Consequently all effects of FOXO on cell fate as described above are held against the light of its impact on organismal lifespan, and are used to further our understanding of lifespan and ageing.
Biological ageing can be defined as a timely decline of functional ability of cells, tissue or organism and changes in lifespan observed in model organisms are thought to reflect at least in part the process of ageing. Many theories have been put forward to explain the phenomenon of ageing, amongst which the so-called 'free-radical theory of ageing' likely best explains the function of DAF-16/FOXO in lifespan. In essence this theory postulates that because organisms use molecular oxygen (O_2) for efficient generation of ATP, they are also continuously exposed to the damaging effects of cellular reactive oxygen species (ROS) that is produced because of O_2 consumption. The accumulating damaging effects of ROS are then believed to underlie the natural process of ageing. Importantly, whereas ageing itself is not a disease, ROS-induced cellular damage is generally considered important in the etiology of almost all age-related disease, including cancer and diabetes. In agreement with the above, DAF-16/FOXO has been shown to regulate a number of genes (e.g. MnSOD, sestrin3, catalase) which are involved in conferring resistance against increased cellular ROS. The apparent importance of DAF-16/FOXO in mediating resistance against increased ROS led to the suggestion that DAF-16/FOXO in return is also subject to regulation by ROS. Indeed, numerous studies by others and us have shown that in contrast to insulin signaling, increased ROS result in activation of DAF-16/FOXO. Most importantly, at least within the context of this review it has been shown in mammalian cells, as well as in *C. elegans* and *D. Melanogaster* that direct JNK-mediated phosphorylation of DAF-16/FOXO mediates activation of DAF-16/FOXO following increased ROS (Fig. 1) [44-46]. In agreement enhanced JNK activity increases lifespan in

C. elegans and *D. melanogaster* and this requires DAF-16 and dFOXO respectively [44, 45]. Although JNK-mediated phosphorylation is important to regulate FOXO after increased ROS, the activity of FOXOs is further tightly regulated by other posttranslational modifications such as acetylation, methylation and ubiquitination, all of which are regulated after changes in cellular ROS. All these PTMs affect FOXO function and this can occur in various ways. FOXOs shuttle between nucleus and cytoplasm and PTMs can shift the equilibrium of this shuttling to either site. Also when bound to DNA, PTMs are required to unfold transcriptional activity through recruitment of co-activators/repressors (e.g. p300/CBP; SIRT). Finally, FOXO protein stability is regulated by PTMs, Thus FOXO activity can be regulated through multiple means and multiple PTMs and this provides a challenge in understanding the interplay between the various PTMs. Here, we will discuss the opposing signals, of activation and inactivation, mediated by phosphorylation on FOXOs.

Signal transduction pathways regulating FOXO phosphorylation status

1. Growth factor signaling

FOXO transcription factors are regulated by the insulin signaling pathway through phosphoinositide 3-kinase (PI-3K) and protein kinase B (Fig. 1). All FOXO members as well as DAF-16 contain 3 conserved PKB phosphorylation sites (Fig. 2). *In vivo* all three sites are phosphorylated following growth factor, in particular insulin and IGF-1, treatment [8, 9, 47, 48]. PKB-induced phosphorylation of FOXO on these three sites results in the binding to 14-3-3 and this correlates with export of FOXO out of the nucleus [10, 48-50]. Shuttling of proteins between nucleus and cytoplasm is a highly regulated process and requires accessory proteins such as importins and exportins [51]. At present there is still little detailed knowledge as to how FOXO shuttling is regulated. Domains within FOXO that could function as nuclear export signal (NES) or nuclear localization signal (NLS) have been assigned but are supported by limited experimental evidence. For FOXO4 a non-classical NLS that surrounds the PKB phosphorylation site Ser-193 has been defined [10]. Shuttling of FOXO4 was shown to be a Ran- and Crm1-dependent mechanism and PKB-mediated phosphorylation of FOXO4 on Ser-193 functionally inactivates the NLS, causing the continuously shuttling transcription factor to be detained in the cytoplasm [10]. The primary sequence of the FOXO4 NLS shows that it is not a classical NLS and indeed none of the classical importins were found to bind FOXO4 and at present the importin responsible for nuclear import is unknown.

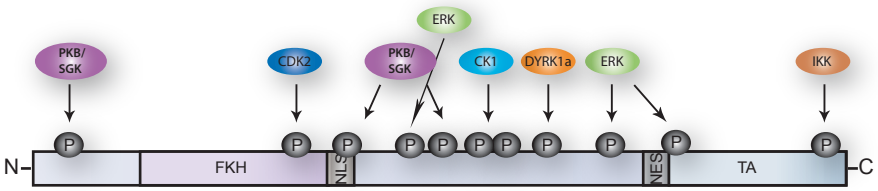
Although all FOXO members harbor a putative NES sequence, its function remains unclear. Nuclear export of Forkheads is sensitive to leptomycinB treatment indicating a Crm1-dependent export mechanism [10]. Deletion of the putative NES clearly affects nuclear export of FOXO4, yet surprisingly Crm-1 binding appears unaffected by phosphorylation and the NES deletion does not prevent binding between FOXO4 and Crm1. This leaves the possibility that an additional protein in complex with Forkhead/Crm1 might provide the required NES.

Serum- and glucocorticoid-inducible kinase (SGK) is highly homologous to PKB and like the family of PKB kinases (PKBa, b and g), the SGK family also consists of three members (SGK1, 2 and 3). SKGs phosphorylate the same consensus phosphorylation site in target proteins (RXRXXS/T) as PKB, and therefore SGK can phosphorylate FOXO3 at the same sites as PKB (e.g. Thr-32, Ser-253 and Ser-315 in FOXO3a) (Fig. 2) [52]. However SGK preferentially phosphorylates Thr-32 and Ser-315, whereas PKB preferentially phosphorylates Thr-32 and Ser-253 in FOXO3a [52].

SGK/PKB mediated phosphorylation not only provides a binding interface for 14-3-3 proteins but PKB-mediated phosphorylation of Ser-319 of FOXO1 has also been shown to act as a docking site for subsequent phosphorylation by Casein Kinase 1 (CK-1). CK-1 recruitment in this way results in

phosphorylation of two additional sites, Ser-322 and Ser-325 in FOXO1 (Fig. 2) [53].
Initial *in vitro* kinase experiments using purified his-tagged PKB indicated phosphorylation of an apparent non-PKB site [8]. Further analysis revealed that a kinase(s) harboring a natural his-tag apparently co-purifies with his-tagged PKB. Dual-specificity tyrosine-phosphorylated and regulated kinase 1A (DYRK1a) was identified in these purified PKB fractions as a natural his-tag expressing contaminant and responsible for phosphorylation of Ser-329 in FOXO1 and Ser-268 in FOXO4 (Fig. 2) [54]. Interestingly, Ser-329 phosphorylation appears constitutive as this is at least not regulated by insulin like growth factor signaling, or by over-expression of PI(3)K. Taken together combined PKB and CK-1 mediated phosphorylation and constitutive DYRK1a phosphorylation results in a consecutive stretch of phosphorylated residues (Ser-319, 322, 325 and 329 in FOXO1) and it is suggested that this negatively charged patch in FOXO greatly enhances nuclear export of FOXO1 in a Ran-Crm1 specific manner [53].

A



B

	FOXO1	FOXO3a	FOXO4	Conserved in:
PKB/ SGK	Thr24 Ser256 Ser319	Thr32 Ser253 Ser315	Thr28 Ser193 Ser258	*C. elegans* *D. melanogaster*
CK1	Ser322 Ser325	Ser318 Ser321	Ser261 Ser264	
DYRK1a	Ser329	Ser325	Ser268	
CDK2	Ser249			
IKK		Ser644		
ERK		Ser294 Ser344 Ser425		

FIGURE 2: Phosphorylation dependent inactivation of FOXO. (**A**) Schematic representation of FOXO domains and phosphorylated residues. (**B**) Kinases involved in phosphorylation and subsequent inactivation of FOXO. Specific phosphorylation sites of the different FOXO members are indicated. Numbering of all phosphorylation sites is for human FOXO. CDK2, cyclin dependent kinase 2; ERK, extracellular signal-regulated kinase; FKH, Forkhead domain; IKK, IkB kinase; NES, nuclear export signal; NLS, nuclear localization signal; TA, transactivation domain.

2. Ikk-dependent signaling

Immunohistochemical analysis of certain tumors revealed apparent FOXO3a localization in the cytoplasm, while no hyperactive PKB/AKT could be detected. This led to the suggestion that in these tumors other kinases, besides PKB, are responsible for relocalizing FOXO3a to the cytosol. This resulted in the identification of IκB kinase (IKK) as being responsible for Ser-644 phosphorylation of FOXO3a

(Fig. 2). The phosphorylation of FOXO3a on Ser-644 seems to be specific for the human and mouse FOXO3a, as this site is not conserved among the other members of the FOXO family. The classical role of IKKs is to regulate NFκB during cytokine-induced and inflammatory responses and in agreement phosphorylation of FOXO3a on Ser-644 is induced after TNFα stimulation. Ser-644 phosphorylation is suggested to inhibit, like PKB through nuclear exclusion of FOXO, followed by poly-ubiquitination and consequent degradation. Thus IKK mediated Ser-644 phosphorylation results in inhibition of FOXO3a transcriptional activity and this may occur in a PKB independent manner, as a mutant of FOXO3a, mutated in all three PKB sites, is still suppressed by IKK [55]. However, PKB has been shown to positively regulate IKK [56], and thus may act upstream rather than downstream of IKK, and Ser-644 phosphorylation would thereby provide an additional mechanism whereby PKB is able to inhibit FOXO3a function.

3. DNA damage signaling

ROS-induced DNA damage is considered a main driving force of ageing. Thus a linkage between DNA damage and FOXO function is to be expected. Initially, it was shown that FOXO activation results in increased GADD45a gene expression [17, 28]. GADD45a has been implicated in DNA repair and in agreement it was shown that FOXO activation results in GADD45a dependent repair of an ex-vivo UV-damaged plasmid [28]. Interestingly it was shown recently that GADD45a has a key role in active DNA demethylation [57]. This may explain how GADD45a facilitates DNA repair, but also indicates that FOXO through GADD45a may contribute to re-activation of genes silenced by DNA methylation.

A high-throughput mass-spectrometry analysis of proteins phosphorylated on serine followed by glutamine (SpQ), the consensus for ATM and ATR-mediated phosphorylation, revealed FOXO1 to be a potential ATM substrate [58]. This would be in accordance with aforementioned possibility that FOXO-induced gene expression may facilitate DNA repair. Subsequently it was shown that FOXO and ATM can interact, but rather than serving as a substrate, FOXO was suggested to be required for ATM to become activated upon DNA damage. Cells in which FOXO3a expression was reduced by siRNA showed complete lack of ATM activation following DNA damage [59]. How FOXO senses DNA damage and how this then is transmitted to ATM remains yet unresolved. Alternatively, a recent report shows that in hematopoietic stem cells, FOXO3a deficiency compromises ATM expression suggesting loss of ATM activation is primarily due to loss of ATM expression [60].

Furthermore, CDK2 is reported to directly phosphorylate FOXO1 on Ser-249 (Fig. 2) [61]. Phosphorylation on this site results in cytoplasmic localization and consequent inhibition of FOXO1 transcriptional activity. Thus DNA damage through inhibition of CDK2 would activate FOXO to either induce a cell cycle arrest and concomitant repair, or to induce cell death. In addition the same group reported CDK1 to also phosphorylate Ser-249 in FOXO1 [62]. This would suggest that FOXO activation following DNA damage occurs at both G1/S and G2/M and that FOXO activation does not link to a specific mode of DNA damage repair. Thus the involvement of FOXO in DNA repair appears general rather then specific. However and in contrast to the above, Yuan *et al.*, reported CDK1 mediated FOXO1 phosphorylation on Ser-249 to activate FOXO1 [63]. Here it was shown that upon CDK1-mediated phosphorylation, FOXO1, at least partially, is released from 14-3-3 and that this mediates cell death in neurons [63]. It should be noted that at least *in vivo* neurons are mostly post-mitotic and this may explain a different mode of action for FOXO in cycling cells. Yet, in addition, the same authors describe a role for FOXO1 in proliferating cells, where upon phosphorylation by CDK1, active FOXO1 regulates the expression of the mitotic regulator Polo-like kinase (PLK) [63]. Thus how these data, of Ser-249 phosphorylation activating or inactivating FOXO1, can be reconciled at the biochemical level is at present not clear.

4. Stress dependent signaling

Next to the many ways identified for the negative regulation of FOXO, more recently, several pathways have been identified to be important in activation of FOXO. One of the kinases described to activate FOXO is, c-Jun N-terminal Kinase (JNK) (Fig. 1). Upon cellular stress, JNK can directly phosphorylate FOXO (on different sites compared to PKB) (Fig. 3) and thereby overrules the negative phosphorylation by PKB, resulting in nuclear translocation and transcriptional activation of, in particular, FOXO4 [46]. The consensus sites for JNK phosphorylation in FOXO4, are not conserved among the other FOXOs, indicating more specified functions for the different FOXO proteins. However, next to the direct phosphorylation of FOXO4 by JNK, JNK is also reported to phosphorylate 14-3-3 proteins and thereby release 14-3-3 from its interaction partners. Indeed, a similar role for JNK is reported for the binding of FOXO to 14-3-3, which is abrogated upon phosphorylation of 14-3-3 by JNK, resulting in translocation of FOXO to the nucleus [64]. Since 14-3-3 binding regulates all FOXO family members, this might provide a more general function of JNK towards activation of FOXO. In addition, JNK mediated activation of FOXO is conserved in both *C. elegans* [45] and *D. Melanogaster* [44], where JNK mediated activation of DAF-16 or dFOXO results in stress resistance and lifespan extension.

Another kinase reported to be important for activation of FOXO3a is MST1. Under conditions of oxidative stress MST1 phosphorylates FOXO3a on Ser-207 (Fig. 3). Upon phosphorylation, FOXO3a is released from 14-3-3 proteins and translocates to the nucleus to promote transcription of its target genes [65]. However, structural analysis has shown that Ser-207 phosphorylation is incompatible with DNA binding [66] and this led to the suggestion that upon nuclear entry Ser-207 needs to be specifically dephosphorylated before FOXO becomes active. The MST1 mediated activation of FOXO is conserved in *C. elegans*, where the MST1 ortholoque, CST1, is important in regulation of DAF-16

A

B

	FOXO1	FOXO3a	FOXO4	Conserved in:
JNK			Thr447 Thr451	*C.elegans* *D.melanogaster*
MST1	Ser207			*C.elegans*
AMPK		Thr179 Ser399 Ser413 Ser555 Ser588 Ser626		*C.elegans*

FIGURE 3: Phosphorylation dependent activation of FOXO. (**A**) Schematic representation of FOXO domains and phosphorylated residues. (**B**) Kinases involved in phosphorylation and subsequent activation of FOXO. Specific phosphorylation sites of the different FOXO members are indicated. Numbering of all phosphorylation sites is for human FOXO. AMPK, AMP- dependent protein kinase.

dependent lifespan extension [65]. Recently, another group has shown that MST1 requires JNK-mediated phosphorylation on Ser-82 for its full activation and activity towards FOXO3a regulation. Mutation of Ser-82 to alanine abrogates the MST1 mediated phosphorylation of Ser-207 in FOXO3a [67]. However, MST1 is also a well-known upstream regulator of stress kinases including JNK and p38 [68]. Thus it could be equally well possible that a Ser-82 mutant of MST1 is impaired in JNK activation and therefore displays reduced ability to regulate FOXO. Clearly, further studies should shed light on the complicated interplay between MST1 and JNK signaling.

5. Nutrient signaling

In situations of low energy levels, the AMP-dependent protein kinase (AMPK) plays an important role in activation of energy-producing pathways and inactivation of energy-consuming pathways. AMPK exerts its function through numerous substrates including FOXO3a [69]. AMPK phosphorylates FOXO3a at six different sites (Fig. 3) and thereby activates its transcriptional program. Unlike JNK- and MST1-mediated regulation of FOXO, AMPK does not regulate FOXO3a subcellular localization. Meaning that FOXO3a needs to be nuclear in order to become activated by AMPK. This can either be a result of lack of growth factors and thereby no PKB/GSK signaling, or activation of the JNK/MST1 signaling pathway to localize FOXO to the nucleus, where AMPK can phosphorylate and further accelerate FOXO mediated transcription. Again this pathway is conserved in *C. elegans*, where upon some forms of dietary restriction, AMPK is activated and mediates DAF-16 dependent stress resistance and lifespan extension [70].

6. Regulation of FOXO activity by protein phosphatases

Because of the tight control of FOXO proteins by phosphorylation, a role for phosphatases in FOXO regulation can be expected. Protein phosphatase 2A (PP2A) has been shown to directly dephosphorylate FOXOs although the various studies differ within the details. Yan *et al.* reported that following knockdown of PP2A, but not PP1, there is increased phosphorylation on the PKB sites Thr-24 and Ser-256 of FOXO1 [71]. In contrast Singh *et al.* showed similar results for FOXO3a yet provided additional evidence that compared to FOXO3a, PP2A did not dephosphorylate FOXO1 and only slightly FOXO4 [72]. A major confounding factor is obviously the ability of PP2A to also dephosphorylate a number of the upstream FOXO regulators, including PKB [73, 74]. However, Singh et al. excluded to some extend such an indirect role of PP2A on FOXO *in vivo*. In addition, dephosphorylation by PP2A apparently affected FOXO function as in presence of PP2A inhibitors; FOXO1 is no longer able to induce apoptosis [71].

The catalytic subunit of PP2A requires targeting subunits to exert its proper function [75]. The PP2A targeting subunit B65epsilon is identified as a binding partner of FOXO3a, yet B65epsilon did not appear to mediate PP2A directed dephosphorylation [72]. Interestingly, the protease calpain has been shown to regulate FOXO3a in part through PP2A [76]. The B56 alpha and gamma targeting subunits of PP2A are *in vitro* substrates of calpain, and calpain regulates B56 alpha stability *in vivo*, suggesting a direct role of calpain in the regulation of PP2A function. However, this was linked to regulation of PKB activity rather than dephosphorylation of FOXO3a directly. Indeed, in this respect it is interesting to note that a genetic screen in *C. elegans* revealed pptr-1, a B56 regulatory subunit of the PP2A holoenzyme to regulate PKB activity in the worm [77]. In agreement mammalian B56beta regulates PKB phosphorylation at Thr 308 in 3T3-L1 adipocytes [77]. Thus PP2A regulates both PKB and FOXO and the concerted action on both is to increase FOXO activity. Likely the relative contribution of PP2A on both determines within the experimental set-up the conclusion as to what player mediates the effect of modulating PP2A activity. In addition resolving what targeting subunit of PP2A is required for its

effect on FOXO may help to further understand the details of dephosphorylating the different FOXO members.

7. Regulation of phosphorylation by methylation

The PKB phosphorylation sites of FOXOs adhere to the consensus sequence for PKB phosphorylation i.e. RXRXXS/T. Recently, it was shown that Arg-248 and -250 within the FOXO1 consensus encompassing the second PKB site (R(248)XXR(250)XS(253)) are subject to methylation by the methyltransferase PRMT1 [78]. Oxidative stress induces PRMT1 binding to FOXO1 and mediates methylation on Arg-248 and Arg250 in mouse FoxO1. Methylation on these sites abrogates PKB mediated phosphorylation on Ser-253. Therefore, PRMT1 mediated methylation of FOXO1 prevents its nuclear exclusion, polyubiquitination and proteosomal degradation [78]. Thus besides dephosphorylation, PRMT1-mediated FOXO methylation presents another mean to reduce phosphorylation on the PKB sites. This involvement of methylation again nicely illustrates the importance of crosstalk between different PTMs to fine-tune FOXO regulation. Interestingly, FOXO3a has been described to directly regulate BTG1, which is a positive regulator of PRMT1, suggesting a positive feedback loop where FOXO potentiates itself via induction of a BTG1/PRMT1 complex and subsequently abrogates PBK mediated phosphorylation [79].

Mechanisms of phosphorylation dependent FOXO regulation

Phosphorylation-dependent control of FOXO ubiquitination and degradation

As illustrated above, crosstalk between PTMs is an important mode of FOXO regulation and phosphorylation of FOXO thus indirectly regulates other PTMs of FOXO. Phosphorylated residues often serve as docking sites for additional regulatory proteins including kinases, as illustrated above for CK1. Phosphorylation of serine/threonine residues followed by a proline are also oftentimes a docking site for the peptidylisomerase PIN1 and indeed also FOXOs bind PIN1 [80]. The consequence of PIN1 binding to proteins is not uniform as its major action is to induce a conformational change in the target protein by inducing a cis to trans isomerization of the peptide bond between the phosphorylated residue and the proline at +1. This conformational change was initially shown to increase dephosphorylation of target proteins (eg c-myc [81]) and as such PIN1 functions in a manner similar to the targeting subunits of phosphates like PP2A. However in case of FOXO no apparent effect was seen on the phosphorylation status of both PKB and JNK sites. In contrast PIN1 binding appeared to affect de-ubiquitination of FOXO through increasing the activity of the deubiquitinating enzyme USP7 towards FOXO [80]. At present there is no clear evidence that acetylation of FOXOs is dependent on FOXO phosphorylation. Recently, the acetyltransferase p300/CBP-associated factor (PCAF) was reported to repress FOXO1 in a PKB dependent manner, since phosphorylation of the PKB site Ser-253 of FOXO1 is required for the interaction between PCAF and FOXO1 [82]. However, inhibition by PCAF occurred independent of its acetyltransferase activity [82]. In contrast to acetylation, ubiquitination of FOXOs is tightly co-regulated with phosphorylation. Originally it was noted that several important substrates of PKB display decreased protein half-life following PKB-mediated phosphorylation [83]. This suggested that PKB phosphorylation in general regulates the recruitment of an E3 ligase to induce polyubiquitination and degradation of its target proteins. The F-box protein Skp2 has been identified as a putative E3 ubiquitin ligase responsible for polyubiquitination of several PKB substrates including FOXOs (Fig. 4A) [84]. Skp2 is a component of SCF^{Skp2} (Skp1, Cul1, F-box protein Skp2) ubiquitin E3 ligase complex that targets the CDK inhibitor $p27^{Kip1}$ and other substrates for ubiquitin-dependent proteolysis [85]. Given the role of FOXO in $p27^{Kip1}$

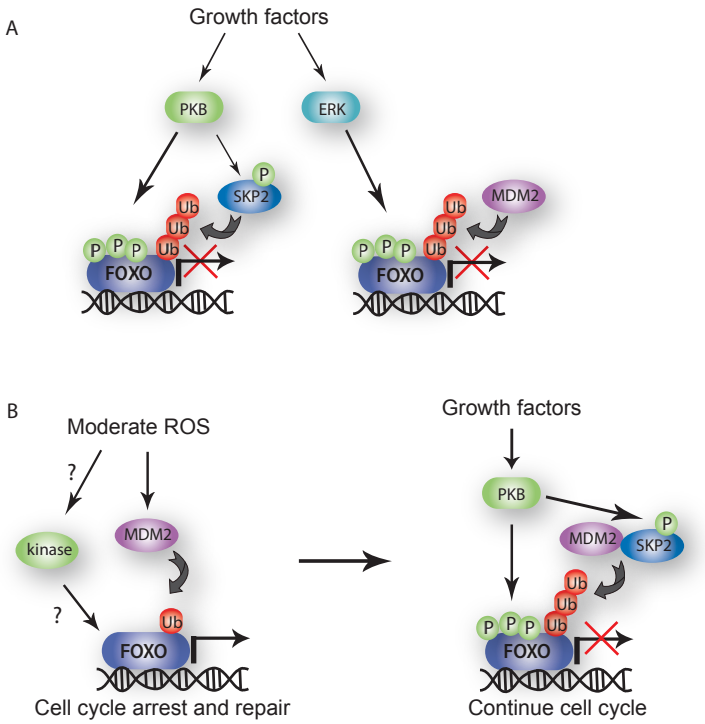


FIGURE 4: FOXO and ubiquitination. (**A**) Upon growth factor signals, FOXOs are phosphorylated by either PKB or ERK, resulting in recruitment of the E3 ligases SKP2 and MDM2, which polyubiquitinate FOXO resulting in its degradation. PKB can also activate SKP2 directly by phosphorylation. (**B**) Model of a switch between MDM2-mediated monoubiquitination versus polyubiquitination, where moderate stress can induce monoubiquitination and thereby activation of FOXO, resulting in cell cycle arrest and potential damage repair. If the damage is repaired and cell cycle can progress, FOXO can again be inactivated by growth factor-induced poly-ubiquitination and degradation. MDM2, murine double minute 2; SKP2, S-phase kinase-associated protein 2.

regulation this already suggested a mode of concerted regulation. Indeed, Skp2 has been shown to respond to PI3K/PKB signaling in multiple ways. Initially it was shown that PI3K activation through loss of PTEN results in increased Skp2 expression, [85] however this lacked a mechanism. Subsequent studies showed that Skp2 binds to PKB and that Skp2 is phosphorylated by PKB at Ser-72. Phosphorylation of Ser-72 results in Skp2 translocation to the cytoplasm, but the consequence of this remains unclear. Gao *et al.* show that in addition to nuclear exit, Ser-72 impairs Cdh1 binding and consequently APC-Cdh1-mediated Skp2 destruction, thereby providing rationale for the observed increase of Skp2 in PTEN negative cells [86]. Lin *et al.* show that Ser-72 phosphorylation results in increased SCF^{skp2} complex formation. Clearly Skp2 when stably in complex with Skp1 and Cul1 will, be it indirectly, protected from interacting with Cdh1 and degradation [87]. Thus in contrast to PKB substrates such as $p27^{kip1}$, $p21^{cip1}$, Tsc2 and FOXO, the consensus is that Skp2 is protected from destruction by PKB-mediated phosphorylation and this results in enhanced Skp2-dependent E3 ligase activity. In agreement with the above, platelet-derived growth factor treatment of chicken embryo fibroblasts (CEFs) transformed through overexpression of activated forms of PI3K and/or PKB, induced a strong reduction in FOXO1 half-life (<1 hr)[88]. Importantly, this also suggests that inhibition of FOXO function through increased degradation is a gain-of-function for cells transformed through increased PI-3K signaling. Huang *et al.* found that, in contrast to FOXO1, FOXO3 and FOXO4 do not interact with Skp2 [84]. However, growth

factor signaling affects FOXO3 stability [83], indicating that a Skp2-dependent degradation of FOXO3, and possibly also FOXO4, can not be ruled out. Clearly, in growth factor signaling through PKB, Skp2 is an important regulator of FOXO stability, but in addition Mdm2 has been suggested as E3 ligase mediating polyubiquitination and degradation of FOXO in growth factor signaling through MAPkinase/ERK (see Fig. 4A). FOXOs can be phosphorylated *in vitro* by several proline-directed kinases including MAPK/ERK [89]. Subsequently, it was shown that MAPK/ERK mediated phosphorylation of FOXO3a on Ser-294, Ser-344 and Ser-425 (Fig. 2), results in increased interaction of FOXO3a with the E3 ligase MDM2, thereby enhancing MDM2 dependent degradation of FOXO3a [90]. In addition, Fu *et al.* describe PKB dependent polyubiquitination of FOXO1 and FOXO3a mediated by MDM2 [91].

Phosphorylation dependent control of FOXO mono-ubiquitination and activation

Whereas MDM2 may mediate polyubiquitination and degradation of FOXO3a during growth factor signaling, MDM2-mediated mono-ubiquitination has been suggested to mediate FOXO activation during increased cellular oxidative stress (Fig. 4B). Following increased ROS, FOXO4 becomes mono-ubiquitinated, and this results in nuclear localization and increased transcriptional activity [92]. Recently, Brenkman *et al.*, found MDM2 to be the E3 ligase responsible for this stress-induced FOXO4 mono-ubiquitination [93].

Thus upon growth factor signaling, FOXO factors become phosphorylated and subsequently polyubiquitinated resulting in degradation. However, upon oxidative stress, where activation of FOXO factors is favored, FOXO4 becomes mono-ubiquitinated by MDM2, which results in its activation. If MDM2 is able to mono-ubiquitinate the other FOXO transcription factors remains to be established, as well as the possible involvement of a kinase, prior to the MDM2 mediated FOXO mono-ubiquitination. Nevertheless, in terms of regulation it is interesting that MDM2 may critically determine the activity status of FOXO. At present the model would be that under conditions of moderate cellular ROS increase, FOXOs become active through MDM2 dependent mono-ubiquitination and that this facilitates repair. If repair has been completed cell cycle progression may resume and growth factors may override the FOXO imposed cell cycle block by inducing MDM2 dependent FOXO degradation. (Illustrated in Fig. 4B)

Integrating opposing signals

Considering that PKB and JNK likely represent the major negative and positive regulatory input on FOXO respectively (Fig. 1), it is of importance to understand the general crosstalk between, and regulation of PKB and JNK activity. Several studies have described various means of crosstalk between PI3K/PKB and JNK. Here we will describe in brief several observations in this respect possibly relevant in understanding FOXO regulation.

Control of JNK signaling occurs through so-called scaffold proteins. These scaffold proteins are responsible of orchestrating the proper assembly of the JNK kinase cascade, which typically consists of a JNKK and a JNKKK (nicely reviewed in [94]). Second, due to their specific cellular locations these scaffold proteins are likely responsible for ensuring that JNK signaling is tailored towards the proper substrates given the stimulus context in which JNK activation occurs.

MST1 belongs to the STE20 group of MAPKKKs and is the mammalian homologue of *Drosopohila* HIPPO. In *D. melanogaster* as well as in mammals MST1/hippo is shown to restrict cell growth and survival and genetics in *Drosophila* has outlined a pathway (nicely reviewed in [95]). Whereas in *Drosophila* Mst1 signals through transcriptional control by Yorkie of cyclinE and DIAPs (Drosophola inhibitor of apoptosis), studies in mammalian cell lines indicate other mediators including, JNK

and p38 pathways through MKK4/7 and MKK3/6 respectively [68], but also H2B and FOXO. PKB can phosphorylate MST1 on Thr-120 and thereby inhibits MST1 function towards JNK as well as its role in apoptosis (Fig. 6A) [96, 97]. In addition, PBK has been reported to phosphorylate MST1 on Thr-387 and thereby inhibits MST1 mediated FOXO3 nuclear translocation and activation [97]. Besides MST1, PKB also phosphorylates other JNKKKs. For example ASK1 is a redox sensitive JNKKK that mediates in part ROS-dependent JNK activation. PKB phosphorylates ASK1 at Ser-83 and thereby inhibits ASK1 (Fig. 6B) [98]. In addition to the inhibition of JNKKKs, PKB is also described to inhibit the JNKK, MKK4 by direct phosphorylation on Ser-78 (Fig. 6C) [99].

Furthermore, PKB has been described to regulate the function of JNK scaffolds. PKB interacts with JIP1, but here the precise functional consequence of this interaction remains unclear. PKB binding to JIP correlates with inhibition of JNK activation (Fig. 6D) [100]. In addition, binding of PKB to JIP1 is also acclaimed to affect PKB activity itself, but both inhibition and activation of PKB have been reported [101, 102]. In this respect at least activation of PKB through binding to JIP1 is most extensively documented. JIP1 binds to the pleckstrin homology [PH] domain of PKB and as such may substitute for PI3P lipids, which otherwise bind the PH domain of PKB and this is essential for PKB activation. Furthermore, it has been shown that PDK1, the kinase responsible for phosphorylation of PKB within its activation loop is also recruited to JIP1. However, PDK1 does not bind JIP1 directly, but through RalGDS an exchange factor for the small GTPase Ral [103]. The existence of a quaternary complex consisting of PKB/RalGDS/JIP1/PDK1 has not yet been demonstrated directly, but if present would indeed suggest that this will result in PKB activation. An interesting consequence of such a mechanism of PKB activation would be that this represents first a mechanism of PKB activation that can occur independent of PI3P lipids and second therefore a possibility to activate PKB in cellular domains devoid of membranes. PI3K independent activation of PKB has been reported but with little detail as to the mechanism involved.

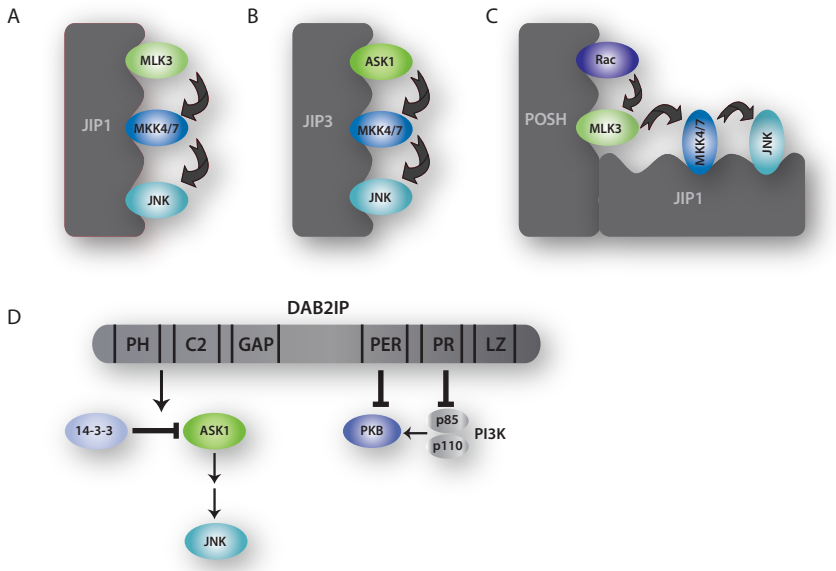


FIGURE 5: Overview of stress regulated JNK scaffold proteins. (**A, B**) JNK interacting proteins 1 and 3 (JIP1/JIP3) both regulate JNK activation by assembling MAPKKks and MAPKKs important for JNK activation. (**C**) POSH together with JIP1 provides another scaffold platform for stress-dependent JNK signaling. (**D**) DAB2IP regulates JNK signaling by stress-dependent activation of ASK1. ASK1, apoptosis signal-regulating kinase 1; C2, protein kinase C-conserved domain; DAB2IP, DOC2/DAB2 interactive protein; GAP, GTPase activating protein; JIP1, JNK interacting protein 1; LZ, leucine zipper; PER, period- like domain; PH, pleckstrin homolo- gy; POSH, plenty of SH3 domains; PR, proline-rich region.

As summarized in Figure 5, JNK activation can occur through multiple different scaffold platforms and JIP1 is not the only JNK pathway scaffold regulated by PKB. Plenty of SH3 domains (POSH) is another scaffold for JNK signaling to which PKB can bind [104, 105]. Interestingly, POSH appears to bind specifically to PKBb and not PKBa, whereas such isozyme preference has not been documented for JIP1. The precise action of PKB in this complex is unclear. Figueroa *et al.* provide evidence that PKB phosphorylates MLK3, a JNKKK, and as such regulates MLK3 binding to POSH and hence JNK activation (Fig. 6e) [104]. Lyons *et al.* provide evidence that PKB phosphorylates Ser-304 of POSH, which lies within the binding domain of POSH for the small GTPase RAC. Consequently, this phosphorylation impairs binding of RAC and subsequent JNK activation (Fig. 6f) [105]. To complicate matters further, interaction between POSH and JIP1 [106] as well as JIP1 and JIP3 [107] has been described and thus under conditions of

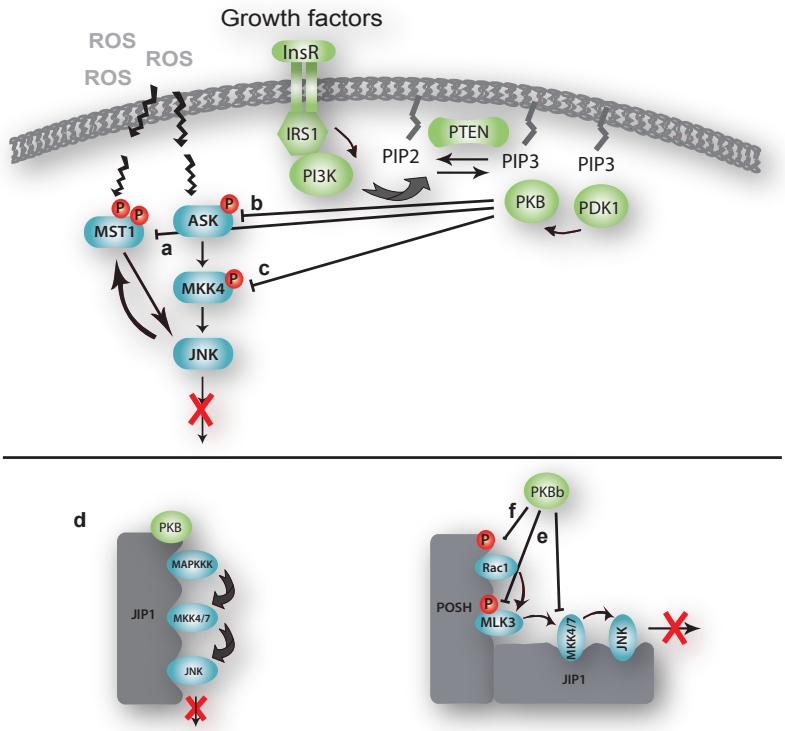


FIGURE 6: Inactivation of JNK signaling by PKB. Schematic overview of PKB mediated inhibition of JNK signaling pathway. PKB phosphorylates several MAPKKK, like MST1 (**A**), ASK1 (**B**) and MLK3 (**E**) and the MAPKK, MKK4 (**C**) subsequently inhibiting their function. Additionally, PKB can regulate several JNK scaffold proteins, like JIP1 (**D**) and POSH (**F**), thereby preventing proper complex assembly and subsequent JNK activation. MLK3, mixed lineage kinase 3; MST1, mammalian STE20-like protein kinase 1.

heterodimerization PKB can also affect JNK scaffolds indirectly. Interestingly, overexpression of POSH, similar to JNK activation, results in extended lifespan of *D. Melanogaster* [108].

As described above and summarized in Figure 6, PBK has many ways to inhibit stress induced activation signals towards FOXOs, however, in situations where survival and proliferation is not favorable, the PKB signaling pathway needs to be turned off. Indeed, there are several examples in which oxidative stress signaling can inhibit the insulin pathway. One example is the direct effect of ROS on PKB kinase activity. Inside its kinase domain, PKB has two cysteins that can form a disulphide bond upon oxidation, which

results in increased binding of PP2A to PKB and subsequent dephosphorylation of PKB on Thr308 and Ser-493 resulting in inhibition of PKB activity (Fig. 7A) [109, 110]. In contrast, PTEN, a lipid PI3-phosphatase that metabolizes PtIns(3,4,5)P3 and inhibits PI3-kinase signaling, is highly sensitive to inactivation *in vitro* by hydrogen peroxide [111]. Thus, on one hand ROS can activate PKB by inhibition of PTEN, but on the other hand it inhibits PKB activity by recruiting PP2A. Possibly, there is a difference in short term and long term ROS, where short term ROS can still activate PKB, as this is still favorable for proliferation, whereas long term ROS will result in inactivation of PKB.

Inhibition of PKB also occurs through activation of JNK, as JNK is able to directly phosphorylate insulin receptor substrate (IRS) (Fig. 7B) [112, 113], thereby inhibiting the insulin pathway. Interestingly, in obese conditions, there is excessive activation of JNK, resulting in phosphorylation and inactivation of IRS and subsequent attenuated insulin signaling and thereby the development of insulin resistance.

Besides inhibition of the insulin pathway, JNK mediates a positive feedback loop by phosphorylation of the JNKKK, MLK3, thereby enhancing the MLK3/JNK signaling (Fig. 7C) [114]. In addition, as already described above, JNK can phosphorylate and activate MST1 (Fig. 7D) [67]. Furthermore, JNK has been implicated in regulation of stability of several of its upstream signaling members, like POSH, JIP1 and MLK3, providing another way of amplifying its own signaling [115].

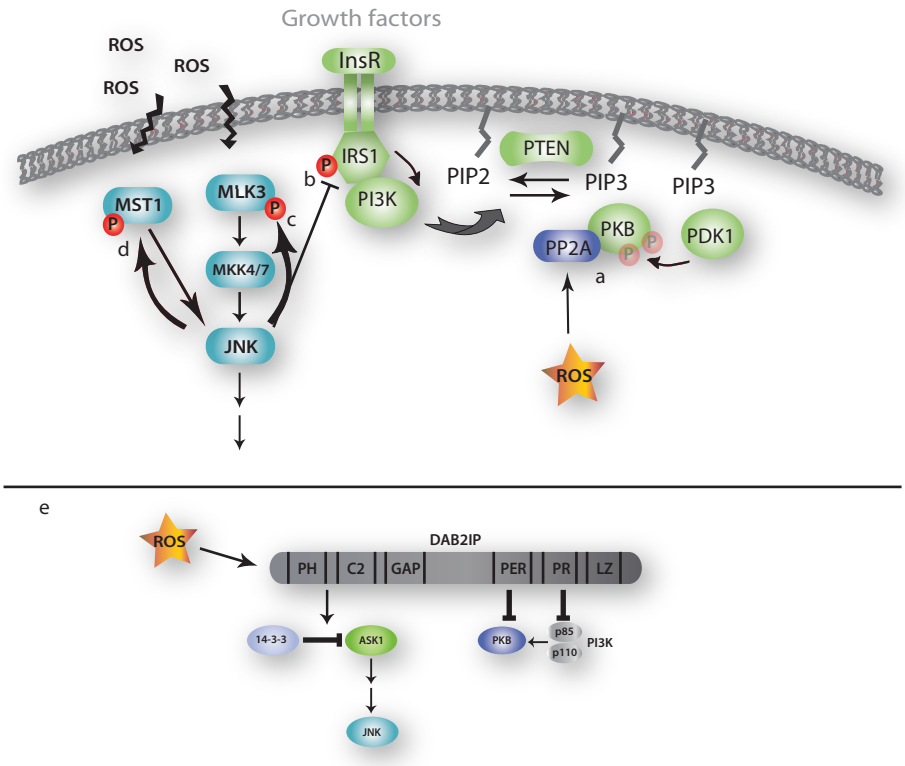


FIGURE 7: Inactivation of PKB signaling by stress signals. Schematic overview of ROS mediated PKB inactivation. Inactivation of PKB can occur directly by ROS, thereby attracting PP2A, resulting in dephosphorylation and inactivation of PKB (**A**) or indirectly via activation of JNK, which subsequently can phosphorylate and inactivate IRS1 (**B**). Activation of JNK results in several positive feedback loops, by direct phosphorylation and thereby activation of MLK3 (**C**) and MST1 (**D**). Additionally, stress signals result in assembly of a scaffolding complex consisting of DAB2IP/AIP1 (**E**), which can directly activate ASK1, by releasing ASK1 from 14-3-3, thereby enhancing JNK signaling. PP2A, protein phosphatase 2A; ROS, reactive oxygen species.

As already mentioned, PKB is able to interfere with the JNK signaling pathway at the level of scaffold proteins. Another, somewhat unique, scaffold protein, called DAB2IP/AIP1 (DOC-2/DAB2 interactive protein or ASK1 interacting proteins), has also been implicated in regulation of JNK, but in addition coordinates inactivation of PKB (Fig. 7E). DAB2IP is originally identified as a member of the RasGAP family and in addition to its GAP activity, it has been identified to be involved in apoptosis by mediating dissociation of ASK1 from its inhibitor 14-3-3, thereby activating JNK signaling [116]. Recently, Xie *et al*. show the ability of DAB2IP to regulate both the inhibition of PI3K/PKB and activation of ASK1 in response to stress signals (Fig. 7E) [117]. So thereby providing another way of inhibiting PKB signaling, while enhancing JNK signaling.

Taken together it is clear that PKB can interfere with many of the signal transduction pathways leading to JNK activation and the other way around, JNK can interfere with many players in the insulin pathway. Further detailed knowledge is needed to understand when, where and how PKB interferes with JNK and JNK interferes with PKB. In addition, detailed knowledge about the JNK signaling cascade(s) that impinge on FOXO is also required to understand how PKB and JNK interfere in FOXO regulation.

Future directions

Even more kinases?

Several high throughput screens, including siRNA screens, have been performed to discover novel regulators of FOXO function. Of relevance to this review is a recent siRNA screen in *Drosophila* Schneider S2 cells dedicated to find kinases/phosphatases regulating dFOXO [118]. This screen yielded numerous hits that provide novel insight into the issues described above. For example knockdown of Inositol-requiring protein 1 (IRE-1) represses dFOXO transcriptional activity. IRE-1 is a stress-activated endonuclease resident in the ER that is conserved in all known eukaryotes. IRE-1 is an essential mediator of all aspects of the unfolded protein response (UPR). UPR gene expression in mammals relies largely on pancreatic ER kinase (PERK) and ATF6. Furthermore, mammalian IRE1 proteins activate JNK by recruiting the TRAF2 protein to the ER membrane independently of their endonucleolytic activity [119]. Thus IRE-1 may couple UPR to FOXO activation by regulating JNK.

Next to IRE-1, this screen identified the TAO1 kinase as a potential FOXO regulator. Initially, TAO1 kinase was suggested to be a checkpoint kinase. However this turned out erroneously, as the employed siRNA against TAO1 displayed Mad2 downregulation as an off-target effect thereby explaining its effect on the M-phase checkpoint. More plausible within the context of FOXO regulation is the observation that TAO1 can impinge on p38 following DNA damage [120].

Also interesting hits of this screen are a number of regulators of inositol lipid metabolism e.g. inositol (myo) -1 (or 4)- monophosphatase 1 (IMPA) NP, Phosphatidylinositol 4-kinase (PI4K), phosphatidylinositol-4-phosphate 5-kinase type I alpha and DAG kinase delta II. As PKB activity is dependent on PI3P lipids these kinases/phosphatases may all indirectly affect PKB activity. Although not a surprising conclusion, it does reveal apparent critical steps in inositol metabolism towards PKB and thus potential targets for small molecules to inhibit PKB and to reactivate FOXO, for example in tumors.

Clearly, our understanding of FOXO regulation in general and regulation through phosphorylation is far from complete. As both kinases and phosphatases are considered being ‘druggable’, further research may yield compounds that can (re)activate FOXO and provide benefits that may come at least somewhat close to the beneficiary effect of activating DAF-16 in *C. elegans*.

Abbreviations

AMPK	AMP-dependent protein kinase
apoB	apolipoprotein B
apoCIII	apolipoprotein CIII
ASK1	Apoptosis Signal-regulating Kinase 1
ATM	Ataxia Telangiectasia Mutated
ATR	ATM and Rad3-related
BTG1	B cell Translocation Gene 1
CDK1/2	Cyclin Dependent Kinase 1/2
CK-1	Casein Kinase 1
DAB2IP	DOC2/DAB2 Interactive Protein
DAF-16	abnormal dauer fomation 16
DYRK1a	Dual specificity tyrosine-phosphorylated and Regulated Kinase 1a
ERK	Extracellular signal-Regulated Kinase
FOXO	Forkhead Box O
GADD45	Growth Arrest and DNA Damage
G6Pase	Glucose-6-Phosphatase
IKK	IκB Kinase
IRE1	Inositol Requiring protein 1
IRS	Insulin Receptor Substrate
JIP1	JNK Interacting Protein 1
JNK	c-Jun N-terminal Kinase
MDM2	Murine Double Minute 2
MLK3	Mixed Lineage Kinase 3
MnSOD	Manganese SuperOxide Dismutase
MST1	Mammalian STE20-like protein kinase 1
MTP	Microsomal Triglyceride transfer Protein
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal
PCAF	p300/CBP-Associated Factor
PDK1	3-Phosphoinositide-Dependent protein Kinase 1
PEPCK	PhosphoEnolPyruvate CarboxyKinase
PERK	Pancreatic ER Kinase
PH	Pleckstrin Homology
PI3K	PhosphoInositide 3-Kinase
PIN1	Peptidyl prolyl Isomerase 1
PKB	Protein Kinase B
PLK	Polo Like Kinase
POSH	Plenty Of SH3 domains
PP2A	Protein Phosphatase 2a
PRMT1	Protein arginine N-MethylTransferase 1
PTEN	Phosphatase and TENsin homolog
PTM	PostTranslational Modification
ROS	Reactive Oxygen Species

SGK	Serum- and Glucocorticoid-inducible Kinase
SKP2	S-phase Kinase-associated Protein 2
TRADD	Tumor necrosis factor Receptor-Associated Death Domain
TRAIL	Tumor necrosis factor-Telated Apoptosis Inducing Ligand
UPR	Unfolded Protein Response
USP7	Ubiquitin Specific Peptidase 7

Thesis outline

A complex network of signaling pathways mediates the balance between FOXO activation and inactivation. Loss of the tumor suppressive function of FOXOs as a result of proteasomal-mediated degradation and cytoplasmic localization often occurs in cancer cells. Therefore, deciphering the signaling pathways that regulate FOXO localization and degradation is important and may lead to the identification of new therapeutic targets for cancer.

This thesis provides further molecular insight in the signaling pathways that regulate FOXO activity and localization. In **Chapter 2**, we show how the small GTPase RALA is involved in FOXO activation. We describe RALA-dependent formation of a scaffold complex consisting of RLF-JIP1-MLK3-MKK4 and JNK, which is necessary to mediate JNK-dependent FOXO activation. We also show evolutionary conservation of RALA/JIP1-mediated FOXO activation in *C. elegans*. Furthermore in **Chapter 3** we show that next to JNK-mediated phosphorylation of FOXO, RALA is also important in regulation of its ubiquitination. It mediates ubiquitination of both FOXO4 and p53 and in addition RALA seems involved in HDMX protein abundance. HDMX functions together with HDM2 as an E3 ligase complex to regulate ubiquitination of p53. Our data suggest that RALA plays a role in regulation of the HDMX-HDM2 complex formation. In **Chapter 4** we used a quantitative proteomics approach to determine the interactomes of FOXO1, FOXO3 and FOXO4 to shed light on their different behavior. This approach allows comparison of the different interaction partners and we discuss possible improvements on the method we used, one of which we applied in **Chapter 6**. In **Chapter 5** we discuss the current literature on CCM1 and propose a dual role for CCM1 in regulation of both adhesion and transcription. In **Chapter 6** we show that indeed CCM1 can act as a transcriptional co-regulator. We demonstrate that CCM1 can bind to FOXO1, FOXO3 and FOXO4 and that localization of FOXO correlates with CCM1 localization to the same compartment. In addition, this nuclear CCM1-FOXO complex interacts with a SWI/SNF chromatin remodeling complex. This SWI/SNF complex contains ARID1A and the histone deacetelyse HDAC1, suggesting a role in chromatin condensation and subsequent transcriptional repression. In agreement, our experiments in **Chapter 6** show an inhibitory role of CCM1 towards FOXO induced changes in transcription. In **Chapter 7** we summarize and discuss the work described in this thesis and propose future research directions.

CHAPTER 2

The small GTPase RALA controls JNK-mediated FOXO activation by regulation of a JIP1 scaffold complex

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Abstract

Forkhead Box O (FOXO) transcription factors are tumor suppressors and increase lifespan of model organisms. Cellular stress, in particular oxidative stress due to an increase in levels of reactive oxygen species (ROS), activates FOXOs through JNK-mediated phosphorylation. Importantly, JNK regulation of FOXO is evolutionary conserved. Here we identified the pathway that mediates ROS-induced JNK-dependent FOXO regulation. Following increased ROS, RALA is activated by the exchange factor RLF, which is in complex with JIP1 and JNK. Active RALA consequently regulates assembly and activation of MLK3, MKK4 and JNK onto the JIP1 scaffold. Furthermore, regulation of FOXO by RALA and JIP1 is conserved in *C.elegans*, where both ral-1 and jip-1 depletion impairs heat shock-induced nuclear translocation of the FOXO orthologue DAF16.

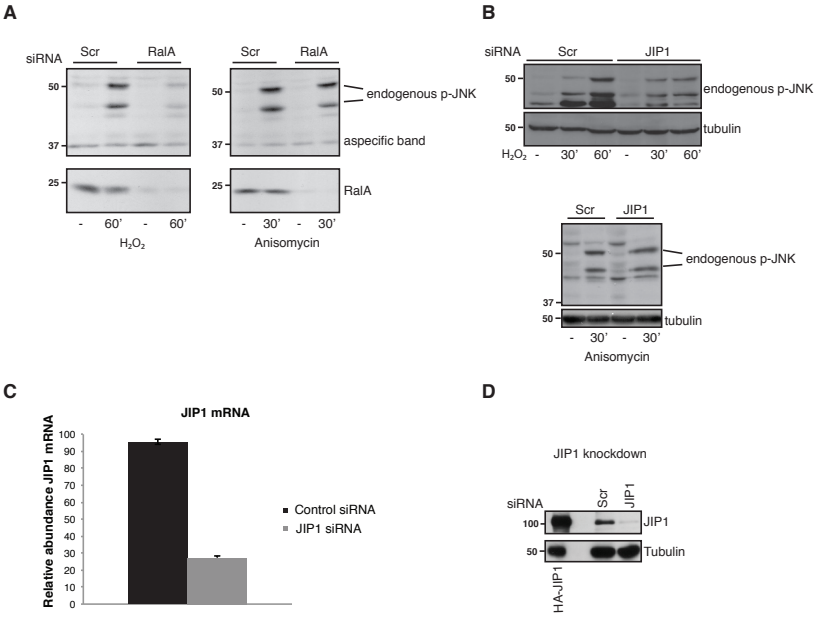
Introduction

The forkhead Box O (FOXO) class of transcription factors are involved in a variety of cellular processes like cell cycle regulation, apoptosis and metabolism (reviewed in [121, 122]). In model organisms FOXO activity affects lifespan as well as age-related diseases like cancer and diabetes (reviewed in [123]). Multiple upstream pathways regulate FOXO activity through post-translational modifications that influence nucleocytoplasmic shuttling of FOXO [124]. FOXOs are negatively regulated by the phosphoinositide-3 kinase (PI3K)/protein kinase B (PKB or AKT) pathway. Activation of PI3K/PKB induces phosphorylation and nuclear exclusion of FOXO, thereby inhibiting FOXO transcriptional activity. In addition, signaling induced by ROS relocates FOXO to the nucleus and thereby potentiates its activation (reviewed in [124]). Previously, we described the importance of JNK-dependent phosphorylation of FOXOs in this response towards ROS [46]. Both the insulin/PI3K/PKB signaling and ROS/JNK signaling pathways towards FOXO have been conserved through evolution in species as diverse as *Caenorhabditis elegans* [45], *Drosophila melanogaster* [44] and *Hydra vulgaris* [125]. In agreement, JNK activity extends lifespan in *C.elegans* [45] and *D. melanogaster* [44] and this requires the FOXO homolog DAF-16.

The JNK signaling pathway is regulated in time and space by the formation of signaling modules. These signaling modules typically consist of a scaffold protein that interacts with proteins that relay the extracellular signal from kinase to kinase, ultimately resulting in phosphorylation of JNK (reviewed in [94, 126]). Examples of scaffold proteins that are able to interact with members of the JNK pathway are Filamin [127], β-Arrestin [128], p130Cas [129] and JIP1, 2, 3 and 4 (reviewed in e.g. [94]). Several extracellular signals can regulate the assembly of different scaffold complexes. For example, the assembly of the Filamin signaling complex containing TRAF2 and MKK4 is induced by TNFα [127] and in hippocampal neurons, it has been shown that JIP1 is required for stress-induced JNK activation [130]. Whereas a scaffolding molecule ensures specificity in terms of the use of upstream regulators of JNK, a recruitment signal is often needed to specify the location of the signaling complex. Small GTPases are known to function as a recruitment signal. For example, Ras-GTP induces dephosphorylation of the MAPK scaffold Kinase suppressor of Ras-1 (KSR), which results in its translocation from the cytosol to the membrane. There it associates with Raf and downstream effector proteins, such as MEK and ERK, facilitating sequential kinase reactions [131]. RAL is a Ras-like small GTPase that cycles between an active GTP-bound and an inactive GDP-bound state. The two RAL isoforms, RALA and RALB, share 85% sequence homology. Several signals result in RAL activation, including growth factor stimulation (EGF and insulin) [132] and Ca^{2+} mobilization [133]. RAL is involved in several physiological and pathophysiological cellular processes, such as exocytosis [134], endocytosis [135, 136], cellular transformation [137-140] and transcriptional regulation [141-143]. RAL regulates activation of several transcription factors including c-Jun [141], NF-kappaB [144], Zonab [145] and FOXO4 [142], however, the molecular mechanism whereby RAL regulates transcriptional activity is largely unexplored. Overexpression of a constitutive active RAL exchange factor or increased ROS levels, results in JNK-dependent phosphorylation and activation of FOXO4 [46]. This suggests a pathway whereby RALA regulates JNK to modulate FOXO transcriptional activity. However, how RALA mediates specificity towards JNK mediated FOXO regulation is poorly understood. Because small GTPases are reported to recruit signaling complexes to confer specificity to signaling, we here addressed the role of JNK scaffold proteins in the regulation of JNK by RALA, and the signaling towards FOXO4. We show that activation of RALA results in the assembly of an active JIP1 scaffold complex consisting of MLK3, MKK4 and JNK. Furthermore, formation of this complex by RALA is necessary for proper JNK signaling towards FOXO4 and subsequent FOXO4 transcriptional activity. Finally, we show the conservation of RAL/JIP1 dependent FOXO regulation in *C. elegans*.

The scaffold protein JIP1 is important for RALA induced JNK activation

Scaffold proteins are important in the assembly of a functional JNK module and can be regulated by small GTPases. Therefore we examined if scaffold proteins can provide the crosstalk between RALA and JNK. We concentrated on JNK interacting protein 1 (JIP1), because JIP1 has been described to selectively bind to JNK and not to other MAP kinases like p38 and ERK [159] and we have no evidence that RALA participates in H_2O_2 induced p38 activation (data not shown and [46]). Furthermore, JIP1 is important in ROS induced JNK activation [130]. Similar to RALA depletion (Fig. 1A), siRNA mediated knockdown of JIP1 inhibited ROS-induced JNK activation (Fig. 1B). Both RALA and JIP1 are required specifically for ROS-induced JNK activation, as anisomycin-induced JNK activation was not affected by knockdown of RALA or JIP1 (Fig. 1A and 1B). The efficiency of the JIP1 siRNA oligonucleotides in NIH3T3 cells was confirmed both by qPCR and western blot (Fig. 1C and 1D). In agreement with the possibility that RALA regulates JIP1, we observe binding of RALA to JIP1. Importantly, this interaction depends on RALA activity. To activate RALA we used expression of RLFCAAX, an active mutant of the RalGEF RLF, in which the Ras binding domain of RLF has been replaced by a CAAX membrane localization motif, resulting in a constitutively active RalGEF [150]. RLFCAAX expression induces binding of RALA to JIP1, while expression of the inactive mutant (RLFΔcatCAAX), carrying a deletion of the catalytic domain does not (Fig. 1E). In addition, H_2O_2 treatment of cells (which activates RALA as we previously published [46]) also induced the interaction of RALA and JIP1 (Fig. 1F). Hence, activation of RALA results in binding of RALA to the JIP1 complex.



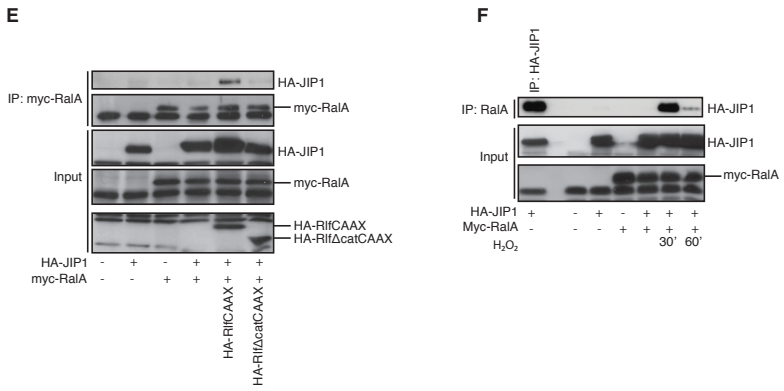


FIGURE 1: The small GTPase RALA regulates JNK activity via the scaffold protein JIP1. (**A**) Knockdown of RALA inhibits ROS-induced JNK activation, but not anisomycin-induced JNK activation. Phosphorylation of JNK (Thr183/Tyr185) and knockdown of RALA are shown. (**B**) Knockdown of JIP1 results in inhibition of ROS-induced JNK activation but not of anisomycin inducd JNK activation. Experiment was carried out as in Figure 1A. (**C**) Knockdown of JIP1 was verified with qPCR. Relative abundance of JIP1 over PBGD is shown. Data are represented as mean +/- SEM. (**D**) and western blot; overexpressed HA-JIP1 is shown in the first lane to control for the position of JIP1. (**E**) JIP1 binds to RLFCAAX-induced active RALA. Myc-RALA was immunoprecipitated and western blots were analyzed for HA (JIP1, RLFCAAX and RLFΔcatCAAX) and myc (RALA). (**F**) ROS-induced interaction of RALA with JIP1. Myc-RALA was immunoprecipitated and western blots were analyzed for HA (JIP1) and myc (RALA).

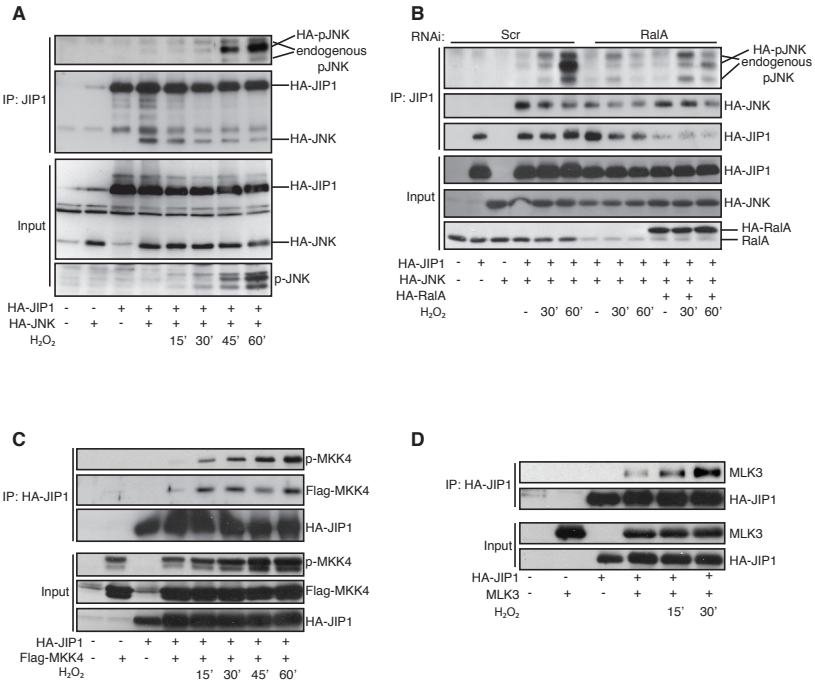
ROS induced assembly of the JNK signaling cascade on the scaffold protein JIP1

Next we looked into the interaction between JNK and JIP1. As shown in Fig. 2A, the interaction of JNK with JIP1, as assessed by co-immunoprecipitation was already evident under basal conditions and treatment of cells with H_2O_2 did not significantly affect binding of JNK to JIP1. However, increased ROS resulted in time-dependent activation of the JNK pool co-immunoprecipitated with JIP1 (Fig. 2A). Consequently, we investigated the role of RALA in activation of JNK on JIP1. Knockdown of endogenous RALA inhibits activation of JNK in complex with JIP1. In addition, we could rescue this inhibition by expression of HA-RALA (Fig. 2B). From these data we conclude that the JNK pool interacting with JIP1 is regulated by RALA following increased ROS.

Scaffold proteins can direct activity of MAP kinases (like JNK) by assembly of the complete MAP kinase cascade complex. Herein, a MAP kinase kinase kinase (MAP(3)K) phosphorylates and activates a MAP kinase kinase (MAP(2)K). The activated MAP(2)K subsequently phosphorylates and activates a MAP kinase (MAPK) (reviewed in [94, 126, 160]). Components of the JNK cascade are MAP(3)Ks; such as members of the MEK kinase and mixed lineage kinases and MAP(2)K members like MKK4 and MKK7. To determine which MAP(2)K is important in signaling from RALA to JNK via JIP1, we analyzed binding of the MAP(2)K MKK4 to JIP1 following H_2O_2 treatment. Binding between JIP1 and MKK4 is rapidly induced upon H_2O_2 treatment (Fig. 2C). Furthermore, MKK4 activation (measured with a phospho-antibody against Ser257/Thr261) was also rapidly induced and increased over a longer time period compared to total MKK4. This suggests not only that H_2O_2 signaling mediates the binding of MKK4 to JIP1, but also results in activation of MKK4 over time (Fig. 2C). We verified the involvement of MKK4 in ROS-induced JNK activation by ectopic expression of either dominant-negative MKK4 (dnMKK4), or wild type MKK4 (wtMKK4). DnMKK4 was able to block the H_2O_2 -induced JNK activation, while wtMKK4 could not (data not shown). As both MKK4 and MKK7 are described to be important for full JNK activation [161, 162] and MKK7 is described to bind JIP1 [159] we tested the involvement of RALA in regulation of MKK7 binding to JIP1. In agreement with the literature we found MKK7 bound to JIP1 (data not shown), however, in contrast to MKK4, the interaction appeared not regulated by RALA. This

does not exclude a role for MKK7, but at least suggests MKK4 to be the main mediator of ROS/RALA signaling towards JNK via the JIP1 scaffold complex.
Next, we tested MAP(3)Ks (MLK3 and ASK1), known to be important in JNK signaling, for interaction with JIP1 following increased ROS. As shown in Fig. 2D, we observed increased binding of MLK3 upon H_2O_2 treatment, suggesting that MLK3 might be important for regulation of RALA mediated JNK activation. Taken together, upon increased ROS, a MAPK module, consisting of MLK3, MKK4 and JNK is assembled onto the JIP1 scaffold protein and subsequently activated.
Subsequently we determined the role of RALA in the assembly and activation of the above outlined ROS-induced JIP1-JNK complex. As shown in Fig. 2B, knockdown of RALA had no effect on JNK binding to JIP1, but reduced the activation of JNK on JIP1. In contrast, upon RALA knockdown we observed a decrease in ROS-induced binding of MKK4 (Fig. 2E) and MLK3 (Fig. 2F) to JIP1. From these data we conclude that the small GTPase RALA regulates binding of both MLK3 and MKK4 to JIP1, which subsequently results in activation of JNK on JIP1. Hence, RALA mediated JNK activation is accomplished through the regulation of a MLK3/MMK4/JNK cascade formed onto the JIP1 scaffold complex in response to ROS, Whether the regulation of this cascade proceeds through an orderly and sequential binding of the individual components, or whether this involves the formation of a complex in which all components are bound simultaneously remains to be determined.

The RALGEF RLF is in complex with JIP1 to regulate RALA-mediated JNK activation
Activation of small GTPases such as RAL usually proceeds through activation of Guanine nucleotide Exchange Factors (GEFs). We observed that assembly of an active JIP1-JNK complex following increased ROS requires RALA. Furthermore, RALA activation by ROS or expression of RLFCAAX induced the binding of RALA to JIP1. Therefore, we tested the involvement of the RALGEF, RLF [150], in mediating



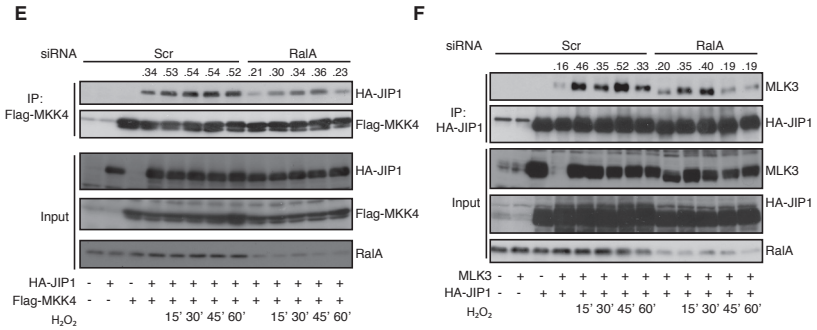


FIGURE 2: RALA regulates assembly of a functional JIP1 scaffold complex consisting of MLK3-MKK4 and JNK. (**A**) ROS-induced activation of co-immunoprecipitated JNK with JIP1. HA-JIP1 was immunoprecipitated with an anti-JIP1 antibody. Western blots were probed for pJNK (Thr183/Tyr185) and HA (JNK and JIP1). (**B**) Ectopically expressed RALA rescues inhibition of JNK activation on JIP1 upon RALA knockdown. HA-JIP1 was immunoprecipitated with anti-JIP1 antibody and western blots were analyzed for pJNK (Thr183/Tyr185), HA (JNK, RALA and JIP1) and endogenous RALA. (**C**) ROS-induced binding and activation of MKK4 on JIP1. Experiment was carried out as in Figure 2A. (**D**) ROS-induced interaction of MLK3 to JIP1. Experiment was performed as in Figure 2A. (**E,F**) RALA mediates ROS-induced interaction of MKK4 (**E**) and MLK3 (**F**) to JIP1. Flag-MKK4 (**E**) or HA-JIP1 (**F**) was immunoprecipitated with anti-Flag or HA antibody and western blots were analyzed for pJNK (Thr183/Tyr185), HA (JIP1), Flag (MKK4), MLK3 and RALA. Numbers indicate ratio bound-JIP1 over immunoprecipitated MKK4 (**E**) and bound-MLK3 over immunoprecipitated JIP1 (**F**).

JIP1-JNK complex assembly induced by RALA. Indeed, RLF interacts with JIP1 however, similar to JNK, this interaction was independent of ROS (Fig. 3A). Because our experiments indicate that activated RALA binds to JIP1, and GEFs such as RLF bind to inactive GTPases, we tested if the presence of RALA was required for the interaction of RLF to JIP1. Upon RALA knockdown RLF is still bound to JIP1, indicating that this interaction, similar to JNK binding to JIP1, occurs independent of RALA (Fig. 3B). Simultaneous binding of RLF and JNK to JIP1 prior to RALA activation might allow rapid and directed activation of RALA towards JNK. To examine if RLF is indeed present in one complex together with JIP1 and JNK, we performed a sequential pull-down. First Flag-RLF was immunoprecipitated and this precipitate of RLF and associated proteins were eluted from the beads and subsequently immunoprecipitated for JIP1. The JIP1 pulldown was assayed for the presence and activity of JNK. As shown in Fig. 3C, immunoprecipitation of RLF resulted in a pulldown of JNK bound to JIP1 and in addition we observed activation of JNK upon H_2O_2 treatment in this trimeric RLF-JIP1-JNK complex. Next to the binding of HA-pJNK to JIP1, we were also able to detect endogenous pJNK in the RLF-JIP1 complex (Fig. 3C).

Activation of RAL is necessary to activate MLK3

Small GTPases are well known to directly activate kinases; a typical example is the Ras-Raf pathway, where Ras activates Raf-1 [163-165]. Therefore we tested if RALA was able to directly activate MLK3, or that this may require additional components. For this purpose, we analyzed activation of MLK3 using a phospho-specific MLK3 antibody, recognizing the phosphorylation sites in its activation loop (Thr277/Ser281). Ectopic expression of RLFCAAX, but not the inactive version RLFΔcatCAAX, was sufficient to induce autophosphorylation of MLK3 (Fig. 4A). Effector proteins of small GTPases such as Raf-1 bind preferentially to the active GTP-bound form of these GTPases [163]. Therefore, we performed a pull-down with GST-RALA, loaded either with GDP or GTP, to see if MLK3 prefers binding to either the GDP or GTP bound form of RALA. As shown in Fig. 4B, MLK3 preferentially binds RAL^{GTP}, suggesting that MLK3 is an effector protein of RALA. To map the region where RALA is binding to MLK3, we constructed several mutants (Fig. 4C, top panel). A mutant of MLK3 that contains only the kinase domain can still interact with endogenous RALA (Fig. 4C), suggesting that RALA interacts with the kinase domain of

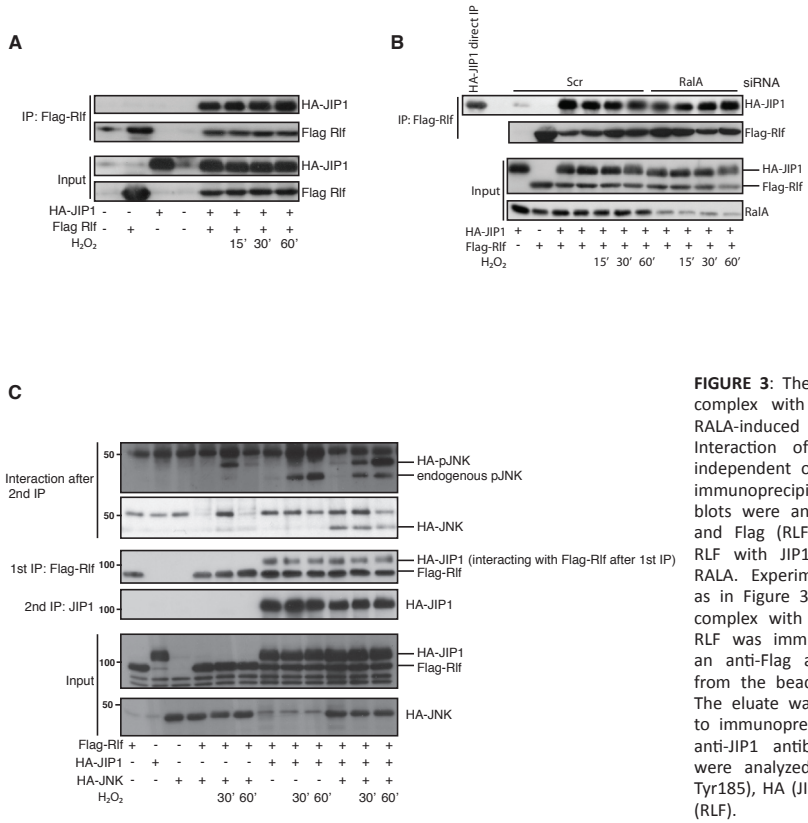


FIGURE 3: The RALA GEF RLF is in complex with JIP1 and mediates RALA-induced JNK activation. (**A**) Interaction of RLF with JIP1 is independent of ROS. Flag-RLF was immunoprecipitated and western blots were analyzed for HA (JIP1) and Flag (RLF). (**B**) Interaction of RLF with JIP1 is independent of RALA. Experiment was performed as in Figure 3A. (**C**) RLF is in one complex with JIP1 and JNK. Flag-RLF was immunoprecipitated with an anti-Flag antibody and eluted from the beads with Flag-peptide. The eluate was subsequently used to immunoprecipitate HA-JIP1 with anti-JIP1 antibody. Western blots were analyzed for pJNK (Thr183/Tyr185), HA (JIP1 and JNK) and Flag (RLF).

MLK3. Finally, we investigated the importance of MLK3 in the assembly of an active JIP1-JNK complex after increased ROS. Upon H_2O_2 treatment, JNK is activated on JIP1, and this activation is delayed in presence of MLK3 RNAi (Fig. 4D).

RALA-JIP1-JNK complex assembly is necessary for ROS induced FOXO4 regulation

As described previously, the Forkhead box O transcription factor FOXO4 is phosphorylated and activated by JNK in a ROS and RAL-dependent manner [46]. To determine the relevance of the above described RALA-JIP1-JNK complex towards FOXO4, we performed knockdown of several components of the identified JNK signaling pathway and analyzed JNK-dependent phosphorylation of FOXO4 with a phosphospecific antibody against two of the JNK sites; phospho-FOXO4-Thr223/Ser226 [166]. Both knockdown of RALA and JIP1 resulted in decreased H_2O_2 -induced FOXO4 phosphorylation (Fig. 5A). In addition, knockdown of MLK3 also decreased FOXO4 phosphorylation (Fig. 5B). Furthermore, overexpression of MLK3 could rescue the decrease in FOXO4 phosphorylation after RALA RNAi (Fig. 5C), showing the ability of MLK3 to act downstream of RALA to regulate JNK-mediated FOXO4 activation. In addition, as expected from the effects on FOXO4 phosphorylation, JIP1 is also required for full FOXO4 activity, as shown by a luciferase reporter (Fig. 5D). FOXO4 overexpression results in increased transcriptional activity measured by 6xDBE-luciferase activity, whereas knockdown of JIP1 inhibits FOXO4 transcriptional activity.

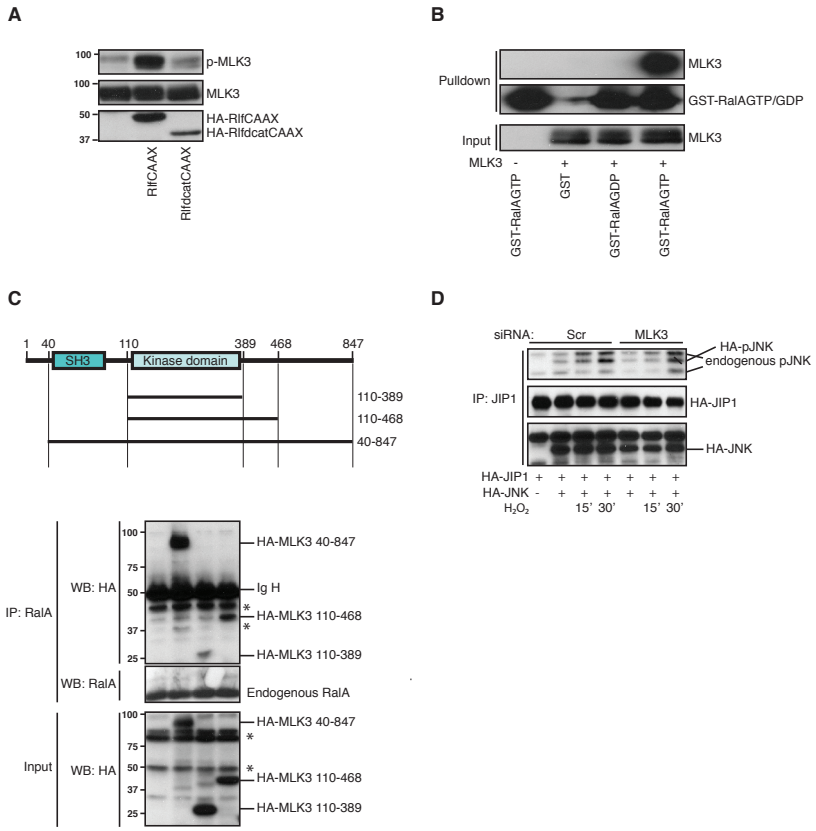


FIGURE 4: MLK3 is important downstream of RALA in activation of the JIP1-JNK complex. (**A**) Activation of RALA results in phosphorylation of MLK3 in its activation loop. HEK293T cells were analyzed for phosphorylation on Thr277/Ser281. (**B**) MLK3 specifically binds to GTP-bound RALA. HEK293T cells were transfected with the indicated constructs and the GST pulldown was performed as described in material and methods. Western blots were probed for MLK3 and GST. (**C**) RALA interacts with the kinase domain of MLK3. HA-tagged MLK3 mutants, as depicted in upper panel, were overexpressed and endogenous RALA was immunoprecipitated. Western blots were analyzed for HA and RALA. Stars indicate background bands. (**D**) Knockdown of MLK3 inhibits ROS-induced activation of JNK on JIP1. HA-JIP1 was immunoprecipitated with JIP1 antibody. Western blots were analyzed for pJNK (Thr183/Tyr185) and HA (JIP1 and JNK).

Conserved role for RALA and JIP1 in stress-induced DAF16 nuclear localization

In both *D. melanogaster* and *C. elegans*, activation of JNK results in nuclear localization of dFOXO/DAF16 and increased lifespan [44, 45]. Based on this notion, we questioned whether FOXO regulation by RALA observed in mammalian cells [46] is evolutionary conserved in *C. elegans*. Therefore, we used a *C. elegans* strain expressing DAF-16::GFP (TJ356). This strain has been shown to respond to environmental stresses, such as heat-shock, by displaying nuclear translocation of DAF-16::GFP [156]. RNAi-mediated knockdown of *ral-1* resulted in a delayed heat shock-induced DAF16 nuclear localization compared to control RNAi (L4440) (Fig. 6A). To confirm that in *C. elegans* heat shock regulates JNK activity and this may involve *ral-1*, we analyzed JNK activity by performing a GST-jun pulldown kinase assay [158] on worm lysates subjected to heat-shock with control or *ral-1* RNAi. Heat shock indeed induces JNK activity [167] and importantly this induction occurred in a *ral-1*-dependent manner (Fig. 6B), confirming the role

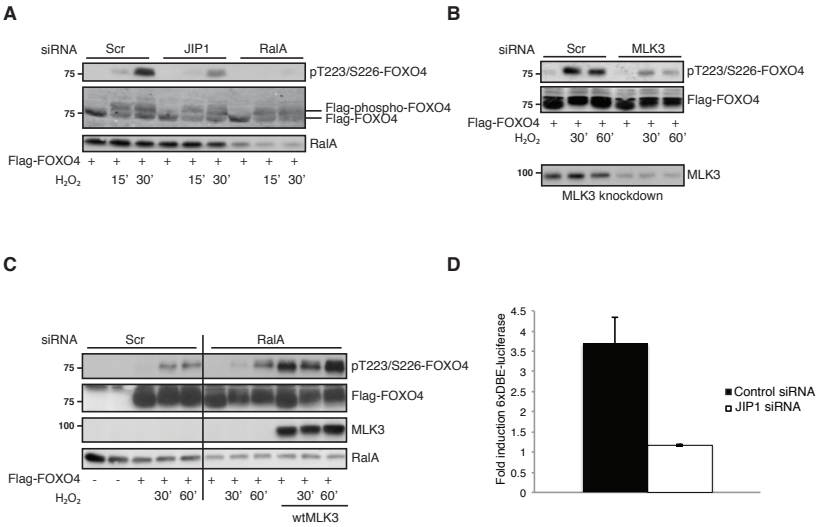


FIGURE 5: The assembly of a functional JIP1 scaffold complex by RALA ensures proper JNK signaling towards FOXO4 (**A**) Knockdown of RALA or JIP1 inhibits FOXO4 phosphorylation on JNK sites (Thr223/Ser226). Flag-FOXO4 was immunoprecipitated from NIH3T3 cells with anti-Flag antibody and western blots were analyzed for phosphorylation of Thr223/Ser226 on FOXO4, Flag (total FOXO4) and RALA. (**B**) MLK3 knockdown inhibits ROS-induced FOXO4 phosphorylation on JNK sites. Experiment was performed as in 5A. Western blots were analyzed for phosphorylation of Thr223/Ser226 on FOXO4 and Flag (total FOXO4). Efficiency of MLK3 siRNA is shown below on overexpressed MLK3, due to bad quality of MLK3 antibody for detecting endogenous MLK3. (**C)** Knockdown of RALA inhibits JNK-induced FOXO4 phosphorylation and MLK3 overexpression rescues this inhibition. Cell lysates were examined for MLK3, RALA and phosphorylation of FOXO4 on the JNK sites (Thr223/Ser226), total FOXO4 levels remained equal. wtMLK3; wild type MLK3. (**D**) JIP1 knockdown inhibits FOXO4 transcriptional activity. NIH3T3 cells were transfected with Flag-FOXO4, tk-renilla-luciferase and 6xDBE luciferase and siRNA against JIP1 or a scrambled control sequence. The graph shows fold induction of FOXO4 over GFP. Data are represented as mean +/- SEM.

of *ral-1* in regulation of JNK signaling upon heat shock. Because we demonstrate the importance of JIP1 in the assembly of a complete signaling complex to mediate JNK activity and regulation of FOXO4 activity in mammalian cells, we questioned whether JIP1 mediated FOXO regulation is also conserved. To this end we crossed the DAF16::GFP line with a *jip-1* mutant line. Subsequently we tested three *jip-1* mutant lines for heat shock-induced translocation of DAF16. All three *jip-1* mutant strains showed delayed nuclear translocation upon heat shock, similar to what we observed with *ral-1* siRNA. A representative example is shown in Fig. 6C. Apparently, the evolutionary conserved regulation of FOXO by JNK observed in *C. elegans*, proceeds through a signaling pathway that consists of the small GTPase RAL and the JNK scaffold JIP1. These data show that not only the well known regulation of FOXO by PI3K/PKB (AKT) signaling is evolutionary conserved, but also that the RALA/JIP1/JNK axis in regulation of the FOXO transcription factor is conserved.

Discussion

Numerous signaling pathways have been shown to modulate FOXO activity. Two main pathways, the PI3K/PKB (AKT) pathway and JNK, appear evolutionary conserved to tightly regulate FOXO activity. In the presence of growth factors the insulin pathway through PI3K/PKB negatively regulates FOXOs, while FOXOs are activated by JNK following oxidative stress. Previously we identified a role for the

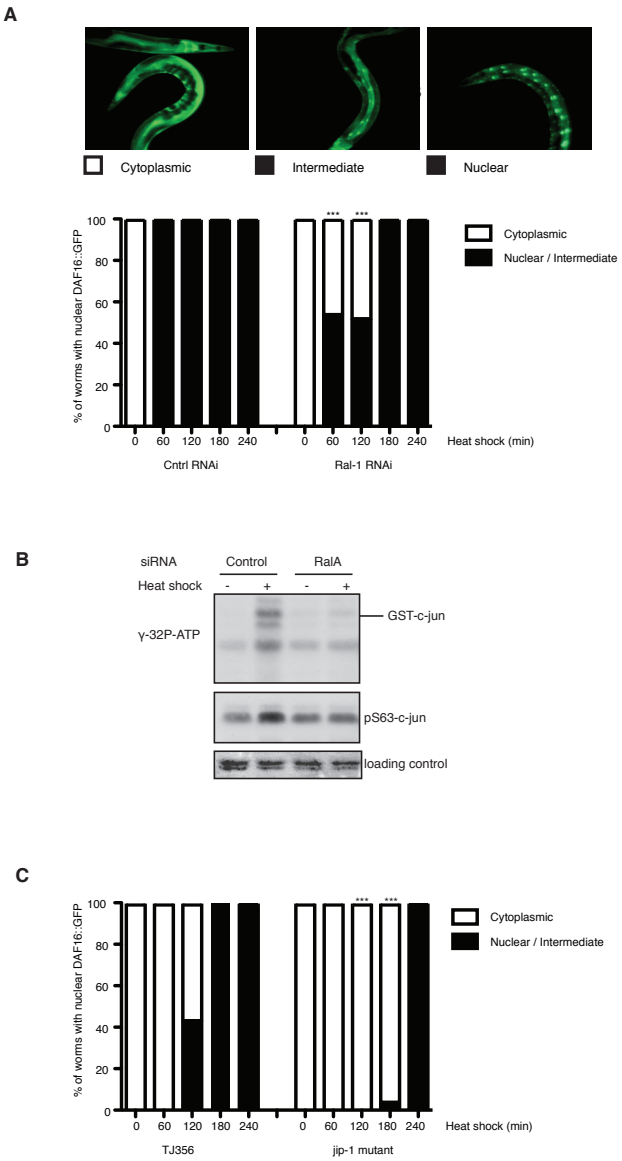


FIGURE 6: Conserved regulation of FOXO by RALA/JIP1 in *C. elegans*. **(A**) *C. elegans ral-1* is important in stress induced nuclear DAF16 translocation. TJ356 (DAF16::GFP) were synchronized and fed with control (L4440) RNAi or *ral-1* RNAi. After they reached adulthood, the worms were exposed to heat stress (33°C) and counted for the presence of nuclear, intermediate or cytoplasmic DAF16::GFP at indicated time points. The upper panel shows representative pictures of cytoplasmic, intermediate and nuclear DAF16 localization. Intermediate and nuclear translocation was pooled (and scored as nuclear) to perform the Fisher's exact test. (p-value = p=0.0002 (***). (**B**) Heat shock-induced JNK activation is mediated by *ral-1*. TJ356 worms were exposed to heat stress and subsequently lysed to perform a GST-c-jun pulldown. C-jun phosphorylation was detected by both anti-phospho-c-jun (Ser63) and autoradiography (γ-32P), Coomassie blue staining of the gel is shown as loading control **(C)** *jip-1* is important for heat shock induced nuclear DAF16 translocation. TJ356 and *jip-1* mutant worms were synchronized and after they reached adulthood, the worms were exposed to heat stress (33°C) and counted for the presence of nuclear, intermediate or cytoplasmic DAF16::GFP at indicated time points. Intermediate and nuclear translocation was pooled (and scored as nuclear) to perform the Fisher's exact test. P-values are 0.0002 for 120 min and <0.0001 for 180 min of heat shock (according to Fisher's exact test).

small GTPase RALA in JNK regulation of FOXO4. However, how RALA regulates JNK-mediated FOXO activation was unclear, therefore we analyzed the pathway that specifies RALA induced JNK activation following increased cellular oxidative stress. We show that upon increased ROS, RALA mediates JIP1-dependent activation of JNK, which results in phosphorylation, and activation of FOXO4. RALA regulates the assembly of a MAPK cascade consisting of MLK3, MKK4 and JNK on the scaffold protein JIP1. Under basal conditions, JIP1 is in complex with RLF and JNK, possibly to allow rapid activation of JNK by RALA. Upon increased ROS, the small GTPase RALA is recruited to JIP1 and activated by RLF, this subsequently results in the recruitment and activation of MLK3. As GTP-bound RALA, but not GDP-bound RALA, binds to MLK3, these results suggest MLK3 to be a potential novel RAL effector. Following

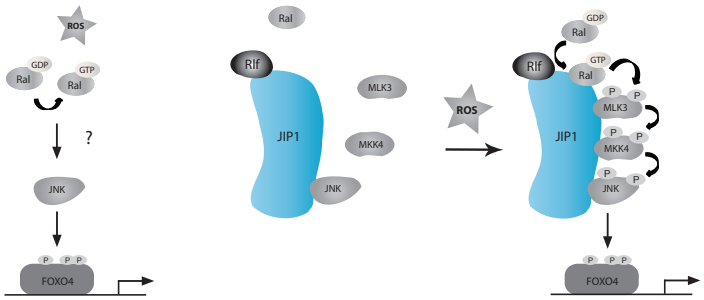


FIGURE 7: Model for RALA dependent FOXO4 regulation. Upon ROS signaling, RALA is activated by RLF on JIP1. Subsequently, the binding and activation of both MLK3 and MKK4 on JIP1 is mediated by RALA, which ultimately results in activation of JNK. Finally, activated JNK can phosphorylate and activate FOXO4. For further details see text.

MLK3 activation, MKK4 is recruited to JIP1 and activated; this subsequently results in phosphorylation and activation of JNK. Active JNK subsequently phosphorylates and thereby activates FOXO4 (depicted in the model in Fig. 7). Finally we show that RALA/JIP1 mediated FOXO regulation is conserved in *C. elegans.* Although it is not entirely clear how heat stress results in an increase in oxidizing conditions, it has been shown that heat stress results in JNK dependent nuclear shuttling of DAF16 [45]. We indeed observed that in *C. elegans* heat-shock induced DAF-16 nuclear translocation as well as JNK activation was reduced following RNAi against *ral-1*. Combined these and our data clearly suggest RALA/JNK/DAF-16 regulation to be conserved.

How does ROS regulate the JIP1 scaffold complex?

We identified the RAL exchange factor RLF and RALA as the components most proximal to ROS. However, the question still remains how ROS activates RLF/RALA. It has been described that GTPases can be sensitive to cysteine oxidation and that this might regulate their activity (reviewed in [168, 169]). Redox-mediated cysteine modification has been suggested to collaborate with and/or enhance the action of guanine nucleotide-exchange factors in the activation of small GTPases. Such a mechanism was proposed for Ras and Rho family members and could explain activation of RALA by ROS. Another possibility is that ROS induce modifications on either JIP1 or RLF, which results in re-localization of the complex to the membrane where RALA is present and becomes activated. Finally, it is possible that JIP1 binding to RLF inhibits RLF function and that ROS-induced modifications might relieve this inhibition. However, we tested if the presence of recombinant JIP1 affected RLF exchange activity *in vitro* and did not observe a change in activity (data not shown). We are currently further exploring these possibilities.

In our study we identified MLK3 as the MAPKKK important for JIP1-mediated JNK activation following increase in ROS. Also for MLK3, redox-mediated cysteine oxidation has been described. Cysteine oxidation of MLK3 results in its dimerization and subsequent activation [170]. However, we found that RALA can activate MLK3 independent of ROS (Fig. 4A). In agreement, also others demonstrated direct activation of MLK3 by small GTPases (Rac and CDC42) [148]. This suggests that MLK3 can be activated by several mechanisms, either via cysteine oxidation or by binding of small GTPases. If MLK3, next to RALA mediated activation, is also regulated by cysteine oxidation in the JIP1 scaffold complex, remains to be investigated.

Potential mechanism of MLK3 activation by RALA

As described by others, the small GTPase Cdc42 can activate MLK3 through binding and membrane targeting [171, 172]. Binding of Cdc42 was mapped to the SH3 region of MLK3 and it was suggested that the binding of $Cdc42^{GTP}$ to MLK3 induces release of its SH3 mediated autoinhibition and subsequently promotes dimerization and (auto) phosphorylation within its activation loop. The mechanism whereby RALA mediates MLK3 activation differs from Cdc42, as we mapped the interaction of RALA to the kinase domain of MLK3. Furthermore, our results show that RALA, in addition to activation of MLK3, also regulates binding of MLK3 to JIP1. Thus, alternative to the mechanism of Cdc42 activation, RALA binding to the MLK3 kinase domain may facilitate binding of the SH3 domain of MLK3 to JIP1 and thereby relieve auto-inhibition indirectly. This and other possibilities as to how RALA is able to regulate MLK3 activity by binding to its kinase domain are currently under investigation.

RALA in D. Melanogaster and C. elegans

Whereas we demonstrate a clear role of RALA in the activation of JNK in both mammalian cells and the nematode *C. elegans*, others have shown in *D. melanogaster* that RALA plays an inhibitory role towards JNK [173, 174]. An important difference between these developmental studies and ours is that we address a post-developmental role of RALA. Importantly, the outcome of JNK signaling is highly context-dependent, either resulting in pro-apoptotic or anti-apoptotic activity. It might be that during development, JNK activity is differently regulated compared to post-development. In addition, whereas we used siRNA against RALA, these studies used a dominant-negative version of RAL (RALN28) to inhibit RAL signaling. Surprisingly, in *D. melanogaster* ectopic expression of dominant-negative RALN28 induces in part similar phenotypes as ectopic expression of dominant-active RALV20 [174]. We indeed observe no difference between siRNA-mediated inhibition of RAL (Fig. 1A) and ectopic expression of RALN28 [46]. However, we were not able to observe activation of JNK when expressing the dominant active version of mammalian RAL (RALV23). RALV23, being a GTP bound form of RAL, does not bind to RALGEFs and therefore potentially does not bind to JIP1. We show here that RAL activation needs to occur at the JIP1 scaffold in order to activate JNK, and therefore RALV23 is probably unable to activate JNK.

At present little is known concerning the role of RAL in *C. elegans* development and/or adult life. A recent study indicates a role for RAL in determining vulval pattern formation during development and this was attributed to a role for RAL in antagonizing RAS-RAF signaling [175]. In addition, a synthetic lethal screen for loss of RAP-1 in *C. elegans* identified RAL as being essential for survival of RAP-1 null worms [157]. In our study we describe an important role for RALA in mediating JNK signaling in *C. elegans*.

Activating vs inhibiting signals towards FOXO

JNK-mediated FOXO phosphorylation is emerging as an important positive regulatory pathway opposing the negative regulation by PI3K/PKB signaling. Interestingly, JIP1 has been described to play an important role in PKB regulation, however current literature is unclear, or maybe even contradictory, as to whether JIP1 acts as a negative or positive regulator of PKB [100, 102, 107]. More importantly there is extensive cross talk between JNK and PKB signaling. For example, PKB has been described to negatively regulate several components of the MAPK signaling complex described here [99, 104, 176]. Whereas JNK is able to negatively regulate PKB, for example through direct phosphorylation and subsequent inhibition of IRS1 [113]. Surprisingly, RALGDS, a RAL exchange factor similar to RLF, is found to recruit PDK1 to PKB via JIP1 and thereby enhances PDK1 mediated PKB phosphorylation and subsequent activation [103]. However, this function of RALGDS does not require its ability to activate RAL. The interplay between PKB and JNK signaling is clearly complex and requires further detailed

and directed studies. Nevertheless, it supports the idea that exchange factors for RAL such as RLF and RALGDS are upstream components of pathways leading to JNK versus PKB respectively and that JIP1 can be of importance in determining the control of negative and positive signaling pathways towards FOXO4.

The cellular response to ROS is tightly controlled and ranges from proliferation to cell cycle arrest and apoptosis, dependent on ROS level, cell type and developmental stage. FOXO plays a central role in this response at the level of transcription and is regulated in multiple ways through ROS mediated post-translational modifications [177]. FOXO activity is probably the most important determinant of organismal lifespan and this has been suggested to be dependent on its regulation of the cellular response to ROS [178]. The pathway we describe here from ROS to FOXO activity illustrates how a specific ROS signal could be fine-tuned to carefully adjust FOXO activity and regulate the appropriate response. Like FOXO, JNK and recently also MKK4 [179] have been implicated in ageing, which suggests that the here delineated RAL/RLF/JIP-1/MLK3/MKK4/JNK signaling cascade towards FOXO could be important for lifespan determination.

Experimental procedures

Cell culture and transfections
HEK293T and NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Cambrex Bioscience, Verviers Belgium), supplemented with 10% fetal bovine serum, Penicillin/streptomycin and 0.05% L-glutamine. Cells were transfected using FuGENE6 (Roche), according to the manufacturer's instructions. Experiments were carried out 48 h after transfection. siRNAs for Scrambled, RALA, MLK3 or JIP1 were transfected using either Oligofectamine (Invitrogen) or INTERFERin (Polyplus Transfection) according to the manufacturer's instructions, experiments were analyzed 72 h after transfection. Cells were treated with 200 μM H_2O_2 or 5 μg/μl anisomycin for the indicated timepoints or left untreated
Plasmids and siRNA oligonucleotides -MKK4 and dnMKK4 in pCMV-Flag were kindly provided by Dr. T. Katada, University of Tokyo [146, 147]. JIP1 in pCDNA3-HA was kindly provided by Dr. T. Tsuzuki. pCDNA3-MLK3 was kindly provided by J. Gutkind [148]. HA-MLK3 mutants were obtained by conventional cloning, more information is available upon request. The following plasmids have been described before: JNK1 in pMT2-HA [149], RALA, RLF, RFLCAAX, RLFΔcatCAAX (deletion of aa 212-327) in pMT2-HA [150], RALA in pCDNA3-myc, GLOFlag3-Flag-FOXO4 [151], 6x FOXO DNA-binding element (DBE)-firefly luciferase and TK-*Renilla* luciferase [24]. All siRNAs used were synthesized by Dharmacon. The target sequence for the scrambled siRNA is: 5'-UAGCGACUAAACACAUCAAUU-3'. The target sequences for RALA [152] and MLK3 [153] were described previously and for knockdown of JIP1 a pre-designed ON-TARGET plus smartpool was used.

Antibodies
The following antibodies were purchased: phospho-JNK (Thr183/Tyr185), phospho-cjun (Ser63), phospho-MKK4 (Ser257/Thr261), phospho-MLK3 (Thr277/Ser281), RALA and MLK3 antibodies from Cell Signaling, anti-JIP1 (Santa Cruz, B-7), anti-JNK1 (Biosource), anti-α-tubulin (Calbiochem) and anti-Flag-M2 (Sigma). Anti-HA (12CA5), anti-Myc (9E10) and anti- Phospho- FOXO4 (Thr223/Ser226) were described before [13, 154].

Immunoprecipitation and Western blot

For immunoprecipitation, cells were lysed in buffer containing 50mM Tris-HCl (pH7.5), 1% NP40, 5mM EDTA, 100mM NaCl, protease and phosphatase inhibitors. Cell lysates were centrifuged for 10 minutes at 14000 rpm at 4ºC and 5% of the supernatant was used as input material. Immunoprecipitation of the proteins of interest was done with protein agarose beads, coupled to the protein or tag specific antibody, for 2hrs at 4ºC. Beads were washed with lysis buffer and resuspended in Laemmli sample buffer. Samples were separated on SDS-polyacrylamide gels and transferred to PVDF-membrane. Western blot analysis was performed on an Odyssey scanner (LI-COR Biosciences) using fluorescently labeled secondary antibodies (Fig. 5A, Flag blot) or by enhanced chemiluminescence (all other figures). Experiments were performed at least 2-5 times of which a representative figure is shown in the manuscript. Blots were quantified with ImageJ.

GST pulldown

Purification of RAL and loading with GppNHp (a non-hydrolysable GTP) or GDP was done as described previously for Rap [155]. Recombinant GST-RALA-GppNHp and GST-RALA-GDP were precoupled to Glutathion Agarose beads for 10 min at 4°C in GST-buffer (50mM Tris-HCl (pH7.5), 50mM NaCl, 5mM $MgCl_2$, 5% glycerol and 5mM DTT) and washed 3 times with RAL buffer (10% glycerol, 1% NP40, 50mM Tris-HCl (pH7.5), 200mM NaCl, 2.5 mM $MgCl_2$, protease and phosphatase inhibitors). Cells were lysed in RAL buffer and centrifuged for 10 min at 4°C, lysates containing HA-JIP1 or MLK3 were incubated with the precoupled beads for 5 min at 4°C. Beads were washed with RAL buffer and resuspended in Laemmli sample buffer, followed by western blotting.

Luciferase reporter assay

Luciferase-based measurement of FOXO activity was performed as described before [46]. Briefly, NIH3T3 cells were transfected with siRNA against JIP1 or scrambled siRNA and the 6xDBE-luciferase reporter construct, together with TK-*renilla*-luciferase as internal control for transfection. Luciferase activity was analyzed with a Dual Luciferase Assay system (MicroLumat Plus LB 96V, Berthold Technologies), according to manufacturer's instructions.

Quantitative RT-PCR

RNA isolation from NIH3T3 cells was performed using the RNeasy mini kit from Qiagen and cDNA was created using iScript cDNA synthesis kit from Bio-Rad. IQ SybrGreen (Biorad) real time qPCR was performed according to manufacturers instructions. Relative abundance JIP1 mRNA was corrected using PGBD mRNA. The following primers were designed to detect JIP1 mRNA: Forward primer: 5'-CGTTCCTCCAGTGCTGAGTC - 3' Reverse primer: 5' - CCGAGGCACAAACCTGAATA - 3' and for detection of PBGD mRNA (used as housekeeping gene), the following primers were designed: Forward primer: 5'- GCCTACCATACTACCTCCTGGCT - 3' Reverse primer: 5'- AAGACAACAGCATCACAAGGGTT - 3'

C. elegans

TJ356 [*Pdaf-16::daf-16-gfp; rol-6*] [156] and VC20318 (nonsense mutation in *jip-1* coding exon) strains were obtained from the Caenorhabditis Genetics Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440) and maintained under standard conditions. RNAi against *ral-1* was kindly provided by F. Zwartkruis and is described previously [157]. TJ356 was crossed with VC20318 to obtain mutant *jip-1* DAF16::GFP strains. We obtained multiple positive lines, of which we used three for the translocation experiments.

Crossings were checked for mutation in the *jip-1* gene by PCR and subsequent sequencing with the following primers:
Forward primer: 5'-ATCAGCGGAAAATCCAGAAA-3' Reverse primer: 5'-CAATGGAGTCCTCGTCGATT-3' TJ356 and *jip-1* mutant worms were synchronized by bleaching and L1 larvae were transferred to RNAi plates containing *E. coli* expressing either control RNAi (L4440) or *ral-1* RNAi [157] or standard agar plates with *E. coli*. Worms were grown until they reached adulthood (indicated by presence of eggs on the plates) and subsequently exposed to heat shock (33°C). Translocation of DAF16::GFP was scored double blind to be nuclear, cytoplasmic or intermediate at least every hour in at least 25 worms per condition using a NIKON SMZ1500 dissection microscope equipped with an epifluorescence setup. Kinase assays using worm lysates were performed as follows: protein lysates of at least 40 worms per condition were incubated with GST-jun [158] for 30 minutes following affinity purification of GST-jun using glutathion beads. After washing 3 times with RAL buffer without $MgCl_2$ (10% glycerol, 1% NP40, 50mM Tris-HCl (pH7.5), 200mM NaCl, protease and phosphatase inhibitors) and 2 times with kinase buffer without ATP (25mM Tri-HCL (pH 7.5), 20mM $MgCl_2$, 2mM DTT), the beads were incubated with kinase buffer (25mM Tri-HCL (pH 7.5), 20mM $MgCl_2$, 2mM DTT, 100µM ATP) for 30 minutes at 30°C. The kinase reaction was stopped by adding 5x concentrated Laemmli sample buffer and heated for 5 minutes at 95 °C. Samples were analyzed by SDS-PAGE, followed by autoradiography. Subsequently, the western blots were analyzed with a phospho-c-jun (Ser63) antibody.

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CHAPTER 3

The role of RALA IN HDM2-mediated protein ubiquitination

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Abstract

Forkhead box O (FOXO) transcription factors are extensively modified by post-translational modifications including acetylation, methylation and ubiquitination. Whereas poly-ubiquitination of FOXOs results in proteasomal breakdown, mono-ubiquitination of FOXOs correlates with FOXO nuclear location and activation. Numerous E3 ligases have been suggested to mediate ubiquitination of FOXOs. Mono-ubiquitination of FOXO4 is mediated by the E3 ligase HDM2. We show here that the small GTPase RALA via activation of JNK is required for HDM2-mediated FOXO4 mono-ubiquitination and for p53 ubiquitination and degradation. We show *in vitro* that JNK can phosphorylate HDM2 and we identified Threonine 218 as the phosphoacceptor site. Mutational analysis showed that loss of phosphorylation or constitutive phosphorylation of Thr-218 did not change HDM2 activity towards FOXO4 or p53. Thus we conclude that JNK-mediated Thr-218 phosphorylation does not regulate HDM2-dependent ubiquitination towards FOXO4 or p53. HDMX/Mdm4 is homologous to HDM2 and is an essential regulator of p53 activity; partly via HDM2 regulation. This function of HDMX appears especially important during cellular stress as illustrated by HDMX regulation by ATM following DNA damage. Our results suggest that RALA plays a role in regulation of HDMX protein levels and hereby potentially mediates HDM2-dependent regulation of FOXO4 and p53 via HDMX. Taken together, RAL/JNK signaling regulates HDM2 function and this does not involve JNK phosphorylation of HDM2 but likely proceeds through the regulation of complex formation between HDM2 and HDMX.

Introduction

Ubiquitination is an important post-translational modification in which an 8 kDa Ubiquitin is covalently attached to proteins. Ubiquitination of proteins involves three classes of enzymes in which the catalytic subunit of an E1 Ubiquitin-activating enzyme forms a thioester bond with the C-terminus of Ubiquitin. Ubiquitin is subsequently transferred from E1 to the catalytic cysteine of an E2 ubiquitin conjugating enzyme. This E2-ubiquitin conjugate then cooperates with an E3 enzyme to transfer ubiquitin to a substrate. There are two families of E3 ligases, the RING E3 ubiquitin-ligases and the HECT E3 ubiquitin-ligases. RING ligases bind both the E2 ligase and substrate protein to transfer ubiquitin to the substrate. HECT ligases first accept ubiquitin from the E2 ligase onto a catalytic cysteine and subsequently transfer ubiquitin to the substrate (reviewed in [180]). A protein substrate can either be mono-, multimono-, poly- or linear-ubiquitinated (reviewed in [181]). Mono- or multimono-ubiquitinated proteins are ubiquitinated on a single or several lysines, resulting in mono- or multimono-ubiquitination respectively [182]. Consecutive rounds of ubiquitination on the substrate-conjugated ubiquitin result in poly-ubiquitin chains on the substrate protein and this is referred to as poly-ubiquitination [180, 182]. Distinct to poly-ubiquitin chains, also linear chains can be assembled on a target protein in a 'head-to-tail' manner by linkages between the C- and N-termini of ubiquitin [183]. Recent findings have indicated that the first ubiquitin moiety can also be fused to the alpha-NH2 group of the N-terminal residue or to cysteins. Ubiquitin contains seven lysines at positions 6, 11, 27, 29, 33, 48 and 63, which can all be utilized during poly-ubiquitination [184]. The position of ubiquitination determines the outcome, for example tightly packed arrangements of K48 chains doom their substrates for proteasome dependent degradation, whereas the stretched-out flexible structure of K63 and linear chain assemblies encode a diverse set of non-degradative signals. The ubiquitin-binding domain-containing effector proteins discriminate between those different poly-ubiquitin chains a feature that is believed to depend on the tertiary structures of the different ubiquitin chains [185, 186].

HDM2 (Human Double Minute)/MDM2 (Mouse Double Minute) is a RING E3-Ubiquitin ligase and extensively studied in its role in p53 regulation [187, 188]. $MDM2^{-/-}$ mice are embryonic lethal and this lethality can be rescued by simultaneous loss of p53 [189, 190]. This indicates p53 is a major target of HDM2 in regulating survival, but does not exclude a role for HDM2 in regulating ubiquitination of other proteins. HDM2 can facilitate poly-ubiquitination and proteasome-dependent degradation of p53 [187, 188, 191, 192], but under certain circumstances HDM2 can also promote mono-ubiquitination resulting in p53 export from the nucleus [191, 193-195]. A second mechanism of HDM2 to inhibit p53, next to ubiquitination, is by masking the DNA binding domain through direct protein-protein interaction [196]. Next to HDM2 another RING domain-containing protein, HDMX/HDM4 is described as an important regulator of p53 [197, 198]. Also $MDMX^{-/-}$ mice die early in embryogenesis and similar to $MDM2^{-/-}$ mice, this can be rescued by co-deletion of p53 [199-201]. HDMX is described to function in a multimeric complex with HDM2 and together they are important for p53 regulation [202-204]. HDM2 stability is controlled by DNA damage-activated post-translational modifications and by its own RING domain. HDMX interacts with the RING domain of HDM2 and thereby potentially mediates regulation of HDM2 stability. Although HDMX contains a RING domain, it seems not to act as an E3 ubiquitin ligase towards p53 [205]. Based on the observation that HDM2-HDMX heterodimers are more effective in p53 ubiquitination [205], it has been suggested that the HDM2/HDMX ligase complex is more efficient in targeting p53 for degradation, compared to HDM2 alone. However, it was also shown that MDMX could compete with MDM2 for p53 binding and thereby prevent degradation of p53 [197, 198]. In addition, one of the most important functions of HDMX is, like HDM2, to directly bind p53 and thereby inhibit its transcriptional

activity [206]. In summary, the exact function of MDMX in controling p53 stability is complex and requires further study. Both HDM2 and HDMX are highly regulated by posttranslational modifications like phosphorylation, which is mediated by kinases activated by DNA damage signals, other stress signals and kinases involved in cell growth and survival signals. DNA damage-induced phosphorylation on HDMX/HDM2 disrupts the complex formation of p53-HDM2/HDMX and subsequently results in p53 stabilization and HDM2/HDMX degradation. In contrast, Protein Kinase B (PBK)/AKT-mediated phosphorylation on HDM2 and HDMX results in their stabilization and subsequent p53 degradation (reviewed in [207]). Although many phosphorylation events disrupt the HDM2-p53 interaction, mouse studies show that blocking phosphorylation on these sites causes only modest defects in p53 function. Therefore it is likely that additional mechanisms are important for p53 function. Indeed, it seems that the molecular ratio of HDM2-HDMX-p53 is important to determine the functional outcome in terms of p53 stability. Low concentrations of GST-MDM2 can mediate mono-ubiquitination of p53 while high concentrations promote poly-ubiquitination of p53 [208]. However how different amounts of HDM2 determine its function as a mono- or poly-ubiquitin ligase was unknown. Recently, Wang *et al*. showed that, unlike GST-tagged MDM2, non-GST full-length MDM2 *in vitro* possesses only weak E3 ligase activity for p53 and results only in mono-ubiquitination of p53, whereas addition of MDMX activated MDM2 and converted MDM2 to a poly-ubiquitination E3 ligase for p53 [209]. Thus it is suggested that HDMX inhibits p53 by enabling HDM2 to convert mono into poly-ubiquitination and hence degradation of p53.

The Forkhead box O (FOXO) family of transcription factors is important in several biological processes like cell cycle arrest, metabolism and stress resistance [122, 124, 177]. FOXOs are extensively regulated by posttranslational modifications such as phosphorylation, acetylation and ubiquitination [210, 211]. Phosphorylation of FOXOs coincides with their ubiquitination. For example PBK-mediated phosphorylation of FOXO1 results in recruitment of SKP2, which mediates poly-ubiquitination, and subsequent degradation of FOXO1 [84]. Furthermore PKB-mediated phosphorylation can also result in MDM2 recruitment and subsequent poly-ubiquitination of both FOXO1 and FOXO3a [212]. Moreover, ERK-mediated phosphorylation of FOXO3 is suggested to result in MDM2-mediated poly-ubiquitination of FOXO3. However, although the authors suggest that ERK-mediated phosphorylation is necessary for binding of MDM2 to FOXO3 and subsequent poly-ubiquitination, the paper lacks experimental proof to support this statement [90]. We have recently shown that MDM2 can mediate mono-ubiquitination and subsequent activation of FOXO4 [93]. FOXO4 mono-ubiquitination and JNK-mediated phosphorylation both coincide with FOXO4 nuclear localization and activation, however it is unknown if phosphorylation is a prerequisite for FOXO4 ubiquitination.

Here we show that JNK activation is indeed important for FOXO4 ubiquitination and that direct JNK-mediated phosphorylation on either FOXO4 or MDM2 is not important for FOXO4 ubiquitination. We identified Threonine 218 as a JNK phosphorylation site on HDM2. Mutational analysis showed that mimicking loss of phosphorylation by substitution of Threonine 218 by an Alanine or mimicking constitutive phosphorylation by replacing it by a Glutamic acid did not change HDM2 E3 ligase activity towards FOXO4 or p53. Either substitution also did not change HDM2 stability or its influence on p300-mediated acetylation of p53 and p300. Therefore, the regulation of FOXO ubiquitination by RAL/JNK signaling is unlikely to proceed through JNK-mediated phosphorylation of HDM2. This leaves other possible modes of regulation. We show that RALA/JNK signaling affects HDM2-mediated regulation of p53 stability and therefore regulation of ubiquitination by RALA/JNK likely occurs through a general rather than a FOXO-specific mechanism. We provide evidence that RAL/JNK signaling likely impinges on HDMX and/or HDMX/HDM2 heterodimerization as RALA activation enhances HDMX stability and inhibits HDM2 induced HDMX breakdown.

Results

RALA-JNK signaling regulates FOXO4 ubiquitination

Protein ubiquitination has been described to often correlate with protein phosphorylation [213]. Moreover, for many proteins, phosphorylation is even a prerequisite for ubiquitination. Our lab has extensively studied the effects of ROS on the activity of FOXO4. Increasing cellular ROS levels induces nuclear accumulation and activation of FOXO4 and this coincides with both phosphorylation [46] and mono-ubiquitination [92] of FOXO4. c-Jun N-terminal Kinase (JNK), a member of the MAPK family, is activated upon H_2O_2 treatment and phosphorylates FOXO4 on the same residues that are phosphorylated under these conditions. Hence, we investigated the possible role of JNK-mediated FOXO4 phosphorylation on the mono-ubiquitination of FOXO4.

We first analyzed ubiquitination of FOXO4 in JNK1,2$^{-/-}$ MEFs and observed almost complete loss of ROS-induced FOXO4 ubiquitination (Fig. 1A). Add-back of JNK1 restored ROS induced FOXO4 mono-ubiquitination, showing an important role for JNK-signaling in regulating FOXO4 mono-ubiquitination. The small GTPase RALA mediates ROS-induced JNK-signaling towards FOXO4 [46, 214]. In agreement, siRNA-mediated reduction of RALA expression also prevented ROS-induced FOXO4 mono-ubiquitination (Fig. 1B). We previously deciphered the pathway from RALA to JNK and identified MLK3 as the MAPKKK in this pathway downstream of RALA [214]. Accordingly, siRNA-mediated knockdown of MLK3 expression inhibited the ROS-induced FOXO4 ubiquitination (Fig. 1C), whereas overexpression of MLK3 rescued the knockdown effect of MLK3 and enhanced basal ubiquitination of FOXO4 (Fig. 1C, last four lanes). Thus the RALA-MLK3-JNK signaling pathway plays an important role in mediating FOXO4 ubiquitination during ROS signaling. FOXO4 mono-ubiquitination is mediated via the E3 ligase HDM2 [93].

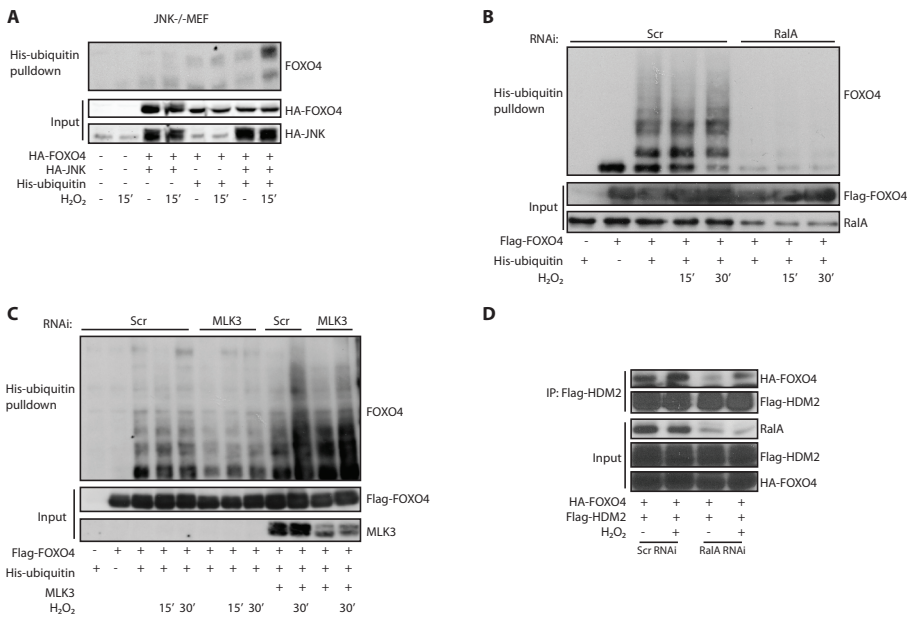

FIGURE 1: RALA-mediated signaling towards FOXO4 is important for FOXO4 ubiquitination. (**A**) Ubiquitination of FOXO4 in JNK1.2$^{-/-}$ MEFs. (**B**) Ubiquitination of FOXO4 with or without RALA siRNA in HEK293T cells (**C**) Ubiquitination of FOXO4 with knockdown of MLK3 in HEK293T cells (**D**) Immunoprecipitation of FOXO4 in HEK293T cells, western blots were analyzed for HA (FOXO4), Flag (HDM2) and RALA.

Hence we determined whether the absence or RALA affects HDM2 binding to FOXO4. Knockdown of RALA inhibited the interaction between FOXO4 and HDM2 (Fig 1D), which suggests showing a role for RALA in regulating the interaction of HDM2 with FOXO4.

JNK phospho-mutants of FOXO4 display increased mono-ubiquitination

The above-described experiments suggest JNK-mediated FOXO4 phosphorylation is involved in FOXO4 mono-ubiquitination. We previously identified seven JNK phosphorylation sites (a Serine or Threonine followed by Proline) in FOXO4 [80]. To assess the requirement of phosphorylation on these sites for ubiquitination, we examined several mutants of FOXO4 in which the Serine/Threonine of two or more of these sites was substituted for an Alanine, for their ubiquitination. All FOXO4 JNK-site mutants showed increased ubiquitination compared to wild type FOXO4 (Fig. 2A). This likely represent increased multimono-ubiquitination, since we performed these experiments with and without a proteasome inhibitor (MG132) and observed no difference in ubiquitination signal (data not shown). Knockdown of RALA inhibits binding of HDM2 to FOXO4; therefore we tested the binding of HDM2 to the FOXO4 mutants. All mutants showed enhanced binding to HDM2, compared to wild type FOXO4, which is in agreement with their increased mono-ubiquitination (Fig. 2B). The de-ubiquitinating enzyme (DUB) USP7 interacts with and de-ubiquitinates FOXO4 [92]. The interaction of USP7 with the various FOXO4 mutants was similar compared to wild type FOXO4 (Fig. 2C), thus the increased mono-ubiquitination of these mutants did not result from decreased USP7 binding.

Taken together, these data suggest that phosphorylation of FOXO4 by JNK is not a prerequisite for FOXO mono-ubiquitination as mutation of the identified JNK sites in FOXO4, to mimic loss of phosphorylation, did does not abrogate the ubiquitination but instead increased the mono-ubiquitination of FOXO4. Therefore, an alternative explanation for these results is that the RALA/JNK pathway regulates the

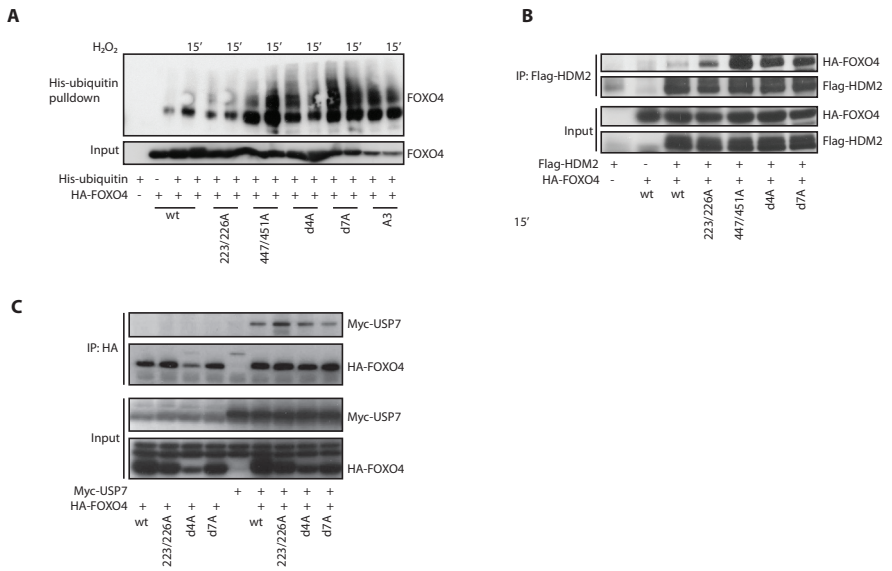

FIGURE 2: JNK-mediated phosphorylation of FOXO4 is not important for FOXO4 ubiquitination. (**A**) Ubiquitination of wild type FOXO4 (wt), or FOXO4 mutated in: Thr-223/Ser-226, Thr-447/Thr-451, Thr-223/Ser-226/Thr-447/Thr-451 (d4A) and Thr-223/Ser-226/Thr-447/Thr-451/Ser-237/Ser-268/Thr-370 (d7A) in HEK293T cells. (**B**) Immunoblot of HEK293T cell-lysates immunoprecipitated for Flag-HDM2; western blots were analyzed for HA (FOXO) and Flag (HDM2). (**C**) Immunoblot of Myc-USP7 pulldown and of lysates from HEK293T cells transfected with myc-USP7, HA-FOXO4 wt, HA-FOXO4 223/226, HA-FOXO4d4A and HA-FOXO4 d7A.

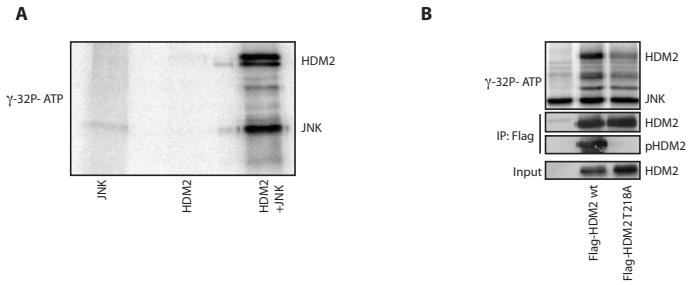

FIGURE 3: JNK phosphorylates HDM2 *in vitro*. (**A**) *In vitro* kinase assay with recombinant HDM2 and JNK. (**B**) *In vivo* kinase assay with Flag-HDM2 wt (wild type) and Flag-HDM2 T218A immunoprecipitated from HEK293T cells and recombinant JNK.

activity of HDM2. To explore this option, we performed an *in vitro* kinase assay, with recombinant JNK and recombinant HDM2. As shown in Fig. 3A, JNK could phosphorylate HDM2 *in vitro*. When examining the HDM2 protein sequence, we identified only one Threonine followed by a Proline (Thr-218), which could potentially be phosphorylated by JNK. We mutated this site in HDM2 to an Alanine to mimic loss of phosphorylation and examined both HDM2 and HDM2^{T128A} in an *in vitro* kinase assay for JNK-mediated phosphorylation. Indeed, the HDM2^{T218A} showed decreased phosphorylation by JNK *in vitro* (Fig. 3B), indicating that this is a potential JNK phosphorylation site in HDM2.

HDM2 and HDM2^{T128A} behave similar in their stability and in FOXO4 binding and ubiquitination

HDM2 protein stability is predominantly regulated by its auto-ubiquitination. Under basal conditions Death domain-associated protein (Daxx) directs the de-ubiquitinating enzyme USP7 to HDM2, which results in HDM2 de-ubiquitination and subsequent stabilization. Upon DNA damage, Daxx dissociates from HDM2, resulting in HDM2 auto-ubiquitination and degradation [215]. We wondered whether JNK-mediated phosphorylation on HDM2 could influence this USP7-mediated de-ubiquitination and stabilization of HDM2. First, the stability of HDM2^{T128A} is not altered compared to wild type HDM2 (Fig. 4A). Also FOXO4 stability was not altered by overexpression of either HDM2 or HDM2^{T128A}, as expected because HDM2 does not induce poly-ubiquitination of FOXO4 (Fig.4B).

Second, the interaction between USP7 and HDM2^{T128A} was not altered compared to HDM2, both in presence and absence of ROS signaling (Fig. 4C). From these data, we conclude that JNK-mediated HDM2 phosphorylation on T218 is not important for the recruitment of USP7 and stability of HDM2.

The prolyl-isomerase PIN1 has been described to inhibit FOXO4 mono-ubiquitination by recruiting USP7 and thereby enhancing de-ubiquitination of FOXO4 [80]. In addition, increased levels of PIN1 inhibit poly-ubiquitination of p53 [216] and PIN1 activity is usually controlled by substrate phosphorylation [217]. Hence, we examined the role of PIN1 in the regulation of HDM2 and determined whether phosphorylation of HDM2 has an effect on its interaction with PIN1. We found PIN1 to interact with both USP7 and HDM2. However, mutating the JNK phosphorylation site in HDM2 had no effect on the binding between HDM2 and PIN1 (Fig. 4D). Also, increasing amounts of PIN1 did not inhibit HDM2 binding to USP7 (Fig. 4E). In short, the stability of HDM2^{T128A} as well as its interaction with USP7 is similar to wild type HDM2. Although the co-immunoprecipitation of PIN1 with HDM2 has not been described previously, it does not affect the interaction of USP7 with HDM2. Therefore, in contrast to what has been described for FOXO4, PIN1 appears not important for regulation of USP7-mediated de-ubiquitination of HDM2. Combining these results, it is not likely that JNK-mediated phosphorylation of HDM2 affects HDM2 protein stability.

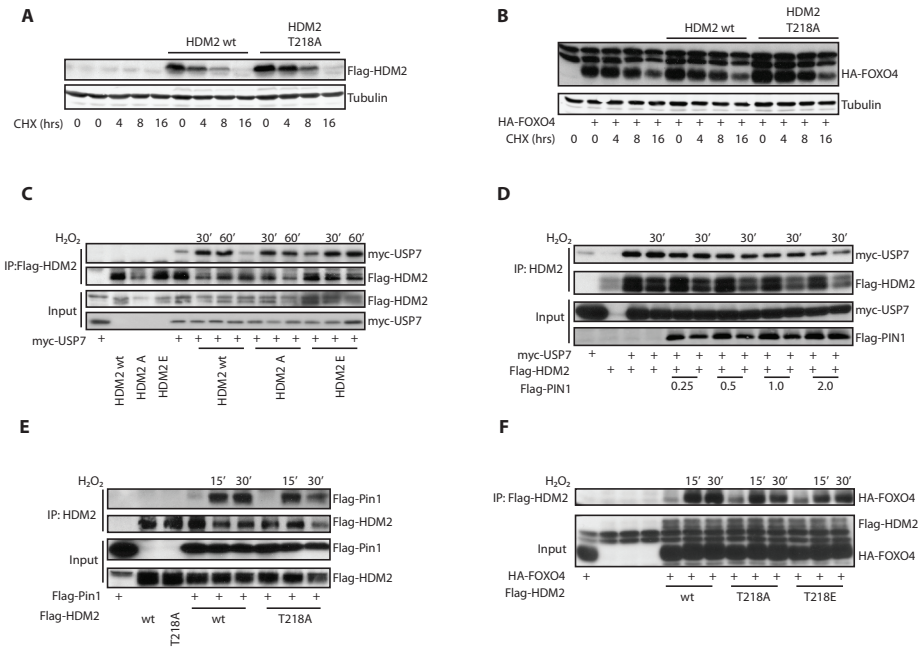

FIGURE 4: Similar function of wild type and mutant HDM2^{T128A}. (**A**) Immunoblots of lysates from HEK293T cells transfected with Flag-HDM2 and Flag-HDM2^{T128A} (HDM2 T218A) (**B**) Immunoblots of HEK293T lysates transfected with HA-FOXO4, Flag-HDM2wt and Flag- HDM2^{T128A} (HDM2 T218A). (**C**) Immunoblots of Flag-HDM2 pulldown and of lysates from HEK293T cells transfected with myc-USP7, Flag-HDM2wt, Flag- HDM2^{T128A} (HDM2 A) and Flag- HDM2^{T128E} (HDM2 E). (**D**) HDM2 pulldown and HEK293T lysates with overexpression of myc-USP7, Flag-HDM2 and increasing concentrations of Flag-PIN1. (**E**) HDM2 pulldown with anti-HDM2 antibody and lysates from HEK293T cells transfected with Flag-PIN1, Flag-HDM2 wt and Flag- HDM2^{T128A} (T218A). (**F**) Immunoblots of Flag-HDM2 pulldown or of lysates from HEK293T cells transfected with Flag-HDM2 wt, Flag- HDM2^{T128A} (T218A), Flag- HDM2^{T128E} (T218E) and HA-FOXO4. wt = wild type; T218A = mutation of Threonine 218 to Alanine; T218E = mutation of Threonine 218 to Glutamic acid. Cells were treated with 200μM H_2O_2 for the indicated time-points or left untreated.

Because RALA is important for the interaction of FOXO4 with HDM2 (Fig. 1D), we determined the importance of JNK-mediated phosphorylation of HDM2 for its interaction with FOXO4. Both the phospho-dead T128A and phospho-mimicking T128E mutants of HDM2 bound to FOXO4 to a similar extend as wild type HDM2 (Fig. 4F). Therefore, JNK-mediated phosphorylation on HDM2 appears not to determine HDM2 interaction with FOXO4.

3

HDM2 and HDM2^{T128A} equally regulate P300

HDM2-mediated ubiquitination of p53 was found mutually exclusive with p300/CBP-mediated acetylation at the same lysines [218]. Furthermore, HDM2 is described to inhibit p300-mediated p53 acetylation [219] and since p300-mediated acetylation is important for p53 activation [220], this provides yet another layer of regulation towards p53. P300 also acetylates FOXO4 and thereby regulates its transcriptional activity [221]. We examined if JNK mediated HDM2 phosphorylation would affect its ability to regulate p300 acetyl transferase activity and subsequent p53 or FOXO4 acetylation. We used p53/Mdm2 double knockout MEFs (p53$^{-/-}$, Mdm2$^{-/-}$ MEFs) to exclude any additional effect of endogenous MDM2. Indeed, HDM2 inhibits the auto-acetylation (and thereby activity) of p300, however mutating the JNK phosphosite in HDM2 to an Alanine, did not affect HDM2-mediated inhibition of p300 acetylation (Fig 5A). Also p300-mediated acetylation of its targets p53 (Fig. 5B) and FOXO4 (Fig. 5C)

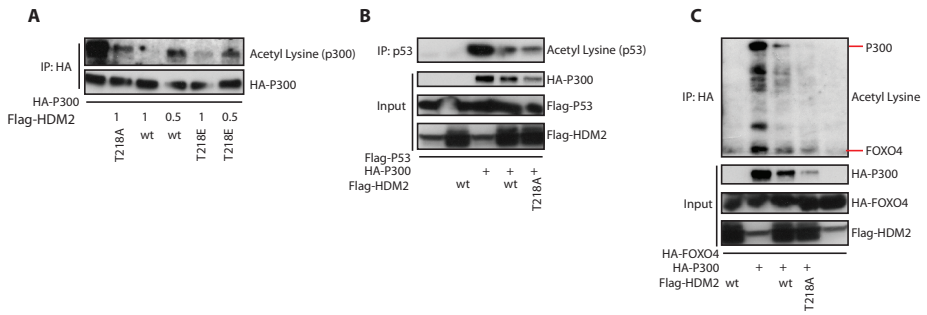

FIGURE 5: Similar effects of wild type and HDM2^{T128A} on p300 regulation. (**A**) Immunoblots of HA-p300 pulldown from DKO MEFs transfected with HA-p300, Flag-HDM2^{T128A} (T218A) and Flag-HDM2 ^{T128E} (T218E), western blots are probed for acetyl-lysine and Flag. (**B+C**) Immunoblots of p53 pulldown (**B**) or FOXO4 pulldown (**C**) and of lysates from DKO MEFs transfected with Flag-p53 (**B**), HA-FOXO4 (**C**), HA-p300, Flag-HDM2 wt and Flag-HDM2^{T128A} (T218A). wt = wild type; T218A = mutation of Threonine to Alanine

was inhibited by wild type HDM2 and to a similar extend by HDM2^{T128A}. Hereby we can conclude that JNK-mediated phosphorylation of HDM2 is not essential for HMD2-mediated regulation of p300 or for p300 function towards its downstream targets.

p53 and FOXO4 are ubiquitinated to a similar extend by both wild type and mutant HDM2

To determine the effect of JNK-mediated phosphorylation on HDM2-mediated ubiquitination of its targets p53 and FOXO4, we compared wild type and mutant HDM2 in their ability to ubiquitinate p53 and FOXO4 in p53$^{-/-}$, Mdm2$^{-/-}$ MEFs. Upon add-back of wild type HDM2, we could greatly enhance FOXO4 mono-ubiquitination (Fig. 6A) and p53 ubiquitination (Fig. 6B), but also add back of mutant HDM2 showed similar induction of ubiquitination of FOXO4 and p53 (Fig. 6A and Fig. 6B). This clearly shows that Thr-218 phosphorylation on HDM2 is not necessary for HDM2-mediated ubiquitination of either p53 or FOXO4.

To determine whether the phosphorylation of HDM2 by JNK might be more important during DNA damage signaling, compared to general ROS signaling, we determined the ubiquitination of FOXO4 upon treatment with Etoposide (a DNA damage reagent). However, Etoposide treatment did not affect FOXO4 mono-ubiquitination and also wild type or mutant HDM2 mediated FOXO4 mono-ubiquitination was not affected by Etoposide (Fig. 6C).

Different HDM2 concentrations have different effects on FOXO4 versus p53 ubiquitination

While performing the above-described experiments, we noticed that only very small amounts of HDM2 could mediate FOXO4 ubiquitination, whereas higher amounts did not result in FOXO4 ubiquitination (Fig. 7A). Therefore we compared the ubiquitination of both FOXO4 and p53 to see whether this was general for all proteins ubiquitinated by HDM2. Low amounts of HDM2 (20-50ng) largely increased FOXO4 ubiquitination, whereas higher amounts (100-200ng) of HDM2 were less efficient in FOXO4 ubiquitination (Fig. 7B). However, this is different for p53 ubiquitination, where low amounts of HDM2 resulted in some increase in its ubiquitination, whereas higher amounts of HDM2 resulted in a substantial increase in p53 ubiquitination (Fig. 7C). Also mutant HDM2 behaved similar in this respect (Fig. 7A and data not shown), indicating that phosphorylation of HDM2 on T218 seems not important in determining the extend of ubiquitination of these two different downstream targets.

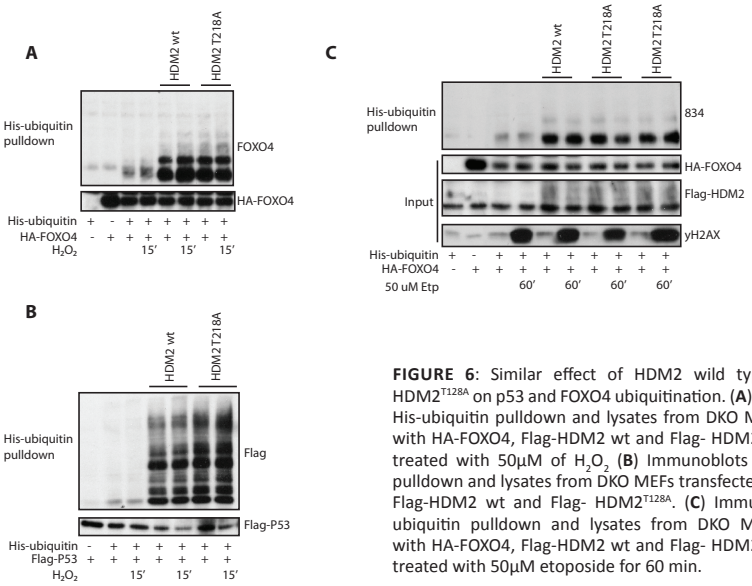

FIGURE 6: Similar effect of HDM2 wild type and mutant HDM2^{T128A} on p53 and FOXO4 ubiquitination. (**A**) Immunoblots of His-ubiquitin pulldown and lysates from DKO MEFs transfected with HA-FOXO4, Flag-HDM2 wt and Flag- HDM2^{T128A}. Cells were treated with 50μM of H_2O_2 (**B**) Immunoblots of His-ubiquitin pulldown and lysates from DKO MEFs transfected with Flag-p53, Flag-HDM2 wt and Flag- HDM2^{T128A}. (**C**) Immunoblots of His-ubiquitin pulldown and lysates from DKO MEFs transfected with HA-FOXO4, Flag-HDM2 wt and Flag- HDM2^{T128A}. Cells were treated with 50μM etoposide for 60 min.

A general role for RALA in mediating protein ubiquitination?

Although we could not identify a role for RALA/JNK signaling in direct regulation of HDM2 function, our data clearly show a role for RALA-JNK in FOXO4 ubiquitination. To test other modes of regulation we first addressed the question whether RALA specifically determines ubiquitination of FOXO or has a more general role in regulating HDM2-mediated protein ubiquitination. As indicated, p53 is the best-described target of HDM2 and p53 stability is for a large part regulated by proteins that affect the interaction of p53 with HDM2. In addition, p53 function is also regulated by JNK signaling. Whether

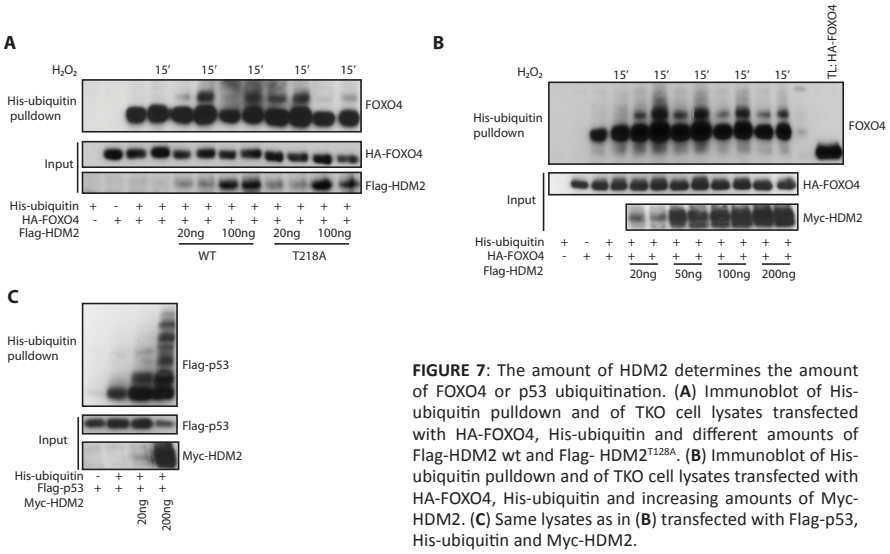

FIGURE 7: The amount of HDM2 determines the amount of FOXO4 or p53 ubiquitination. (**A**) Immunoblot of His-ubiquitin pulldown and of TKO cell lysates transfected with HA-FOXO4, His-ubiquitin and different amounts of Flag-HDM2 wt and Flag- HDM2^{T128A}. (**B**) Immunoblot of His-ubiquitin pulldown and of TKO cell lysates transfected with HA-FOXO4, His-ubiquitin and increasing amounts of Myc-HDM2. (**C**) Same lysates as in (**B**) transfected with Flag-p53, His-ubiquitin and Myc-HDM2.

JNK potentiates or inhibits p53 activity depends on the context. During DNA damage signaling, JNK phosphorylates p53 on Thr-81, which results in stabilization and activation of p53 [222], whereas in unstressed conditions JNK mediates HDM2-p53 complex formation and subsequent p53 degradation [223]. Thus, we tested whether knockdown of RALA would affect HDM2-mediated p53 ubiquitination. Similar as for FOXO4, siRNA of RALA in HEK293T cells inhibited the HDM2-mediated p53 ubiquitination (Fig. 8A). However in TKO cells ($p53^{-/-}$, $Mdm2^{-/-}$, $Mdmx^{-/-}$ MEFS), RALA knockdown increased the HDM2-mediated p53 ubiquitination (Fig. 8B). The difference between these experiments might be explained by the presence of HDMX. Whereas the TKO cells lack endogenous MDMX and MDM2, in HEK293T cells both are present, although at low levels. Add-back of HDMX in the TKO cells resulted in a minor decrease of HDM2-mediated p53 (Fig. 8B) and FOXO (data not shown) ubiquitination. Previously, others have reported different outcomes of HDMX on HDM2-mediated ubiquitination among different cell lines [198]. Furthermore, HEK293T cells contain the SV40 large T antigen and adenovirus E1A/E1B proteins, all of which have been described to inhibit p53 function. Therefore, ubiquitination of p53 might be differently regulated in these cells.

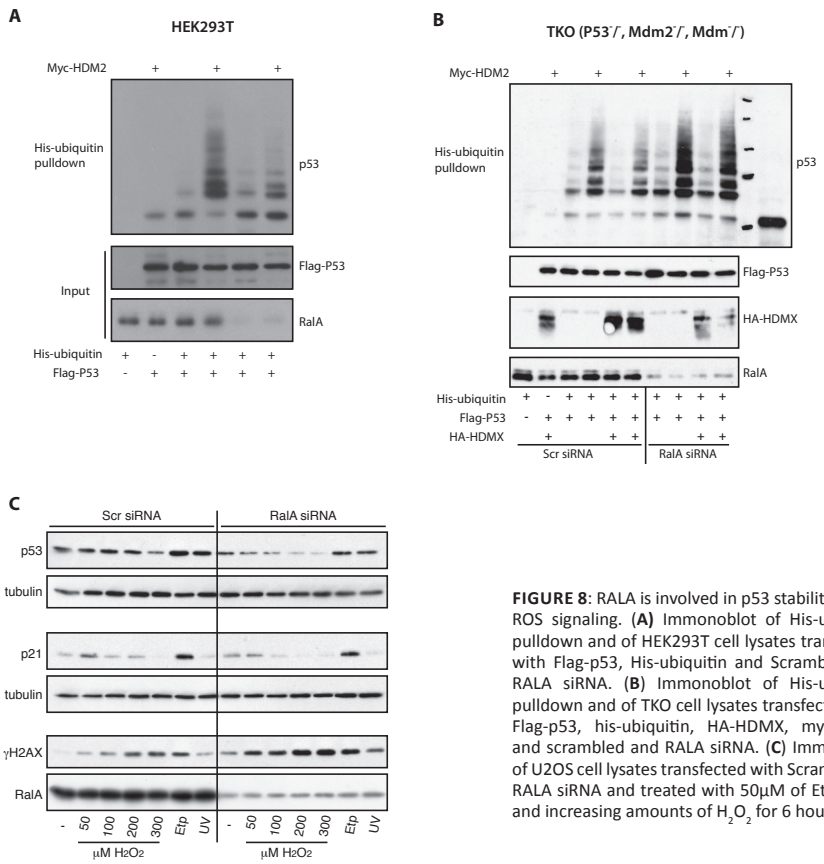

FIGURE 8: RALA is involved in p53 stability during ROS signaling. (**A)** Immonoblot of His-ubiquitin pulldown and of HEK293T cell lysates transfected with Flag-p53, His-ubiquitin and Scrambled and RALA siRNA. (**B**) Immonoblot of His-ubiquitin pulldown and of TKO cell lysates transfected with Flag-p53, his-ubiquitin, HA-HDMX, myc-HDM2 and scrambled and RALA siRNA. (**C**) Immunoblot of U2OS cell lysates transfected with Scrambled or RALA siRNA and treated with 50μM of Etoposide and increasing amounts of H_2O_2 for 6 hours.

Because the effect of RALA siRNA on p53 ubiquitination remained inconclusive, we determined the stability of p53 during stress signals with or without RALA siRNA. Treatment of U2OS cells with increasing amounts of H_2O_2 had minor effects on p53 levels, only treatment with 300μM H_2O_2 decreased p53 abundance. In contrast, Etoposide treatment (DNA damaging reagent) stabilized p53, as described before. Knockdown of RALA, decreased p53 protein levels throughout the H_2O_2 concentration range, whereas the Etoposide-induced increase in p53 levels remained unaffected (Fig. 8C). Also $p21^{Cip1}$ protein levels were affected by RALA siRNA (Fig. 8C). Whether this is because of degradation of p53 or a direct effect on the stability of $p21^{Cip1}$ remains to be established. From these data we suggest that RALA plays a role in controlling p53 protein levels during oxidative stress signals. Whether this is reflected by the ubiquitination of p53 in these cells remains to be investigated.

Control of HDMX by RALA?

As described above, HDM2 E3 ligase activity can be controlled by HDMX. Because RALA-JNK signaling affects HDM2-mediated regulation of p53 and FOXO4, we questioned if RALA has a function in HDMX regulation. We first determined whether HDMX could be phosphorylated by JNK *in vitro*. Indeed, addition of recombinant JNK to recombinant HDMX resulted in appearance of multiple bands, suggesting that HDMX can be a JNK substrate (Fig. 9A). However, mutational analysis is necessary to confirm this observation. To test if the RALA-JNK pathway has an effect on HDMX protein levels, we activated RALA by co-transfection of its active guanine nucleotide exchange factor (GEF), RLF (RlfCAAX)[150]. Expression of RlfCAAX inhibited the HDM2-mediated degradation of HDMX, whereas the catalytic inactive mutant (RlfΔcatCAAX) did not (Fig. 9B). Furthermore, low amounts of stress stabilized HDMX; but the HDM2-mediated degradation of HDMX was not affected (Fig. 9C+D). Surprisingly, when we examined the effect of RALA activation by co-transfection of RlfCAAX on HDMX alone in TKO cells (lacking endogenous MDM2) (Fig. 9E), we observed stabilization of HDMX. As HDM2 is not present in these cells, this would suggest that in absence of HDM2, there is another E3 ligase responsible for regulation of HDMX protein levels. Because RALA knockdown affects p53 protein levels in U2OS cells (Fig. 8C), we also determined the effect of RALA activation on HDMX protein levels in U2OS cells. Co-expression of RlfCAAX resulted in an increase of HDMX protein level, even upon co-transfection of HDM2 (Fig. 9F). Taken together, RALA activation seems to affect HDMX abundance. Whether RALA directly affects HDMX stability or other mechanisms affecting protein expression remains to be determined.

3

Discussion

Both ROS-induced phosphorylation and ubiquitination are important for FOXO4 activity, however the connection between these posttranslational modifications has not been investigated. Here we show that the small GTPase RALA regulates HDM2-induced FOXO4 ubiquitination and provides a link between JNK-mediated phosphorylation and HDM2-mediated ubiquitination. *In vitro* JNK can phosphorylate HDM2 on Thr-218. However, extensive analysis of this phosphorylation site in HMD2 showed similar effects of HDM2 and mutant $HDM2^{T128A}$ in all assays tested and therefore excludes a role of this phosphorylation site in the E3 ligase activity of HDM2 towards p53 and FOXO4. Others have shown a role for CDK2 (cyclin-dependent kinase 2) in phosphorylating murine MDM2 on the same site as we identified. Phosphorylation on this site was shown to weaken the interaction of MDM2 with p53

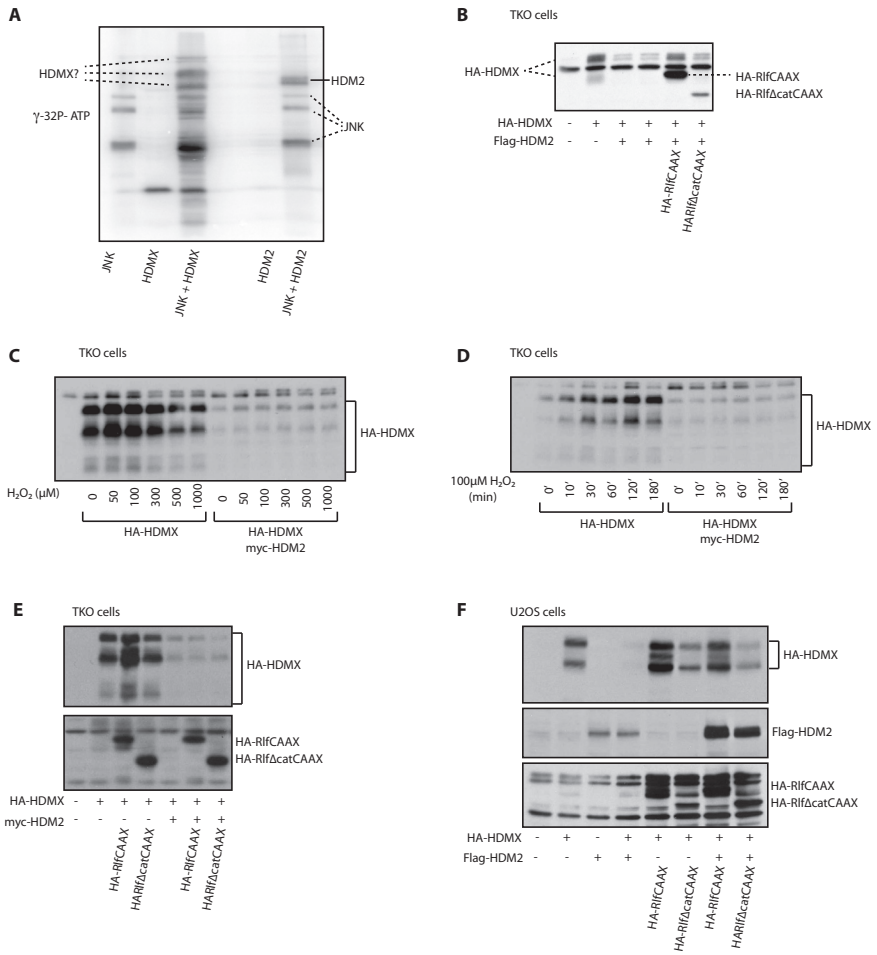

FIGURE 9: RALA affects HDMX protein abundance. (**A**) *in vitro* kinase assay with recombinant JNK, HDM2 and GST-HDMX pulldown (**B**) Immunoblot of TKO cells transfected with HA-HDMX, Flag-HDM2, HA-RlfCAAX and HA-RlfΔcatCAAX. (**C, D** and **E**) Immunoblots of TKO cell lysates transfected with HA-HDMX and myc-HDM2. Cells were treated with increasing amounts of H_2O_2 for 30 minutes (**C**) or 100 μM H_2O_2 for increasing time-points (**D**) or co-transfected with HA-RlfCAAX and HA-RlfΔcatCAAX (**E**). (**F**) Immunoblot of U2OS cells transfected with HA-HDMX, Flag-HDM2, HA-RlfCAAX and HA-RlfΔcatCAAX.

and it is suggested that Cyclin G can attract PP2A to dephosphorylate MDM2 and thereby inhibit its interaction with p53 [224, 225]. However these experiments were performed with GST-MDM2 which has been shown to result in MDM2-unrelated ubiquitination signals, due to the GST-tag [209]. Furthermore, no mutational analysis for Thr-218 (Thr-216 in mice) was performed and no ubiquitination assays for p53 or MDM2 are shown. Analysis of human HDM2 showed that Cyclin G affected Ser-166 phosphorylation, whereas Thr-218 is not mentioned [225]. Moreover, Zhang *et al.* have shown that CDK2 is not able to phosphorylate human HDM2, probably because it lacks the CDK consensus sequence

and the cyclin recognition motif (CRM) [224]. Thus it remains unclear whether CDK2 has a role in HDM2 regulation via Thr-218 phosphorylation and our analysis shows no essential role for Thr-218 in HDM2 E3 ligase activity.

Is RALA important in determination of HDM2-mediated mono- versus poly-ubiquitination?
We identified RALA to be important for regulation of the E3 ligase activity of HDM2 towards its downstream targets. We show that RALA activation or loss of expression affects p53, p21 and HDMX protein levels. Stability is determined by poly-ubiquitination and as mentioned above HDMX is an important cofactor for HDM2 activity towards downstream targets. However how and when HDMX regulates HDM2 function appears highly context dependent and complex. Our data suggest that RALA might be involved in regulation of HDMX levels. HDMX is described to promote HDM2-dependent poly-ubiquitination of p53, and therefore it will be important to determine if RALA may regulate the shift from HDM2-mediated mono-ubiquitination towards HDM2-mediated poly-ubiquitination of p53. Additional experiments are necessary to determine if RALA indeed mediates the stability of HDMX protein levels. Therefore, future experiments will include cyclohexamide experiments and determination of HDMX (poly)-ubiquitination.

Does RALA mediate stability of HDMX by changing its localization?
Another important mode of regulation of HDMX/HDM2 is by alteration of their subcellular localization. Upon DNA damage, Chk2-mediated phosphorylation of MDMX on S367 is important for stimulating 14-3-3 binding which promotes MDMX nuclear translocation and MDM2-mediated degradation [226, 227]. Also ATM mediated phosphorylation of HDMX results in its nuclear localization and subsequent HDM2-mediated degradation [228, 229]. Under basal conditions, CDK2/Cdc2^{p34} phosphorylates HDMX on Serine 96 and thereby mediates nuclear export of HDMX [230]. Export of HDM2 is mediated by HDMX and mutating Ser-96 in HDMX results in nuclear localization of HDM2 and is suggested to result in degradation of HDM2. However, the effect of CDK2 inhibition on HDMX stability in absence of HDM2 was not determined. Serine 96 is followed by a Proline and therefore also a potential JNK phosphorylation site. Our data suggest that upon RALA activation (and thereby also JNK activation) HDMX levels are affected. Whether this is through RALA-mediated effects on protein stability/ubiquitination or through other mechanisms that affect protein expression remains to be determined. Based on the data from Elias *et al.*, RALA activation would potentially result in cytoplasmic localization of HDMX and HDM2. We determined the localization of wild type and T218A mutant HDM2 and both were localized in the nucleus (data not shown). However, we performed these experiments in U2OS cells, while Elias *et al.* performed their experiments in MDMX$^{-/-}$, p53$^{-/-}$ DKO cells. We are currently performing experiments to determine whether RALA can affect localization of HDMX/HDM2.

Proposed model for RALA-mediated regulation of HDMX/HDM2
Our data show that RALA is important in FOXO4 mono-ubiquitination. Furthermore, RALA activation seems to affect HDMX and p53 protein abundance and p53 ubiquitination. During DNA-damage signaling, JNK-mediated phosphorylation of p53 results in its stabilization and activation [222], whereas in unstressed conditions JNK mediates HDM2-p53 complex formation and subsequent p53 degradation [223]. In the latter the authors suggest that phosphorylation deficient JNK is sufficient to target p53 for degradation and they indicate that depending on the cell cycle stage there is a preference for JNK-p53 complexes (G0/G1) or HDM2-p53 (G2/M) complexes. Based on our data combined with literature we propose a model in which RALA determines localization of the HDMX/HDM2 complex (Fig. 10). We suggest that by activation of JNK the pool of a HDMX-HDM2 complex in the cytoplasm is increased, where HDM2-mediated mono-ubiquitination of FOXO occurs resulting in FOXO nuclear translocation and activation. Coinciding with this, ROS-induced RALA-JNK activation might cause p53 phosphorylation and subsequent stabilization. Knockdown of RALA or low levels of ROS will then result

in decreased JNK-mediated phosphorylation of p53, FOXO4 and HDMX and subsequently decreased FOXO mono-ubiquitination and increased p53 poly-ubiquitination. Future experiments to determine the localization of HDMX/HDM2 upon RALA activation or inhibition are essential to decipher how exactly the activity of RALA-JNK is involved in these processes. Additionally, also the cellular compartment where FOXO4 mono-ubiquitination occurs needs to be further investigated.

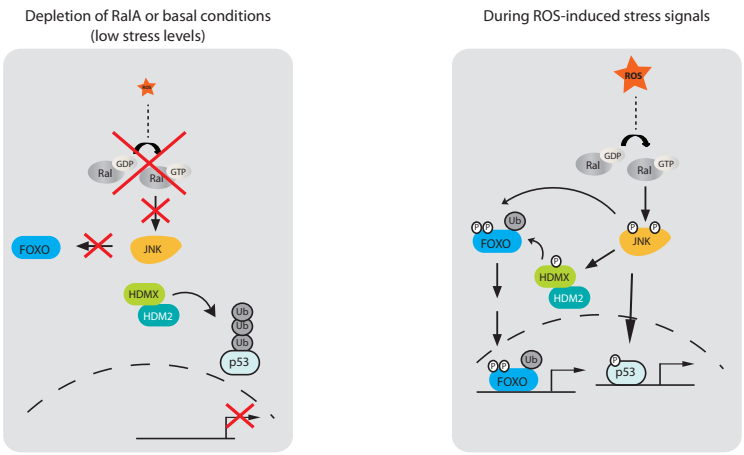

FIGURE 10: Suggested simplified model of how RALA might influence HDM2/HDMX-mediated protein ubiquitination. For further details see text.

Experimental procedures

Cells and reagents

HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Cambrex Bioscience, Verviers Belgium), supplemented with 10% fetal bovine serum, Penicillin/streptomycin and 0.05% L-glutamine. $P53^{-/-}$, $MDM2^{-/-}$ (DKO) cells and $p53^{-/-}$, $MDM2^{-/-}$, $MDMX^{-/-}$ (TKO) cells were kindly provided by A.G. Jochemsen and described previously [198]. They were maintained in DMEM with high glucose (4,5 g/L), supplemented with 10% fetal bovine serum, Penicillin/streptomycin and 0.05% L-glutamine. HEK293T cells were transfected with FuGENE6 (Roche), DKO and TKO cells with Lipofectamine 2000 (Invitrogen), both according to the manufacturer's instructions. siRNA oligo's were transfected with HiPerFect (Qiagen) for 48hrs. Cells were treated with 200μM H_2O_2 for immunoprecipitation experiments and with 50μM H_2O_2 for the ubiquitination assays for the indicated time-points or cells were left untreated. Cyclohexamide and MG132 were purchased (Sigma and Biomol respectively).

Constructs and antibodies

HA-FOXO4, Flag-p53, Flag-HDM2 [93], HA-p300 [221], Flag-pin1 [80], Myc-usp7 [92], His-ubiquitin [92] and HA-FOXO4 mutants Thr223/Ser226, Thr447/Thr451, d4A: Thr223/Ser226/Thr447/Thr451 and d7A: Thr223/Ser226/Thr447/Thr451/Ser237/S268/
Thr370 [80] were described previously. Mutant HDM2 was created with site-directed mutagenesis using the following primers: (T218A Forward 5' – AGTGAATCTACAGGGGCGCCATCGAATCCGGAT – 3', T218A Reverse 5'– ATCCGGATTCGATGGCGCCCCTGTAGATTCACT –3') and (T218E Forward 5' –AGTGAATCTACAGGGGAGCCATCGAATCCGGAT –3' T218E Reverse 5'– ATCCGGATTCGATGG CTCCCCTGTAGATTCACT -3') using full length HDM2 cDNA.
Recombinant JNK was purchased from Upstate/Millipore (Catalogue nr: 14-327M) and recombinant His_6-HDM2 from Boston Biochem. GST-HDMX was kindly provided by A.G. Jochemsen. The following antibodies were purchased: anti-MDM2 (Santa Cruz, H-221), anti-MDMX (Millipore, 8C6), anti-RALA (Cell Signaling), anti-p53 (Santa Cruz, DO-1), anti-acetyl-lysine (Cell Signaling) and anti-FlagM2 (sigma). The following antibodies were described previously: anti-FOXO4 (834)[13], anti-HA (12CA5) and anti-Myc (9E10) [13].

Immunoprecipitation and Western blot

For immunoprecipitation, cells were lysed in buffer containing 50mM Tris-HCl (pH7.5), 1% NP40, 5mM EDTA, 100mM NaCl, protease and phosphatase inhibitors. Cell lysates were centrifuged for 10 minutes at 4°C and the supernatant was used as input material. Immunoprecipitation of the proteins of interest was done with protein agarose beads, coupled to the protein or tag specific antibody, for 2hrs at 4°C. Beads were washed with lysis buffer and resuspended in Laemmli sample buffer. Samples were separated on SDS-polyacrylamide gels and transferred to PVDF-membrane. Western blot analysis was performed by enhanced chemiluminescence. Experiments were performed at least 2-5 times of which a representative figure is shown.

Protein stability assay

HEK293T cells were transfected with wild type or mutant Flag-HDM2 with or without HA-FOXO4. Cells were treated with 10 μg/μL of cycloheximide for the indicated time points.

Ubiquitination assay

The ubiquitination assays were essentially performed as described previously [92]. Briefly, HEK293T cells, DKO ($P53^{-/-}$,$MDM2^{-/-}$) or TKO ($P53^{-/-}$,$MDM2^{-/-}$, $MDMX^{-/-}$) cells were transfected with the indicated constructs and 48 hrs post transfection the cells were left untreated or treated as indicated and lysed in urea lysis buffer. Ubiquitinated proteins were precipitated using Ni-NTA agarose beads and analyzed by western blot.

Kinase assay

Recombinant JNK with or without recombinant HDM2 was incubated with kinase buffer (25mM Tri-HCL (pH 7.5), 20mM $MgCl_2$, 2mM DTT, 100µM ATP) and 5µCI of γ-P [ATP] for 30 minutes at 30°C. GST-HDMX was bound to Prot G/A beads and subsequently incubated with recombinant JNK in kinase buffer with 5µCI of γ-P [ATP] for 30 minutes at 30°C.

For the *in vivo* kinase assay, Flag-HDM2 and Flag-HDM2 T218A were immunoprecipitated with FlagM2 affinity beads (Sigma) from HEK293T cells. Immunoprecipiteded HDM2 was eluded from the beads with 200µg/ml 3x Flag-peptide (Sigma) and subsequently incubated with recombinant JNK in kinase buffer for 30 minutes at 30°C. The kinase reaction was stopped by adding 5x concentrated Laemmli sample buffer and incubation for 5 minutes at 95°C. Samples were analyzed by SDS-PAGE, followed by autoradiography.

CHAPTER 4

Identification of FOXO1, 3 and 4 specific interaction partners by a quantitative proteomics approach

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Abstract

The Forkhead box O group of transcription factors is largely redundant in a number of biological functions, such as their tumor suppressive function, yet the individual knockout mice display very different phenotypes. To establish whether the interactome of FOXO1, FOXO3 and FOXO4 is important in determining their distinct functions, we applied Stable Isotope Labeling of Amino acids in Cell culture (SILAC) followed by immunoprecipitation and quantitative mass spectrometry to identify interacting proteins of FOXO1, FOXO3 and FOXO4. In addition, we explored changes in interacting partners of FOXO4 upon H_2O_2 treatment, which is has been described to result in FOXO4 activation. Using this approach, we predominantly identify several isoforms of the 14-3-3 proteins, which are involved in the shuttling and cytoplasmic localization of FOXO1, FOXO3 and FOXO4. This likely reflects the relative low abundance of other FOXO interactors. Additional optimization is required to allow low abundant interaction partners to be identified.

Introduction

Forkhead box O (FOXO) transcription factors are important in a variety of biological processes in which they often have redundant functions. In mice and human there are four FOXO family members identified, FOXO1, FOXO3, FOXO4 and FOXO6. FOXO1, FOXO3 and FOXO4 bind the same DNA target sequence to mediate transcription. Their expression patterns are in general overlapping, however FOXO1 is higher expressed in white and brown adipose tissue, FOXO3 in liver and FOXO4 in muscle [4, 231, 232]. In cell culture-based systems FOXO1, FOXO3 and FOXO4 behave similarly in biochemical assays, in their regulation of target genes and they bind the same DNA sequence [4]. In contrast, FOXO6 is more distantly related, its expression is restricted to the brain and unlike the other members, FOXO6 is not regulated by nucleo-cytoplasmic shuttling [233, 234].

Whereas the functional redundancy became clear upon development of a conditional *Foxo1*$^{-/-}$, *Foxo3*$^{-/-}$, *Foxo4*$^{-/-}$ mouse model where loss of all three *Foxo* alleles was required for the development of lymphoblastic thymic lymphomas [235], individual knockout mice for *Foxo1*, *Foxo3* or *Foxo4* display very different phenotypes suggesting that besides their redundant functions they also possess distinct characteristics. *Foxo1* knockout mice are embryonic lethal due to defects in angiogenesis [236, 237], whereas knockouts of *Foxo3* and *Foxo4* are viable. *Foxo3*-deficient mice display age-dependent infertility, abnormal follicular development and spontaneous T-cell activation and lymphoproliferation [237-239]. *Foxo4* knockout mice exhibit no clear phenotype [237]. These differences may in part be due to differences in cell- or tissue-specific expression of the FOXOs. However, it can also indicate differential regulation of the different FOXO family members.

To determine whether the different characteristics are mediated through interactions with different interaction partners, we performed stable isotope labeling of amino acids in cell culture (SILAC) followed by immunoprecipitation and quantitative tandem mass spectrometry analysis (MS/MS). SILAC is a metabolic labeling technique that allows quantitative proteomics. Cell lines are grown in media lacking a standard essential amino acid, but supplemented with an isotopically labeled form (non-radioactive) of that amino acid. Using metabolic labeling, “Heavy” or “Light” labeled amino acids like lysine and/or arginine, are incorporated into newly synthesized proteins. The difference in mass between peptides containing “Heavy” or “Light” amino acids is detected by mass spectrometry analysis and therefore allows identification of differentially regulated proteins [240].

We performed GFP-pulldowns on lysates of cells expressing GFP-FOXO1, GFP-FOXO3 or GFP-FOXO4 grown in “Heavy” or “Light” SILAC medium, followed by quantitative tandem mass spectrometry (MS/MS) in order to establish the interactome of each FOXO member. We consistently identified several isoforms of the known FOXO interactor, 14-3-3. However we could not identify other FOXO-specific interaction partners, therefore our approach needs to be refined for the comparison of FOXO-specific interaction partners.

Results

A quantitative proteomic approach to identify FOXO4 interaction partners

Previous studies have shown that immunoprecipitation of GFP-tagged proteins with GFP-Trap-A beads provide an excellent approach to analyze the protein interaction network when combined with SILAC followed by quantitative mass spectrometry [241, 242]. To establish whether this approach will be a useful tool to examine the FOXO interactome we performed our initial experiments in A14 cells (NIH3T3 cells ectopically expressing the insulin receptor [243]) with stable expression of GFP-FOXO4 (A14-GFP-

FOXO4). Due to overexpression of the insulin receptor, FOXO4 localization is predominantly cytosolic. A14 (control cells, not containing GFP-FOXO4) and A14-GFP-FOXO4 cells were grown in "Light" or "Heavy" SILAC medium and as a control, a label-swap, or "reverse," experiment was performed. After nuclear-cytoplasmic fractionation, immunoprecipiation of GFP-tagged FOXO4 was performed on all fractions. Following incubation and washes, beads were combined and bound proteins were on column digested with trypsin and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Fig. 1A). Equal expression of GFP-FOXO4 in both heavy and light SILAC culture medium grown cells was confirmed by western blot (Fig. 1B). Precipitated proteins display a high Heavy/Light (H/L) ratio in the forward experiment and low H/L ratio in the reverse experiment (e.g. FOXO4 in the upper right quadrant of the scatterplot), background proteins have a ~1:1 ratio in both pull-downs. Specific FOXO4-interacting proteins will show in the same quadrant as FOXO4.

When we precipitate GFP-FOXO4 from A14-GFP-FOXO4 cells under basal conditions, we predominantly identified a family of known FOXO-interacting proteins: the 14-3-3 proteins (indicated in scatterplot with: YWHAx, in which 'x' can be replaced by G, B, Q, H, etc.). These proteins bind to FOXO4 in both the cytoplasmic and nuclear fractions (Fig. 1C and 1D). In addition we identified the acetyltransferases CBP and p300, both described to mediate acetylation of FOXO transcription factors [244], as specific interactors in the cytoplasm. Moreover, we identified several heat shock proteins (HSPs) binding to FOXO4 (Grp75, Grp78, Hsc70) (Fig. 1C and 1D). Heat shock proteins counteract the toxicity of abnormal proteins by facilitating protein turnover. Their presence in MS pulldowns is therefore often considered unspecific. However, in *Drosophila melanogaster*, expression of HSPs is induced upon oxidative stress through JNK-mediated dFOXO activation. Whether their presence in our pulldowns is unspecific due to ectopic expression of GFP-FOXO4 or specific for FOXO4 remains to be determined.

The interaction of FOXO with 14-3-3 proteins keeps FOXOs inactive. Therefore, activation of FOXO by, for example, H_2O_2 might lead to the identification of other interaction partners. The interaction of FOXOs with other proteins is often determined by posttranslational modifications. Treatment of cells with H_2O_2 results in several posttranslational modifications on FOXO4, like JNK-mediated phosphorylation and MDM2-mediated ubiquitination and results in nuclear localization of FOXO4 [46, 80, 93]. Furthermore, a multitude of proteins have been described to specifically interact with FOXO4 upon H_2O_2 treatment, including the prolyl isomerase PIN1 and β-catenin [80, 245]. Therefore we compared untreated with H_2O_2 treated A14-GFP-FOXO4 cells. Surprisingly, upon H_2O_2 treatment, the number of FOXO4-specific interactors was not increased (Fig, 1E and 1F). None of the described H_2O_2-mediated interaction partners of FOXO4 were identified as specific interactor. In the cytoplasmic fraction we only identified tropomyosin 2 and 4 (Tpm2 and Tpm4), which are members of the actin filament binding protein family, with H/L ratios higher compared to background (Fig. 1E). In the nuclear fraction, no proteins showed a H/L ratio above background (determined with boxplot statistics, data not shown) (Fig. 1F). However, some proteins with a slightly increased ratio above background and consistently identified in multiple pulldowns, were of particular interest. For example members of the SWI/SNF complex, BAF53A, BAF57 and BAF170 (shown in blue in Fig. 1F), were identified with H/L ratios different than background. This is in line with a recent publication in *C. elegans*, where the FOXO homologue DAF16 was found to interact with the same members (BAF155/170 and BAF57) of the SWI/SNF complex [246]. Furthermore, two other members of a different chromatin remodeling complex, Ruvbl1 and Ruvbl2, were also identified as FOXO4-specific interactors upon H_2O_2 treatment. However in other SILAC experiments (data not shown), we identified these proteins as FOXO-specific binding partners also under basal conditions.

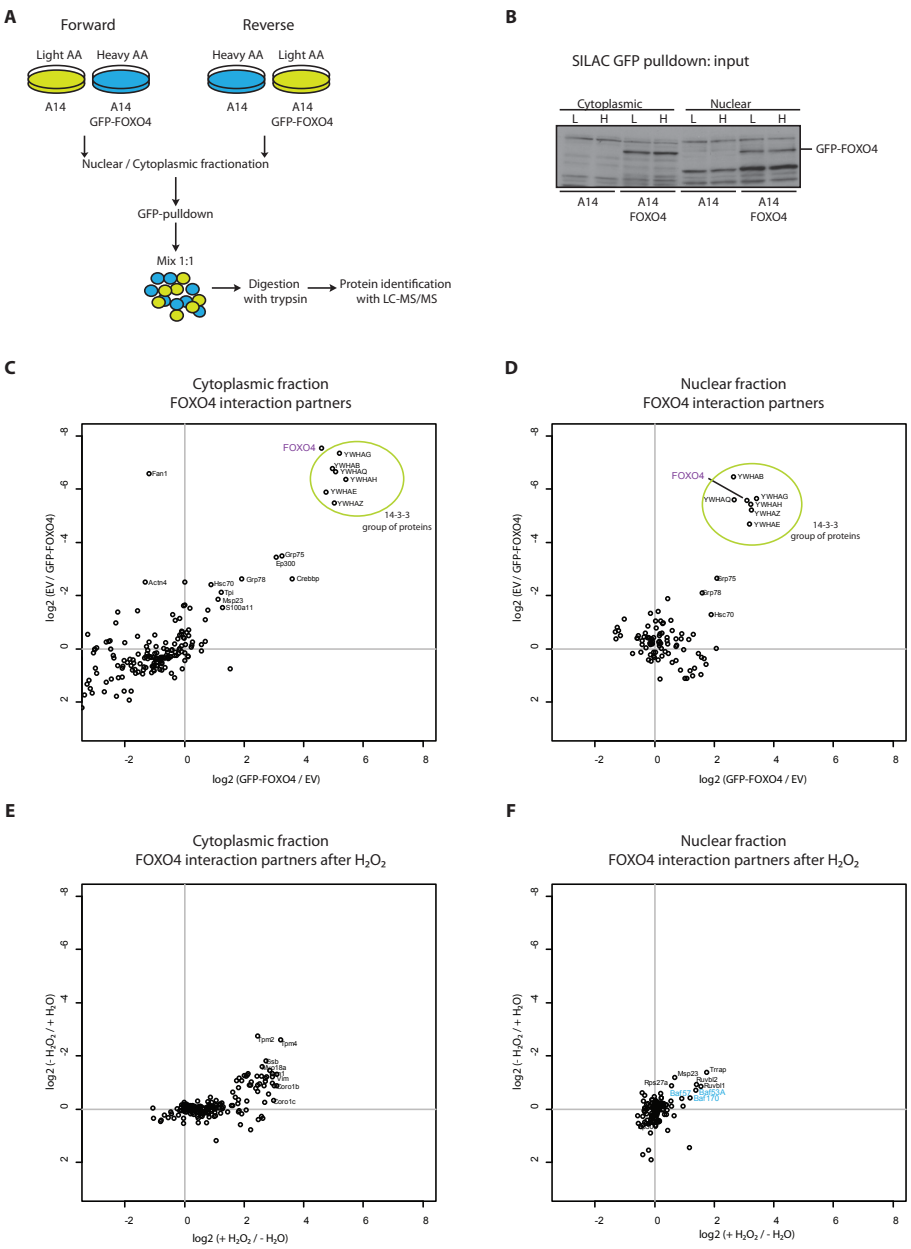

FIGURE 1: Identification of FOXO4 interaction partners using SILAC. (**A**) Description of the workflow used for the SILAC experiments. (**B**) Immunoblot of lysates from the nuclear and cytoplasmic fraction of A14 or A14-GFP-FOXO4 cells in SILAC medium labeled with heavy (H) or light (L) isotopes. (**C-F**) Scatterplot of GFP-FOXO4 pulldown in A14 or A14-FOXO4 cytoplasmic (**C**) or nuclear (**D**) extracts under basal conditions or after H_2O_2 treatment (**E** and **F**). Specific interactors have a high H/L forward ratio and low H/L reverse ratio and will appear in the right upper quadrant of the scatterplot.

Also Trrap is consistently identified with a slightly increased H/L ratio. Trrap is a histone acetyltransferase co-factor involved in recruitment of histone acetyltransferease (HAT) complexes to chromatin during transcription (reviewed in [247]). Taken together, our approach does not clearly identify specific interactors of FOXO4 other than 14-3-3 and heat shock proteins.

Generation of inducible FOXO1, FOXO3 and FOXO4 cell lines

The A14 cell line we used for our initial SILAC experiments constitutively expresses GFP-FOXO4. In other cells, overexpression of FOXO results in cell cycle arrest, whereas these cells grow normally. This can either be due to high insulin signaling and subsequent FOXO inactivation, or the cells are adapted to the presence of FOXO4. Therefore we decided to generate tetracycline-inducible cells lines for FOXO1, FOXO3 and FOXO4 to determine whether this would result in the identification of more specific interactors. We used a GFP-containing tetracycline-inducible vector, as GFP-pulldowns with GFP-Trap-A beads show minimal background in mass spectrometry analysis [241, 242]. The vector we used for generation of FOXO-inducible cell lines contains Biotin, Flag and EGFP sequences under a pCMV/2xTetO2 promotor (depicted in Fig. 2A). After cloning, expression of the construct was confirmed in HEK293T cells (Fig. 2B). To determine whether these constructs remain functional with

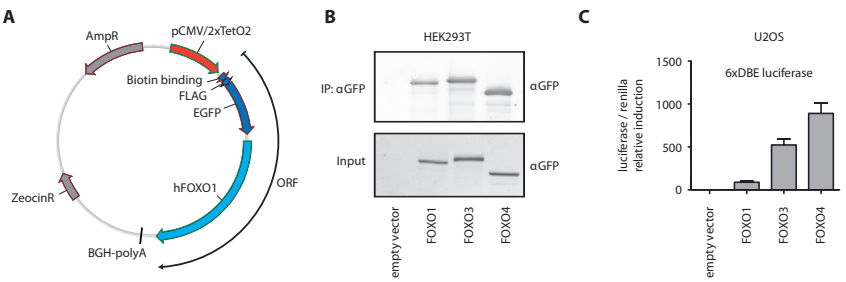

FIGURE 2: Stable cell lines of tetracyclin-inducible GFP-tagged human FOXO1, 3 and 4. (**A**) Vector map of pBioPS-FLAG-EGFP with human FOXO1. Tetracylin-induced expression results in N-terminal Biotin, Flag and EGFP-tagged (BFE) FOXO. (**B**) Immunoblots of GFP-pulldown and input of HEK293T cells expressing BFE-FOXO1, BFE-FOXO3 and BFE-FOXO4. (**C**) Luciferase reporter assay in which luciferase expression is driven by 6 canonical Forkhead DNA binding elements (6xDBE) in U2OS cells overexpressing pBioPS-FLAG-EGFP with indicated human FOXOs.

respect to their ability to induce transcription, they were tested in a transcription reporter assay using a 6x FOXO-specific DNA binding element (6xDBE) driven luciferase (Fig. 2C). Surprisingly although all three constructs have comparable expression levels in HEK293T cells (Fig. 2B), their activity differs markedly in the luciferase assay (Fig. 2C).

Characterization of FOXO1, FOXO3 and FOXO4 monoclonal cell lines

After generation of monoclonal cell lines with the above-described constructs, we tested the expression of individual clones of cells expressing GFP-FOXO1, GFP-FOXO3 and GFP-FOXO4 upon induction with doxycycline for 16 hours (Fig. 3A). Based on the immunoblot analysis, we choose one monoclonal cell line for each FOXO with comparable expression levels, to further characterize for functionality (A13, B13 and C13). Activation of luciferase activity is shown in each monoclonal cell line upon induction with doxycycline (Fig. 3B). However although the expression levels of all three FOXOs seemed equal, the activity largely differs. FOXO4 seems most potent to activate the 6xDBE promotor activity, whereas FOXO1 is hardly able to induce luciferase expression. Further analysis of the monoclonals by immunofluorescence showed remarkable differences in localization of the three

FOXOs (Fig. 3C). Whereas FOXO1 is predominantly cytoplasmic under basal conditions, FOXO4 is mainly nuclear and FOXO3 shows both nuclear and cytoplasmic staining. Upon inhibition of the PI(3)K pathway with LY294002, FOXO1 and FOXO3 shift towards nuclear localization (Fig. 3C, Dox+LY). This difference in localization explains the difference in luciferase activity shown in Fig. 3B, as the amount of

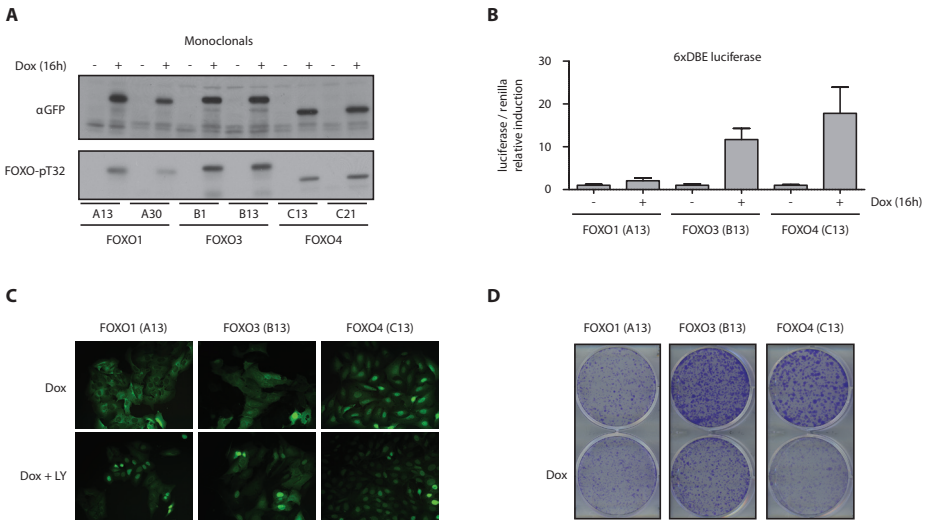

FIGURE 3: Characterization of inducible FOXO monoclonal cell lines. (**A**) Immunoblot of U2OS TET-inducible cell lines with FOXO1 (A13 and A30), FOXO3 (B1 and B13) and FOXO4 (C13 and C21). Cells were treated with doxycyclin and immunoblots were probed for GFP and FOXO phosphorylation on a PKB phosphorylation site (Thr24-FOXO1, Thr32-FOXO3 and Thr28-FOXO4). (**B**) Luciferase reporter assay using the 6xDBE-luciferase plasmid in monoclonal cell lines. (**C**) Fluorescence microscopy on indicated monoclonal cell lines. Cells were treated with doxycycline alone or in combination with the PI(3)K inhibitor LY294002. (**D**) Colony assay on indicated monoclonal cell lines. Cells were treated with doxycycline or left untreated.

activity is reflected by the amount of nuclear localization. To determine functionality of the FOXOs in a different way, the ability of colony formation was tested for each monoclonal cell line. Upon induction with doxycycline, FOXO4 and FOXO3 were the most prominent inhibitors of colony formation, whereas induction of FOXO1 made no difference in the amount of colonies (Fig, 3D), which corresponds with their transcriptional activity measured in the luciferase assay.

Comparison of FOXO1, FOXO3 and FOXO4 interaction partners by a SILAC approach

The identification of FOXO-specific interaction partners can provide valuable information to determine whether the FOXO family members are regulated by a specific set of interacting proteins. To identify specific interaction partners for FOXO1, FOXO3 and FOXO4, we performed GFP pulldowns followed by quantitative mass spectrometry. A13 (FOXO1), B13 (FOXO3) and C13 (FOXO4) monoclonal cell lines were grown in “Light” or “Heavy” SILAC medium and as a control, a label-swap, or “reverse,” experiment was performed. Equal expression of GFP-FOXO1, GFP-FOXO3 and GFP-FOXO4 in both heavy and light SILAC culture medium was confirmed by western blot (Fig. 4A). 16 hours before lysis, half of the cells were treated with doxycyline to induce FOXO1, FOXO3 or FOXO4 expression. A GFP-pulldown was performed on all conditions. Following incubation and washes, beads were combined (untreated heavy labeled with doxycycline treated light labeled and vice versa) and bound proteins were digested with trypsin and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

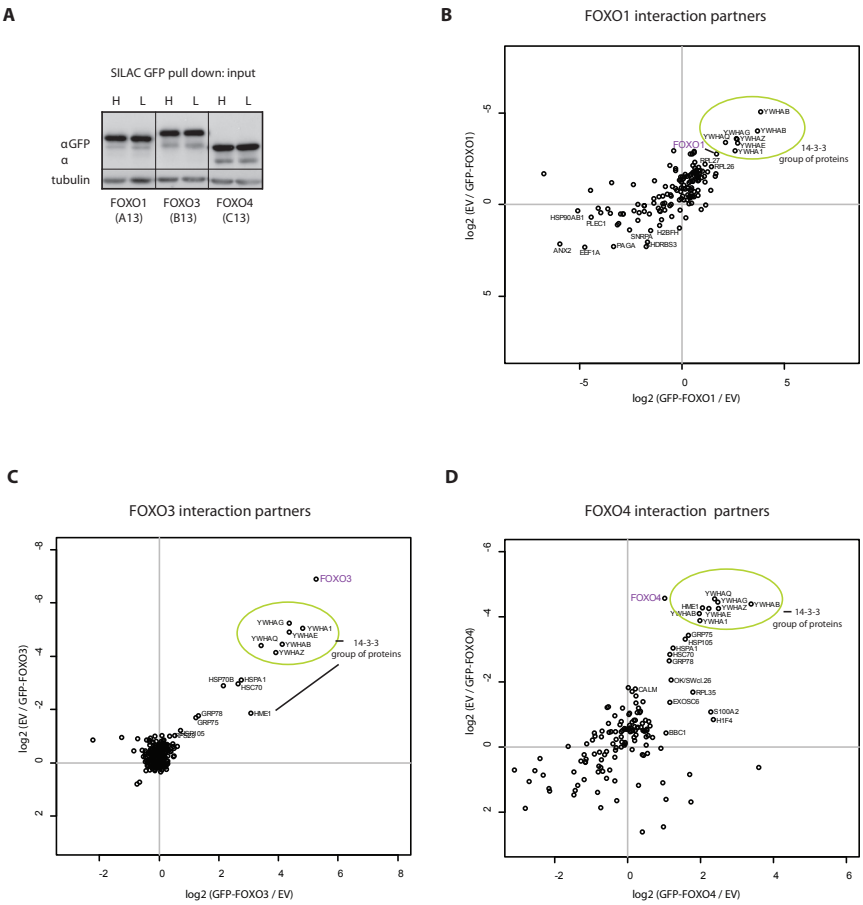

FIGURE 4: Identification of FOXO1, FOXO3 and FOXO4-specific interaction partners. (**A**) Immunoblot of lysates from monoclonal cell lines in SILAC medium labeled with heavy (H) or light (L) isotopes. (**B**-**D**) Scatterplots of GFP-pulldowns for FOXO1 (**B**), FOXO3 (**C**) and FOXO4 (**D**), showing the identified interactors for each FOXO. Specific interactors have a high H/L forward ratio and low H/L reverse ratio and will appear in the right upper quadrant of the scatterplot.

Scatterplots are shown for each monoclonal cell line, showing specific interactors in the upper right quadrant (high H/L ratio in forward experiment and low H/L ratio in the reverse experiment). Similar as shown for FOXO4 in Figure 1, all FOXOs show specific interaction with the 14-3-3 (YWHAx) proteins (Fig. 4B-4D) and FOXO3 and FOXO4 with heat shock proteins (GRP78, GRP75 and HSC70). Furthermore, only the FOXO3-inducible cell line showed a clear distinct 'cloud' of background proteins, whereas the other two cell lines lack the distinction of such a clear background cloud. Therefore, the identification of specific interactors is difficult. Again, besides the 14-3-3 group of proteins, this approach does not lead to the identification of FOXO-specific interaction partners.

Discussion

FOXO1, FOXO3 and FOXO4 are largely redundant in a number of biological functions, such as their tumor suppressive function [235], yet the individual knockout mice display very different phenotypes. In part this may be due to differences in cell- or tissue-specific expression of the FOXOs. For example FOXO3 is predominantly expressed in metabolic tissues such as liver and muscle, whereas FOXO1 is predominantly expressed in adipose tissue [4]. However, these different phenotypes may also indicate that individual FOXOs have distinct functions. To address this question, we generated monoclonal cell lines expressing GFP-FOXO1, GFP-FOXO3 or GFP-FOXO4. Indeed, these cell lines show differences between the individual FOXO members. Firstly, when comparing FOXO localization at equal expression level the localization of each FOXO member differs. Whereas FOXO1 is almost exclusively localized in the cytosol, FOXO4 display a predominant nuclear localization and FOXO3 an intermediate localization. The transcriptional activity, as measured by a luciferase reporter, reflects the difference in localization. Whereas the predominantly nuclear FOXO4 is a potent activator of the 6xDBE promotor, similar levels of the predominantly cytoplasmic FOXO1 are unable to induce expression from this promotor (Fig. 3). Based on these observations we performed quantitative mass spectrometry experiments in order to identify the interaction partners of the different FOXOs. Although we consistently identified FOXO-specific interactions with several isoforms of 14-3-3, other interactions were not consistent or did not contain H/L ratios above background. Taken together, our approach did not result in the identification of a sufficient amount of interaction partners in order to draw conclusions on the differential regulation of FOXO1, FOXO3 and FOXO4.

There are several explanations as to why we were not able to detect FOXO-specific interactors:
First, FOXO transcription factors are tightly regulated by a variety of posttranslational modifications. Therefore, a number of interacting proteins that would be expected to show up in a FOXO interactome are enzymes like kinases, phosphatases, E3-ligases and acetyltransferases. Indeed, we identified the acetyltransferases p300 and CBP to interact with FOXOs, but identification was not consistent. One reason why a very limited amount of these modifying enzymes were identified is because these interactions of enzymes with their targets are usually transient, so in order to detect these, exact timing is essential. Alternatively, these transient interactions can be chemically fixed through cross-linking methods (reviewed in [248]), however this lowers the identification rate of the MS analysis.
The posttranslational modification itself is also important for interaction with other proteins. The clearest example is the 14-3-3 group of proteins, which bind phosphorylated serine/threonine residues. We observed numerous 14-3-3 isozymes to bind to FOXOs in agreement with many previous studies, showing for example 14-3-3 binding to FOXOs phosphorylated by PKB/AKT [9, 249, 250]. It would be of interest to study whether 14-3-3 isozyme specificity represents specific function or whether these are redundant interactions. In addition 14-3-3 acts as a dimer [251, 252], which can accommodate two phosphorylated residues and therefore these dimers can function to generate inter-protein and intra-protein-bridges. For example, in *C. elegans* 14-3-3 mediates SIR-2.1 (SIRT1) binding to DAF-16 [253, 254]. Given the number of 14-3-3 isoforms that we observe to bind, it is surprising that these binding events apparently do not result in co-recruitment of FOXO interacting proteins. Similar to 14-3-3, binding of the prolyl isomerase PIN1 in general requires phosphorylation and PIN1 binding to FOXO is dependent on JNK-mediated phosphorylation [80]. Surprisingly, whereas 14-3-3 is easily detected PIN1 was not detected, even when pulldowns were performed on lysates of cells treated with H_2O_2 to activate JNK and other stress signaling pathways.
Second, compared to many other protein classes, transcription factors in general are known to bind a

large number of regulatory and accessory proteins. Protein-protein interactions are mostly considered to involve structured interactions, however FOXOs are only structured within their DNA binding domains. The regions with little to no structures are referred to as intrinsically disorganized regions (IDRs) [255]. These domains enable transcription factors to bind a variety of interaction partners and are thereby enable them to integrate upstream signaling processes towards downstream transcriptional control. The IDRs of FOXOs are under control of a diverse set of posttranslational modifications [211], which is suggested to be essential for binding of a large number of co-factors required for FOXO function. The ability to interact with such a diversity of proteins suggests that many of these interactions occur at low stoichiometry. This would provide an explanation for the minimal amount of interaction partners we identified in our experimental set up, as these low abundant complexes will not distinguish from the background as H/L ratios will remain low. Furthermore, we used GFP-tagged FOXO constructs for our pulldowns and it is possible that the GFP tag prevents binding with a subset of interaction partners, although when tested for localization and transcriptional activation, the constructs behave similar to untagged FOXO (Fig. 3).
In Chapter 6 we show that a simultaneous pulldown of two interacting proteins results in identification of a large complex of interacting proteins. This suggests that under normal conditions these protein complexes indeed exist at low stoichiometry and by creating a 1:1 ratio between the protein of interest (FOXO) with its co-factor by simultaneous pulldown of both results in the identification of several specific interaction partners.

Recently, our group has performed pulldowns followed by quantitative tandem mass spectrometry (MS/MS) to screen for cysteine-disulfide-dependent interaction partners of FOXO4 [256]. These pulldowns are performed under non-reducing conditions and the samples are treated with iodoacetamide in order to mask all free cysteines. Subsequently, the cysteine mutant of FOXO4 was compared to a mutant with only one cysteine. The comparison of mutants harboring only one cysteine to a mutant without any cysteines seems essential in this approach to identify a multitude of cysteine-disulphide-dependent interaction partners of FOXO4. In addition, because cysteine bonds are covalent, this approach allows very stringent washing, not only reducing background binding proteins, but also removing the highly abundant 14-3-3 proteins. The success of this approach does stress the need to minimize the difference between 'control' and 'treated' samples. Therefore, in future experiments comparing FOXO phosphosite-mutants to wildtype FOXO might be a solution. For instance, comparing wildtype FOXO with a previously described constitutively active mutant (mutated in its PKB phosphorylation sites and therefore not interacting with 14-3-3 proteins, referred to as FOXOA3) would provide an opportunity to separate interactors from background. Similarly, treating cells with the PKB inhibitors VIII or LY294002 might also reduce interaction with 14-3-3 proteins and allow identification of other interaction partners. To summarize, the variety of possible complexes and their relatively low abundance hamper the identification of FOXO-specific interaction partners, therefore minimizing differences between 'control' and 'treated' samples might provide a solution in future experiments.

Experimental Procedures

Cell culture and transfections

U2OS cells stably expressing TetR (U2OS-UTRM10 cells, [257]) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (tetracycline free, LONZA), L-glutamine and antibiotics at 37°C. A14 [243] and A14-GFP-FOXO4 (kindly provided by M. van Triest) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS L-glutamine and antibiotics at 37°C. A14-GFP-FOXO4 cells were treated with 200 μM H_2O_2 for 30 minutes. Transient transfection of plasmid DNA was performed using FuGene transfection reagent (Promega) according to the manufacturer's instructions. U2OS cells harboring constitutive expression of a TET-repressor element were transfected with pTON-BioPS-FLAG-EGFP-FOXO1/FOXO3/FOXO4. Monoclonal cell lines were then derived using limited dilution seeding of Zeocin selected (400 mg/mL (Invitrogen)) cells in 96-wells plates and consecutive expansion. Individual clones were isolated and tested for expression upon doxycyline (Sigma, 1 μg/mL).

Constructs and antibodies

The following antibodies were purchased: anti-Flag (Flag-M2, Sigma), anti-α-tubulin (Calbiochem), anti-FOXO1-pT24/ FOXO3-pT32 (Cell signaling). Rabbit-anti-GFP was kindly provided by Prof. G. Kops. pTON-BioPS-FLAG-EGFP (generated by Marvin Tanenbaum) was adapted to become Gateway compatible by Fried Zwartkruis. The following constructs were described previously: 6x FOXO DNA-binding element (DBE)-firefly luciferase and TK-*Renilla* luciferase [24]. Gateway entry vectors for human FOXO1, FOXO3 and FOXO4 were generated according to manufacturers protocol (Invitrogen) with the following primers:

hFOXO1 FW: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCGAGGCGCCTCAGGTGGTG
hFOXO1 RV: GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGCCTGACACCCAGCTATG
hFOXO3 FW: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCAGAGGCACCGGCTTCCCCG
hFOXO3 RV: GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGCCTGGCACCCAGCTCTG
hFOXO4 FW: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAGAATTCAGCCACAGAAGGCC
hFOXO4 RV: GGGGACCACTTTGTACAAGAAAGCTGGGTCCTATCAGGGATCTGGCTCAAAG

Gateway entry clones were transferred into destination vector pBioPS-FLAG-EGFP, a kind gift from Fried Zwartkruis (Gateway compatible vector, adapted from pTON-bioPS-FLAG-EGFP generated by Marvin Tanenbaum). pBioPS-FLAG-EGFP is an inducible expression plasmid under the control of the strong CMV promoter and two tetracycline operator sites (TetO2) and contains Biotin-binding, FLAG and EGFP sequences resulting in an N-terminally tagged fusion protein.

Fluorescence microscopy

A13 (FOXO1), B13 (FOXO3) and C13 (FOXO4) monoclonal cell lines were plated on coverslips and treated overnight with doxycycline and/or 10 μM LY294002 (Enzo Life Sciences) or left untreated. Cells were fixed with 4% paraformaldehyde and blocked with 1% BSA. Fluorescence was captured using a Zeiss Axioskop microscope.

Luciferase assay

Luciferase-based measurement of FOXO activity was performed as described before [46]. Briefly, A13 (FOXO1), B13 (FOXO3) and C13 (FOXO4) monoclonal cell lines were transfected with the 6xDBE-luciferase reporter construct, together with TK-*renilla*-luciferase as internal control for transfection. Cells were treated with 1 μg/mL doxycycline for 16 hrs or left untreated. Luciferase activity was

analyzed using a Dual-Luciferase Reporter Assay System (Promega) in a MicroLumat Plus LB 96V (Berthold Technologies), according to manufacturer's instructions.

Colony formation assay

A13 (FOXO1), B13 (FOXO3) and C13 (FOXO4) monoclonal cell lines were seeded in six well plates with or without the addition of 1 µg/mL doxycycline. 10 days after seeding, cells were fixed in methanol, stained with 0.5% crystal violet in 25% methanol and washed with demi-water to determine the amount of colonies formed.

SILAC labeling

A13 (FOXO1), B13 (FOXO3) and C13 (FOXO4) monoclonal cell lines were cultured in medium consisting of 500 ml SILAC Dulbecco's Modified Eagle Medium without arginine, lysine and glutamine (PAA, E15-086), supplemented with 1% L-Glutamine, Penicillin/Streptomycin, 10% dialized FBS (Gibco), 73 µg/ml L-Lysine (light/K^0 (Sigma, A6969) or heavy/K^8 (Sigma, 608041 or Silantes, 211603902)) and 29.4 µg/ml arginine light/R^0 (Sigma, A6969) or heavy/R10 (Sigma, 608033 or Silantes, 201603902)). Cells were cultured in SILAC medium until labeling efficiency exceeded 95% after which cells were expanded and harvested in lysis buffer (50mM Tris-HCl, 1% Triton TX-100, 1.5mM $MgCl_2$, 300mM NaCl, 1mM DTT and protease and phosphatase inhibitor cocktail) or harvested in PBS to generate nuclear and cytoplasmic extracts.

Nuclear-cytoplasmic fractionation

The fractionation was essentially done as described previously [258]. Briefly, cells were trypsinized and washed two times with PBS. Using a hypotonic buffer, the cells were swollen, after which the cells were lysed by dounce homogenizing in the presence of 0.15% NP40 and complete protease inhibitors. After centrifugation, the pellet consisting of nuclei was lysed by 90 min incubation in 2 volumes of nuclear lysis buffer (420mM NaCl, 20mM HEPES pH 7.9, 20% v/v glycerol, 2mM $MgCl_2$, 0.2mM EDTA, 0.1% NP40, complete protease inhibitor w/o EDTA (Roche) and 0.5mM DTT). After centrifugation, the supernatant containing the soluble nuclear extract was aliquoted and snap frozen until further usage. Protein concentrations of the nuclear and cytoplasmic fractions were determined using the Biorad Protein assay.

GFP pulldowns SILAC

After fractionation, GFP-pulldowns were performed with GFP-Trap_A beads (gta-20, Chromotek) in lysis buffer (50mM Tris-HCl, 1% Triton TX-100, 1.5mM $MgCl_2$, 300mM NaCl, 1mM DTT and protease and phosphatase inhibitor cocktail) with addition of 50 µg/ml EtBr to prevent protein-DNA-protein interactions. Proteins were eluted from the GFP-beads with 0.1M Glycine pH2.5 and subsequently trypsinized on FASP columns (30 kD columns). The FASP procedure is performed as described previously [259].

Mass Spectrometry

Peptides were separated using an EASY-nLC (Proxeon) connected online to an LTQ-Orbitrap Velos mass spectrometer (Thermo) as described [260]. Raw data were analyzed using MaxQuant version 1.2.2.5 and mapped using protein database IPI human V3.68 fasta (monoclonal cell lines) or IPI mouse V3.68 fasta (A14/A14-GFP-FOXO4). Resulting protein groups were further analyzed using Perseus.

Identifications were filtered for standard contaminants, reverse hits, number of peptides (>1) and unique peptides (>0). Ratios were logarithmitized (log2) and groups (consisting of forward and reverse) were defined.Scatter plots were made using a custom R script.

CHAPTER 5

A generalized view on CCM1 function: Adhesion complexes and transcriptional control

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Adherens junctions and tight junctions regulation

During embryonic development, endothelial cells form the network of blood vessels, essential for transport of nutrients, fluids, circulating cells, gasses and hormones to almost all tissues in our body. A tight monolayer of endothelial cells is lining the inner site of all vessel types and regulates the exchange of solutes and fluids between blood and tissue and controls entry of leukocytes in the surrounding tissue. The ability of endothelial cells to properly regulate cell-cell adhesions between themselves and neighboring cells is essential for regulation of all these functions. Endothelial cells have two specialized types of junctions to regulated cell-cell contacts, called adherens junctions (AJ) and tight junctions (TJ). Adherens junctions confer cell-to-cell contacts and tight junctions regulate the paracellular passage of ions and solutes. Proper formation of these junctions is important for tissue integrity, leukocyte extravasation, vascular permeability and angiogenesis. In both types of junctions, adhesion is mediated through transmembrane proteins, such as cadherins and nectins in AJs and claudins, occludins and JAMs (Junction Adhesion Molecules) in TJs. AJs are formed at the early stages of intercellular contacts and are followed by the formation of TJs. AJs are suggested to influence the formation of TJs, as in some cases, in absence of AJs, TJs are not formed. Intracellular signaling mediated by the transmembrane proteins in AJs and TJs is mediated by a diverse set of signaling proteins. For example, in TJs intracellular signaling can be mediated by ZO-1, ZO-2 and ZO-3

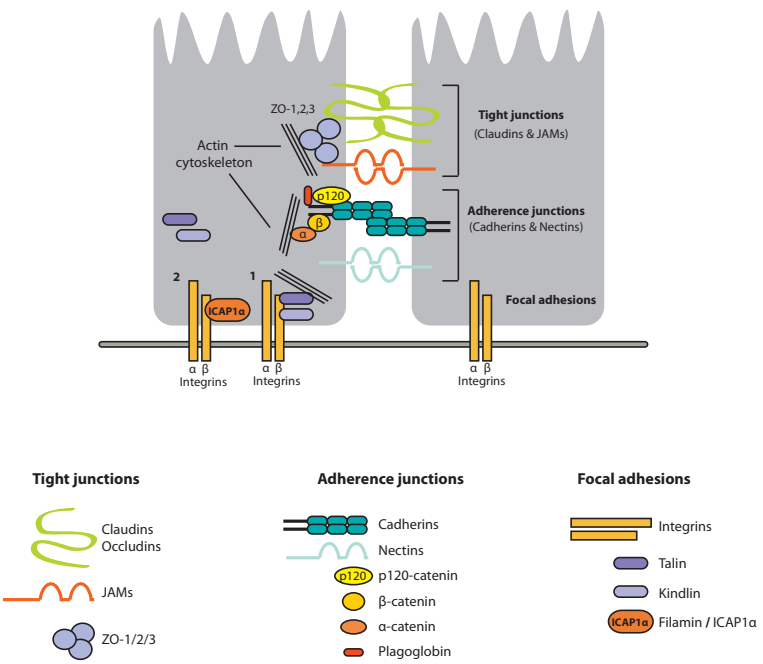

FIGURE 1: A simplified representation of the important mediators of cell-cell and cell-matrix adhesion in endothelial cells. The adherens junctions consist of the Claudin and JAM families of transmembrane proteins, which are connected to the actin cytoskeleton via the ZO-family of proteins. Tight junctions consist of the catenin and nectin families of transmembrane proteins, which are connected to the cytoskeleton via the β-catenin interaction to α-catenin. Integrin mediated-cell-cell or cell-matrix interactions at focal adhesion sites is established via interaction of talins and kindlins to actin bundles. This can be inhibited by binding proteins such as filamin and ICAP1α to the β-integrin tail. For further details see text.

(zonula occludens) and in AJs this is mediated by the Catenins, in particular p120-catenin, β-catenin, α-catenin and plakoglobin (γ-catenin). β-catenin interacts directly with the cytoplasmic tail of the cadherins. α-catenin can interact with β-catenin and the actin cytoskeleton, although this interaction seems mutually exclusive. (reviewed in [261]) (Depicted in Fig. 1). Cell-cell adhesion in endothelial cells is mediated by Vascular Endothelial (VE)-cadherin. The interaction between VE-cadherin and p120-catenin/ β-catenin is tightly regulated by (de-) phosphorylation and binding of p120-catenin to VE-cadherin inhibits the internalization of VE-cadherin [262]. Tyrosine phosphorylation of VE-cadherin reduces the interaction with p120-catenin and might therefore induce its internalization, resulting in disruption of AJs. Next to internalization, VE-cadherin is also regulated by cleavage [263] and through up or downregulation of its expression [264, 265].

Integrin signaling

The interaction of cells to the extracellular matrix (ECM) and the link with the ECM to the actin cytoskeleton at focal adhesion sites is mediated by the transmembrane glycoprotein called integrin (Fig. 1). Integrins consist of dimers containing an α- and β-chain. There are 18 α- and 8 β-integrins and the combination of those determines the interaction with specific ECM proteins and the subsequent downstream signaling event (Hynes 2002). Activation of integrins occurs through both outside-in and inside-out signaling. Outside-in activation is mediated by extracellular stimulation, resulting in a conformational change that allows interaction with several cytoplasmic proteins. Inside-out activation is mediated by the interaction of intercellular activators such as talin and kindlin (Fig. 1 (1)) and also results in an open conformation. Inactive integrins adopt a closed conformation, which inhibits recruitment of extracellular ligands and intracellular proteins (reviewed in [266]). Several proteins are reported to compete with intercellular activators for binding to integrins and thereby inhibit integrin activation. For example ICAP1α can compete with talin and kindlin for binding to β1-integrins (Liu et al 2013). Filamin is another inhibitory protein of integrins, it interacts with the NXXY motif in β integrin tails and thereby inhibits talin binding (Fig. 1 (2), reviewed in [266]). Crosstalk between the adherens junctions and integrin signaling is postulated to be important for proper development and tissue architecture, however the molecules and molecular mechanisms involved are still ill defined. Mainly engagement of integrins with ECM proteins is reported to affect cadherin-containing adherens junctions, whereas cadherins that regulate integrin function its much less explored. Most of the crosstalk between cadherens and integrins is mediated by small GTPases, non-receptor kinases, cell surface receptors and alterations of the actin network (reviewed in [267]).

Regulation of junctions by small GTPases

Adherens junctions, tight junctions and focal adhesions are highly regulated by small GTPases belonging to the RAS superfamily of small G proteins. These small GTPases act as molecular switches by cycling between an active GTP-bound and inactive GDP-bound form. They are tightly regulated by GTPase activating proteins (GAPs), which stimulate hydrolysis of GTP (inactivation) and guanine nucleotide exchange factors (GEFs), which stimulate GTP loading (activation) [268]. The small GTPase RAP1 is a member of the RAS super family important in the promotion of cell-cell adhesion through regulation of the formation and maturation of cell-cell contacts via stimulation of the adhesive function of VE-cadherin [269]. In return, VE-cadherin is necessary for the recruitment of MAGI-1, a scaffold for the RAP1 guanine nucleotide-activating factor (GEF) PDZ-GEF. In addition, RAP1 activates the clustering

of integrins to mediate cell adhesion to the extracellular matrix and promotes cell spreading. RAP1 is also suggested to mediate crosstalk between adherens junctions and integrin signaling, in which RAP1 is activated upon E-cadherin internalization and trafficking along the endocytic pathway. This endocytosis-dependent activation of RAP1 is required for the formation of integrin-based focal adhesions [270, 271].

Also RALA, the small GTPase described in **Chapter 2**, is important in tight junction regulation, via a GTP-dependent interaction with ZONAB (ZO-1-associated nucleic acid-binding protein). ZONAB is a Y-box transcription factor that regulates expression of genes in a cell density-dependent manner [272]. Upon increase in cell density, the amount of the RALA-ZONAB complex increases, resulting in in release of transcriptional repression by ZONAB [145].

RHO GTPases also belong to the RAS superfamily of small GTPases and promote the formation of stress fibers and increase endothelial permeability [273]. RHO induces stress fibers via activation of myosin light chain (MLC), which interacts with actin and slides along actin filaments causing contractility. MLC is regulated by myosin light chain kinases (MLCKs) and RHO kinases. RHO can induce RHO kinase-mediated phosphorylation of MLC [274] and alternatively, RHO kinase can phosphorylate and thereby inactivate myosin light chain phosphatase, which dephosphorylates MLC [275, 276]. The effect of RHO activity on endothelial permeability is less clear and is suggested to involve a fine balance between RHO and RAC, another member of the RHO family of small GTPases. Improvement of endothelial barrier function can be achieved by low RHO, high RAC activity, whereas decreased barrier function is accomplished by high RHO, low RAC activity. Although low RHO activity is beneficial for the endothelial barrier [277], long term inactivation of RHO can also result in increased permeability [278]. Similarly, RAC activity is required for endothelial barrier function, whereas long term activation of RAC results in stress fiber formation and junction breakdown [273].

Dual role of proteins in adhesion complexes and transcription

In general, there are two mechanisms whereby junction complexes activate intracellular signals: by regulation of signaling cascades or via shuttling of specific proteins between adhesions sites at the plasma membrane and the nucleus. The latter type of signaling proteins are called NACos; proteins that can localize to the Nucleus and Adhesion Complexes [279]. β-catenin is an extensively studied example with such a dual localization. β-catenin stability is predominantly regulated by Wnt signaling. In absence of Wnt signaling, β-catenin is targeted for degradation by a multiprotein destruction complex consisting of the scaffold proteins Axin and Adenoma Polyposis Coli (APC), the serine/threonine kinases Casein Kinase 1 (CK1) and Glycogen Synthase-3β (GSK-3β) and the protein phosphatase 2A (PP2A). Phosphorylation of β-catenin by CK1 and GSK-3β target β-catenin for β–TRCP-mediated ubiquitination and degradation by the proteasome [280] (Fig. 2A). Activation of Wnt signals result in inactivation of GSK3β activity and stabilization of β-catenin which subsequently mediates transcription via the TCF (T cell factor) / LEF (lymphocyte enhancer binding factor 1) family of transcription factors (Fig. 2B). In absence of Wnt signaling, TCF/LEF transcription factors bind groucho and act as transcriptional repressors. Whereas in presence of Wnt signals, β-catenin displaces groucho and binds other co-factors to form a transcriptionally active complex with TCF/LEF [281] (Fig. 2A and 2B).

In absence of a Wnt stimulus, the majority of β-catenin is located at the plasma membrane where it binds to the cytoplasmic domain of type 1 cadherins. Association of E-cadherin to β-catenin prevents proteosomal degradation of both proteins.

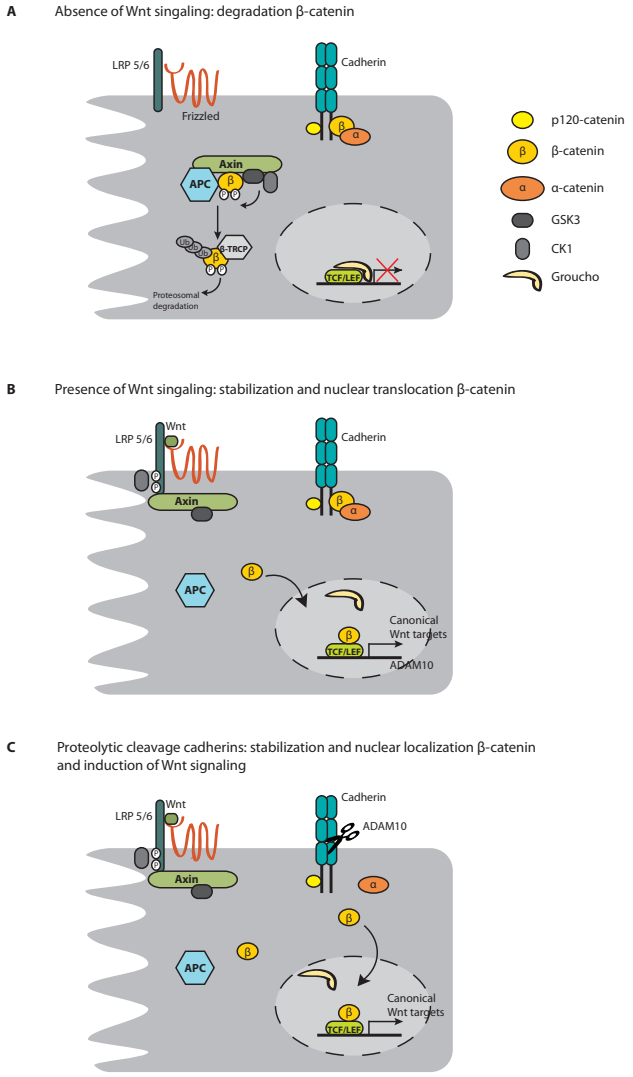

FIGURE 2: Dual role of β-catenin in adhesion complexes and transcription regulation. (**A**) In absence of wnt signaling, β-catenin is degraded by the APC-destruction complex. (**B**) In presence of wnt signaling, degradation of β-catenin by APC is prevented and β-catenin activates TCF/LEF-mediated transcription. (**C**) Disruption of E-cadherin signaling by for example ADAM10-mediated cleavage of E-cadherin, results in nuclear translocation of β-catenin and subsequent activation of the Wnt signaling pathway.

β-catenin shields a PEST sequence motif on E-cadherin, which when available is recognized by an ubiquitin ligase that marks E-cadherin for degradation [282], whereas E-cadherin prevents binding of APC and Axin to β-catenin and thereby prevents degradation of β-catenin.
Based on various studies, it is believed that there is crosstalk between cadherin-mediated cell adhesion and canonical Wnt signaling. For example proteolysis of cadherins by ADAM10 results in nuclear translocation of β-catenin and subsequent activation of Wnt/ β-catenin target genes like cyclin D and c-Myc [283, 284] (Fig. 2C). Furthermore, ADAM10 is identified as a target gene of TCF/ β-catenin [285], providing a feed forward loop towards activation of Wnt target genes.
Next to TCF/LEF-mediated transcription, β-catenin also mediates transcription via other transcription factors, like the Forkhead box O (FOXO) family. During reactive oxygen species (ROS) signaling, β-catenin switches form TCF/LEF towards FOXO-dependent transcription [245, 286]. Furthermore, in absence of VE-cadherin signaling, β-catenin relocates to the nucleus and together with FOXO1 mediates inhibition of Claudin-5 transcription [287]. In this manner, FOXO acts as a mediator between adherens and tight junctions. Also p120-catenin [288] and the ZO proteins [289] have dual functions in both junction and transcription regulation [290, 291].

Cerebral Cavernous Malformation

Defects in proper formation of the endothelial monolayer can cause major problems in for instance (neo)vasculogenesis or the formation of the blood brain barrier. Patients with cerebral cavernous malformations (CCM) have vascular malformations predominantly in the brain and sometimes in the skin [292] and retina [293]. This can cause a variety of problems like severe neurological symptoms such as focal defects (20-45%), migraine-like headaches (6-52%), seizures (23-50%) and/ or brain hemorrhages (9-56%), however about 40% of the cases are asymptomatic. CCMs are characterized by a cluster of dilated blood vessels in which each individual vessel is lined with a layer of endothelium[294, 295]. The cerebro-vascular lesions are thought to be the result of defective endothelial cell junctions [296, 297]. The prevalence of CCM has been estimated to be 0.1-0.5%, based on cerebral magnetic resonance imaging (MRI) and autopsy studies of large cohorts of patients [298]. Both sporadic (80%) and familial (20%) forms of CCM have been identified. Due to studies investigating patients with sporadic and familial CCM, it is found that familial CCM patients develop larger numbers of lesions and suffer more frequently from symptoms like seizure and hemorrhage. From these data, a two-hit hypothesis has been suggested for the pathogenesis of CCM [299, 300].
The first gene identified related to CCM patients is called KRIT1 (Krev-interaction trapped 1) or CCM1. Later on, two other genes were found to be associated with CCM, CCM2/OSM1 (osmosensing protein 1)[301, 302] and CCM3/PDCD10 (programmed cell death 10) [303]. Over 150 different germline mutations are identified in either one of these genes, predominantly resulting in loss of function. To date it has been established that the three CCM proteins can form a complex [304-306]. How and whether disruption of this complex of CCM1, CCM2 and CCM3 is involved in the pathogenesis of CCM is still highly unknown.

CCM in model organisms

All three CCM genes are well conserved among both vertebrates and non-vertebrates [307] and subsequently many attempts have been made to mimic the CCM phenotype. Mice that lack *Ccm1* or *Ccm2* die in mid-gestation with vascular defects [308-310]. Ccm1 is ubiquitously expressed until E10.5,

at which point the expression becomes restricted to neural and epithelial tissues [311, 312]. Endothelial-specific ablation of *Ccm2* results in lethality at mid- gestation due to impaired embryonic angiogenesis and endothelial-specific deletion of *CCM1* produces hemorrhagic vascular lesions in the cerebellum and retina that resemble CCMs [309, 313, 314]. However, neuronal and smooth muscle cell-specific deletion of *Ccm2,* does not affect vascular development [309]. Also for *Ccm3*, both constitutive and tissue-specific deletion gave similar phenotypes [315]. Mice with heterozygous knockout of *Ccm1* or *Ccm2* do not develop CCM-like vascular lesions in the brain with any useful frequency, which makes these mice unsuitable to study CCM pathogenesis. Because of the suggestions of a two-hit hypothesis for the disease phenotype of CCM patients, other mice studies used mice lacking either p53 [316] or *Msh2* [317] in addition to heterozygosity of CCM1. These mice have high mutation frequencies and were therefore chosen to function for second hit generation. Indeed, these mice develop CCM-like lesions, indicating that these mice have a second mutation resulting in CCM-like lesions. The combined data of the existing CCM mice models all indicate an important role for CCM in endothelial barrier function and vasculogenesis, but until now they do not provide sufficient insight into the molecular function of the CCM proteins in CCM pathogenesis.

Similar to mice, all three CCM proteins are expressed in zebrafish. Depletion of zebrafish CCM1 (Santa), CCM2 (valentine) and CCM3 (ccm3a and ccm3b) results in a dilated heart phenotype combined with vascular defects [318-321]. Interestingly this phenotype is similar to that of heart of class (heg) mutations, suggesting that they are functioning in the same molecular pathway (Kleaveland, 2009). HEG1 is a transmembrane protein of unknown function that is expressed specifically in the endothelium and endocardium. Also in mice, CCM2 and HEG1 were found to interact genetically. $Heg1^{-/-}$; $Ccm2^{lacz/+\ mice}$, like $Ccm2^{-/-}$ mice, have severe cardiovascular defects and die early in development [322]. It is also shown that in human umbilical vein ECs (HUVECs) CCM1 needs HEG1 to localize to endothelial cell-cell junctions [323]. Recently, a novel gene with sequence identity to *ccm2*, *ccm2l*, was described in zebrafish and mice. Whereas the Ccm2L knockout mice are viable with no gross cardiovascular defects [324], in zebrafish injection of ccm2l morpholino results in cardiac dilation [325]. In both mice and zebrafish, knockdown of ccm2l in addition to mutations in the heg-CCM pathway enhances heart defects. Because not all CCM patients have mutations in the coding regions of CCM1, CCM2 or CCM3, it is likely that more genes are involved in the pathogenesis and CCM2L is an interesting candidate in this perspective.

Molecular details of CCM1

Of the three CCM proteins, KRIT1/CCM1 is the first protein identified related to CCM [326, 327] and is most extensively studied compared to CCM2 and CCM3. Our research in **Chapter 6** focuses on CCM1 among the other CCM proteins in relation to FOXO function, therefore I will focus on the molecular details described for CCM1.

CCM1 was first identified in a yeast two-hybrid screen for interaction partners of the small GTPase RAP1 [328]. As RAP1 plays an important role in cell-cell adhesion [329], cell-matrix adhesion [330] and cell polarity [331] the interaction with CCM1 led to the suggestion of a role of CCM1 in maintaining junction integrity together with RAP1 [332]. In the past couple of years, several groups indeed show a role for CCM1 in the junctions. CCM1 is found in complex with β-catenin and RAP1 at the junctions and loss of CCM1 results in release of β-catenin from VE-cadherin in AJs [333] (Fig. 3 (1)).

CCM1 in integrin signaling

Next to RAP1, an integrin binding protein was identified to interact with CCM1, called ICAP1α (integrin

cytoplasmic domain-associated protein 1) [334, 335]. ICAP1α binds to the cytoplasmic domain of integrin β1 and thereby prevents binding of talin [336-338] and kindlin [339, 340]. Binding of talin and kindlin to integrin β1 is essential for proper integrin mediated cell adhesion and formation of focal adhesions [341], hence inhibition of this binding disrupts proper cell adhesion. Binding of ICAP1α to β1-integrins is mutually exclusive with its binding to CCM1 [334, 335]. Suggesting a model where CCM1 prevents binding of ICAP1α to β1-integrins and preserves proper talin- and kindlin-mediated integrin signaling [342] (Fig. 3 (2)). ICAP1α mediates stability of CCM1 by binding the NPXY motif of CCM1, resulting in an open and more stable conformation [343]. Furthermore, CCM1 is shown to interact with microtubules [343, 344] and from this a model is suggested where CCM1 is brought towards the plasma membrane via microtubules [343, 344], where subsequently RAP1 and ICAP1α can capture CCM1. Indeed, Liu et al. show that RAP1 binding to CCM1 releases CCM1 from microtubules, enabling the translocation to cell-cell junctions [345].

Inhibition of RHO signaling

Recently, several groups have reported an inhibitory role of CCM1 towards RHO signaling [346, 347]. Activation of RHO results in ROCK-mediated phosphorylation of several substrates involved in regulation of actin cytoskeletal dynamics, like myosin light chain and LIM kinase. Furthermore, ROCK is described to phosphorylate Occludin and Claudin-5 in brain endothelium and thereby enhances

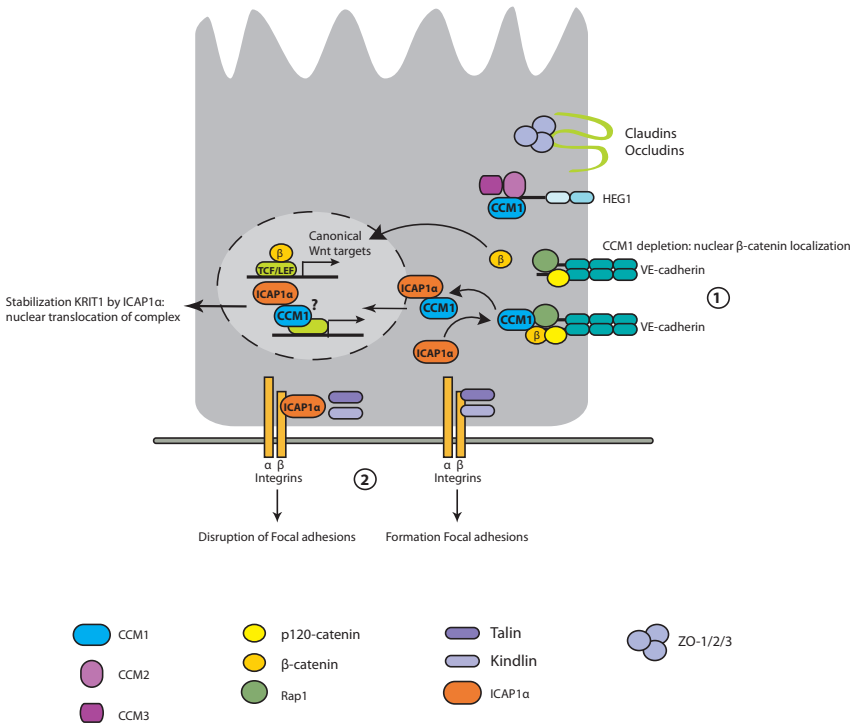

FIGURE 3: Molecular details of CCM1 biological function. Loss of CCM1 results in release of β-catenin from VE-cadherin and subsequent activation of TCF/LEF-dependent transcription (1). Interaction of ICAP1α to β1-integrins, disturbs focal adhesions by preventing binding of talin and kindlin. CCM1 inhibits binding of ICAP1α to β1-integrins and ICAP1α stabilizes CCM1 followed by nuclear translocation of the complex (2). CCM1 is located to the plasma membrane through interaction with the HEG1 transmembrane receptor.

leakiness [348]. Inhibition of RHO by CCM1 is beneficial to keep endothelial cells in a quiescent state and maintain the endothelial monolayer. In addition, Nd1-L, an actin binding protein that negatively regulates RHO activity, is reported to induce cytoplasmic localization of CCM1, providing an extra layer of CCM1-mediated RHO regulation [349]. Also for both CCM2 and CCM3 an inhibitory role towards RHO has been described [346, 347, 350]. How inhibition of RHO/ROCK by the CCM proteins is achieved is not known.

Inhibition of angiogenesis

As described above, loss of VE-cadherin signaling results in weakened cell contacts, but it also results in initiation of angiogenesis [351]. Loss of CCM1 results in release of β-catenin from VE-cadherin, subsequent nuclear translocation and transcriptional activation, ultimately resulting in cell cycle re-entry and potential activation of angiogenesis. Regulation of angiogenesis is mostly regulated by the Notch signaling pathway [352]. In mammals, there are four Notch receptors (Notch1-4) and five ligands (DLL1, DLL3-4 and Jagged 1-2). DLL4-Notch can inhibit endothelial sprouting by inhibition of excessive tip-cell formation and is shown to inhibit sprouting in culture cells, animal embryos and during tumor angiogenesis [352-355]. Interestingly, loss of CCM1, CCM3 or ICAP1α impairs DLL4-Notch signaling, resulting in excessive angiogenesis [356-358]. Furthermore, induction of DLL4-NOTCH signaling by CCM1 results in increased PKB signaling and inhibition of ERK. Also protein lysates from human CCM1 lesions show increased phospho-ERK levels [356], indicating that CCM1 suppresses ERK activation. Altogether this suggests that CCM proteins activate DLL4-Notch signaling and thereby inhibit excessive angiogenesis, but also here molecular details are lacking on how CCM proteins activate DDL4-Notch signaling.

CCM in cell polarity

Next to maintaining the endothelial monolayer, adherens junctions are also important for maintaining cell polarization and lumen formation. In various cell types and organisms, cell polarity is established by a protein complex consisting of: the partitioning defective (PAR) proteins PAR-3 and PAR-6 and atypical protein kinase C (aPKC) [359, 360]. PAR-3 assembles PAR-6, aPKC and the RAC1 guanine nucleotide exchange factor TIAM1 [361, 362]. PAR-6 can interact with proteins from other cell polarity complexes like Crumbs and Pals1 from the CRB3–Pals1–PATJ (Pals1-associated tight junction protein) and Lgl (Lethal giant larvae) from the Scribble-Disc large (Dlg)-Lgl complex [363-366]. In vertebrate epithelial cells, the PAR complex is localized to the tight junctions and disruption of this complex result in defects in tight junctions and polarity. VE-cadherin is co-distributed with members of the Par polarity complex, like Par-3 and Par-6 [367]. Integrin β1-matrix interactions at the basal EC surface regulate PAR-3 expression and junctional localization. Loss of CCM1 results in loss of apicobasal polarity and disturbance of proper vascular lumen formation, indicating an important role for CCM1 in polarity [368]. However how CCM1 regulates polarity is unknown. Because integrin β1-matrix interactions regulate PAR-3 expression, it is possible that ICAP1a together with CCM1 plays a role polarity. Alternatively, serine threonine kinase (STK) 24, STK25 and mammalian sterile twenty-like 4 (MST4) were identified as interaction partners of CCM3 in a yeast-two-hybrid screen [304, 369-371]. Combined with the connection of MST4 with LKB1 function in cell polarity [372] this indicates a potential role for CCM3 in cell polarity.

Endothelial to mesenchymal transition

Loss of apicobasal polarity and cell-cell contacts is also associated with the induction of endothelial to mesenchymal transition (EndMT). EndMT is characterized by the acquisition of mesenchymal- and stem cell-like characteristics by the endothelium [373, 374]. By use of an endothelial-specific tamoxifen-inducible *Ccm1* loss of function mice (iCCM1), it was demonstrated that the endothelial cells lining the vascular

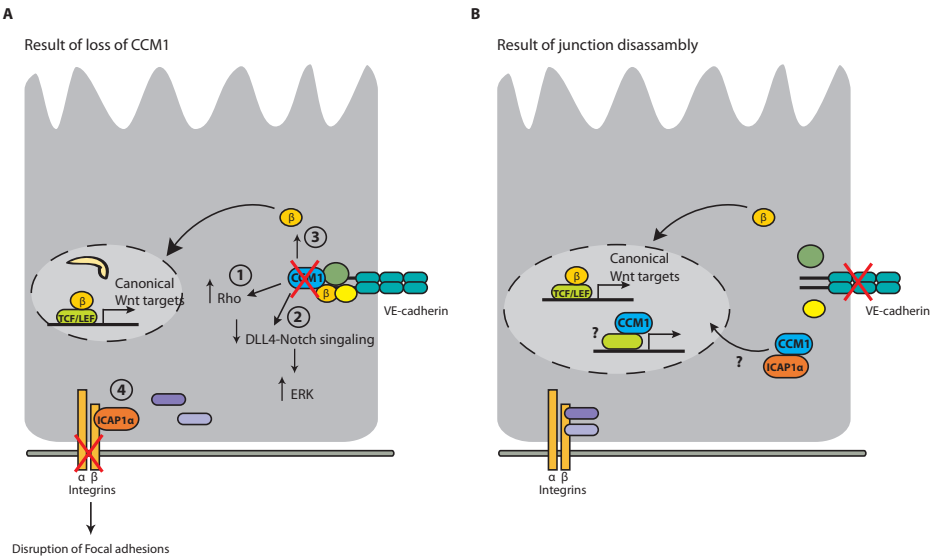

FIGURE 4: (**A**) Loss of CCM1 results in activation and inhibition of several pathways ultimately resulting in increased proliferation and angiogenesis. (**B**) Suggested model in a situation where cadherin signaling is disrupted. See text for more details.

lesions associated with CCM, showed highly disorganized VE-cadherin expression and upregulated N-cadherin expression [375]. Furthermore, CCM1 downregulation in lung and brain microvascular endothelial cells showed increased proliferation and enhanced invasive/sprouting capacitiy, which is mediated by Notch inhibition and subsequent BMP6 (bone morphogenetic protein 6) upregulation. Upregulation of BMP6 activates transforming growth factor-β (TGF-β) and BMP signaling pathways and results in increased EndMT [375]. As Wnt/β-catenin signaling plays an important role in EndMT in myocardial cells [376] and loss of CCM1 enhances β-catenin-dependent activation of the Wnt signaling pathway [333], this might be another pathway that contributes the EndMT phenotype.

Spatial regulation of CCM1

Although most functions described for CCM1 take place in the cytoplasm/plasma membrane, CCM1 is also found in the nucleus. Of the known interaction partners for CCM1, CCM2 and Nd1-L are described to mediate cytoplasmic localization, whereas ICAP1α mediates nuclear translocation of CCM1 [377]. ICAP1α-mediated nuclear translocation of CCM1 is dominant over the cytoplasmic localization induced by CCM2.

What is the function of nuclear CCM1?
Nuclear localization of CCM1 can be induced by co-transfection of ICAP1α, however the mechanism behind this translocation is still unknown. As described above, loss of CCM1 or VE-cadherin results in nuclear translocation of β-catenin and subsequent increase of β-catenin-dependent transcription regulation. Whether loss of VE-cadherin or β-catenin also results in CCM1 nuclear localization will be important to determine to get more insight into the signals that mediate nuclear CCM1.

In *C. elegans*, it is shown that KRI-1 (*C. elegans* CCM1) is important for nuclear localization of DAF16 (*C. elegans* FOXO), which results in subsequent life-span extension during lack of germ-line signals [378]. Whether CCM1 is also important in regulation of mammalian FOXO localization has not been described yet, however, recently a function for CCM1 in regulation of ROS homeostasis via FOXO is shown [379]. CCM1 knockout MEFs (Mouse embryonic fibroblasts) show increased ROS levels and decreased levels of FOXO1 and SOD2 (super oxide dismutase 2). The authors suggest a stabilizing effect of CCM1 on the stability of FOXO1. However they also show increased PBK-mediated FOXO phosphorylation upon add-back of CCM1. As described earlier in this thesis (Chapter 3), phosphorylation of FOXO by PKB results in ubiquitination and degradation of FOXOs, which makes the conclusions of the authors questionable. Also others have shown increased PKB phosphorylation upon CCM1 overexpression in HUVEC cells [356], whereas this is not shown for CCM3 [358]. Based on these data it is unclear what effect CCM1 could have on FOXO function and if this involves the nuclear localization of either FOXO or CCM1.
Interestingly, FOXO1 and FOXO3a also have an important function in the regulation of angiogenesis. Foxo1$^{-/-}$ mice die from severe vascular defects [236, 237] and inducible Foxo 1, 3 and 4 knockout mice show up-regulation of Sprouty and PBX1 among others, as FOXO-regulated mediators of endothelial cell morphogenesis and vascular homeostasis [235]. Furthermore, in endothelial cells it is shown by microarray analysis that FOXO1 induces many genes associated with vascular destabilization and apoptosis [380]. For example Angiopoietin-2 (Ang-2) is an important FOXO1 target [380]. Ang-2 is an antagonist of the receptor tyrosine kinase Tie2 and its activating ligand Ang-1. Tie2 and Ang-1 are required for vascular development [381-383]. Ang-1 promotes survival of endothelial cells by activation of PKB and subsequent inhibition of FOXO1 [380]. Hence regulation of Ang-2 by FOXO inhibits its inhibition through Ang-1 mediated PKB activation.

Concluding remarks

All experimental work performed to unravel CCM1 function point towards its importance in maintaining a tight endothelial monolayer. In model organisms, all knockouts described for CCM1 display endothelial defects. All molecular work described for CCM1 is based on the use of knockout MEFs or siRNA-mediated knockdown in endothelial cell lines. From these experiments a couple of conclusions can be drawn (summarized in Fig. 4A).

1. Loss of CCM1 results in increased RHO activity and leakiness of the endothelial barrier.
2. Next to induction of RHO activity, loss of CCM1 results in decreased activity of the DLL4-Notch pathway, resulting in increased proliferation. This is also indicated by increased activity of ERK in CCM patient material.
3. Loss of CCM1 disturbs the adherens junctions due to release of β-catenin from VE-cadherin. Release of β-catenin from VE-cadherin stimulates its function as transcription co-factor and thereby enhances activation of the Wnt pathway and induction of proliferation.
4. Loss of CCM1 results in binding of ICAP1α to the β1-integrin tail and subsequent disruption of focal adhesions.
5. Loss of CCM1 results in increased ROS due to decreased FOXO levels and subsequent decreased SOD-mediated ROS scavenging.

These data provide us with information on what can be the result upon loss of CCM, however they provide little molecular details on CCMs mode of action, especially regarding the nuclear function of CCM1. Based on the knowledge gained from other junctional proteins and on the observed nuclear

localization of CCM1, we suggest a role of CCM1 in transcription regulation. We propose a similar role as described for β-catenin, which upon release of cadherins, translocates to the nucleus to mediate transcription. Furthermore, ICAP1α shows a stabilizing function towards CCM1 and induces its nuclear localization. Therefore examining the circumstances in which ICAP1α interacts with CCM1 and mediates KRIT1 nuclear localization will provide more clues on the nuclear function of CCM1 (suggested model in Fig. 4B).

In **Chapter 6** of this thesis, we provide evidence for a transcriptional co-factor function of CCM1 and we propose a similar role for CCM1 as described for β-catenin: a dual role in both junction maintenance and transcription regulation. However in contrast to β-catenin-mediated activation of transcription, we show that CCM1 mediates inhibition of FOXO-induced transcription.

CHAPTER 6

The adhesion protein CCM1 acts as a transcriptional co-regulator for FOXO

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Abstract

Cerebral cavernous malformation is a disease caused by defects in the endothelial monolayer. Mutations in the Krev-interaction trapped 1 (KRIT1)/CCM1 gene are associated to this disease. CCM1 is important in maintaining junctional integrity and cell polarity. Research on CCM1 function has focused on its role in maintaining cell adhesion through participation in adherens junctions. However, CCM1 is also observed to be present in the nucleus, but molecular details on a possible nuclear function of CCM1 are lacking. The Forkhead box O (FOXO) family of transcription factors mediates a variety of cellular processes like cell cycle arrest, stress resistance and apoptosis and also plays an important role in endothelial function. Here we identify a role for nuclear CCM1 in repression of FOXO-mediated transcription of target genes. FOXO and CCM1 interact, which is important for nuclear translocation of CCM1. Binding of CCM1 to FOXO in the nucleus facilitates interaction with the SWI/SNF chromatin-remodeling complex to FOXO. Mass-spectrometry analysis reveals that CCM1/FOXO bound SWI/SNF complex, in addition to HDAC1, specifically harbors the ARID1A subunit. This particular composition of the SWI/SNF complex plays a role in chromatin condensation and subsequent transcription inhibition. Interestingly, overexpression of CCM1 results in inhibition of FOXO-mediated induction of target genes. Furthermore, FOXO-induced apoptosis in breast cancer cells is repressed by CCM1. Taken together, our observations provide a novel role for CCM1 in the nucleus, where it acts as a negative transcriptional co-regulator of FOXO.

Introduction

Epithelial and endothelial cells communicate to their neighbors and underlying extracellular matrix via large protein complexes in adherens junctions (AJ) and tight junctions (TJ). This information determines several aspects of cell behavior like cell proliferation and differentiation. Endothelial cells present in a continuous monolayer are in a 'resting state', contain highly organized junctions and do not respond to growth factors. Whereas endothelial cells, which are ready to proliferate, are responsive to growth factors and contain disorganized junctions. Defects in the endothelial monolayer are implicated in many diseases like cerebral cavernous malformation (CCM). Patients suffering from CCMs, are characterized by cavities in the brain due to deficiencies in the endothelial monolayer [294-297]. CCM1/KRIT1 is one of the first proteins found to be associated with CCM[326, 327]. CCM1 is a Rap1 effector and interacts with E-cadherin and β-catenin at adherens junctions and is important for maintaining junction integrity [328, 333]. Most research on CCM1 is focused on its role in maintaining the endothelial barrier. Several CCM1-mediated processes are proposed to be involved in this function, like inhibition of the Rho pathway [346, 347], activation of DLL4-Notch signaling [356] and regulation of the β1-integrin interacting protein ICAP1α [334, 335].
DAF16, the *Caenorhabditis elegans* Forkhead Box O (FOXO) orthologue, is an important regulator of lifespan; both the absence of germ line signals or reduced insulin signaling result in a DAF16-dependent lifespan-extension [384, 385]. Genetic screens have identified genes, amongst which *kri-1*, that are required for DAF-16 dependent lifespan extension following germ cell ablation. *Kri-1* encodes the KRIT1/CCM1 orthologue in *C. elegans* and more detailed analysis suggested that KRI-1 is important for DAF16 nuclear localization in absence of germ line signaling and subsequently important in DAF16-mediated lifespan extension of *C. elegans* [378]. In mammalian cells, CCM1 is described to maintain reactive oxygen species (ROS) homeostasis via regulation of FOXO1 and FOXO3 [379]. FOXO transcription factors are involved in many cellular processes like metabolism, cell cycle arrest, apoptosis and ROS scavenging (Reviewed in [122, 177, 386]. CCM1 knockout mouse embryonic fibroblasts (MEFs) display high amounts of ROS levels which can be rescued by add back of CCM1, subsequent FOXO stabilization and upregulation of the ROS scavenging protein super oxide dismutase 2 (SOD2) [379]. How CCM1 influences FOXO function and whether this requires interaction between FOXO and CCM1 is unknown.

Signals from junctions are transmitted towards the cell interior via two different mechanisms: by regulation of intercellular signaling cascades or via shuttling of proteins between adhesions sites at the plasma membrane and the nucleus. Proteins involved in the latter type of signaling are called NACos; proteins that can localize to the Nucleus and Adhesion Complexes [279]. For example, ZO-1, a tight junction protein, sequesters the transcription factor ZONAB in the cytoplasm and thereby inhibits proliferation. ZONAB, when present in the nucleus, interacts with the cell cycle regulator CDK4 and controls expression of cell cycle regulators like cyclin D1 and PCNA [272]. In adherens junctions, β-catenin binds to E-cadherin and α-catenin, whereas in the nucleus β-catenin mediates transcription via for example TCF or FOXO transcription factors [245, 281, 286]. Several junction proteins are described to act as NACos. However, some proteins with nuclear localization, including CCM1, do not have an identified function for this localization.
In agreement with its potential nuclear localization CCM1 harbors a putative nuclear localization sequence (NLS). Mutation of this sequence reduced the nuclear localization of CCM1 to ~10% [306]. Furthermore a role for ICAP1α in mediating nuclear localization of CCM1 has been described. The β1-integrin binding protein ICAP1α affects CCM1 stabilization through binding to the NPXY motive in CCM1, which results in an open, more stable conformation of CCM1 [343]. ICAP1α binds to the

cytoplasmic domain of integrin β1 and thereby prevents binding of talin [336-338] and kindlin [339, 340]. This subsequently inhibits proper formation of focal adhesions. ICAP1α acts as a negative regulator of integrin function by competing with kindlin for binding to the β1-integrin tail. This suggests that integrin activation and concomitant release of ICAP1α results in CCM1 nuclear translocation if free, non-E-cadherin bound CCM1 is present. What function is mediated by nuclear CCM1 is however at present completely unknown.

Here we show that CCM1 interacts with FOXO1, FOXO3 and FOXO4 and that ICAP1α stabilizes the interaction of CCM1 to FOXO4. Nuclear localization of CCM1 does not depend on its NLS sequence, but requires the presence of FOXO in the nucleus. Ectopic expression of CCM1 mediates a general inhibition of FOXO4 target gene regulation as shown by microarray analysis. Furthermore loss of CCM1 enhances FOXO-induced activation of apoptosis and inhibition of colony formation capacity. Finally, we show that the CCM1-FOXO complex interacts with the switch/sucrose nonfermenting (SWI/SNF) chromatin remodeling complex and Histone Deacetelase 1 (HDAC1), indicating that CCM1 is important to recruit the SWI/SNF complex to induce chromatin remodeling in order to inhibit FOXO function. Combining these results, we have found a novel role for CCM1 as a transcriptional co-regulator and therefore suggest that CCM1 has a dual role in maintenance of junction complexes and regulation of transcription.

Results

ICAP1α-induced stabilization of the CCM1-FOXO complex

Both in *C. elegans* and in mammalian cells CCM1 is described to influence FOXO function, yet molecular details are largely lacking. Therefore, we questioned whether FOXO and CCM1 could interact. Indeed CCM1 co-immunoprecipitated with FOXO1, FOXO3 and FOXO4 (Fig. 1A and Fig. 1B). During Wnt signaling, increased stability of β-catenin is believed to be the key regulatory step to enable nuclear translocation of β-catenin. Because ICAP1α has been shown to act as an important determinant for CCM1 stabilization, we questioned whether ICAP1α could have a similar function on stabilization and localization for CCM1. Expression of wildtype CCM1 is increased when ICAP1α is co-expressed (Fig. 1C, compare lane 2 with 4). This is dependent on the interaction between CCM1 and ICAP1α, because a mutant of CCM1 that cannot bind ICAP1α (CCM1 NPXY) was not affected by ICAP1α expression (Fig. 1C, lane 3 versus lane 5). This suggests that complex formation between CCM1 and ICAP1α serves to increase CCM1 stability. We subsequently tested whether ICAP1α had an effect on the CCM1-FOXO4 complex formation. Upon ICAP1α co-expression the level of CCM1 co-immunoprecipitated with FOXO4 increased and in addition we found both CCM1 and ICAP1α to co-immunoprecipitate with FOXO4. Importantly, ICAP1α does not appear to interact with FOXO4 alone (Fig.1D), which suggest that FOXO4 can interact with the CCM1- ICAP1α complex through its interaction with CCM1. In addition, ICAP1α stabilized the CCM1-FOXO4 interaction (lane 5 versus lane 7). Stabilization of β-catenin is important for its nuclear localization. Therefore, we next determined whether ICAP1α-induced stabilization of CCM1 could influence CCM1 localization. The localization of ICAP1α or CCM1 alone is both in the cytoplasm and nucleus (Fig. 1E and 1F respectively), whereas in presence of ICAP1α, CCM1 clearly shows increased nuclear localization (Fig. 1G). Because, ICAP1α increases the CCM1-FOXO interaction and in *C. elegans* CCM1 is described to be important for FOXO nuclear localization, we determined the localization of CCM1 and FOXO. Both FOXO4 and CCM1 alone are distributed both in the cytoplasm and nucleus (Fig. 2A and 2B). Surprisingly, ectopic expression of both proteins resulted in nuclear

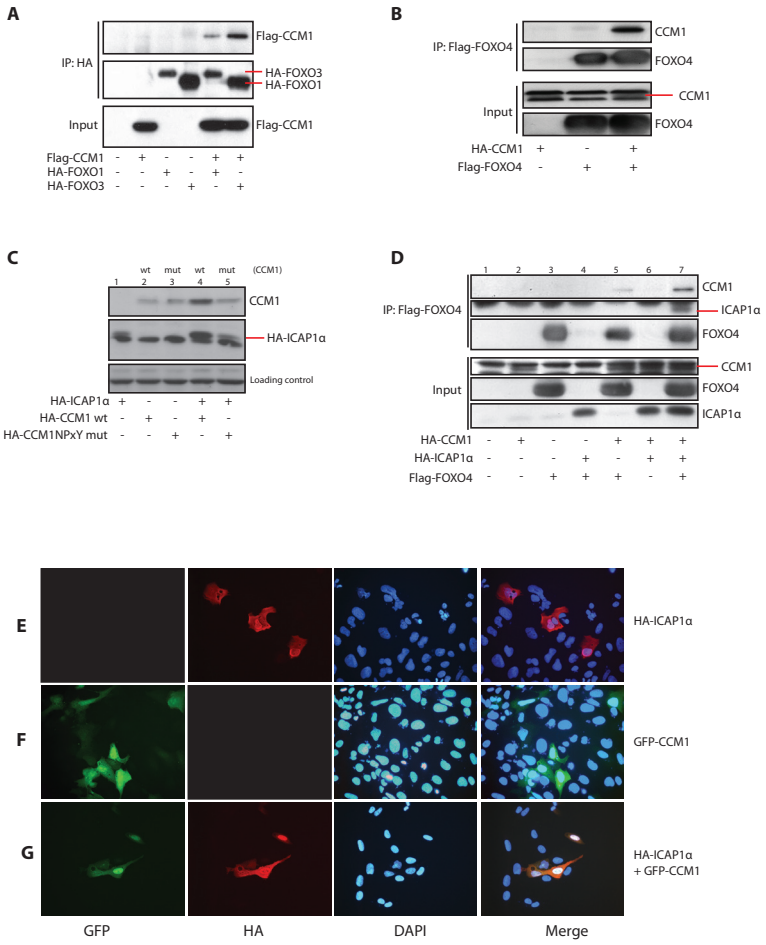

FIGURE 1: ICAP1α mediates CCM1 stabilization and localization. (**A**) Immunoblot of Flag-pulldown and of HEK293T cell lysates transfected with Flag-FOXO4 and HA-CCM1. (**B**) Immunoblot of HA-pulldown and of HEK293T cells transfected with HA-FOXO1, HA-FOXO3 and Flag-CCM1. (**C**) Immunoblot of lysates from HEK293T cells transfected with HA-ICAP1α, HA-CCM1 wild type (wt) and HA-CCM1 mutated in its NPXY motif (mut) (**D**) Immunoblot of Flag-pulldown and HEK293T cells transfected with Flag-FOXO4, HA-CCM1 and HA-ICAP1α. Numbers indicate lane number. (**E-F**) Immunostaining of U2OS cells transfected with HA- ICAP1α and/ or GFP-CCM1 for HA and DAPI.

localization of CCM1 in cells where FOXO4 was also localized in the nucleus (Fig. 2C). This is similar in the situation where ICAP1α is co-expressed with CCM1. Furthermore, nuclear localization of CCM1 is not necessary for translocation of FOXO4 to the nucleus, because expression of CCM1dNLS (mutated in its nuclear localization signal ^{377}) did not affect the ability of FOXO4 to translocate to the nucleus (Fig. 2E). In contrast, nuclear localization of FOXO4 is essential for nuclear translocation of CCM1, because expression of FOXO4dNLS did no longer result in the predominant nuclear CCM1 staining as observed in Fig. 2A (Fig. 2D). Importantly this is not caused by disruption of the CCM1/FOXO4 interaction because the NLS mutants can still interact (Fig. 2F). Taken together, CCM1 localization correlates with FOXO localization and changing FOXO localization is followed by CCM1 relocalization.

In addition, neither inhibition nor stimulation of the nuclear localization of FOXO4 (by activation of PKB with insulin treatment or JNK by H_2O_2 treatment, respectively) affected the ability of FOXO4 to interact with CCM1 (Fig. 2G). Thus, in summary, the interaction between FOXO and CCM1 is independent of the cellular localization of the proteins. Furthermore, CCM1 and FOXO co-localize and translocation of FOXO to the nucleus enables CCM1 nuclear localization.

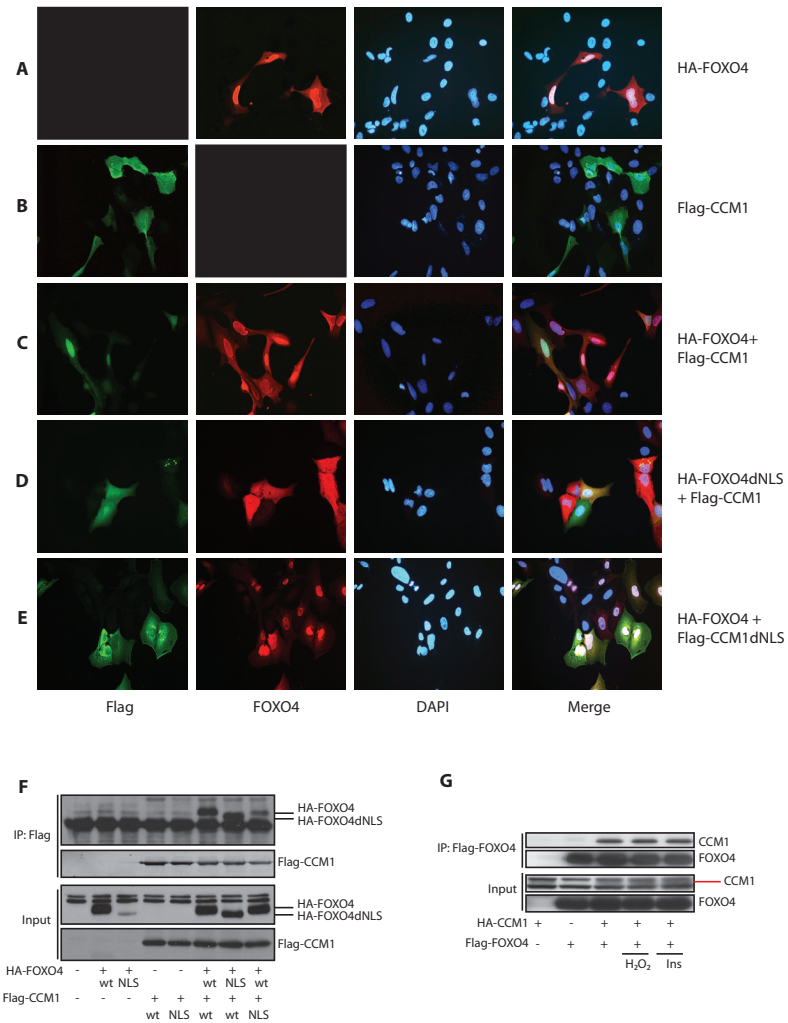

FIGURE 2: Nuclear FOXO4 mediates nuclear translocation of CCM1. (**A-E**) Localization of FOXO4 and CCM1 in U2OS cells transfected as indicated with wild type or mutant HA-FOXO4 and/or Flag-CCM1. Cells were immunostained for FOXO4 and/or Flag-CCM1 and nuclei were visualized with DAPI. (**F**) Immunoblot of Flag-pulldown and lysates from HEK-293T cells transfected with HA-FOXO4, Flag-CCM1, HA-FOXO4 with a deletion in its NLS signal (HA-FOXO4dNLS) and Flag-CCM1 with mutations in its NLS signal (Flag-CCM1dNLS). (**G**) Immunoblot of Flag-pulldown and lysates from HEK293T cells transfected with HA-CCM1 and Flag-FOXO4. Cells were treated with 200μM H_2O_2 for 30 min or 5ug/ml Insulin (Ins) for 30 min.

CCM1 inhibits a subset of FOXO4-mediated target gene regulation

The nuclear localization of CCM1 suggests a potential role in transcription regulation. As shown, FOXO nuclear localization is essential for CCM1 nuclear localization, therefore CCM1 might be important in FOXO-mediated transcription. This indicates that FOXO might integrate junctional status into an appropriate proliferative response by the use of such a regulation.

To test this hypothesis, we performed a microarray analysis where we compared FOXO4-induced gene regulation with or without CCM1 co-expression. Upon expression of FOXO4, there are several genes significantly up or down regulated as indicated by the blue line in Fig. 3A. Upon co-expression of CCM1, we observed a clear shift on the FOXO4-regulated genes, as indicated by the red dots, which represent the individual genes that were regulated by FOXO4 (Fig. 3A). The FOXO-induced genes were in general less induced upon CCM1 expression and this was similar for the genes that were repressed by FOXO4. Thus nuclear localization of CCM1 results in repression of FOXO4–induced target genes. Hence, this suggests that release of CCM1 from adherens junctions, followed by nuclear localization, results in repression of FOXO-mediated target gene regulation. To further delineate on which target genes CCM1 influences FOXO-mediated transcription, we choose a set of genes that were identified in our lab as FOXO targets [387]. To enhance the window in which we observe optimal FOXO activation, we used a FOXO1-inducable U2OS cell line (described in Chapter 4). Upon inhibition of the PKB pathway, by treatment with either LY294002 or VIII, induction of these genes via endogenous FOXO activation is observed. This is greatly enhanced upon induction of FOXO1 expression (Fig. 3B-3E). Interestingly, upon inhibition of PBK, induction of target gene transcription by FOXO1 is decreased after expression of CCM1. In summary, our microarray data combined with qPCR of selected target genes show an inhibitory effect of CCM1 on FOXO-induced target gene regulation.

Loss of CCM1 enhances FOXO-induced apoptosis in breast cancer cells

FOXO transcription factors can oppose the growth stimulating effects mediated by PKB through induction of cell cycle arrest. Furthermore in a number of cell types FOXOs can induce apoptosis by induction of pro-apoptotic genes like Bim, Bcl-6 and Fas-ligand [9, 19-21]. In some cell types, the induction of cell cycle arrest is followed by cell death, both induced by FOXO. In endothelial cells, FOXO induces apoptosis and counteracts the pro-survival actions of PKB [388, 389]. To determine if CCM1 repression of FOXO-induced transcription can affect FOXO function we used p53, E-cadherin mutant breast cancer cells (Kep1.11: K14cre; $Trp53^{F/F}$, $Cdh1^{F/F}$ [390]). We previously observed that induction of wild type or constitutively active FOXO3 (FOXO3A3, mutated on the PKB/AKT phosphorylation sites) induces apoptosis in these cells (unpublished data). Upon induction of either FOXO3 or FOXO3A3 expression, we observed an increase in apoptosis, measured by uptake of propidium iodine, which was enhanced after knockdown of CCM1 (Fig. 4A and B). To further decipher the repressive function of CCM1, we looked at FOXO-induced inhibition of cell growth, measured by colony formation capacity. Upon treatment with doxycycline, both FOXO3 and FOXO3A3 inhibited cell growth (Fig. 4C and D). Furthermore, loss of CCM1 enhanced the FOXO-induced inhibition of cell growth (Fig 4C and D). Knockdown of CCM1 was verified with qPCR (Fig. 4E). Taken together, loss of CCM1 enhances both FOXO-induced apoptosis and inhibition of cell growth and this shows that CCM1 has an inhibitory role towards FOXO.

Quantitative analysis of CCM1-FOXO4 interaction partners

To determine how CCM1 can influence FOXO-mediated transcription of target genes, we made use of a pulldown approach combined with quantitative mass spectrometry in order to identify interaction partners of the CCM1-FOXO complex. We used the tetracyclin-inducible FOXO1 U2OS cell line in order

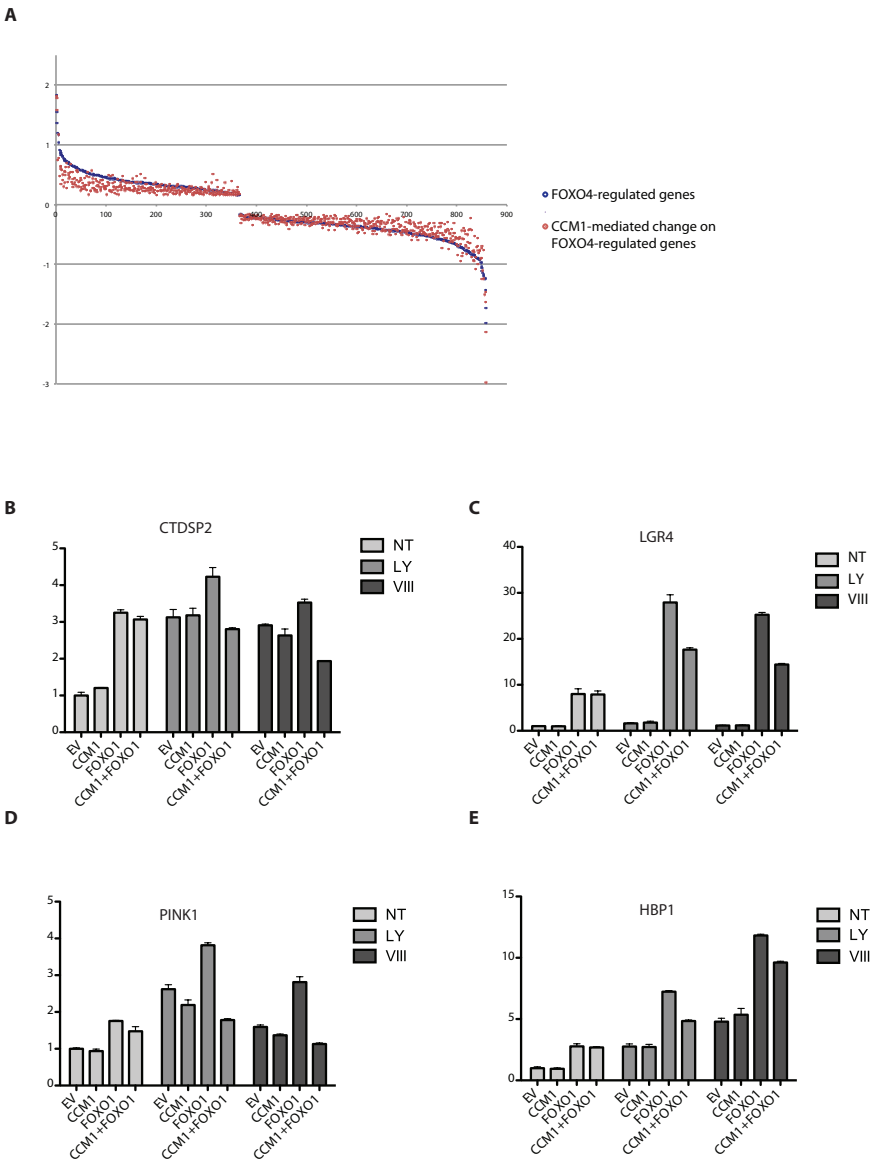

FIGURE 3: CCM1 inhibits FOXO-induced target gene regulation. (**A**) FOXO-mediated up- and downregulated genes with a p-value of less than 0.05 are shown in a scatterplot. The red dots indicate the effect of overexpression of CCM1 on the FOXO-regulated genes. The x-axis represent the number of each gene and the y-axis shows the fold-induction or repression of each gene. (**B**-**E**) A13 cells were treated with doxycycline for 16 hrs and 10μM LY294002 or 10μM VIII for 8hrs or left untreated. Graphs show relative abundance of CTDSP2 (**B**), LGR4 (**C**), PINK1 (**D**) and HSP1 (**E**) over tubulin. Each graph shows a representative figure of at least 3 experiments.

to compare nuclear with cytoplasmic localization of the complex. Under basal conditions FOXO1 is localized predominantly in the cytoplasm, whereas upon inhibition of the PI3K/PBK (AKT) pathway with LY294002, FOXO1 shifts towards nuclear localization (Chapter 4). CCM1 localization follows FOXO1 localization; therefore we can easily manipulate the localization of the CCM1-FOXO1 complex.

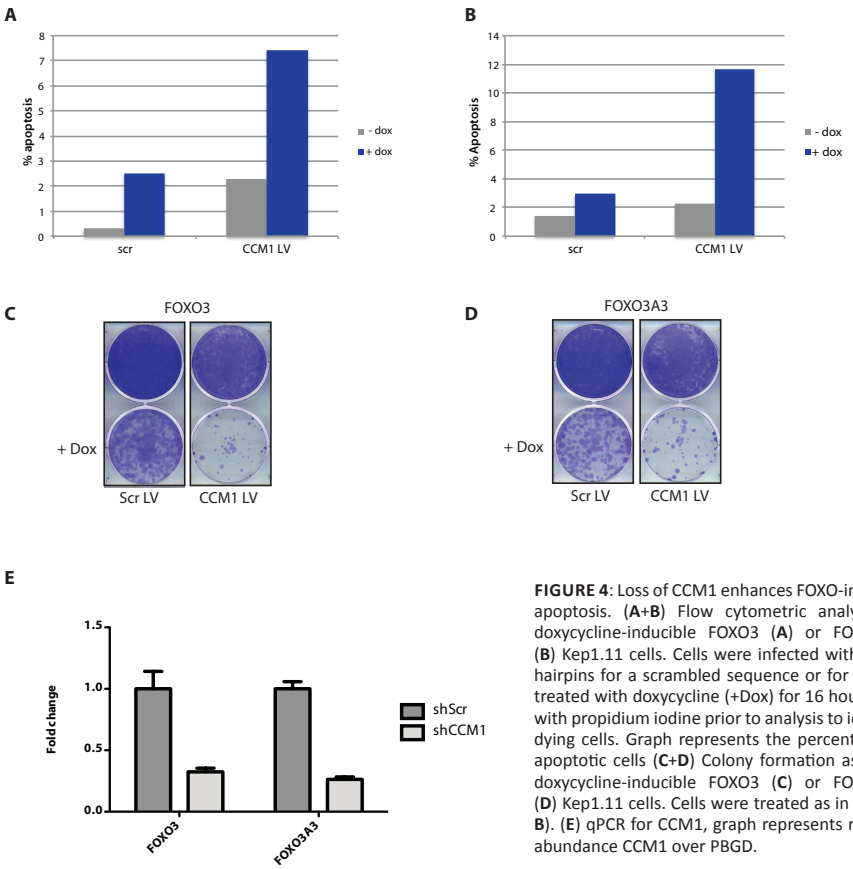

FIGURE 4: Loss of CCM1 enhances FOXO-induced apoptosis. (**A+B**) Flow cytometric analysis of doxycycline-inducible FOXO3 (**A**) or FOXO3A3 (**B**) Kep1.11 cells. Cells were infected with short hairpins for a scrambled sequence or for CCM1, treated with doxycycline (+Dox) for 16 hours and with propidium iodine prior to analysis to identify dying cells. Graph represents the percentage of apoptotic cells (**C+D**) Colony formation assay of doxycycline-inducible FOXO3 (**C**) or FOXO3A3 (**D**) Kep1.11 cells. Cells were treated as in (**A** and **B**). (**E**) qPCR for CCM1, graph represents relative abundance CCM1 over PBGD.

To identify interaction partners for CCM1, we performed a Flag-pulldown combined with quantitative mass spectrometry. Cells grown in “Light” or “Heavy” SILAC medium were transfected with either empty vector or Flag-CCM1. As a control, a label-swap, or “reverse,” experiment was performed. Upon nuclear-cytoplasmic fractionation, a Flag-pulldown was performed on all conditions. Following incubation and washes, beads were combined and bound proteins were on column digested with trypsin and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Raw MS data were analyzed using MaxQuant [391]. Specific interactors are distinguishable from background proteins by their Heavy/Light (H/L) ratio. Proteins binding selectively to Flag-CCM1 have a high ratio in the forward pull-down and a low ratio in the reverse pull-down, while background proteins will have a ~1:1 ratio in both pull-downs. In the cytoplasmic fraction we could not identify specific CCM1 interactors (Fig. 5A). On the contrary, for nuclear CCM1 we identified the known interaction partner ICAP1α, bound to CCM1 (Fig. 5B). The identification of ICAP1α bound to CCM1 in the nuclear fraction, confirmed previous publications [377] and our own observations (Fig. 1G) on ICAP1α-mediated nuclear translocation of CCM1. Although we did not find other interaction partners, the identification of ICAP1α nicely confirmed that we used a valid method to identify CCM1 interaction partners.

Next we aimed to identify specific cytoplasmic and nuclear interactors of the CCM1-FOXO1 complex. Therefore, we performed a simultaneous pulldown for CCM1 and FOXO1, which allowed purification

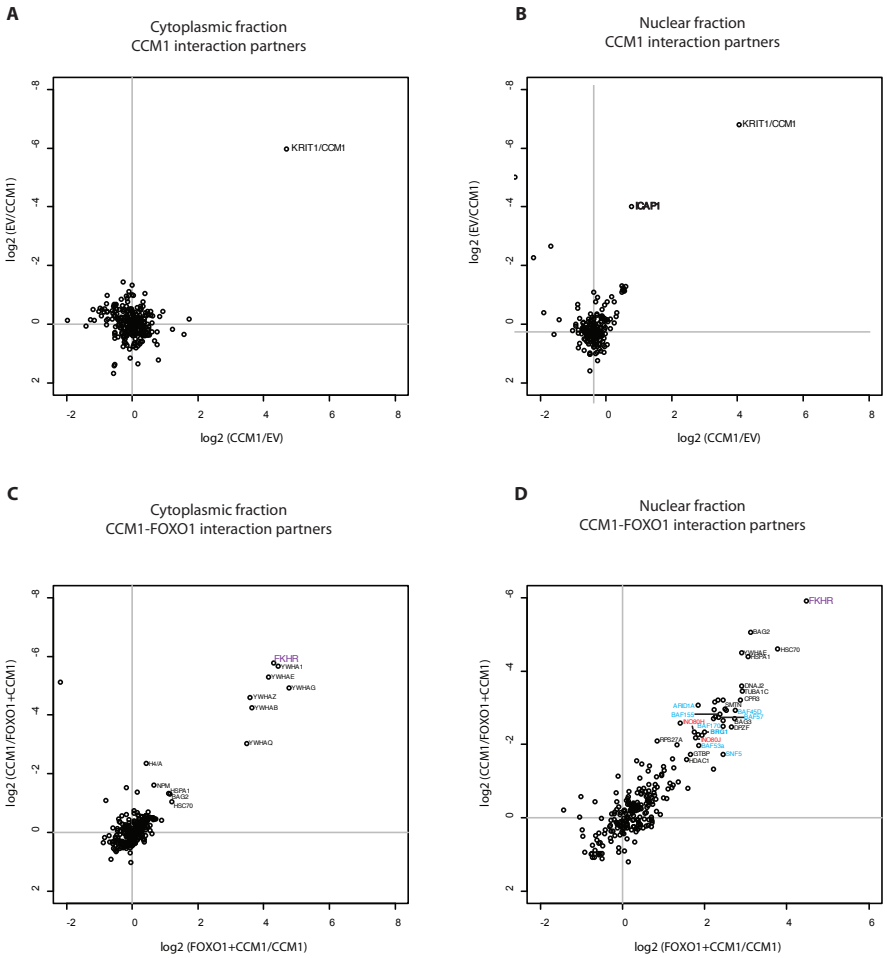

FIGURE 5: Nuclear CCM1-FOXO complex interacts with the SWI/SNF chromatin remodeling complex. (**A**-**D**) Proteins interacting with CCM1 (**A**-**B**) in the cytoplasmic (**A**) and nuclear (**B**) fraction or with CCM1-FOXO1 (**C**-**D**) in the cytoplasmic (**C**) and nuclear (**D**) fraction identified by quantitative tandem mass spectometry. Each dot in the scatterplots represents the SILAC ratios of an interacting protein. Specific interactors lie within the upper right part of the scatterplot.

of the proteins in a ~1:1 ratio. In addition, nuclear localization of the CCM1-FOXO1 complex was ensured by inhibition of the PKB pathway (by treatment with LY294002). We compared the CCM1 pulldown with the CCM1-FOXO1 pulldown in order to find specific interactors for the CCM1-FOXO1 complex. First we identified FOXO1, which confirms the efficiency of the pulldown (Fig. 5C and 5D). Because CCM1 is immunoprecipitated in both conditions, it has a 1:1 H/L ratio in both the forward and reverse experiment and therefore is present in the background. Next to FOXO1, we identified several 14-3-3 proteins, which are known FOXO1 interacting proteins and important to keep FOXO inactive in the cytoplasm. In the cytoplasmic fraction, these are the proteins predominantly found to interact, but not specific for the CCM1-FOXO1 complex, as FOXO1 alone also interacts with these proteins (Chapter 4). In the nuclear fraction we identified several CCM1-FOXO complex-specific interactors (Fig. 5D).

The most predominant proteins identified are part of the SWI/SNF chromatin remodeling complex (Fig. 6A, indicated in blue and with arrows). The interaction between CCM1, FOXO1 and two of the SWI/SNF subunits, BAF155 and BAF57, was confirmed by co-immunoprecipitation (Fig. 6C and 6D). Importantly, an interaction between FOXO1 and endogenous BAF57 and BAF155 was observed in absence of CCM1. In contrast, the presence of FOXO1 is essential for binding of CCM1 to the SWI/SNF subunits. Interestingly, upon inhibition of PKB (by LY294002 treatment), CCM1 could interact with BAF57 and BAF155 even in absence of ectopic FOXO1 expression. As PKB inhibition causes nuclear localization of FOXO, which results in translocation of CCM1 to the nucleus (Fig. 2C), this interaction might occur via endogenous FOXO.

The SWI/SNF chromatin remodeling complex consists of a large group of proteins which mediate or inhibit access of the transcription machinery to the DNA by repositioning of histones (reviewed in [392]). Interestingly, we identified ARID1A as part of this complex to specifically interact with CCM1-FOXO1. ARID1A interaction with the SWI/SNF chromatin complex is mutually exclusive with ARID1B binding and is suggested to mediate the repressive function of the SWI/SNF complex [393].

A

Gene Names	Ratio H/L Forward CCM1+FOXO1 nucleus	Ratio H/L Reverse CCM1+FOXO1 nucleus
FOXO1	4.470667	-5.90047
HSC70	3.773996	-4.608584
BAG2	3.113684	-5.048114
HSPA1	3.05266	-4.397023
TUBA1C	2.914163	-3.458687
YWHAE	2.903482	-4.498701
DNAJ2	2.900181	-3.589372
DNAJA2	2.877607	-3.213303
YWHAQ	2.795248	NaN
TUBB	2.770914	-3.319622
→ BAF45D	2.749642	-2.924892
BAG3	2.732616	-2.697593
DPZF	2.644895	-2.481082
YWHAB	2.57238	NaN
TUBB2C	2.519794	-2.929171
SMTN	2.489466	-2.961283
→ SNF5	2.456832	-1.730057
YWHAZ	2.453386	-2.643496
→ BAF170	2.442227	-2.496387
NaN	2.4422	-3.204366
→ BAF155	2.373732	-2.825312
→ BAF57	2.335883	-2.733507
TUBB3	2.328693	-3.201443
CAD	2.265557	-2.750882
DNAJB6	2.243821	-3.163203
HSP105	2.236309	-2.950313
ABPL	2.218998	-2.696658
C21orf50	2.212725	-1.327891
→ BRG1	2.004322	-2.340952
LMN2	1.957766	NaN
AKAP8L	1.935196	-2.24578
→ BAF53A	1.85587	-1.978805
P4HA	1.847676	-2.267635
→ ARID1A	1.844546	-3.060578
→ BAF60A	1.814632	NaN
INO80J	1.78031	-2.178339
INO80H	1.74386	-2.331409
GTBP	1.652922	-1.736533
NOL5A	1.588517	-0.8020843
HDAC1	1.55277	-1.591614
HSP27	1.399281	-2.577336
VIM	1.361544	-0.9837604
ATP5A	1.327515	-1.981537
DXS423E	1.248535	-1.359934
HSPC120	1.234072	-0.8912682
MTA1L1	1.224596	NaN
CHD4	1.215368	NaN
ERBA2L	1.213192	-1.605113

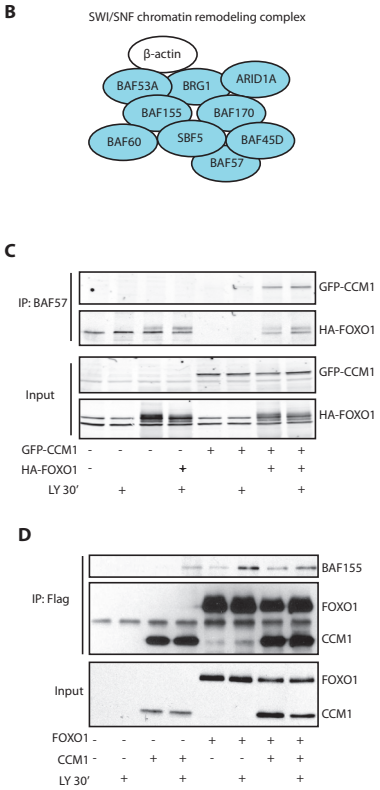

FIGURE 6: Interaction of CCM1/FOXO1 with the SWI/SNF chromatin remodeling complex. (**A**) Table summerizing proteins that interact with CCM1/FOXO1 in the nucleus identified by quantitative mass spectometry. Proteins shown have a high forward (H/L) ratio and low reverse (H/L) ratio. Members of the SWI/SNF complex are indicated in blue. Members of the INO80 complex in red. FOXO1 is indicated in purple. (**B**) representation of the SWI/SNF complex with in blue all proteins found in the SILAC pulldown. (**C**) Immunoblot of BAF57 pulldown and lysates from HEK293T cells transfected with GFP-CCM1 and HA-FOXO1. Cells were treated with 10μM LY294002 (LY) for 30 minutes. (**D**) Immunoblot of Flag-pulldown of lysated from tetracyclin-inducible FOXO1 U2OS cells transfected with Flag-CCM1. Cells were treated with 10μM LY294002 (LY) for 30 minutes.

Furthermore, we also identified HDAC1, a histone deacetylase that is also important to induce condensation of chromatin and thereby inhibits transcription. Taken together, we identified an ARID1A-containing SWI/SNF chromatin remodeling complex. This complex specifically interacts with the CCM1-FOXO1 complex and this provides an explanation on how CCM1 can mediate repression of FOXO transcription factors.

Discussion

Various junction-related proteins are demonstrated to participate in both maintenance of junction integrity and regulation of transcription. Here we show such a dual function for CCM1, which is localized to the nucleus by the FOXO transcription factors where it inhibits FOXO-mediated transcription and regulation of apoptosis. Furthermore, we show that this CCM1-FOXO complex binds to the SWI/SNF chromatin-remodeling complex. Most importantly, the CCM1-FOXO complex interacts with the ARID1A-containing SWI/SNF-complex, which is shown for c-Myc gene expression to be involved in repression of transcription rather than activation [393]. Moreover, we also identified HDAC1 as a specific interactor for FOXO/CCM1 implicating that the presence of CCM1 mediates the interaction with a transcriptional repressing chromatin remodeling complex (depicted in Fig. 7). Interestingly, not all FOXO target genes are affected by CCM1 overexpression. This could be caused by insufficient sensitivity of the assay or a need for additional players. However, it might also indicate that the here-identified CCM1-FOXO-SWI/SNF axis of transcriptional regulation controls a specific subset of target genes. Future research will be directed towards unraveling the specificity of CCM1 on the regulation FOXO and identification of the molecular mechanisms controlling this regulation.

Role of FOXO transcription factors in chromatin remodeling

Recently, the *C. elegans* orthologue of FOXO, DAF16, is shown to accomplish transcription via the SWI/SNF chromatin remodeling complex [246]. Based on their results it is suggested that FOXO acts as a pioneer factor. Pioneer factors bind condensed chromatin and via recruitment of protein complexes they mediate chromatin opening and activate transcription. Although for DAF16 it is not shown whether it binds to open or closed chromatin regions, based on the interaction with SWI/SNF components it is suggested that DAF16 mediates recruitment of chromatin remodelers to initiate chromatin opening and therefore acts as a pioneer factor. Our group determined that FOXO3 binds preferentially to open chromatin regions containing active enhancers, which suggests that FOXO does not act as a pioneer factor (A. Eijkelenboom, personal communication). There are several explanations that can rationalize these differences. In *C. elegans*, DAF16 activation is achieved by knockout of *daf2*, the insulin receptor in *C. elegans*. Therefore, there is a 'steady state', more prolonged period of DAF16 activation accomplished, whereas in our lab, we look at transient activation of FOXO. Possibly the actions as a pioneer factor are more relevant in a more prolonged activation of FOXO, whereas they are not involved in transient transcriptional changes. Also, it is proposed that the DNA binding of pioneer factors within the chromatin landscape is not substantially different from non-pioneer transcription factors [394]. Therefore, many transcription factors potentially function as a pioneer factor for only a subset of regions. Interestingly, the *C. elegans* genome only encodes one ARID1 gene, which can mediate both SWI/SNF-mediated activation and inhibition of transcription. Therefore, the existence of ARID1A and ARID1B seems the have evolved in higher organisms and possibly allows further fine-tuning of transcription.

Remarkably, we identified only a subset of the SWI/SNF complex to interact with FOXO4 upon ROS

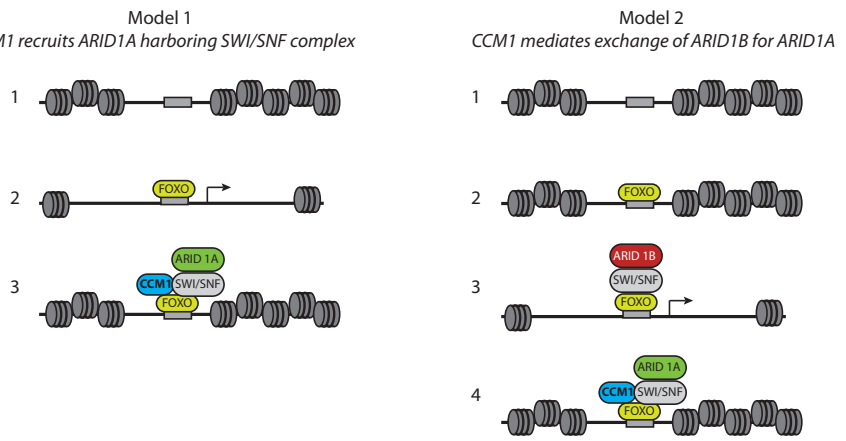

FIGURE 7: Model for CCM1-mediated repression of FOXO. **Model 1**: CCM1 recruits an ARID1A containing SWI/SNF complex. FOXO binding to specific DNA elements (DBE) within the genome determines FOXO-induced gene regulation (1). However, FOXO binding only occurs when these elements are in a relative chromatin-free environment. FOXO binding by itself may result in further chromatin opening (2). Following release of CCM1 e.g. by disruption of junctions, CCM1 may translocate to the nucleus to recruit an ARID1A containing SWI/SNF complex. This functions to repress FOXO-mediated gene transcription due to HDAC1 co-recruitment, histone deacetylation and consequent chromatin condensation (3). **Model 2**. CCM1 mediates exchange of an ARID1B activating SWI/SNF complex to an ARID1A repressing SWI/SNF complex. In this model and as suggested in *C. elegans*, binding of the chromatin remodeler SWI/SNF is required for DAF-16/FOXO mediated gene activation. This likely results in further opening of chromatin (3). Based on data obtained with c-myc this may be an ARID1B harboring SWI/SNF complex. Following release of CCM1 e.g. by disruption of junctions, CCM1 may translocate to the nucleus to facilitate or force the exchange of the ARID1B component for ARID1A. This turns the SWI/SNF complex from an activator to a repressor complex. Similar as in model 1 this repression of FOXO-mediated gene transcription is due to HDAC1 co-recruitment, histone deacetylation and consequent chromatin condensation (4).

signaling (Chapter 4). Together with the DAF16 data form *C. elegans* this suggests that FOXOs can interact with SWI/SNF complex members. At present we have not yet performed mass spectrometry experiments for all FOXO members and with inhibition of the PKB pathway. Therefore, we cannot conclude whether FOXO alone also binds an ARID1A-containing SWI/SNF complex. However, we propose several models that require further investigation.

Our suggestion is that FOXO alone does not bind the full SWI/SNF complex, whereas CCM1 allows inhibition of FOXOs through attraction of an ARID1A-containing SWI/SNF complex (Fig. 7, Model 1). Alternatively, FOXO interacts with an ARID1B containing complex in order to initiate transcription and CCM1 exchanges this interaction for an ARID1A-containing complex (Fig. 7, Model 2). To test both hypotheses, additional SILAC pulldowns for FOXO1, FOXO3 and FOXO4 upon inhibition of the PKB pathway are essential to establish their nuclear interaction partners. Furthermore, determination of chromatin condensation with and without CCM1 by, for example, the amount of histone acetylation, will provide useful information.

Localization of FOXOs in 'resting' endothelial cells

An important question that remains to be answered is which conditions determine the nuclear localization of the CCM1-FOXO complex. Can this, for instance, be initiated by disruption of junctions? Resting endothelial cells are shown to be in a quiescent state, induced by upregulation of $p27^{kip1}$[395], which is a well-known FOXO target [12, 13]. Therefore it is possible that FOXO is nuclear in resting endothelial cells in order to mediate cell cycle arrest/quiescence. Upon disruption of the junctions

induced by, for example, inflammation or angiogenesis, endothelial cells need to change their transcriptional program. This is illustrated by, for instance, the nuclear localization of β-catenin and ZONAB, which both activate a proliferative response. Under these circumstances, it will be favorable to inhibit FOXO transcription factors in order to inhibit cell cycle arrest/quiescence. It is likely that during the process of disorganized junctions, the cell harbors increased stress. Stress is dominant over growth factor signaling towards FOXO and therefore results in FOXO activation [46]. Hence, next to PKB activation, additional mechanisms in order to inhibit FOXO activity are necessary and our data provide a potential mechanism via CCM1. It will be of importance to determine FOXO localization in resting endothelial cells compared to active endothelial cells. In vitro, this can be mimicked by growing endothelial cells in a monolayer (resting cells) or sparse (active cells). Furthermore, we assume CCM1 to localize to the nucleus upon disruption of junctions, however this also needs to be determined.
Taddei *et al.* demonstrated a role for FOXO in crosstalk between adherens junctions (AJ) and tight junctions (TJ) in epithelial cells. They show that upon loss of VE-cadherin in AJs, FOXO1 together with β-catenin translocate to the nucleus and inhibit transcription of claudin-5 [287]. Claudin-5 is a transmembrane protein necessary for the formation of tight junctions. Our results show that CCM1 mediates inhibition of FOXO activity rather than activation of FOXO suppressive function. Furthermore, our lab and also others have shown that FOXO acts predominantly as a transcriptional activator rather than repressor [246, 387]. Therefore, FOXO repressive functions are possibly indirect and involve additional proteins. Moreover, our lab and others have shown that β-catenin activates FOXO activity under conditions of oxidative stress. It is not shown that loss of β-catenin results in loss of FOXO-mediated inhibition of claudin-5, therefore this inhibition might also occur via CCM1. Upon loss of VE-cadherin CCM1 is also released from AJs. Here we show that CCM1 inhibits regulation of target gene transcription by FOXO (Fig. 3). This would clarify how inhibition of Claudin-5 is achieved without direct repression of target genes by FOXO, which according to data from our group and others rarely happens. Thus, we propose that upon loss of VE-cadherin CCM1 is released and translocates to the nucleus where FOXO is present due to diminished PI(3)K/PKB signaling and increased stress signals. In the nucleus CCM1 subsequently mediates repression via the SWI/SNF complex, which eventually results in repression of FOXO-mediated transcription.

Endothelial to mesenchymal transition

Several situations, like inflammation or induction of angiogenesis require loss of cell-cell junctions. Inflammatory signals can, for example, regulate endothelial permeability by altering the stability and localization of VE-cadherin [261]. It has been suggested that presence of inflammatory cells in CCM lesions are associated with increased risk of hemorrhage [396]. However, heterozygous loss of CCM1 does not promote leukocyte migration or adhesion [397]. Another process whereby cell-cell contracts are disrupted is during epithelial to mesenchymal transition (EMT); a malignant situation whereby epithelial cells lose apicobasal polarity and cell-cell contacts and gain mesenchymal phenotypes with increased migratory and invasive capabilities. Furthermore, a role for CCM1 in inhibition of Endothelial to mesenchymal transition (EndMT) has recently been reported. It was shown that upon loss of CCM1 endothelial cells display characteristic of EndMT. They lose VE-cadherin expression and gain high levels of N-cadherin. Furthermore, several typical mesenchymal markers were upregulated like; SLUG, ID1 and α SMA. In addition, the Notch signaling pathway is inhibited, resulting in increased BMP6 expression, which strongly contributes to the observed EndMT [375]. Our observation that CCM1 mediates repression of FOXO, leads to the suggestion that FOXO might play a role in EMT. Loss of CCM1 in combination with loss of cell-cell contacts would suggest activation of FOXO. Therefore, determination of a role for FOXO in regulation of EMT-related gene transcription will be of interest.

In summary, we have provided new insight in the regulation of CCM1 by FOXO and visa versa, the regulation of CCM1 on FOXO transcriptional activity and provide a novel function for CCM1 as transcriptional co-regulator. We have shown that the CCM1 inhibits FOXO-mediated transcription of a subset of target genes. We suggest that this inhibition occurs through the interaction with a SWI/SNF chromatin remodeling complex.

Experimental Procedures

Cells and reagents

HEK293T and U2OS cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Cambrex Bioscience, Verviers Belgium), supplemented with 10% fetal bovine serum, Penicillin/streptomycin and 0.05% L-glutamine. A13 (Tetracyclin-inducable GFP-Flag-FOXO1 U2OS) cells were maintained in tetracyclin-free Dulbecco's modified Eagle's medium, supplemented with 10% tetracyclin-free fetal bovine serum, Penicillin/streptomycin and 0.05% L-glutamine. K14cre; $Trp53^{F/F}$, $Cdh1^{F/F}$ (Kep1.11, [390]) with tetracyclin-inducible FOXO3 or FOXO3A3 cell lines were kindly provided by M. Hornsveld and cultured in DMEM-F12 medium containing 10% fetal bovine serum (FBS; ICN), 100 IU/ml penicillin, 100 µg/ml streptomycin, 5 ng/ml insulin, 5 ng/ml epidermal growth factor (EGF) (all Invitrogen Life Technologies), and 5 ng/ml cholera toxin (Sigma).

Transfections in HEK293T and A13 cells were performed with FuGENE6 (Roche), according to the manufacturer's instructions and experiments were performed 48hrs posttransfection. U2OS were transfected with the calcium phosphate method and after 24 hours, the transfection medium was replaced with fresh medium. Cells were incubated at 37°C for an additional 24hrs. For knockdown experiments in Kep1.11 cells, cells were infected with lentiviral shRNA constructs for CCM1 and Scrambled from the Sigma Mission shRNA library. Infected cells were selected with puromycin for two days and after 4 days the experiments were performed.

Antibodies and constructs

Flag-CCM1NLS#2 construct (KKKRKK mutated to KAAAAK) was kindly provided by Prof. D. Marchuk and described previously [306]. Flag-CCM1 wild type was made with site directed mutagenesis from the Flag-CCM1 NLS#1 mutant. HA-CCM1 was cloned into the gateway destination vector according to the manufacturer's instructions using full length CCM1 cDNA with the following primers: Forward 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTGAATTCGAAATCCAGAAAACATAGAAGATG-3' and Reverse: 5'- GGGGACCACTTTGTACAAGAAAGCTGGGTGCGGCCGCATTATCATGAATTTCTTTCAGTGGGCAT-3'.

GFP-CCM1 was cloned from full length CCM1 cDNA into the pIC113 vector with the following primers: Forward 5'-CTGTACACTGACTCGAGGGAAATCCAGAAAACATAG-3' and Reverse: 5'-TGATAGTCACGTCGACC TATCATGAATTTCTTTCAG-3'.

Flag-FOXO4, HA-FOXO4, HA-FOXO1, HA-FOXO3 and HA-FOXO4dNLS were described previously [92].

The following antibodies were used: anti-HA (12CA5), anti-FOXO4 (834) and anti- Phospho- FOXO4 (Thr223/Ser226) were described before [13, 154]. The following antibodies were purchased: anti-FlagM2 (Sigma), anti-pT32 (Cell Signaling), anti-HA (Santa Cruz), anti-GFP and anti-BAF57 (Bethyl Laboratories). Anti-BAF155 was kindly provided by Prof. P. Verrijzer. The following reagents were purchased: LY294002 (Merck) and VIII (Santa Cruz).

Immunoprecipitation and Western blot

For immunoprecipitation, cells were lysed in buffer containing 50mM Tris-HCl (pH7.5), 1% NP40, 5mM EDTA, 100mM NaCl, protease and phosphatase inhibitors. Cell lysates were centrifuged for 10 minutes at 14000 rpm at 4°C and 5% of the supernatant was used as input material. Immunoprecipitation of the proteins of interest was done with protein agarose beads, coupled to the protein or tag specific antibody, for 2hrs at 4°C. Beads were washed with lysis buffer and resuspended in Laemmli sample buffer. Samples were separated on SDS-polyacrylamide gels and transferred to PVDF-membrane. Western blot analysis was performed by enhanced chemiluminescence. Experiments were performed at least 2-5 times of which a representative figure is shown in the manuscript.

Immunofluorescence

U2OS cells were plated and transfected on coverslips. Immunostaining was performed 48hrs after transfection. Cells were fixed with 4% paraformaldehyde and blocked with 1% BSA. Cells were incubated with anti-HA (12CA5), anti-FOXO4 (834) or anti-FlagM2 (sigma), followed by goat anti-mouse IgG conjugated to Alexa488 or goat anti-rabbit conjugated to Alexa568. Nuclei were visualized by 4',6-diamidino-2-phenylindole (DAPI). Fluorescence was captured with a Zeiss Axioskop microscope with a 40x Plan-Neofluar oil objective. Images were taken using Axiovision Zeiss software.

Colony formation assay and apoptosis measurements

Kep1.11-FOXO3/FOXO3A3 cells were infected with shRNA constructs against a scrambled sequence or against CCM1 and selected for infection with puromycin for two days. After 2 days recovery from the puromycin selection, we seeded 100.000 cells on six well plates with or without doxycycline. After 2 days, cells were harvested for RNA to check knockdown by qPCR and cells were stained with propidium iodine and measured in FACS to determine the amount of apoptosis.

At 7 days after seeding, cells were fixed in methanol, stained with 0.5% crystal violet in 25% methanol and washed with demi-water to determine the amount of colonies formed in presence of absence of CCM1.

Quantitative RT-PCR

RNA isolation from Kep1.11-FOXO3/FOXO3A3 or A13 cells was performed using the RNeasy mini kit from Qiagen and cDNA was created using iScript cDNA synthesis kit from Bio-Rad. IQ SybrGreen (Biorad) real time qPCR was performed according to manufacturers instructions. Relative abundance CCM1 mRNA was corrected using PGBD mRNA. The following primers were designed to detect: mCCM1 mRNA: Forward 5'AGCTGGGAGATGCTGGTACT-3' and Reverse 5'-CAAGGCCAGCCTGTTTTGTG-3' and for detection of PBGD mRNA (used as housekeeping gene): Forward: 5'- GCCTACCATACTACCTCCTGGCT - 3' Reverse: 5'- AAGACAACAGCATCACAAGGGTT - 3'. The following primers were designed to detect hCTDSP2: Forward 5'-CATTGCTAAGTCGGATCTGC-3' and Reverse: 5'-CCACACAGATCCTTCCTTGAT-3', hLGR4: Forward 5'-GCTGAGTGCTTTGCAGTCTTT-3' and Reverse 5'-CGTCAAGCTGTTGTCATCCA-3', hPINK1: Forward 5'-GAACATCTCGGCAGGTTCCTC-3' and Reverse 5'-GTTGCTTGGGACCTCTCTTG-3', hHSP1: Forward 5'- GTGATGAACACATGGAGCTTG-3' and Reverse 5'-ACATGCCAGATTGGGTAGGA-3' and hTubulin: Forward 5'- TACACCATTGGCAAGGAGAT-3' and Reverse 5'- AACCAAGAAGCCCTGAAGAC-3'.

Microarray analysis

For the microarray experiments, four biological replicates of cDNA made from mRNA isolated from HEK293T cells expressing empty vector, HA-FOXO4, Flag-CCM1, HA-FOXO4+Flag-CCM1 were hybridized to a pool of cDNAs from ten biological replicates of HEK293T cells expressing an empty

vector control. All RNA amplification and labeling procedures were performed in 96-well plates (Abgene) on a customized Sciclone ALH 3000 workstation (Caliper LifeSciences), supplemented with a PCR PTC-200 (Bio-Rad Laboratories), SpectraMax 190 spec- trophotometer (Molecular Devices) and a magnetic bead locator (Beckman). Labeled cRNA product was purified with RNAClean (Agencourt, GC biotech) according to the manufacturer's protocol. Hybridizations of spotted oligo- arrays (Human Operon version2 onto Codelink glass) were performed on a HS4800Pro Hybstation (Tecan). Hybridized slides were scanned on an Agilent scanner (G2565AA) at 100% laser power, 30% photo multiplier tube. After data extraction using Imagene 7.5 (BioDiscovery), print tip Loess normalization was performed on mean spot intensities. Data was analyzed using ANOVA (R version 2.2.1/MAANOVA version 0.98-7) (http://www.r-project.org). In a fixed effect analysis, sample, array and dye effects were modeled. *P* values were determined by a permutation F2 test, in which residuals were shuffled 5,000 times globally. Genes with $P < 0.05$ after family-wise error corrections were considered significantly changed and used for analysis in a scatterplot.

SILAC labeling A13 cells

A13 cells were cultured in medium consisting of 500 ml SILAC Dulbecco's Modified Eagle Medium without arginine, lysine and glutamine (PAA, E15-086), Glutamine, Penicillin/Streptomycin, 1x Non-essential amino acids, sodium pyruvate, 73 µg/ml L-Lysine (light/K^0 (Sigma, A6969) or heavy/K^8 (Sigma, 608041 or Silantes, 211603902)) and 29.4 µg/ml arginine (light/R^0 (Sigma, A6969) or heavy/R10 (Sigma, 608033 or Silantes, 201603902)). Cells were cultured in SILAC medium until labeling efficiency exceeded 95% after which cells were expanded and harvested to generate nuclear and cytoplasmic extracts.

Nuclear-cytoplasmic fractionation

Cells were treated for 16hrs with doxycycline and 30 min with 10µM LY294002 or left untreated prior to fractionation. The fractionation was essentially done as described previously [258]. Briefly, cells were trypsinized and washed two times with PBS. Using a hypotonic buffer, the cells were swollen, after which the cells were lysed by dounce homogenizing in the presence of 0.15% NP40 and complete protease inhibitors. After centrifugation, the pellet consisting of nuclei was lysed by 90 min incubation in 2 volumes of nuclear lysis buffer (420mM NaCl, 20mM HEPES pH 7.9, 20% v/v glycerol, 2mM $MgCl_2$, 0.2mM EDTA, 0.1% NP40, complete protease inhibitor w/o EDTA (Roche) and 0.5mM DTT). After centrifugation, the supernatant containing the soluble nuclear extract was aliquoted and snap frozen until further usage. Protein concentrations of the nuclear extracts were determined using the Biorad Protein assay.

Flag pulldowns SILAC

After fractionation, Flag-pulldowns (Flag-M2 beads, Sigma) were performed in lysis buffer (50mM Tris-HCl, 1% Triton TX-100, 1.5mM $MgCl_2$, 300mM NaCl, 1mM DTT and protease and phosphatase inhibitor cocktail) with addition of 50µg/ml EtBr to prevent protein-DNA-protein interactions. Proteins were eluted from the Flag-beads with 0.1M Glycine pH2.5 and subsequently trypsinized on FASP columns (30kD columns). The FASP procedure is performed as described previously [259].

Mass Spectrometry

Peptides were separated using an EASY-nLC (Proxeon) connected online to an LTQ-Orbitrap Velos mass spectrometer (Thermo) as described [260]. Raw data were analyzed using MaxQuant version 1.2.2.5 and mapped using protein database IPI human V3.68 fasta (monoclonal cell lines) or IPI mouse V3.68 fasta (A14/A14-GFP-FOXO4). Resulting protein groups were further analyzed using Perseus. Identifications were filtered for standard contaminants, reverse hits, number of peptides (>1) and unique peptides (>0). Ratios were logarithmitized (log2) and groups (consisting of forward and reverse) were defined. Scatter plots were made using a custom R script.

CHAPTER 7

Summarizing Discussion

Summarizing Discussion

The FOXO family members are highly redundant in their tumor suppressive function [235], which suggest that loss of all FOXO alleles is unlikely to occur in cancer cells. In contrast, general loss of the tumor suppressive functions of all FOXOs as a result post-translational regulation, like for example proteasomal degradation and changes in cellular localization, is often observed (reviewed in [398]). Thus, deciphering the signaling pathways that regulate FOXO localization and degradation is important in order to understand more of its biological functions.

Regulation of FOXO activation and localization

The Forkhead box O group of transcription factors are highly regulated by posttranslational modifications [211]. As described in **Chapter 1**, the balance between these modifications is important to determine the functional outcome of FOXO signaling. For example, PKB-mediated phosphorylation of FOXO inhibits its DNA binding and also mediates nuclear export through induction of binding with the 14-3-3 protein family [9, 249, 250]. Furthermore, activation of PKB results in phosphorylation and inhibition of multiple proteins involved in FOXO activation. The most important ROS-induced activating signaling pathway towards FOXO4 involves the small GTPase RALA and the MAPK JNK [46, 214]. Although inhibition of FOXO via PKB is extensively studied and most of the players resulting in PKB activation and FOXO inhibition are known, the proteins involved in the signal from RALA towards JNK were completely unknown. In **Chapter 2** we describe the identification of a complete signaling cascade in between RALA and JNK. We identified the scaffold protein JIP1 as important mediator to relay signals from RALA to JNK. Furthermore, we show that RALA is important for the assembly of a functional scaffold complex consisting of JIP1-MLK3-MKK4-JNK. Interestingly, JNK-mediated phosphorylation on FOXO is only described for FOXO4. However three of the seven JNK sites identified in FOXO4 are conserved in FOXO1 and FOXO3 (Fig. 1). Furthermore, stress-induced nuclear localization of FOXO1 and FOXO3 is also JNK dependent although this is thought to occur via JNK-mediated phosphorylation of the 14-3-3 proteins [64]. This subsequently results in release of FOXO and nuclear localization. It will be interesting to determine whether JNK also phosphorylates FOXO1 and FOXO3 and if this involves the same RALA-JIP1 complex as we describe in **Chapter 2.** Alternatively, the RALA-JIP1-JNK complex might also be involved in phosphorylation of the 14-3-3 proteins and thereby activate FOXOs by an additional mechanism.

In **Chapter 4** we highlight the differential regulation of FOXO1, FOXO3 and FOXO4. Although all three FOXOs are in general regulated similarly, they behave very differently under basal conditions. Whereas FOXO4 is mainly nuclear, FOXO1 is completely cytoplasmic and FOXO3 shows an intermediate localization. Indeed, the individual *Foxo* knockout mice show completely different phenotypes [236-239]. Although most people often relate these differences to tissue-specific expression patterns of each FOXO, differential regulation is also a likely explanation for this observation. Interestingly, our lab has identified multiple importins to interact with FOXO4 [256] and FOXO3 (personal communication) in a cysteine disulphide-dependent manner. It will be interesting to determine whether each FOXO member has a preference for a different importin. Notably, the interaction of FOXO4 with transportin-1 is ROS dependent, but is suggested to be JNK independent. Therefore, JNK might be involved in regulation of FOXO export. We have shown in *C. elegans* that both IMB-2 (transportin 1) and RALA (**Chapter 2**) are important for stress-induced nuclear localization of DAF16 [214, 256]. To further investigate the role of JNK in IMB-2 dependent nuclear DAF16 localization, experiments with combined knockdown of IMB-2 and RALA will provide useful information, Additionally, to determine whether the RALA-JIP1-JNK pathway is involved in export of FOXO/DAF16 examination of nuclear retention of FOXO in, for example, RALA or

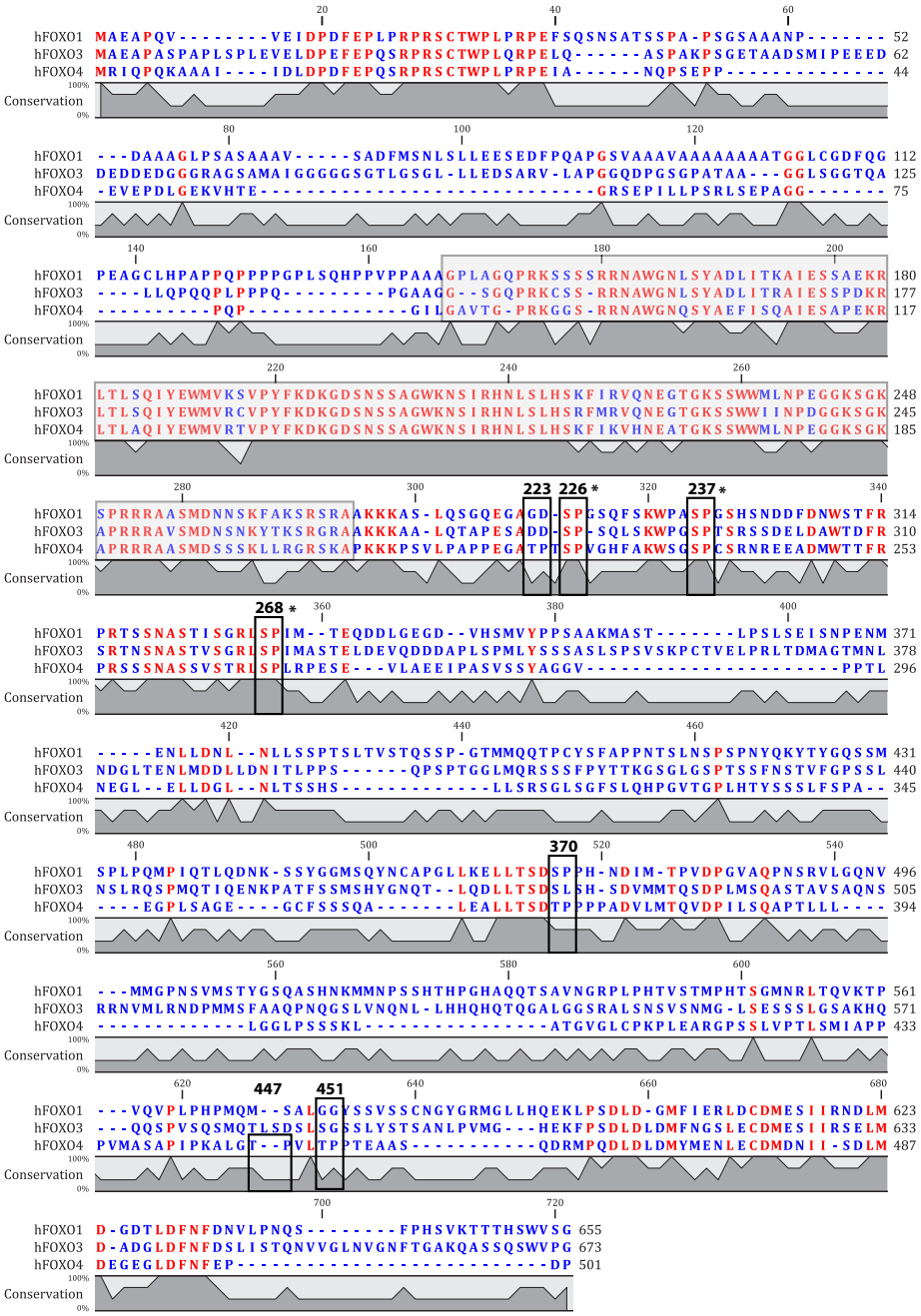

FIGURE 1: Alignment of human FOXO1, FOXO3 and FOXO4 protein sequences. For clarity FOXO6 is left out. Grey area indicates the DNA binding domain ([3]. Black boxes depict the JNK-phosphorylation sites with amino acid numbers related to the FOXO4 sequence. Asterisks show the conserved JNK phosphorylation sites among the FOXOs. Red amino acids indicate the conserved residues among FOXO1, FOXO3 and FOXO4. The grey bar shows the percentage of conservation.

JIP1 knockout cells will provide useful information. We have performed some preliminary experiments to determine retention time of DAF16 in the nucleus upon heat shock. Indeed, *jip1* mutant worms and RALA siRNA showed enhanced export of DAF16 (data not shown), indicating that they are important for nuclear retention of DAF16. Furthermore, if this pathway is specific for FOXO4, this might provide a rationale for the different localization of the FOXO family members.

To gain more insight into the differential regulation of the FOXO family members, we performed SILAC combined with pulldowns and quantitative tandem mass spectrometry in **Chapter 4** in order to determine specific interaction partners for each FOXO member. We predominantly identified several 14-3-3 isoforms. As mentioned above, this group of proteins is involved in relocalization and inactivation of FOXOs. This suggests that under basal conditions, FOXOs are kept inactive and are predominantly bound to 14-3-3 proteins. With this in mind, activation of FOXO by inhibition of the PKB pathway would provide a tool to identify other interaction partners than 14-3-3 proteins. Indeed, upon activation of JNK (by treatment with H_2O_2), we identified additional proteins to interact with FOXO4, although the Heavy/Light ratios were just above background. Other, more drastic methods to activate FOXO might increase those ratios. For example, treatment with the PKB pathway inhibitors LY and VIII or mutation of the PKB phosphorylation sites in FOXO. Another important explanation for the low number of identified interaction partners is the presence of highly unstructured domains in FOXOs. Because of its flexible structure, interaction with multiple proteins is possible. Therefore, the presence of multiple low stoichiometry proteins complexes can occur. These low stoichiometry complexes are difficult to identify with our approach, because their H/L ratio will not increase above background ratios.

In **Chapter 6**, we established another approach to identify novel interaction partners of FOXO. We identified an interaction between the junction protein CCM1 and FOXO and subsequently performed quantitative mass spectrometry to find interaction partners of this complex. In order to extract the complex from the pool of background proteins, we performed a simultaneous pulldown of CCM1 and FOXO1. In this manner, we create a ~1:1 ratio of the complex and avoid the problem of low stoichiometry complexes we encountered in our previous approach. Furthermore, we inhibited the PKB pathway by treatment with LY in order to activate FOXO and relocate the complex to the nucleus. Indeed, this approach improved the identification of novel interaction partners. We found specific interaction of the SWI/SNF chromatin remodeling complex to the CCM1-FOXO1 complex.

RALA-mediated regulation of ubiquitination

Signaling of the E3-ligase complex consisting of HDM2-HDMX is extensively studied for their activity towards p53. The main function of HDMX is believed to be inhibition of the transcriptional activity of p53 [206]. Furthermore, biochemical assays have shown that HMDX can enhance the stability of HDM2 through interaction with its RING domain. However, whether this also occurs in vivo remains unknown to date. HDM2 can act both as a mono-ubiquitin and poly-ubiquitin E3-ligase [187, 188, 191-193, 195, 399]. HDMX is suggested to determine which function HDM2 fulfills (Wang et al 2011). Both proteins are extensively studied with respect to their regulation by posttranslational modifications. Phosphorylation can affect their ubiquitination and subcellular localization (reviewed in [207]). During DNA damage, p53 activity is essential and therefore inhibition of HDM2-HDMX is required. This can occur by multiple mechanisms including degradation and changes in cellular localization. In **Chapter 3**, we elaborate on the regulation of FOXO by HDM2. Previously, our lab has shown that ROS-induced mono-ubiquitination of FOXO4 is dependent on HDM2 [93]. We have shown in **Chapter 3**, that RALA is important for the HDM2-dependent FOXO mono-ubiquitination. Furthermore, RALA is involved in regulation of HDM2-dependent p53 ubiquitination and potentially p53 stability. Because of the general involvement of RALA in

downstream targets of HDM2, we wanted to determine if RALA affects HDM2 activity. We identified a MAPK consensus site in HDM2 and *in vitro* this site can be phosphorylated by JNK. We aimed to determine the function of this phosphorylation site by inhibiting or mimicking its phosphorylation. However, we did not observe any changes in HDM2 stability or activity. This can have several reasons; first, this phosphorylation site is not important for HDM2 function in the assays that we used. Second, if only a subset of HDM2 is phosphorylated on this site, it will be difficult to observe differences among wild type and mutant HDM2. Third, although there is only one typical MAPK consensus site in HDM2, it is possible that JNK can also phosphorylate other sites. We have performed mass spectrometry on recombinant HDM2 to determine the phosphorylation site and identified multiple peptides that were phosphorylated by recombinant JNK (data not shown). These peptides did not harbor the typical JNK consensus site, however they might still be involved in JNK-dependent HDM2 regulation.

Because HDMX can function together with HDM2 to regulate p53, we also investigated the role of RALA in HDMX regulation. Surprisingly, RALA activation seems to affect the abundance of HDMX in our assays. Although further research is essential to determine whether RALA affects HDMX stability or any other mechanism affecting protein expression, it is interesting to speculate on a potential role of RALA in HDMX regulation. As mentioned above, an important mode of regulation of the HDMX-HDM2 complex is changing their cellular localization. Under basal conditions, HDMX is localized in the cytoplasm. Upon DNA damage signals it relocates to the nucleus where it is degraded by HDM2 [229, 400]. Ultimately, this results in activation of p53. We observed in **Chapter 2** that RALA-JNK signaling affects FOXO localization. Therefore, activation of JNK via RALA might also affect HDMX or HDM2 localization and, for example, retain HDMX in the cytoplasm. This might provide an alternative mechanism to inhibit HDMX-dependent p53 inhibition. Alternatively, activation of JNK might affect the interaction between HDMX and HDM2. In case of FOXO, retaining HDMX and subsequently HDM2 in the cytoplasm might result in FOXO mono-ubiquitination and subsequent nuclear localization. If FOXO mono-ubiquitination indeed takes place in the cytoplasm is unknown and needs to be determined. Future experiments to resolve the mechanism of how RALA might regulate HDM2-HDMX are necessary to answer these questions.

FOXOs involved in maintenance of 'resting state' endothelial cells?

In **Chapter 6** we identified CCM1 as a novel regulator of FOXO. Interestingly, CCM1 is involved in the maintenance of junctions and therefore provides a link between junctions and FOXO signaling. Previously, others have shown a role for FOXOs in regulation of proteins involved in endothelial homeostasis [235]. Furthermore, as mentioned in **Chapter 4**, *Foxo1* knockout mice are embryonic lethal due to vascular defects [236, 237]. Thus our identification of CCM1 as interaction partner of FOXO provides further information on the role of FOXO in endothelial function. 'Resting' endothelial cells are in a quiescent state, predominantly mediated via the upregulation of $p27^{kip1}$ [395]. Furthermore, compared to 'motile' endothelial cells, 'resting' cells display higher levels of PKB activation. Whether this increased PKB activity results in complete FOXO inhibition remains to be determined. Because of the increased $p27^{kip1}$ levels, it is possible that a proportion of FOXO is still active in order to mediate $p27^{kip1}$ upregulation. Upon disruption of the endothelial monolayer due to inflammation or induction of angiogenesis, induction of ROS often occurs. As shown in **Chapter 2** and by others [46], ROS results in a RALA-dependent activation of FOXO. The ROS–induced activation of FOXO is dominant over PKB-mediated inhibition [46]. Whereas high levels of ROS require FOXO activity in order to scavenge ROS, induce cell cycle arrest or induce apoptosis (reviewed in [177]), in the case of, for instance, angiogenesis, a different response is required. Therefore, mechanisms to inhibit FOXO in presence of ROS signals are essential. We have shown in **Chapter 6**, that CCM1 might provide such an additional mechanism

of FOXO inhibition. Nuclear localization of FOXO coincides with nuclear localization of CCM1 and expression of CCM1 induced inhibition of a subset of FOXO target genes. In agreement, knockdown of CCM1 enhances FOXO-induced apoptosis and inhibition of cell growth. Finally, the nuclear CCM1-FOXO complex interacts with a SWI/SNF chromatin remodeling complex, containing ARID1A and HDAC1. The SWI/SNF complex can promote both transcriptional activation and inhibition depending on the association with activator versus repressor complexes. In proliferating cells it has been shown to stimulate Myc-dependent transcription of target genes, whereas during differentiating-associated cell cycle arrest the SWI/SNF complex inhibits Myc-dependent transcription [393]. Interestingly, the mutually exclusive components ARID1A and ARID1B seem to determine whether the complex associates with activating or inhibitory complexes. We have shown binding between an ARID1A-containing SWI/SNF complex to the CCM1-FOXO complex and observe a general decrease in expression of FOXO activated genes. In addition, we identified HDAC1 as specific interactor of the CCM1-FOXO complex. Taken together this suggests that CCM1-FOXO interacts with an inhibitory SWI/SNF complex. Recently, the FOXO orthologue in *C. elegans* is found to interact with two components of the SWI/SNF complex (BAF155/170 and BAF57)[246]. This interaction seems necessary for DAF16 to regulate gene transcription. Knockdown of these components inhibits DAF16-dependent gene regulation. Our data suggests that the presence of CCM1 results in the assembly of an inhibitory SWI/SNF complex and mediates inhibition of FOXO. Further research is necessary to decipher whether FOXO alone interacts with an activating SWI/SNF complex, like described for DAF16 and if the presence of CCM1 can shift this complex towards an inhibitory ARID1A-containing complex (Fig. 7, **Chapter 6**). *C. elegans* CCM1 (Kri-1) is involved in nuclear localization of DAF16 [378] and DAF16 is regulated by members of the SWI/SNF complex in order to activate transcription. We suggest that CCM1 mediates inhibition of FOXO via the SWI/SNF complex. Therefore, investigation of DAF16-mediated transcriptional control via SWI/SNF members in situations where KRI-1 is involved in DAF16 regulation will show if the regulation suggested in our model is conserved in *C. elegans*. However, the separate ARID1 members have emerged later in evolution and are not yet present in *C. elegans*. In *C. elegans,* ARID1 can mediate both inhibitory and activating functions of the SWI/SNF complex. Thus, SWI/SNF-mediated regulation of DAF16 might be differently regulated.

Which signals mediate nuclear CCM1?

We have shown in **Chapter 6** that nuclear CCM1 mediates FOXO inhibition, however the question still remains under which circumstances CCM1 relocates to the nucleus? More specifically, when, why and how is CCM1 released from adherens junctions? Or are there different pools of CCM1, for example, a junction-bound pool and a nuclear pool. We have shown in **Chapter 6** that ICAP1α mediates stability and nuclear localization of CCM1. Furthermore, in **Chapter 5**, we describe the function of ICAP1α in integrin signaling. ICAP1α binding the β1-integrin tail inhibits the interaction of the integrin activators talin and kindlin [336-340]. Therefore, activation of integrin signaling might result in ICAP1α release from integrins. We suggest that this 'free' ICAP1α would then be available to bind and stabilize CCM1 and subsequently enhance the CCM1-FOXO4 complex formation. For this model activation of integrin signaling would then also result in release of CCM1 from adherens junctions and thus make CCM1 available to interact with ICAP1α. Future experiments are essential to test this hypothesis although we have performed some preliminary experiments that strengthen this model. When cells are grown on fibronectin in order to activate the integrin receptors, we observed increased binding between CCM1 and FOXO (data not shown). However, to determine whether this is ICAP1α dependent, we need to perform additional knockdown experiments. Next to the interaction, it will also be important to determine localization of the CCM1-FOXO complex upon activation of integrin signaling.

Concluding remarks

Regulation of the Forkhead box O family members is complex and involves many players. In addition, the response of FOXOs is highly dependent on the cellular context. Therefore, the identification of novel processes in which FOXOs are regulated is important to understand more of its biological functions. In this thesis we describe a novel player involved in FOXO inhibition and identify a unique role for the adhesion protein CCM1 in transcription regulation. Furthermore, we applied quantitative proteomics to determine the FOXO interactome. We also identified critical players involved in the ROS induced activation of FOXO. Taken together, we have expanded our knowledge of FOXO regulation and physiologically relevant processes in which FOXOs have an important role.

ADDENDUM

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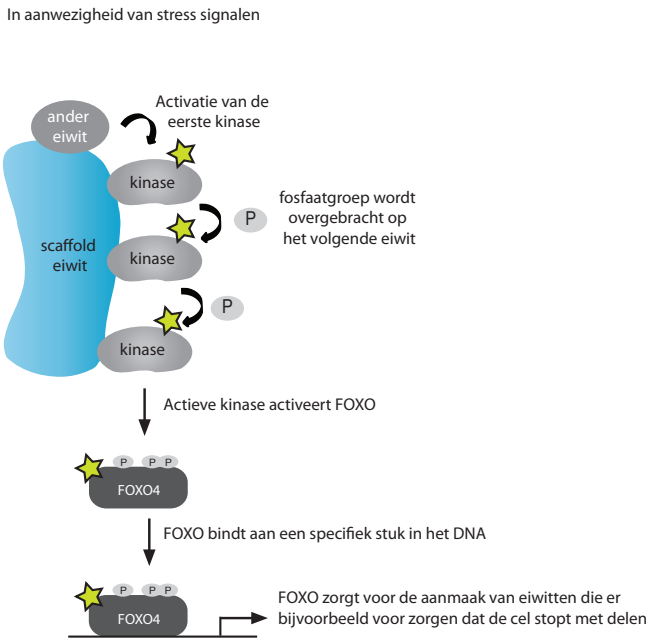
Nederlandse samenvatting

Ons lichaam is opgebouwd uit miljarden cellen die ieder een eigen functie hebben. De eigenschappen van deze cellen liggen vast in ons genetisch materiaal, ook wel DNA genoemd. Het DNA is onder te verdelen in kleinere stukken die we genen noemen. Deze genen bevatten informatie om eiwitten te maken, dit zijn specifieke onderdelen van de cel. Eiwitten hebben allerlei functies in de cel, zo zorgen ze onder andere voor transport en het doorgeven van signalen. Om de genen te kunnen 'lezen' en eiwitten te maken, heb je ook eiwitten nodig. Het speciale type eiwitten dat hiervoor nodig is noemen we transcriptiefactoren. Met behulp van transcriptiefactoren kunnen we de informatie van een specifieke set genen vertalen. Dit proces noemen we 'transcriptie' en leidt uiteindelijk tot de aanmaak van eiwitten. Er bestaat een grote diversiteit aan transcriptiefactoren, die elk een heel specifiek deel van ons DNA lezen.

In dit proefschrift beschrijf ik mijn onderzoek naar een transcriptiefactor, genaamd Forkhead Box O (FOXO). FOXO is verantwoordelijk voor een grote hoeveelheid verschillende processen in ons lichaam. De activiteit van FOXO wordt geregeld door een verscheidenheid aan signalen, waaronder groeifactoren. Activatie van FOXO zorgt onder andere voor het stoppen van de celdeling. Als cellen genoeg groeifactoren krijgen willen ze gaan delen, dus dan zou je FOXO eerst moeten uitzetten. Dit is inderdaad wat er gebeurd: in aanwezigheid van groeifactoren wordt FOXO door andere eiwitten uitgezet en kunnen de cellen gaan delen. Als er echter schadelijke signalen voor de cel aanwezig zijn, is het van belang om FOXO aan te zetten. In dit geval moeten de cellen namelijk stoppen met delen omdat de kans aanwezig is dat ze beschadigd zijn. Wanneer beschadigde cellen toch gaan delen, kan dit lijden tot allerlei ziekteverwekkende situaties, waaronder het ontstaan van tumorcellen. Het is daarom van groot belang om FOXO activiteit goed onder controle te houden. Daarvoor moeten we precies weten welke signalen zorgen voor het aan- of uitzetten van FOXO. Het onderzoek in dit proefschrift beschrijft onder andere welke eiwitten betrokken zijn bij het aanzetten van FOXO in schadelijke situaties van hoge stress. Daarnaast beschrijven we een nieuwe situatie waarbij het van belang is om FOXO uit te zetten.

Signaaltransductie
De activiteit van transcriptiefactoren wordt door een hele 'keten' van andere eiwitten geregeld. Dit is vergelijkbaar met een schakelketting. De eerste schakel wordt aangezet door een signaal en geeft dit door aan de volgende schakel, die geeft het weer door aan de volgende en ga zo maar door. We noemen zo'n schakelketting een 'signaaltransductieroute'. Het is van groot belang om te weten welke schakels er precies zitten tussen het eerste signaal en het eindpunt (de transcriptiefactor FOXO). Signalen die deze schakelketting kunnen starten zijn onder andere de hierboven genoemde groeifactoren en stressfactoren. Wanneer we precies weten wat er tussen dit eerste signaal en FOXO in zit, kunnen we zo'n signaaltransductieroute aan- of uitzetten en daarmee de activiteit van FOXO regelen.

Kinases & scaffold eiwitten
Belangrijke eiwitten in de signaaltransductieroute van FOXO zijn kinases. Kinases zijn enzymen die een fosfaatgroep op een eiwit zetten. Hiermee verstoren ze onder andere de interactie met andere eiwitten en kunnen ze de structuur van het eiwit veranderen. Bij een signaaltransductie route zijn vaak kinases betrokken. Hierbij wordt de eerste kinase geactiveerd door een signaal of een ander eiwit. Vervolgens activeert de geactiveerde kinase de volgende kinase, die op zijn beurt de volgende kinase activeert. Om dit te kunnen doen moeten kinases wel bij elkaar in de buurt zitten.



FIGUUR 1: Ilutratie van de werking van een signaaltransductie route. Signalen van buiten de cel, zoals stress, activeren de route door activatie van het eerste eiwit. Dit eiwit activeert vervolgens de eerste kinase die op zijn beurt de volgende weer activeert. De laatst geactiveerde kinase activeert het eindpunt van de keten: FOXO. FOXO zorgt er vervolgens voor dat er eiwitten aangemaakt worden die de celdeling stoppen.

Daarvoor zijn er eiwitten in de cel die als platform dienen voor de kinases, zodat ze op een specifieke locatie in de cel elkaar kunnen activeren (weergegeven in Fig. 1). Deze eiwitten heten 'scaffold-eiwitten' en in **Hoofdstuk 2** beschrijven we het belang van een scaffold-eiwit in de regulatie van FOXO.

Adhesie van cellen

Een belangrijke functie die alle cellen, op verschillende manieren en in meer of mindere mate, kunnen uitvoeren is dat ze zich kunnen hechten aan elkaar of aan de omgeving. Deze functie noemen we adhesie. De huid is een voorbeeld van een plek waar adhesie van cellen zeer belangrijk is. Doordat ze aan elkaar hechten vormen ze een barrière en beschermen ze het lichaam tegen bijvoorbeeld infecties van buitenaf. In de cel zijn veel verschillende eiwitten betrokken bij dit proces. In **Hoofdstuk 5** bespreken we de functie van één van deze eiwitten en in **Hoofdstuk 6** laten we zien dat dit eiwit FOXO kan remmen.

Inhoud van dit proefschrift

Met het onderzoek in dit proefschrift hebben we onderzocht welke eiwitten FOXO kunnen regelen en wanneer dit gebeurt. In **Hoofdstuk 1** beschrijven we welke signaalroutes belangrijk zijn om FOXO aan of uit te zetten. Daarnaast beschrijven we hoe deze routes invloed op elkaar kunnen hebben. Eiwitten die betrokken zijn bij het aanzetten van FOXO kunnen bijvoorbeeld tegelijkertijd eiwitten remmen die belangrijk zijn om FOXO uit te zetten. Andersom komt ook voor: eiwitten die FOXO uitzetten kunnen eiwitten remmen die FOXO aanzetten. Dit geeft gelijk aan hoe complex de regulatie van FOXO is. In **Hoofdstuk 2** laten we zien dat we een hele signaaltransductieroute hebben gevonden die nodig is om

FOXO aan te zetten in de situatie dat een cel stress ondergaat. We laten zien dat een scaffold-eiwit genaamd JIP1 nodig is om een heleboel andere eiwitten, met name kinases, bij elkaar te brengen zodat deze elkaar kunnen activeren. Uiteindelijk is de laatste geactiveerde schakel (JNK) nodig om FOXO te activeren. In **Hoofdstuk 3** onderzoeken we een andere eiwit aanpassing die belangrijk is voor de regulatie van FOXO, genaamd ubiquitinatie. Bij ubiquitinatie wordt een ubiquitine groep op eitwitten gezet. Dit wordt gedaan door een enzym, en in het geval van FOXO is dit het enzym HDM2. In Hoofdstuk 3 laten we zien dat een ander eiwit genaamd RALA hier een belangrijke rol bij speelt. Hoe RALA hier precies bij betrokken is, is nog niet geheel duidelijk, maar we suggereren dat RALA een eiwit reguleert dat betrokken is bij de activiteit van HDM2.

De FOXO eiwitten zijn onderverdeeld in FOXO1, FOXO3, FOXO4 en FOXO6. FOXO6 komt voornamelijk voor in hersencellen. FOXO1, FOXO3 en FOXO4 komen daarentegen in vrijwel alle cellen van ons lichaam voor. Ze worden ook op een vergelijkbare manier geactiveerd en geremd. Ondanks deze overeenkomsten zijn er ook verschillen tussen deze drie FOXOs. Om de oorzaak van deze verschillen te achterhalen, beschrijven we in **Hoofdstuk 4** een methode die we gebruiken om te kijken welke eiwitten binden aan FOXO1, FOXO3 en FOXO4.

In **Hoofdstuk 5** suggereren we dat een eiwit betrokken bij de adhesie van cellen, genaamd CCM1, ook betrokken is bij een ander proces. Hiervoor moet het eiwit naar een andere locatie in de cel. In **Hoofdstuk 6** laten we zien dat deze verandering van locatie geregeld kan worden door FOXO. Het resultaat hiervan is dat zowel CCM1 als FOXO in de celkern zitten. In de celkern zit het DNA en dat is de plek waar FOXO zijn rol als transcriptiefactor vervult. De aanwezigheid van CCM1 in de kern zorgt ervoor dat FOXO geremd wordt. Hiermee laten we zien dat een eiwit dat tot nu toe alleen bekend was vanwege zijn rol in adhesie, ook betrokken is bij de regulatie van FOXO.

In **Hoofdstuk 7** vatten we de bevindingen van het onderzoek in dit proefschrift samen en bediscussiëren we dit aan de hand van de huidige wetenschappelijke literatuur.

Curriculum Vitae

Maaike Carolina Wilhelmina van den Berg werd geboren op 6 juli 1983 te Utrecht. In 2001 behaalde zij haar VWO diploma aan het Revius Lyceum Doorn met het profiel Natuur & Gezondheid. In September 2001 begon zij aan de studie Biomedische Wetenschappen aan de Universiteit Utrecht die ze in juni 2006 afrondde. Tijdens deze opleiding heeft Maaike een onderzoeksstage gedaan in het Hubrecht Instituut in Utrecht in het laboratorium van Prof. Dr. Ronald Plasterk onder begeleiding van Prof. Dr. Robin May. Gedurende deze stage verhuisde Prof. Dr. Robin May naar Birmingham in het Verenigd Koninkrijk om zijn eigen lab op te starten. Maaike besloot daarom haar stage te vervolgen in Birmingham. Daar heeft ze nog 4 maanden stage gelopen op de afdeling Molecular Pathobiology aan de Universiteit van Birmingham. Aansluitend heeft ze een tweede onderzoeksstage gedaan in het laboratorium van Prof. Dr. Linde Meyaard onder begeleiding van Dr. Robert Jan Lebbink in het Wilhelmina Kinder Ziekenhuis in Utrecht. In september 2006 is Maaike gestart als onderzoeker in opleiding in het laboratorium van Prof. Dr. Boudewijn Burgering op de afdeling Molecular Cancer Research in het Universitair Medisch Centrum Utrecht, waar ze heeft gewerkt aan het onderzoek beschreven in dit proefschrift. Begin 2014 zal Maaike haar onderzoekscarrière voortzetten als postdoctoraal onderzoeker in het laboratorium van Prof. Dr. Paul Martin aan de Universiteit van Bristol in het Verenigd Koninkrijk.

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&

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- Maaike -

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