Regulation of Forkhead BOX O tumor suppressors by Reactive Oxygen Species

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Cover: A colo829 melanoma cell that has become senescent upon expression of FOXO4. Picture taken by Peter de Keizer and modified by Remko Burger

Invitation: Strings of a squashracket visualizing the many processes that are regulated by FOXO signaling described in this thesis. Picture by Niels van den Broek

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Regulation of Forkhead BOX O tumor suppressors by Reactive Oxygen Species

Regulatie van Forkhead BOX O tumor suppressors door Reactieve Zuurstof Soorten

(met een samenvatting in het Nederlands)

Proefschrift

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Abbreviations

4-OHT	4-hydroxytamoxifen
Ac	Acetyl Moiety
ATP	Adenosine Triphosphate
AKT	Ak mouse strain transforming
BRAF	RAS Activated Factor-isoform B
BrdU	5-bromo-2-deoxyuridine
cAMP	Cyclic Adenosine Monophosphate
CDK	Cyclin Dependent Kinase
CENP-F	Centromere Protein F
CHX	Cycloheximide
CK1	Casein Kinase 1
CKI	CDK inhibitor
Cu/ZnSOD	Copper/Zinc Superoxide Dismutase
DA	Dalton
DAF	Dauer Arrest Phenotype
DBD	DNA Binding Domain
DBE	DNA Binding Element
DUB	De-ubiquitinating enzyme
DYRK1	Dual specificity tyrosine phosphorylated and regulated kinase-1
ECL	Enhanced Chemical Luminescence
EGF	Epidermal Growth Factor
ERK	External signal Regulated Kinase
FOXO	Forkhead Box O (Other)
FOXM1	Forkhead Box M (Mitosis) 1
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
H_2O_2	Hydrogen Peroxide
H_2DCFDA	2’7’-dichlorodihydrofluorescein diacetate acetyl ester
HAT	Histone Acetyl Transferase
HAUSP	Herpes virus-Associated Ubiquitin-Specific Protease
HC	Heavy Chain
HDAC	Histone Deacetylase
H/MDM2	Human/Murine Double Minute 2
IGF	Insulin-like Growth Factor
IGFBP	IGF Binding Protein
IIS	Insulin/IGF Signaling
IKK	IκB kinase
IL	Interleukin
IP	Immunoprecipitation
JNK	Jun N-terminal Kinase
kDA	Kilo-Dalton
MAPK	Mitogen Activated Protein Kinase
MEK	MAPK/ERK Kinase
MEF	Mouse Embryonic Fibroblast
MITF	Microphtalmia-Induced Transcription Factor
MLL	Myeloid Lineage Leukemia

MnSOD	Manganese Superoxide Dismutase
NAC	N-Acetyl Cysteine
NES	Nuclear Export Signal
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear Localization Signal
$O_2^{\cdot -}$	Superoxide Anion
$OH^{\cdot -}$	Hydroxyl Radical
OIS	Oncogene Induced Senescence
P	Phosphate Moiety
p53	Tumor Suppressor Protein of 53 kDa
$p16^{ink4a}$	CDK Inhibitory Protein of 16 kDa
$p21^{cip1}$	CDK Inhibitory Protein of 21 kDa
$p27^{kip1}$	CDK Inhibitory Protein of 27 kDa
PAX	Paired Homeobox
PCNA	Proliferating Cell Nuclear Antigen
PDK1	Phosphoinositide-Dependent Kinase 1
PH	Pleckstrin Homology
PI3K	Phosphatidyl Inositol 3-Kinase
Pin1	Peptidyl-Prolyl Isomerase Interacting with NIMA
PKB	Protein Kinase B
pRb	Retinoblastoma tumor suppressor protein
PTEN	Phosphatase and Tensin homolog deleted from chromosome 10
PTM	Post Translational Modification
RAS	Rat Sarcoma
ROS	Reactive Oxygen Species
RING	Really Interesting New Gene
SA-β-GAL	Senescence Associated Acidic β-galactosidase
SAHF	Senescence Associated Heterochromatin Foci
SASP	Senescence Associated Secretory Phenotype
SCF	Skp1, Cullin-1, F-Box protein
S.d.	Standard Deviation
SEM	Standard Error of the Mean
SGK	Serum and Glucocorticoid dependent Kinase
shRNA	Short Hairpin RNA
Sir	Silent information regulator
siRNA	Small interfering RNA
SIRT	Sirtuin
SKP2	S-phase Kinase-Associated Protein 2
SMAD	Small / Mothers Against Decapentaplegic
TAP	Tandem Affinity Purification
TGFβ	Transforming Growth Factor β
TPA	12-O-tetradecanoyl-phorbol-13 acetate
TL	Total cell Lysate
Ub	Ubiquitin Moiety
USP	Ubiquitin-Specific Protease
WW	Double Tryptophan binding domain
wt	Wildtype

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General introduction

The risk on developing cancer increases with age[1,2]. Moreover, many processes that affect the onset of aging, such as altered proliferation, metabolism and stress resistance, are also frequently deregulated in cancer[3,4]. The molecular mechanisms that prevent the onset of aging may therefore be partially related to those that suppress carcinogenesis[5,6].
Accumulation of cellular damage by Reactive Oxygen Species (ROS) has been implicated in tumor progression and accelerates the aging process[7-9]. Forkhead Box O transcription factors, FOXOs, are activated in response to elevations in cellular ROS, however their molecular regulation is only partially unraveled[10]. In this thesis, we describe how FOXO activity is affected by ROS signaling and address their role in tumor suppression. Furthermore, we show how FOXO regulation can be modulated by Pin1, HDM2, p53 and, indirectly, FOXM1. Importantly, a prominent function of ROS-induced FOXO activation is uncovered in the process of oncogene induced senescence (OIS) by $BRAF^{V600E}$. This establishes an antagonistic role for FOXOs in a pleiotropy between tumor suppression and aging.

ROS induced damage accelerates the onset of aging and increases the chance on tumorigenesis

ROS comprise a compilation of oxygen-derivatives including the highly reactive superoxide anions ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$) and the more abundant but less reactive hydrogen peroxide (H_2O_2)[11]. ROS can be generated directly or indirectly as byproducts of mitochondrial energy production[12]. When present at low concentrations, ROS stimulate cellular proliferation through propagation of growth factor signaling and activation of the DNA synthesis machinery[13,14]. However, when ROS levels rise above a threshold, the cellular interior can be damaged through oxidation of proteins and lipids and through induction of DNA breaks[15].

To prevent damage from ROS, cells express a host of ROS scavenging enzymes. The superoxide dismutases MnSOD and Cu/ZnSOD for instance convert $O_2^{\cdot -}$ into H_2O_2 (Ref[16]). H_2O_2 itself is not directly toxic, however, H_2O_2 can be processed to $OH^{\cdot}$ radicals which in turn are very reactive[17]. To prevent this conversion, H_2O_2 can be reduced into water by cytoplasmic glutathione peroxidases and the peroxisomal catalase[11]. Together, these enzymes ensure that ROS levels remain within safe limits.

Cells can respond differently to cellular ROS, dependent on the concentration. For example, an acute, sublethal dose of ROS induces a temporal cell cycle arrest to allow ROS scavenging and, if required, repair. When scavenging is incomplete or the levels of ROS are of a chronic nature, the arrest will become permanent and lead to a state of senescence[18] (Fig. 1). At even higher concentrations the induction of damage may exceed repair to which cells respond by undergoing apoptosis[19,20]. Finally, when ROS levels are too high to allow time for the execution of apoptosis cells may die from necrosis. Together, these countermeasures protect the organism against cells that may otherwise become harmful through ROS induced cellular damage. This benefit comes at a cost, however, as permanent growth arrest or apoptosis decreases the pool of cells that are capable of rejuvenation. Thereby, excessive ROS contribute to premature aging and the inherent reduction in lifespan.

The today commonly accepted theory that first addressed the role of ROS in aging is referred to as the “Free Radical Theory of Aging”[8]. Summarized, this theory defines that in time damage by free radicals builds up as a consequence of lifelong metabolism and the inability to scavenge these radicals by antioxidants. Several reports furthermore support the theory with experimental

A B C D E

Consequence of:	- Growth factor stimulation	- (Changes in) Metabolism - Mild DNA Damage	- Oncogenic mutations - Telomere shortening - proloned DNA damage	- C + lack of repair mechanisms	
Results in:	- Proliferation	- Temporary cell cycle arrest - Repair	- Permanent cell cycle arrest (senescence) - Repair	- Apoptosis	- Necrosis

Fig 1: Cellular responses to intracellular ROS
Dependent on the levels of intracellular ROS cells can respond differently. This can either result in proliferation, or induce a variety of countermeasures to ensure cellular and organismal protection. **a)** Upon growth factor stimulation low levels or ROS are generated due to increased metabolism. These low levels of ROS allow propagation of signal events that result in proliferation. **b)** When ROS levels rise above a threshold the cellular interior can be damaged. To allow scavenging of excessive ROS, proliferating cells undergo a temporal cell cycle arrest, which can not be bypassed by growth factor stimulation. **c)** Several factors can lead to chronic elevation in cellular ROS, such as telemomere shortening and oncogenic mutation. This can result in a permanent state of arrest termed senescence. **d)** If ROS levels increase too far cells are unable to repair cellular damage sufficiently and will undergo apoptosis. This ensures organismal protection against cells which may become malfunctioned and possibly tumorigenic. **e)** When ROS levels are even further increased cells are not able to respond in time to undergo apoptosis and the large amount of damage will cause them to die because of necrosis.

evidence. For instance, selection for longevity in fruit flies showed a strong correlation with increased expression of Cu/ZnSOD and MnSOD as well as delayed cellular senescence[21]. Moreover, mutations in the electron transport chain of *Caenorhabditis elegans* that results in failure to catalyze electron transport leads to oxygen hypersensitivity and decreased lifespan[22]. In addition, several mice have been generated in which ROS scavenging enzymes are knocked out. Mice that lack MnSOD die shortly after birth with severe damage to metabolically active tissues[23,24], effects that can be partially rescued by MnSOD mimetics[25]. Catalase knockout mice develop normally but are susceptible to ROS producing injuries[26]. Whether this leads to premature aging has not yet been determined.
Conversely to loss of scavenging capacity, overexpression of ROS scavenging enzymes protects against ROS-induced damage and increases lifespan in various model organisms including fruit flies (Cu/ZnSOD and MnSOD)[27-30], yeast (MnSOD)[31] and mice (mitochondrially targeted Catalase)[32]. Although context dependent, MnSOD mimetics can also prolong lifespan[33,34]. Together this indicates that acute or chronic build-up of ROS decrease lifespan due to premature onset of aging and that antioxidant scavenging could be beneficial for longevity and the delay of the aging phenotype.
Although cells respond to elevations in cellular ROS by undergoing the above mentioned

countermeasures, elevated cellular ROS levels have also been correlated with tumor proliferation[9,35]. Tumor progression driven by oncogenes as RAS[36-38] and c-Myc[39] is even dependent on ROS production and expression of MnSOD can reverse cellular transformation[40,41]. Thus, next to accelerating the onset of aging, ROS increase the chance on tumorigenesis.

G1/S cell cycle checkpoint mechanisms and their (de)regulation in cancer and aging

In adult organisms, the majority of undifferentiated somatic cells reside in a dormant state, referred to as quiescence or G0. Their proliferation can be triggered by growth factor stimulation. Through members of the small GTPase family RAS these activate a variety of downstream signaling events including the linear cascades of PI3K-PDK1-PKB (also known as AKT) and RAF-MEK-ERK (Fig. 2a)[42]. These pathways initiate cell cycle progression in part through increased expression of Cyclin-D and repression of $p27^{kip1}$ (Ref[43]).

In the absence of growth factor signaling the retinoblastoma protein, pRb, blocks cell cycle progression through direct interaction with the transcription factor E2F and attenuation of its transcriptional activity (Fig. 2b)[43]. Growth factor signaling stimulates Cyclin-D production, that, through association with the cyclin dependent kinases (CDKs) 4 and 6, promotes initial phosphorylation of pRb[44,45]. This allows partial activation of E2F to induce Cyclin-E transcription, which in turn induces hyperphosphorylation of pRb through activation of CDK2. Once hyperphosphorylated, pRb is fully dissociated from E2F, which can then induce transcription of the DNA synthesis program. There are several points in this signaling cascade where the progression can be halted in case of unfavorable conditions. This involves activation of various CDK inhibitors (CKIs), including $p27^{kip1}$, $p21^{cip1}$ and $p16^{ink4a}$ (Fig. 2b), which can be activated by distinct stimuli.

$p27^{kip1}$

Next to limited Cyclin-D expression, hypophosphorylation of pRb in the absence of growth factors is mediated by the activation of the CKI $p27^{kip1}$. $p27^{kip1}$ binds CDK4/6 and, although context dependent, inhibits their activation by Cyclin-D[46,47]. Furthermore, $p27^{kip1}$ can repress CDK2 activation by cyclin-E[48], thus inhibiting G1/S transition at two distinct levels.

As a consequence of growth factor stimulation $p27^{kip1}$ is inactivatedthrough the activities of PKB. PKB can inhibit $p27^{kip1}$ directly through phosphorylation, which subsequently targets $p27^{kip1}$ for poly-ubiquitination and proteasomal degradation[49]. Additionally, PKB signaling indirectly inhibits $p27^{kip1}$ expression by reducing its transcription[50]. In absence of mitogenic stimuli $p27^{kip1}$ expression and nuclear accumulation is increased, consequently inducing cell cycle arrest. Importantly, this arrest is temporal and can be reversed upon stimulation with growth factors.

$p27^{kip1}$-/- mice show decreased lifespan and predisposition to develop neoplasms, establishing the role of $p27^{kip1}$ as a tumor suppressor[51]. Homozygous *$p27^{kip1}$* deletions are very rare in tumors. However, loss or inactivation of one allele is sufficient to promote tumorigenesis, identifying $p27^{kip1}$ as a haplo-insuficcient tumor suppressor. The functional relationship between $p27^{kip1}$ and Cyclin-D has become clear with the observation that $p27^{kip1}$ depletion rescues Cyclin-D deficiency in mice[52]. In parallel, Cyclin-D overexpression or $p27^{kip1}$ downregulation are frequently observed in a large variety of tumors, including breast cancer and melanoma[53,54]. Indeed, loss of $p27^{kip1}$ correlates with cancer progression and is used as a prognostic marker for survival[55] (See also Chapter 2). In contrast, in oncogenic RAF transformed melanomas $p27^{kip1}$

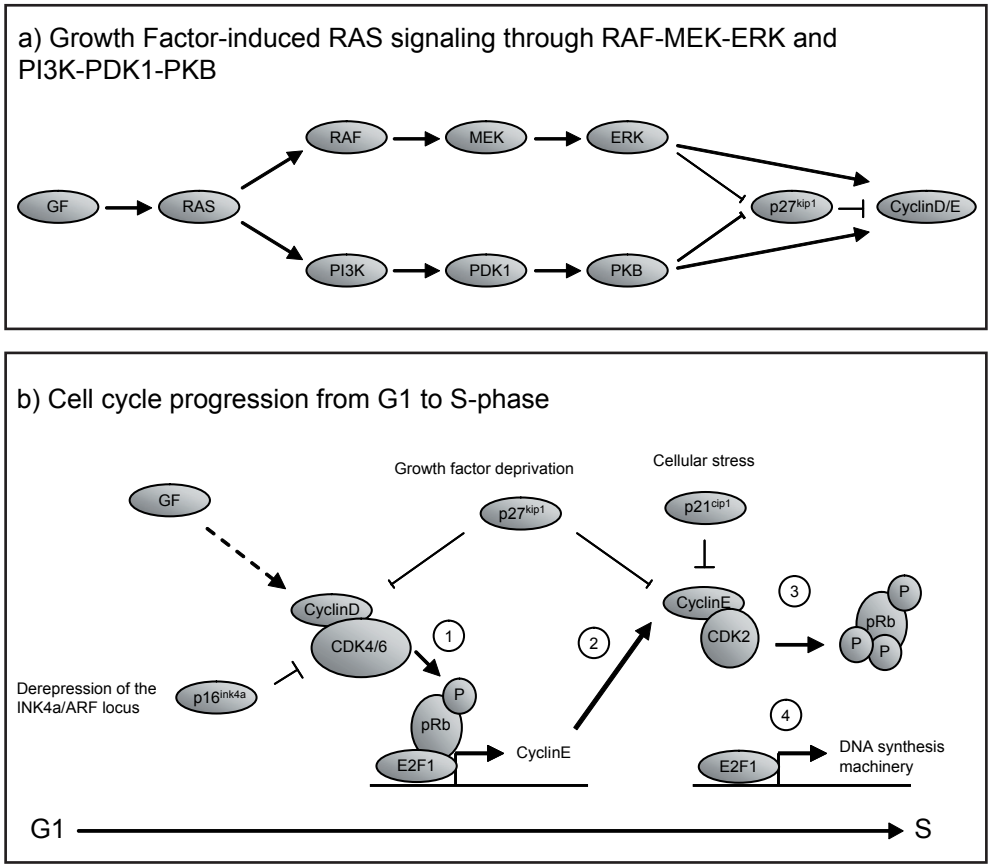

Fig 2: Simplified scheme of G1/S progression and arrest
In absence of nutrients and growth factors, the transcription factor E2F is sequestered by hypophosphorylated pRb transcription of the DNA synthesis machinery is prevented. **a)** Upon growth factor stimulation, such as insulin or EGF, the GTPase RAS is activated and induces Cyclin-D/E activation through the individual downstream signaling cascades of RAF-MEK-ERK and PI3K-PDK1-PKB. Interference with these pathways allows expression of $p27^{kip1}$ and a subsequent arrest in cell cycle progression. **b)** Following Cyclin-D expression the CDK4/6 complex becomes active and induced the initial phosphorylation of pRb (1). E2F is partially released from the repressive effect of pRb and induced transcription of Cyclin-E (2). Cyclin-E binding subsequently actives CDK2 and induces hyperphosphorylation of pRb (3). In this state pRb fully dissociated from E2F, allowing transcription of proteins that regulate DNA synthesis (4).

expression is downregulated due to increased degradation[56], however, $p27^{kip1}$ downregulation in oncogenic RAF or RAS mutated melanocytes does not correlate with proliferation per sé due to expression of other CKIs as $p16^{ink4a}$ and $p21^{cip1}$ that are activated in this background (See also Chapter 4).

p21^{cip1}

Like p27^{kip1}, p21^{cip1} can interact with the Cyclin-D/CDK4/6 and Cyclin-E/CDK2 complexes[43,46]. A difference to p27^{kip1} is that, rather than by growth factor deprivation, p21^{cip1} activation can be induced by oxidative stress[57], genotoxic stress[58] or through cytokines[59]. Next to Cyclin-E/CDK2 binding, p21^{cip1} can also interact with the Proliferating Cell Nuclear Antigen (PCNA). PCNA is the sliding clamp of the eukaryotic DNA polymerases and p21^{cip1} directly impairs PCNA dependent DNA replication and subsequent cell cycle progression[60]. p21^{cip1} expression can be stimulated by (hyper)activation of the RAF-MEK-ERK and the PI3K-PDK1-PKB pathways[61], however in response to the latter p21^{cip1} appears to be dissociated from CDK2[61], CDK4/6 and PCNA[62], allowing cell cycle progression.

p21^{cip1} has especially been studied in the background of p53 signaling. In response to DNA damage p21^{cip1} is transcribed in a p53 dependent manner[58]. Conversely, p21^{cip1} is essential for p53-mediated cell cycle arrest in this setting. Next to cell cycle arrest, p53 can induce apoptosis through various target genes[63]. Whether p53 signaling results in cell cycle arrest or apoptosis, depends on the severity of DNA damage. Nonetheless, in absence of p21^{cip1}, p53 activation results in apoptosis[64], indicating that p21^{cip1} is required to prevent p53-induced apoptosis.

Through these effects on cell cycle arrest and repression of apoptosis, p21^{cip1} plays a dual role in tumor suppression. On one hand, *p21^{cip1}-/-* mice show increased susceptibility for tumor development[65] and *p21^{cip1}-/-* MEFs show accelerated proliferation, coupled to defective (DNA damage-induced) G1-arrest[66]. On the other hand, p21^{cip1} rescues p53-mediated apoptosis induced by DNA damage, thereby ensuring survival[65,67]. In case tumor cells bypass p21^{cip1} mediated cell cycle arrest they therefore benefit from p21^{cip1} expression by remaining viable. Thus p21^{cip1} is a tumor suppressor, but through repression of apoptosis can promote tumorigenesis as well.

p16^{ink4a}

Like p27^{kip1} and p21^{cip1}, p16^{ink4a} can interact with CDKs 4 and 6 and prevent their activation by cyclin-D, but p16^{ink4a} does not interact with Cyclin-E/CDK2[68]. By sequestering CDK4/6, p16^{ink4a} can trigger the dissociation of p21^{cip1} and p27^{kip1} from this complex however, which subsequently do inhibit the Cyclin-E/CDK2 complex[69]. How p16^{ink4a} expression is induced under physiological conditions is not fully understood[69], although it is clear that p16^{ink4a} expression can be induced by oncogenic signaling (see below).

p16^{ink4a}-/- mice are susceptible to tumor formation[70,71], identifying a role in tumor suppression. Especially with regard to melanoma the importance of p16^{ink4a} has been well studied. For instance, in some families with inherited predisposition to melanoma p16^{ink4a} was lost[72]. Moreover, p16^{ink4a} expression is lost in ~50% of all melanomas, primarily in the more malignant types and correlates with poor prognosis[73]. However, in non-tumorigenic naevi p16^{ink4a} is expressed in a mosaic pattern[74,75], whereas the cells which they are comprised of are typically all oncogenically mutated. Thus, p16^{ink4a} mutation alone is insufficient to initiate tumorigenesis.

Together, p27^{kip1}, p21^{cip1} and p16^{ink4a} induce cell cycle arrest in the G1-phase and have been implicated in tumor suppression. While p21^{cip1} and p16^{ink4a} are activated in response to cellular damage or oncogenic signaling, p27^{kip1} arrests cell cycle progression in response to growth factor deprivation. The role of p27^{kip1} in cell cycle arrest by oncogenic signaling is not fully understood. We address the differential regulation of these CDK inhibitors in response to oncogenic stimuli in Chapter 4.

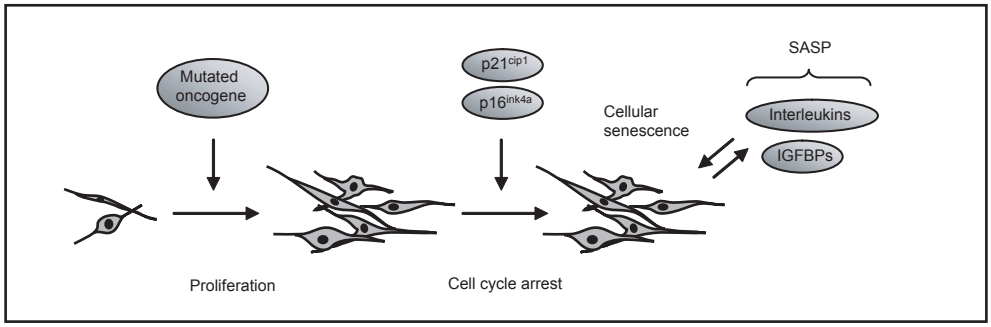

Fig 3: Generalized schematical overview of oncogene induced senescence
Cells that have obtained a hyperactivating mutation in an oncogene, such as observed for the RAS or RAF isoforms, show a brief burst in proliferation. Subsequently, the CDK inhibitors $p21^{cip1}$ and $p16^{ink4a}$ are activated which block cell cycle progression accompanied by secretion of chemokines and cytokines, such as Interleukins and IGFBP family members. This results in a secretory phenotype (SASP), which is required for maintenance of the senescence.

Mutational activation of oncogenes is insufficient to induce tumorigenesis due to activation of oncogene induced senescence (OIS).

In contrast quiescence, senescence is a permanent state of cell cycle arrest[76]. The first observation of cellular senescence was made by Hayflick and Moorhead, who noticed that in contrast to cancer cells, proliferation of human fibroblasts is halted after several rounds of division *in vitro*[77]. This type of senescence is also referred to as replicative senescence[76]. Conditions that lead to senescence are nowadays termed the "Hayflick factors"[76] and include telomere shortening, genotoxic and/or oxidative stress and activation of CKIs such as $p16^{ink4a}$ or $p21^{cip1}$ (Ref[78]). Although there are differences, these factors are also closely connected. For instance, each cell cycle division telomeres shorten, until they reach a minimal critical length. This induces oxidative and genotoxic stress and activates the DNA damage response machinery, which induces senescence through p53-mediated $p21^{cip1}$ transcription[79].

A fundamentally different type of senescence can be triggered by permanent activation of oncogenes. Oncogene activation is required for tumor progression, however their mutation alone is insufficient, as next to undergoing apoptosis, mutated cells can enter a permanent state of cell cycle arrest termed oncogene induced senescence (OIS)[80]. As is for instance the case for naevi, an *in vivo* example of OIS, these cells can remain viable for years without proliferating[74]. Initially, the *in vivo* relevance of OIS was controversial[81], however it is nowadays clear that OIS represents an important mechanism to suppress tumor progression[75,82,83] and re-activation of the OIS response may be clinically useful as a treatment for cancer. Importantly, although transformation by oncogenes as Myc and RAS is driven by ROS (see above), the production of ROS is also essential for the induction of OIS[39,84]. Whether dependency on elevated ROS levels is a common denominator for all types of OIS is not yet clear, as the mechanisms that induce OIS are diverse and differ at least partially for each oncogene[85-87]. An oncogene that triggers OIS when mutated is the Ser/Thr kinase BRAF.

The Ser/Thr kinase BRAF is an oncogene and its mutational activation can induce OIS

The RAF family of Ser/Thr kinases comprises three isoforms (A,B and C-RAF (or Raf-1), respectively). Oncogenic BRAF mutations are present in ~7% of all human tumors with high occurrence in thyroid carcinoma, colorectal cancer, ovarian cancer[88] and especially melanoma (~70%)[89]. In contrast, A- and CRAF mutations in cancer are rarely observed. Likely this is explained by the fact that BRAF can be activated in a one-step mechanism, whereas A- and CRAF activation requires an additional modification[90].

Over 40 mutations in BRAF have been identified, primarily in its kinase domain[91]. BRAF is inhibited through an auto-inhibitory loop, which interacts with the kinase domain through a hydrophobic interaction. The most predominant BRAF mutation is found at amino acid 600[89], in which the hydrophobic Val is replaced with the acidic Glu (V600E). This results in release of the auto-inhibitory loop[91] and a significant increase in downstream signaling towards MEK and ERK[89]. Oncogenic mutation of BRAF is insufficient to drive tumorigenesis due to induction of OIS[92,93]. $BRAF^{V600E}$ can induce OIS when either $p21^{cip1}$ or $p16^{ink4a}$ is inhibited individually[74,94,95], however inactivation of both leads to a strong suppression of OIS[96,97]. Thus, in the background of $BRAF^{V600E}$ signaling, $p21^{cip1}$ and $p16^{ink4a}$ regulate two independent cell cycle inhibitory responses and are functionally redundant for the induction of OIS.

As for BRAF, mutations in the three RAS isoforms, H-, K-, and N-RAS, are frequently observed in cancer[98]. Both downstream signaling of BRAF and RAS involves the MEK-ERK signaling pathway and also oncogenic activation of RAS can result in $p21^{cip1}$ and $p16^{ink4a}$ expression[93]. Regardless of these similarities, there are clear differences in OIS induction by both oncogenes. For instance, $HRAS^{G12V}$ expression in primary melanocytes induces senescence through the ER-associated unfolded protein response, whereas $BRAF^{V600E}$ does not[86]. This response is dependent on regulation of the PI3K/PKB pathway that is activated by $HRAS^{G12V}$, but not $BRAF^{V600E}$. Recently, it has been observed that activation of the PKB pathway is indeed required for $HRAS^{G12V}$ induced OIS[99]. Clearly however, $BRAF^{V600E}$ mutation alone can induce senescence, suggesting that PKB activity is not essential for OIS per se. Thus, there are fundamental differences in the mechanisms that drive senescence by oncogenic RAS and RAF.

Following the initial $p21^{cip1}$/$p16^{ink4a}$ induction, replicative and oncogene induced senescence are associated with a second, more gradual response through secretion of chemokines and cytokines. This results in a Senescence Associated Secretory Phenotype (SASP)[100]. Cytokines play important roles in inflammation and signal in an autocrine or paracrine fashion. Importantly, although SASP is important for the maintenance of senescence it is not sufficient and, CKI expression is still required for cell cycle arrest to occur[75].

The Forkhead box O (FOXO) transcription factor family

The family of Forkhead transcription factors plays a central role in processes as diverse as development, apoptosis, metabolism, differentiation and proliferation[101,102]. Members of this family are grouped in subclasses based on similarities within their DNA binding domain[102]. A subfamily that is of interest to the crosstalk between tumor suppression and aging is the Forkhead Box O subfamily, FOXO. In mammals, this family comprises four known members, FOXO1, 3a, 4 and 6. FOXO2 is identical to FOXO3a[103], FOXO3b represents a pseudogene[104] and FOXO5 is an ortholog expressed solely in the zebrafish *Danio rerio*[102]. FOXO1, 3a and 4 are ubiquitously expressed, although their expression levels vary between tissues and cell types. For instance, FOXO1 is highly expressed in adipose tissue, FOXO3a in the liver and neurons

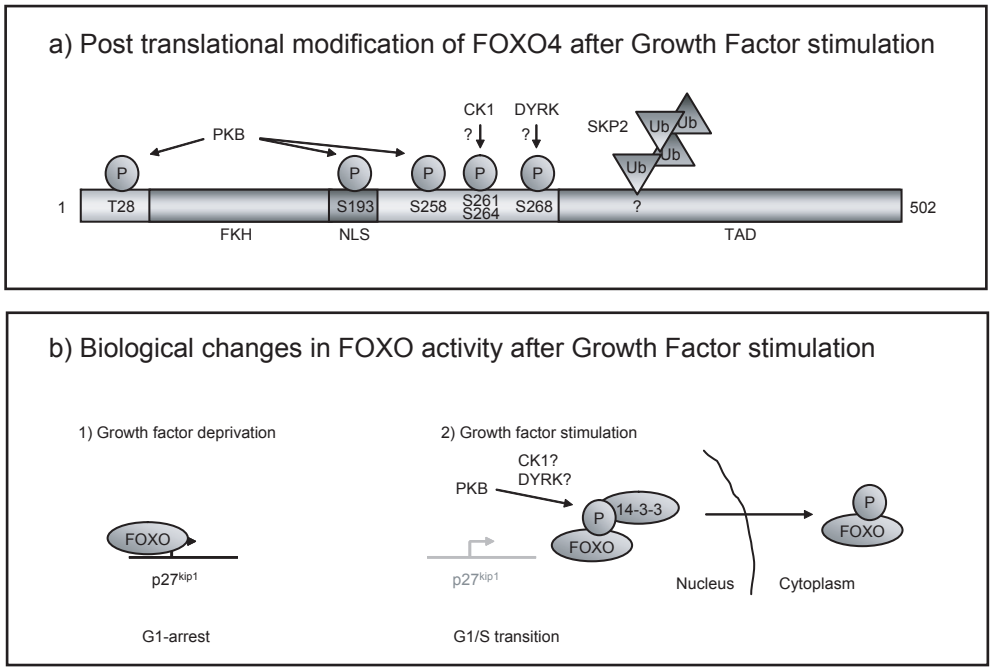

Fig. 4: Overview of post translational modifications of FOXO4 and changes in its biological activity upon growth factor stimulation
a+b) Stimulation with growth factors as insulin and EGF induce phosphorylation of FOXOs on three residues. In FOXO4 these include Thr28, Ser193 and Ser358, which are conserved in its homologs. Following PKB mediated phosphorylation FOXOs can be bound by the nuclear exportin 14-3-3, which facilitates their cytoplasmic relocalization. Subsequently, FOXOs can be ubiquitinated by the E3-ligase SKP2 and targeted for proteasomal degradation. Expression of the FOXO target gene $p27^{kip1}$ is decreased, allowing cell cycle progression.

and FOXO4 is more abundant in skeletal muscle and cardiac tissue[105]. FOXO6 expression is restricted to the brain[106].
Mice knocked out for individual *foxo* genes show distinct phenotypes. *foxo1-/-* mice die *in utero* due to angiogenic defects[105]. In contrast, *foxo3a-/-* mice are viable, but infertile[107,108] and no clear developmental defects were observed for *foxo4-/-* mice to date[109]. Irrespective, as far as known, all three homologs regulate similar target genes and bind to the same DNA sequence TTGTTTA/C[105,110]. Although not all effects up- and downstream of the individual FOXOs are similar for the other homologs, their molecular regulation is to a large extend similar[10]. Effects on their signaling are therefore typically generalized for the three classical homologs.

FOXOs regulate lifespan
Initial data on a biological function of FOXOs was established for the FOXO ortholog in *C. elegans*, DAF-16[111]. When environmental conditions for growth are unfavorable, juvenile *C. elegans* enter a stage of developmental arrest referred to as the dauer stage[112,113]. In this dormant state, which is DAF-16-dependent, the nematode is resistant to oxidative stress through

increased MnSOD levels[114]. Intriguingly, it was observed that deletion of the insulin receptor ortholog *daf-2* extended lifespan in a Daf-16/FOXO dependent manner[115-117]. Later on, similar effects were observed in other organisms[118].
Several reports investigated the role of FOXOs in human lifespan. First of all, genetic variations in FOXO genes are important in the regulation of lifespan in aged individuals through association with altered disease-risk[119]. Second, old women were found to express more FOXO3a mRNA compared to young[120]. Finally, in comparative studies between young versus centenarian populations (people aged 100 or older) in Japan[121] and Germany[122], some polymorphisms in FOXO3a were associated with exceptional old age. These data suggest that also in humans FOXO activity is connected to longevity.

FOXOs are inhibited by proliferation signals
At the molecular level, the observation that *daf-2* deletion increased lifespan through *daf-16* suggested that FOXOs are downstream targets of insulin/IGF signaling (IIS). As mentioned above, growth factors such as insulin and IGF can trigger activation of the linear cascade consisting of PI3K, PDK1 and PKB (See fig. 2a). Indeed, IIS represses DAF-16 through activation of the PI3K and PKB orthologs AGE-1 and AKT1/2, respectively[116,117,123,124]. Also, the effect of DAF-16 on lifespan is lost upon deletion of the PTEN homolog DAF-18, which is a phosphatase that competes with PI3K[125]. Together this identified DAF-16 as a target of IIS, directly inhibited by PKB.
Genes that are essential for growth and development are highly conserved between species[126] and later on, FOXOs were identified as downstream targets of IIS through direct phosphorylation by PKB in *Drosophila melanogaster*[127,128] and in mammals[129-131]. PKB can phosphorylate Thr28, Ser193 and Ser258 in FOXO4 and the equivalent residues in FOXO1 and FOXO3a[129,130,132,133] (Fig. 3). This results in nuclear exclusion through binding of the export carrier 14-3-3[134] and consequently inhibition of their potential to engage target gene transcription. In FOXO6 the latter PKB motif is not conserved and although its transcriptional activity is inhibited by PKB, FOXO6 remains located inside the nucleus[135].
Additional layers of regulation have been identified that follow PKB-mediated phosphorylation. For instance in case of FOXO1, IGF stimulates phosphorylation on the three PKB sites and subsequently by CK1 on two residues in the Nuclear Export Sequence[136]. Phosphorylation of these residues increases binding to the nuclear exportin Ran and results in cytoplasmic relocation of FOXO1. Whether this mechanism is conserved for FOXO3a and FOXO4 is unknown although these residues are conserved (Ser261 and Ser264, respectively). Similarly, FOXO1 can be phosphorylated on a conserved residue by the dual specificity kinase DYRK, also leading to cytoplasmic relocalization[137]. FOXO1 was recently identified as a substrate for methylation on at least two Arg residues adjacent to one of the PKB sites[138] (Arg248 and 250, preceding the PKB/SGK target site Ser253). Methylation on these residues prevents PKB-mediated phosphorylation, however the biological context in which these modifications are mode is not yet understood.
In response to growth factor deprivation FOXOs induce cell cycle arrest through induction of $p27^{kip1}$, whereas expression of other CKIs, such as $p21^{cip1}$ and $p16^{ink4a}$, is unaffected or repressed[50,139]. Conversely, inactivation of FOXOs allows cells to re-enter the cell cycle program again, an effect similar to what has been observed for $p27^{kip1}$ (Ref[140]). Although FOXOs can also directly repress expression of Cyclin-D[141], the inhibitory effects of FOXOs on cell cycle

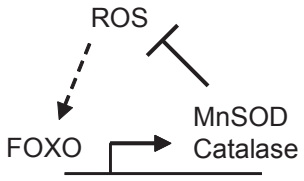

Fig. 5: Feedback loop of ROS mediated FOXO activation
Cellular ROS can lead to activation of FOXOs, which subsequently induce transcription of MnSOD and Catalase. These enzymes scavenge cellular ROS, providing a negative feedback loop towards FOXO activation.

progression are significantly repressed in *p27^{kip1}-/-* MEFs, indicating that p27^{kip1} is indeed an important FOXO target to mediate cell cycle arrest. The importance of *p27^{kip1}* as a FOXO target gene *in vivo* was recently further established when conditional *foxo1, 3a* and *4* triple knockout mice showed a significant decrease in p27^{kip1} mRNA expression[142]. Thus, in response to growth factor deprivation, FOXOs accumulate in the nucleus and induce a temporal, reversible cell cycle arrest through p27^{kip1}, not p21^{cip1} or p16^{ink4a} (Fig. 4).

FOXOs are activated by elevations in cellular ROS to induce a cell cycle arrest and ROS scavenging

Next to growth factor deprivation, FOXOs can be activated by elevations in cellular ROS[143]. This mode of regulation can occur in the presence of growth factors, indicating that signaling towards FOXOs by elevated ROS is dominant over the repressive effects of growth factors. Following their activation, FOXOs protect cells against ROS-induced damage through transcriptional activation of the DNA repair protein GADD45[144] and the ROS scavengers MnSOD[143] and Catalase[145]. This allows for an adaptive response to excessive ROS, whereby FOXO signaling is eventually repressed again through a negative feedback mechanism (Fig. 5). To allow time for ROS scavenging and damage repair to occur, FOXOs induce cell cycle arrest in G1-[146,147] or G2-phase[148]. Whether this is p27^{kip1} dependent is still unclear. FOXOs can be post translationally modified in response to ROS by various moieties that change their ability to transactivate target gene transcription. Thus far known, these modifications include phosphorylation, ubiquitination and acetylation, which can partially occur simultaneously (Fig. 6).

Phosphorylation

FOXO4 can be phosphorylated by the stress kinase JNK through a pathway that involves the small GTPase Ral[146,147]. This phosphorylation occurs at least on two residues in the C-terminus, Thr447 and Thr451, however, JNK can still phosphorylate FOXO4 when these residues are mutated. This points out the presence of additional JNK-target sites, an issue that is addressed in Chapters 2 and 4. Also in other organisms, FOXO signaling can be regulated by JNK. In *C. elegans*, for instance, JNK can directly phosphorylate DAF-16, which leads to increased lifespan[149]. Moreover, in *Drosophila*, the ortholog dFOXO represses cell cycle progression in response to JNK activation through a CKI ortholog[150,151]. Thus, JNK signaling towards FOXOs is evolutionarily conserved and can induce stress resistance and longevity.

Other studies showed that upon ROS signaling, FOXO3a and FOXO1 can become phosphorylated through MST1 signaling[152,153]. This decreases 14-3-3 binding, thereby increasing nuclear accumulation and transcriptional activity. It has been suggested that MST1 phosphorylates FOXO3a and 1 directly, however MST1 also induces JNK activation[154]. It is therefore yet unclear whether the effects of MST1 occur directly or indirectly.

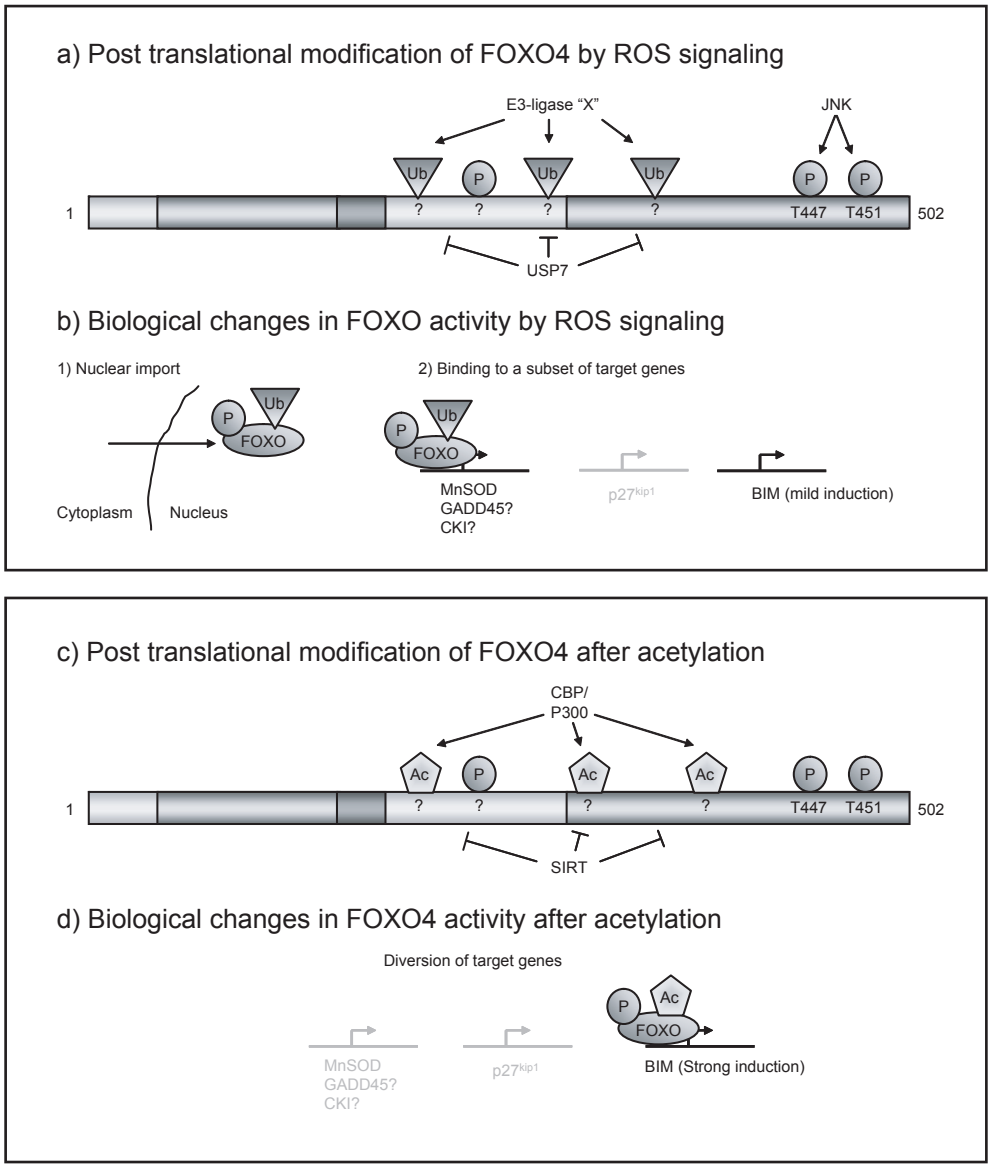

Fig. 6: Overview of post translational modifications on FOXO4 and changes in its biological activity upon ROS signaling

a+b) ROS signaling induces activation of JNK and a yet unknown E3-ubiquitin ligase. JNK induces phosphorylation on at least two residues on FOXO4. Whether this is conserved for fof its homologs is yet unknown. In parallel, FOXOs become mono-ubiquitinated and are located to the nucleus, where they induce transcription of a subset of genes different from those affected by nutrient deprivation. **c+d)** In a distinct response to ROS, FOXOs can become acetylated by P300/CBP, inducing a shift in target gene recognition. Acetylation of FOXO4 can be reversed by SIRT.

Ubiquitination

FOXOs are relatively stable proteins with a reported half-life of 8-10 hours[131,155]. FOXO turnover can be increased in response to growth factor stimulation by SKP2-mediated poly-ubiquitination and subsequent proteasomal degradation[156]. In response to ROS signaling FOXO stability does not notably change[155], nonetheless FOXOs are strongly ubiquitinated in the presence of excessive ROS. This type of ubiquitination reflects a fundamentally different modification than the poly-ubiquitination that results in proteasomal breakdown. Rather, FOXOs are mono-ubiquitinated on multiple residues. Mono-ubiquitination of some transcription factors, e.g. p53[157], has been shown to result in changes in subcellular distribution and in case of FOXOs, mono-ubiquitination leads to increased nuclear localization[155]. Later on, these ubiquitin moieties can be removed again by the de-ubiquitinating enzyme USP7/HAUSP, repressing its transcriptional activity. The mechanism by which USP7 mediates FOXO de-ubiquitination is still elusive. Also, through which E3-ligase ROS signaling induces FOXO ubiquitination is not yet known. These issues are addressed in Chapters 2 and 3.

Acetylation

Ubiquitination of FOXOs in response to cellular ROS is a relatively fast process (visible as early as 5 minutes post H_2O_2 treatment)[155]. Additionally FOXOs can be modified by acetylation, which occurs in a much slower response (around 60 minutes post H_2O_2 treatment)[158]. The enzymes that are responsible for acetylation of FOXOs are the acetyl transferases P300 and CBP. Conversely, de-acetylation of FOXOs can be mediated by the deacetylase SIRT1[158,159]. Lys199 and Lys211 of FOXO4 can both be ubiquitinated and acetylated. However, since the residues that are ubiquitinated and acetylated are only partially known it remains to be determined whether ubiquitination and acetylation of FOXOs are fully mutually exclusive[10].

The effect of acetylation on the biological activity of FOXO has not been without controversy, with reports showing activation[160,161] and inactivation of FOXO signaling[158] albeit on different target genes. Later it was shown that acetylation of FOXOs induces a genome-wide shift in target gene transactivation, explaining these data[162]. On a smaller scale co-expression of p300/CBP or knockdown of SIRT1 represses FOXO-induced $p27^{kip1}$ and GADD45 expression, whereas expression of BIM, an apoptotic FOXO target, is increased[159]. The consensus is however that SIRT1 activates the FOXO response towards ROS scavenging targets and cell cycle arrest[158]. The effects of SIRT1 on FOXO signaling is evolutionarily conserved and the *C. elegans* ortholog Sir2 increases lifespan in a *daf-16* dependent manner[163]. Since in the daf-16 dependent dauer stage proliferation is repressed and MnSOD levels are increased[114], this indeed suggests that de-acetylation by SIRT1 in mammals regulates FOXO target gene transcription in favor of cell cycle arrest and ROS scavenging.

Partner binding of FOXOs in response to ROS signaling

Next to post-translational modification, ROS signaling also induces interaction of FOXOs to a variety of proteins that modify their transcriptional activity. β-catenin is a downstream target of the canonical Wnt signaling cascade that regulates transcriptional activity of TCF and thereby induces proliferation[164]. This pathway is prone to hyperactivation and increased TCF activity is observed in a number of tumors. H_2O_2 represses TCF signaling and in response to this stimulus β-catenin was found to enhance the tumor suppressive function of FOXO[165]. Furthermore, FOXO binding to β-catenin represses the transcriptional activity of TCF[166]. Together these events divert

the cellular response from proliferation towards cell cycle arrest.
A different binding partner of FOXOs in response to ROS signaling is p53[167]. Although FOXOs and p53 both repress tumor progression their regulation appears opposite. In response to nutrient deprivation FOXO induces expression of SIRT by binding and inhibiting the repressive effect of p53 on its transcription[168]. SIRT thereby activates FOXO signaling and represses p53 signaling. Thus, in this background FOXO signaling inhibits p53. Conversely, in response to genotoxic stress p53 signaling is activated and induces activation of SGK, thereby repressing FOXO[169]. Also on other levels p53 and FOXO are conversely regulated. USP7 for instance activates p53 through de-ubiquitination[170], whereas it represses FOXO[155]. Additionally, P300 activates p53 signaling in response to genotoxic stess[171], while it represses the quiescence response of FOXO[158]. The functional outcome of the interaction between FOXO and p53 in response to elevated ROS levels is described in Chapter 5.

TGFβ signaling can induce p21^{cip1} transcription through FOXOs
Most studies have focused on the regulation of FOXO signaling in response to either growth factor deprivation or cellular ROS. However, FOXOs are also regulated through other stimuli that are not directly linked to these processes. One of these is TGFβ, which can induce a cell cycle arrest through p21^{cip1} (Ref[59,172]). TGFβ stimulation results in activation of a pathway involving SMAD2 and 3, ultimately leading to complex-formation of SMAD4 with various other transcriptional activators or repressors[43]. For a subset of immediate responses to TGFβ, SMAD4 interacts with FOXOs[59,173], which (upon c-myc inactivation) leads to increased transcription of GADD45 and p21^{cip1}. Whether FOXOs also regulate p21^{cip1} expression in response to other stimuli is unclear and is addressed in Chapter 4.

FOXO transcription factors are functionally redundant tumor suppressors
The observation that FOXO signaling can induce cell-cycle arrest, immediately suggested that FOXOs fulfill a role in tumor suppression[174]. Indeed, low expression of FOXO is associated with poor prognosis in a variety of tumors, including ovarian cancer[175] and prostate cancer[176]. Furthermore, tumor progression has been correlated with FOXO inactivation in a subset of breast tumors[177] and Paclitaxel treatment of sensitive breast cancer tumors induces FOXO mediated apoptosis, thereby repressing tumorigenesis[178]. Several examples have been reported in which deregulation of FOXO signaling promotes tumorigenesis. Initially, a potential role for FOXOs in this process was observed in malignancies where chromosomal translocations cause fusion of a *foxo* gene with that of another transcription factor. This is for instance the case for PAX3/7-FOXO1 chimera[179] or MLL-FOXO4[180], which strongly promote tumorigenesis. Together, these cases suggested that deregulation of FOXO signaling affects tumor progression. The conclusive evidence that established FOXOs as *bona fide* tumor suppressors however, came from a study in which conditional knockout of *foxo1, 3a* and *4* in mice shows strong lymphoma development[181]. Importantly, individual knockout mice of *foxo3a* or *foxo4* are viable and do not lead to a significant tumor prone phenotype[108,109,182]. Also, conditional knockout combinations of two *foxo* genes only produced mild effects on lymphogenesis[181]. This identified FOXOs as functionally redundant tumor suppressors. Thus, FOXOs play a role in both tumor suppression and aging, two seemingly independent processes. In Chapter 4, we describe an additional role of FOXOs in OIS, thereby further connecting these processes.

In contrast to FOXOs, FOXM1 enhances cell cycle progression

Opposite to FOXOs, cell cycle progression is positively influenced by members of the FOXM1 family. This family comprises three isoforms, FOXM1a, b and c[102]. FOXM1a is not conserved in mice and not transcriptionally active. Its function is still unclear. In contrast, FOXM1b and c are transcriptionally active[183]. As for FOXOs, signaling by FOXM1b and c is typically generalized.

FOXM1 is expressed in cycling cells and degraded by the APC/C complex at the end of cytokynesis[184,185]. *foxm1-/-* mice die *in utero* with developmental defects and contain less cells than wildtype embryos at the same stage due to diminished DNA replication and failure to enter mitosis[186]. Also, MEFs isolated from these mice show defects in the execution of mitosis[187]. In contrast to depletion, activation of FOXM1 results in accelerated G2/M transition[187,188]. FOXM1 expression is triggered in the G1/S-phase, where it is subsequently stabilized and activated through Cyclin-E/CDK2 mediated phosphorylation to promote G1/S transition[189]. Later, in G2/M phase FOXM1 is further phosphorylated by various kinases, resulting in increased stability and transcription of genes that regulate mitotic entry and progression, such as Cyclin-B1, CENP-F and Aurora-B[187].

FOXM1 contributes to G1/S transition by inducing SKP2-mediated ubiquitination and proteasomal breakdown of $p27^{kip1}$ and $p21^{cip1}$ (Ref[190,191]). *foxm1-/-* MEFs senesce prematurely with increased $p21^{cip1}$ expression[190]. Conversely, FOXM1 overexpression can protect against H_2O_2 induced senescence through downregulation of $p21^{cip1}$ (Ref[192]). These data suggest that FOXM1 plays a protective role against cellular senescence through induction of SKP2-mediated downregulation of $p21^{cip1}$.

Expression of FOXM1 strongly correlates with proliferation[193] and is frequently found upregulated in human carcinomas, including breast cancer and melanoma[194,195]. In contrast, FOXM1 depletion has been reported to suppress tumor progression[194,196-198] and targeting FOXM1 may be an interesting strategy to halt tumorigenesis. Thus, although FOXOs and FOXM1 are members of the same family of transcription factors they regulate G1/S transition and proliferation in an opposing fashion. Furthermore, although ROS can regulate FOXO signaling, no effects on FOXM1 signaling have yet been reported. A role for FOXM1 in ROS signaling is described in Chapter 7.

The peptidyl-prolyl isomerase Pin1 promotes cell cycle progression

Reversible phosphorylation of Ser/Thr residues followed by Pro constitutes a major cellular signaling mechanism[199]. The peptide bond connecting two neighboring amino acids can adopt either a *cis* or a *trans* conformation[200]. Local constraint mostly determines which state is preferred. Since a *trans* conformation is energetically more favorable, the far majority of the amino acid backbones are in this state. Pro is an exception to this rule, since for its imide bond the difference in fee energy is much smaller[201]. Especially for surface accessible Pro residues the majority of the imide bonds are in *cis* conformation. Interconversion between these unique states results in a change in the secondary structure of the protein, which may modify its functional properties.

Cis-to-*trans* conversion is a slow process that can be catalyzed by isomerases. Pin1 is a functionally unique isomerase that interacts only with phosphorylated Ser/Thr-Pro motifs, making it a peptidyl-prolyl isomerase[202]. Pin1 is a relatively small protein that consists of two domains; a substrate binding domain for which two Trp residues are critical (WW-domain), and a rotamase domain that catalyzes the *cis-trans* conversion (Fig. 7).

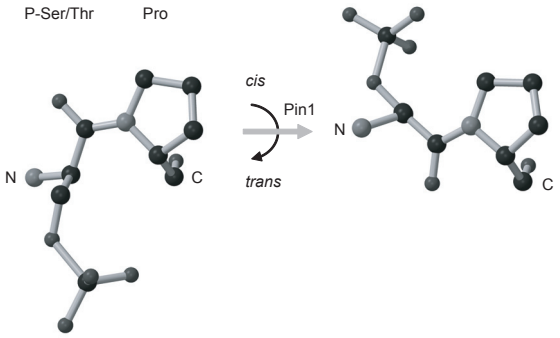

Fig. 7: Ball-and-stick model visualizing Pin1-mediated *cis*-to-*trans* isomerization
The peptide backbone of Ser/Thr residues followed by Pro occurs primarily in *cis* conformation. Pin1 can induce a conformational state to *trans* through isomerization of the peptide bond. Model generated in collaboration with Holger Rehmann

Pin1 is transcribed in response to E2F activation[203] and its activity correlates with cell cycle progression. Expression of Pin1 has been associated with tumor progression in a number of cases[204], especially breast cancer[205,206]. Pin1 regulates a variety of substrates that are also directly or indirectly connected to FOXO signaling, For instance, Pin1 induces stabilization of β-catenin by interfering with the interaction of its destruction complex[207]. Moreover, Pin1 is required for the acetylation and activation of p53 in response to genotoxic stress[208]. Although these proteins affect FOXO signaling, it is unknown whether Pin1 regulates FOXO activity directly, which will be elaborated on in Chapter 2.

Thesis outline
In this thesis, we provide new mechanistic insights in the regulation of FOXO signaling in response to elevated levels of cellular ROS. Furthermore, we describe how these changes modulate the effect of FOXOs on cell cycle progression and discuss how this may affect tumor suppression and aging. In cycling cells, FOXOs can be phosphorylated by elevated cellular ROS. Although acetylation and ubiquitination can be reversed, it was still unclear how the activation of FOXOs by ROS-induced phosphorylation could be counteracted. In Chapter 2, we show that ROS-induced phosphorylation on multiple Ser/Thr residues followed by a Pro generates a docking surface on FOXO4 for the peptidyl-prolyl isomerase Pin1. Thereby, Pin1 inhibits the transcriptional activity of FOXO4, as seen in diminished expression of its target genes Gadd45, $p27^{kip1}$ and $p21^{cip1}$. Pin1 is overexpressed in a wide variety of tumors and we show that high Pin1 levels inversely correlate with $p27^{kip1}$ expression in a panel of breast tumors, adding further molecular detail to the functioning of this disease and providing a possible entry for therapeutic intervention.
Although (multi)mono-ubiquitination of FOXOs is clearly an important event in their regulation by ROS, the E3-ligase that induces FOXO mono-ubiquitination was still unknown. In chapters 3 and 5, it is demonstrated that HDM2 regulates FOXO4 mono-ubiquitination in response to ROS. When expressed at low levels, HDM2 expression stimulates the transcriptional activity of FOXO4. In contrast, higher expression of HDM2 decreases FOXO4 expression and thereby

impairs the repressive effects of FOXO4 on cell cycle progression and proliferation. This may reflect a mechanism to repress FOXO activation by ROS signaling. Nonetheless, mono-ubiquitination of FOXOs increases their activity, indicating that endogenous HDM2 is required for FOXO activation in response to elevated ROS levels.

Oncogenic (B)RAF signaling has been shown to induce $p21^{cip1}$ expression and subsequent cell cycle arrest. In Chapter 4 we identify FOXOs as key regulators of this process. We show that oncogenic $BRAF^{V600E}$ induces FOXO4 phosphorylation through a linear pathway of $BRAF^{V600E}$-MEK-ROS-JNK signaling. Importantly, this results in a $p21^{cip1}$-dependent cell cycle arrest, independent of $p27^{kip1}$, suggesting a stimulus-dependent differential regulation of $p27^{kip1}$ and $p21^{cip1}$ by FOXOs. Oncogenic mutation of BRAF frequently occurs in melanocytes and results in oncogene induced senescence, OIS, at least in part through $p21^{cip1}$. We show that this pathway is conserved endogenously and that ectopic expression of FOXO4 in a $BRAF^{V600E}$-expressing melanoma cell line with low $p21^{cip1}$ levels (re-)induces $p21^{cip1}$-dependent OIS. This effect is conserved for other melanoma cells that express oncogenic BRAF, but not for melanoma cells that express oncogenic RAS or in which both are wildtype as is shown in an addendum to this chapter. Through their role in OIS, these data identify FOXOs as regulators in an antagonistic pleiotropy between tumor suppression and aging.

p53 and FOXO signaling oppose each other in response to growth factor deprivation or genotoxic stress (See above). However, 21^{cip1} is also a well known transcriptional target of p53. In Chapter 5, we show that FOXO4 and p53 interact in response to signaling by oncogenic BRAF. Moreover, FOXO4 mono-ubiquitination and nuclear localization is increased and p53 is phosphorylated on a residues associated with apoptosis and senescence, Ser46, seemingly independent of the DNA damage response pathway. Finally, we show that knockdown of FOXOs or p53 in the melanoma cell line with low $p21^{cip1}$ expression also used in Chapter 4 results in a significant increase in apoptosis.

Finally, in Chapter 6, we address the role of another Forkhead transcription factor, FOXM1, in ROS signaling. In contrast to FOXOs, FOXM1 activation promotes cell cycle progression and is upregulated in multiple types of cancer including melanoma[195]. We show that also FOXM1 is a downstream target of $BRAF^{V600E}$ signaling and that, in contrast to FOXOs, FOXM1 is activated by Pin1. FOXM1 activation inhibits G1/S arrest through transcriptional activation of the E3-ligase SKP2, which promotes proteasomal degradation of for instance $p21^{cip1}$ (Ref[191]). FOXM1 is highly expressed in numerous melanoma cell lines and primary tumors[209] and we show that in the cell line with low $p21^{cip1}$ expression, used in Chapters 4 and 5, FOXM1 and SKP2 levels are elevated. Thus, we propose a model in which regulation of FOXO4 and FOXM1 in response to $BRAF^{V600E}$ determines the fate of cell cycle progression. Finally, we discuss the findings presented in this thesis in light of tumor suppression and aging in Chapter 7.

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The Peptidyl-isomerase Pin1 regulates $p27^{kip1}$ expression through inhibition of Forkhead Box O tumor suppressors

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The peptidyl-isomerase Pin1 regulates p27^{kip1} expression through inhibition of Forkhead Box O tumor suppressors

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Abstract

The Forkhead box O (FOXO) protein family is an evolutionarily conserved subclass of transcription factors recently identified as bona fide tumor suppressors. Preventing the accumulation of cellular damage due to oxidative stress is thought to underlie its tumor-suppressive role. Oxidative stress, in turn, also feedback controls FOXO4 function. Regulation of this process, however, is poorly understood but may be relevant to the ability of FOXO to control tumor suppression. Here, we characterize novel FOXO4 phosphorylation sites after increased cellular oxidative stress and identify the isomerase Pin1, a protein frequently found to be overexpressed in cancer, as a critical regulator of p27^{kip1} through FOXO4 inhibition. We show that Pin1 requires these phosphorylation events to act negatively on FOXO4 transcriptional activity. Consistent with this, oxidative stress induces binding of Pin1 to FOXO, thereby attenuating its monoubiquitination, a yet uncharacterized mode of substrate modulation by Pin1. We have previously shown that monoubiquitination is involved in controlling nuclear translocation in response to cellular stress, and indeed, Pin1 prevents nuclear FOXO4 accumulation. Interestingly, Pin1 acts on FOXO through stimulation of the activity of the deubiquitinating enzyme HAUSP/USP7. Ultimately, this results in decreased transcriptional activity towards target genes, including the cell cycle arrest gene *p27^{kip1}*. Notably, in a primary human breast cancer panel, low p27^{kip1} levels inversely correlated with Pin1 expression. Thus, Pin1 is identified as a novel negative FOXO regulator, interconnecting FOXO phosphorylation and monoubiquitination in response to cellular stress to regulate p27^{kip1}.

Introduction

Forkhead box O (FOXO) transcription factors, consisting of mammalian FOXO1, FOXO3a, FOXO4, and FOXO6 are important downstream targets of the evolutionarily conserved phosphoinositide-3-kinase/Akt (PKB) signaling pathway (1, 2). Akt/PKB negatively regulates FOXO activity through direct phosphorylation by inducing their nuclear exclusion (3). FOXOs play a critical role in longevity, first shown in the nematode *Caenorhabditis elegans*, which had an extended life span upon deletion of the *daf-2*/insulin receptor (reviewed in ref. 4). This requires the FOXO orthologue *daf-16* and is characterized by an increase in stress resistance,

consistent with the notion that resistance to cellular stress correlates with longevity (5).
Stress resistance is also closely related to the onset of age-related diseases such as cancer (5, 6). Indeed, FOXOs were recently shown to be tumor suppressors in a number of cancers (1, 7). Mice depleted for FOXO1, FOXO3, and FOXO4 are characterized by the appearance of thymic lymphomas and hemangiomas (8). Intriguingly, cellular stress, in turn, also changes FOXO activity towards its target genes, thus allowing for an adaptive response to cellular stress (reviewed in ref. 9). Regulation of this process, however, is poorly understood but may be relevant to the ability of FOXO to control tumor suppression.
FOXOs regulate a number of transcriptional targets involved in stress resistance, survival and cell proliferation (reviewed in ref. 7). A key transcriptional FOXO target is the cell cycle arrest gene *p27^{kip1}* (8, 10). The cyclin-dependent kinase inhibitor p27^{kip1} is a haploinsufficient tumor suppressor that regulates the entry of cells from quiescence to cell cycle through inhibition of CDK2 (11). Interestingly, activation of FOXO in cells induces cell cycle arrest and quiescence, involving p130 and p27^{kip1} expression (12). Re-entry into the cell cycle involves down-regulation of p27^{kip1}, a process that is poorly understood but is thought to involve the phosphorylation and degradation of p27^{kip1} (11). In human cancer, expression of p27^{kip1} is often found deregulated and numerous therapies are being developed to restore its function. Localization defects and degradation are thought to be the main cause of p27^{kip1} deregulation. However, it was recently shown that p27^{kip1} levels in human cancer are transcriptionally regulated as well, albeit through unknown mechanisms (13). Regulation of protein activity often involves signaling through posttranslational modifications. These modifications either induce a structural change in the protein, thereby altering its activity, or induce the exposure of sites recognized by regulatory proteins. Pin1 is a peptidyl-prolyl isomerase that specifically recognizes phosphorylated serines and threonines flanked by a COOH-terminal proline residue (14). Pin1-mediated isomerization induces conformational changes in the peptide backbone. This leads to the altered function of its protein substrates and has been shown to be involved in numerous processes, including the regulation of cell proliferation and death (14). Pin1 is found to be overexpressed in many human cancers and is linked to tumorigenesis (15).
Here, we identify a novel regulatory pathway for FOXO signaling. In response to cellular stress, FOXOs are phosphorylated and recognized by Pin1. Evidence is provided that Pin1 negatively regulates FOXO monoubiquitination at the level of deubiquitination through HAUSP/USP7. This inhibits nuclear FOXO translocation in response to hydrogen peroxide–induced stress and ultimately leads to decreased transcription and expression of FOXO4 transcriptional targets, including *p27^{kip1}*. Notably, in a panel of primary human breast cancers, we found an inverse correlation between low 27^{kip1} levels and Pin1 expression.

Results

Identification of seven phosphorylated Ser/Thr-Pro sites on FOXO4

To better understand the FOXO4 adaptive response under conditions of cellular stress, a search for posttranslational modifications was initiated on FOXO4 by employing tandem mass spectrometry (MS). Flag-FOXO4 was purified from hydrogen peroxide–treated human embryonic kidney 293T (HEK293T) cells and subjected to proteolytic digestion. Five novel phosphorylated sites for FOXO4 were characterized, in addition to the previously characterized oxidative stress–sensitive phosphorylation sites Thr^{447}/Thr^{451} (20), suggesting that at least some of these seven sites are regulated through oxidative stress signaling (Fig. 1; Supplementary

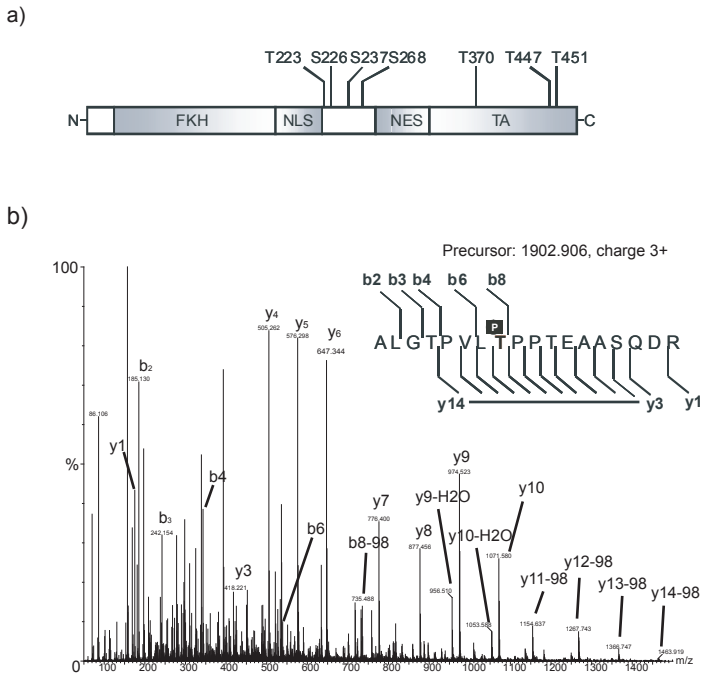


Fig. 1: FOXO4 is phosphorylated on multiple Ser/Thr-Pro sites in conditions of oxidative stress
a) Schematic representation of FOXO4, with the MS-identified phosphorylated residues. FKH, Forkhead domain; NLS, nuclear localization signal; NES, nuclear export signal; TA, transactivation domain. **b)** Representative tandem MS sequence of a FOXO4 tryptic peptide spanning amino acids 444 to 461. b and y ions are shown as well as the -98 Da H_3PO_4 loss and the phosphorylated Thr^{451} residue.

Table S1). TiO_2 columns, which specifically enrich for phosphorylated peptides (18), were used in MS analysis and confirmed these sites. In addition, we identified two double-phosphorylated peptides, Thr^{447}/Thr^{451} and Thr^{223}/Ser^{226} (Supplementary Table S1), indicating that FOXO4 can be multiphosphorylated.

Interestingly, all these sites consisted of phosphorylated Ser/Thr residues, followed by a Pro residue. These phosphorylated Ser/Thr-Pro motifs in proteins are potential recognition sequences for the peptidyl-prolyl isomerase Pin1, which has been proposed to regulate protein function through catalyzed *cis-trans* isomerization, thereby changing the local structure of its substrates (21). This prompted us to test the hypothesis that FOXO4 activity may be regulated by Pin1.

The FOXO4 transcriptional activity on *p27^{kip1}* is inhibited by Pin1

Previously, we have shown that FOXO4 regulates transcriptional activity of the tumor suppressor gene *p27^{kip1}* (10). Real-time reverse transcriptase qPCR was performed to test if Pin1 could affect FOXO4 transcriptional activity. Expression of FOXO4 increased the abundance of $p27^{kip1}$ mRNA by approximately 3-fold (Fig. 2a), similar to previous findings (10). Interestingly, co-

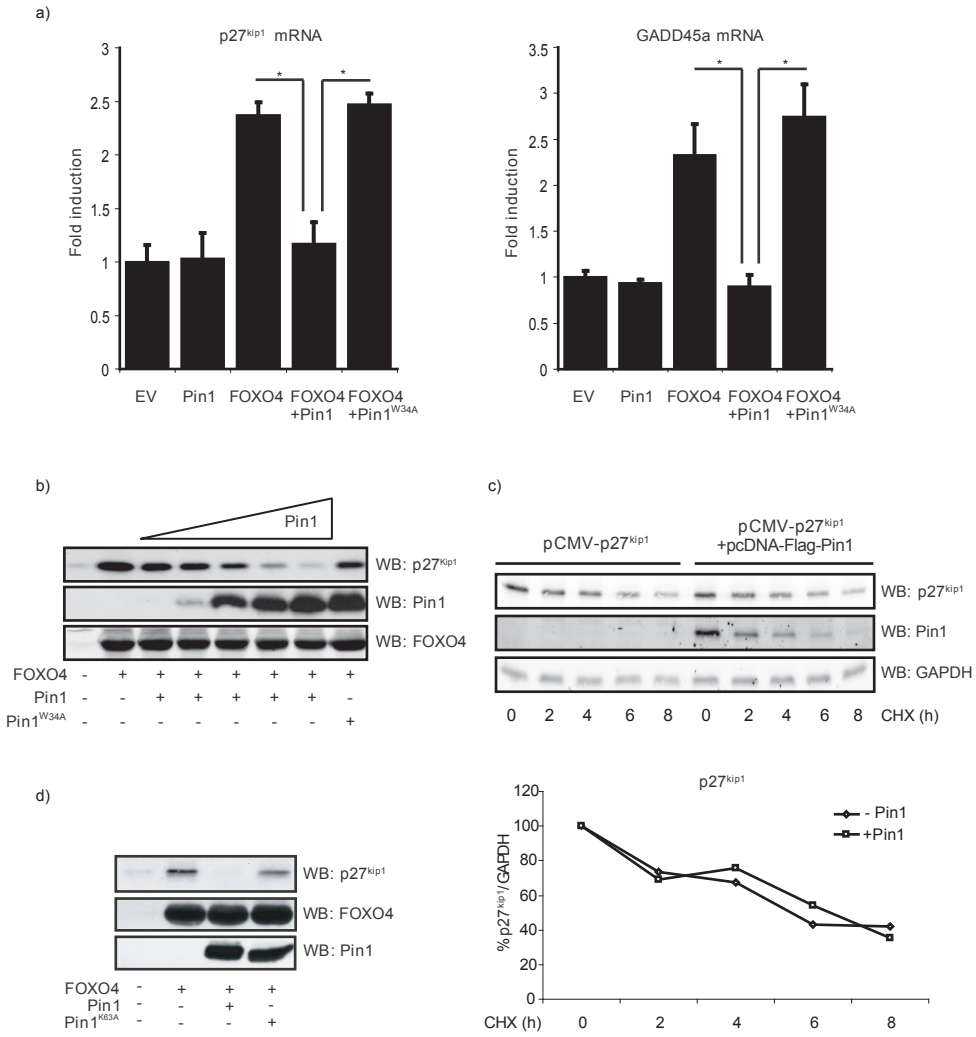


Fig. 2: Pin1 inhibits FOXO4-induced p27kip1 expression
a) Pin1 inhibits FOXO4 transcriptional activity. A14 cells were transfected as indicated together with pBabePuro. Total RNA extracted from puromycin-resistant cells was subjected to real-time reverse transcriptase-qPCR, using specific oligonucleotides for p27kip1 or Gadd45a mRNA. Samples were normalized against PBDG mRNA. *Columns*, mean of triplicates; *bars*, SD (*, $P < 0.05$, *t* test). **b)** Transient expression of Pin1 reduces p27kip1 protein levels. HEK293T cells were transfected as indicated together with pBabePuro. Puromycin-selected cells were analyzed by Western blotting. **c)** Pin1 has no direct effect on p27kip1 protein levels. HEK293T cells were transfected with a CMV-driven p27kip1 construct and/or Flag-Pin1. Prior to lysis, cells were treated for the indicated times with cycloheximide. Protein levels relative to GAPDH were detected and quantified (representative experiment). **d)** The Pin1 effects are dependent on its isomerase activity. HEK293T cells were transfected as indicated together with pBabePuro. Puromycin-selected cells were analyzed by Western blotting.

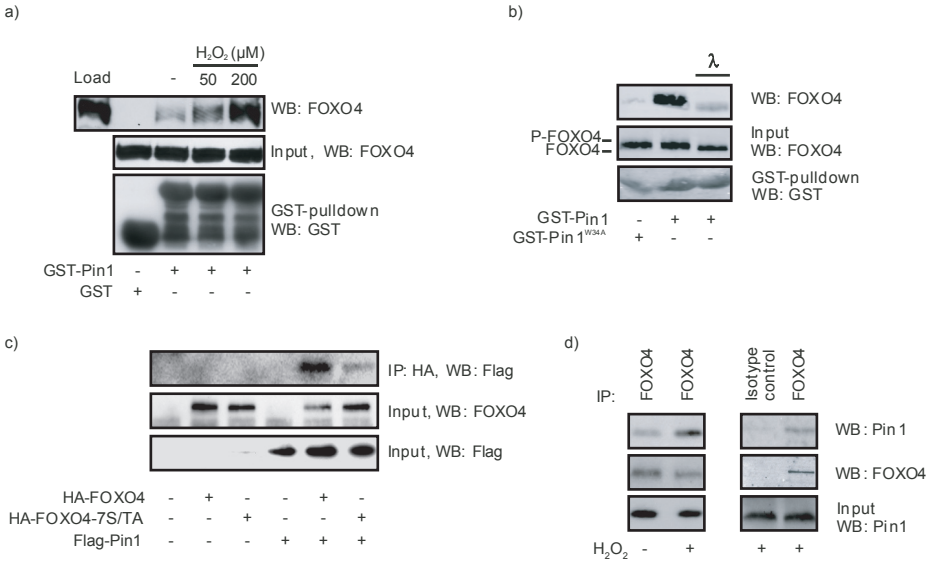
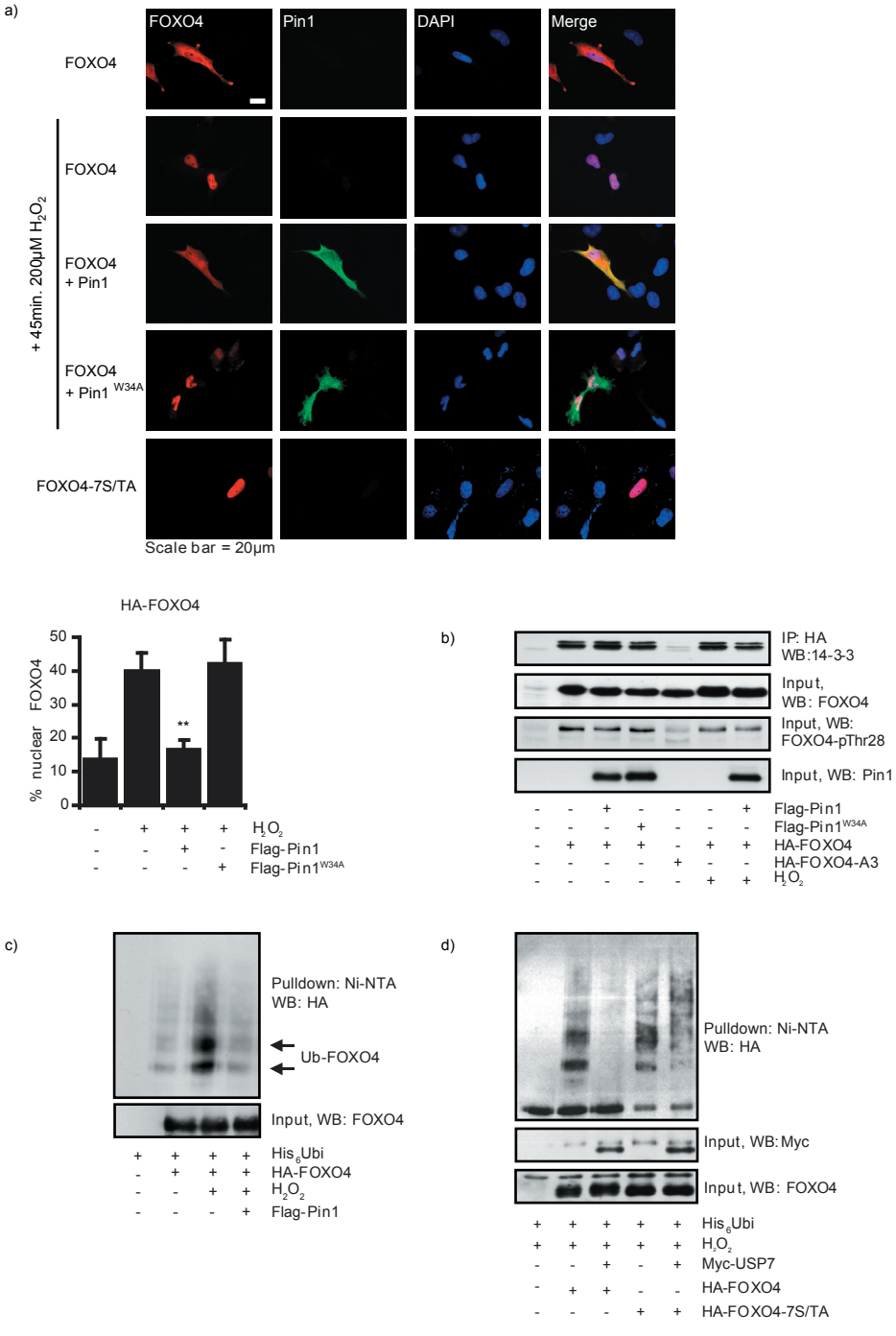


Fig. 3: Pin1 interacts with FOXO4 *in vitro* and *in vivo*
a) FOXO4 binds Pin1 in a GST pulldown assay. HEK293T cells were transfected with empty vector or Flag-FOXO4. Cells were treated for 1 h as indicated and lysates were subjected to a GST pulldown assay. **b)** FOXO4 dephosphorylation prevents Pin1 binding. HEK293T cells were transfected with Flag-FOXO4. One hour prior to lysis, cells were treated with 200 μmol/L of H_2O_2. FOXO4 was purified with an anti-Flag antibody, eluted off with Flag peptide, and treated with lambda phosphatase (λ) for 30 min, or left untreated and subjected to GST pulldown. **c)** Multiple phosphorylation sites are involved in FOXO4-Pin1 binding. HEK293T cells were transfected as indicated, stressed with 200 μmol/L of H_2O_2 for 1 h, and subjected to coimmunoprecipitation. **d)** Endogenous FOXO4 and Pin1 interact. HEK293T cells were treated as indicated, lysed, and either immunoprecipitated with a specific antibody for FOXO4 (N19) or an isotype control.

expression of Pin1 inhibited the FOXO4-induced increase in $p27^{kip1}$ mRNA levels (Fig. 2a). Pin1 harbors two functional domains, an NH_2-terminal WW domain, involved in substrate interaction (22), and a COOH-terminal PPIase domain critical for its isomerase activity (23). The Pin1 mutant $Pin1^{W34A}$, which fails to bind phosphorylated substrates (23), no longer inhibited $p27^{kip1}$ transcription, indicating that the WW domain is required for the Pin1-mediated $p27^{kip1}$ down-regulation. Similar results were obtained by using primers specific for the *Gadd45a* gene (Fig. 2a, right), another FOXO transcription target (24).
To determine if $p27^{kip1}$ protein levels change accordingly, FOXO4 was transiently expressed in the absence or presence of Pin1 and the mutant $Pin1^{W34A}$. Consistent with the reverse transcriptase qPCR results and previous data (10), FOXO4 increased $p27^{kip1}$ protein expression. However, Pin1 co-expression, but not mutant $Pin1^{W34A}$, abolished this increase in a dose-dependent manner (Fig. 2b). Similar results were found for $p21^{cip1}$ expression (Supplementary Fig. S1), another FOXO4 transcriptional target (25). Aside from FOXO-regulated transcription of $p27^{kip1}$, Pin1 could also affect $p27^{kip1}$ expression at other posttranscriptional levels. To test whether Pin1 could also have a direct effect on $p27^{kip1}$ protein stability, we determined the $p27^{kip1}$ half-life time

by performing cycloheximide experiments. Co-expression of Pin1 did not change the p27^{kip1} half-life (Fig. 2c), indicating that Pin1 does not affect p27^{kip1} protein stability.
Finally, we wanted to address if the regulation of p27^{kip1} is mediated by the isomerase activity of Pin1. For this, we co-expressed FOXO4 and a previously described isomerase-defective Pin1 mutant, Pin1^{K63A} (21). Whereas Pin1 inhibited FOXO4-induced p27^{kip1} expression, this mutant did not (Fig. 2d), indicating that the isomerase activity of Pin1 is required for Pin1-mediated inhibition of FOXO4 transcriptional activity. Taken together, these results show that Pin1 expression inhibits FOXO4 transcriptional activity, which is dependent on both the Pin1 substrate interaction domain and the isomerization domain. This prompted us to test if Pin1 and FOXO4 physically interact.

Cellular stress induces Pin1 binding to phosphorylated FOXO4
Glutathione-S-transferase (GST) pulldown experiments were performed to examine if Pin1 could physically interact with FOXO4. As shown in Fig. 3a, GST-Pin1, but not GST alone, specifically precipitated FOXO4 from HEK293T cell lysates. Interestingly, treatment of cells with increasing amounts of hydrogen peroxide prior to lysis strongly enhanced this interaction in a dose-dependent manner. In addition to cellular stress generated by hydrogen peroxide, we tested other stressors as well. GST pulldown experiments were performed on FOXO4-expressing cells that were treated with anisomycin, which is known to activate stress-activated protein kinases (26), doxorubicin and UV, both used to induce DNA damage. Binding of FOXO4 increased only when cells were treated with hydrogen peroxide or anisomycin, indicating specificity for these stressors (Supplementary Fig. S2). Next, GST pulldown experiments were performed to test if Pin1 could also interact with FOXO3a, a closely related FOXO family member. Like FOXO4, FOXO3a was found to bind to Pin1 (Supplementary Fig. S3), suggesting that the interaction is conserved among the FOXO family members. Moreover, consistent with the inability to reduce FOXO4 transcriptional activity, the Pin1^{W34A} mutant, incapable of binding Pin1 substrates, could no longer interact with FOXO4 and FOXO3a (Fig. 3b; Supplementary Fig. S3).
The above experiments indicate that Pin1 interacts with FOXO4 via its WW domain, an interaction that is enhanced in response to hydrogen peroxide. As mentioned, Pin1 binding is specific for phosphorylated Ser/Thr-Pro sites, of which MS on FOXO4 identified seven. Therefore, we analyzed whether the Pin1-FOXO4 interaction is actually dependent on the phosphorylation of FOXO4 on these sites. Pretreatment of hydrogen peroxide–treated FOXO4 with lambda phosphatase, leading to FOXO4 dephosphorylation as indicated by the loss of reduced motility in SDS-PAGE, abolished the ability of FOXO4 to interact with Pin1 (Fig. 3b), indicating that FOXO4 needs to be phosphorylated for its interaction with Pin1.
Next, we set out to determine which phosphorylated FOXO4 Ser/Thr-Pro sites are involved in the Pin1-FOXO4 interaction. Mutational analysis of single phosphorylation sites to Ala residues did not result in significantly decreased Pin1 binding upon peroxide stress, indicating that multiple phosphorylation sites are likely to be involved (data not shown). Further analysis using combinations of multiple Ala mutations consistently showed that the Pin1-FOXO4 interaction decreased progressively (data not shown) and is impaired for the FOXO4 mutant in which all putative Pin1 binding sites, as identified by MS, are mutated to Ala and is therefore referred to as FOXO4-7S/TA (Fig. 3c).
Finally, coimmunoprecipitation assays on endogenous FOXO4 and Pin1 in HEK293T cells showed that Pin1 specifically interacts with FOXO4 *in vivo*, an interaction that is increased


a)
FOXO4
Pin1
DAPI
Merge
FOXO4
+ 45min. 200μM H_2O_2
FOXO4
FOXO4 + Pin1
FOXO4 + Pin1 W34A
FOXO4-7S/TA
Scale bar = 20μm
HA-FOXO4
% nuclear FOXO4
50
40
30
20
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- + + + H_2O_2
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b)
IP: HA
WB:14-3-3
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- - - - + - - HA-FOXO4-A3
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d)
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+ + + + + His_6Ubi
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- + + - - HA-FOXO4
- - - + + HA-FOXO4-7S/TA

after hydrogen peroxide–induced stress (Fig. 3d). Taken together, these results show that Pin1 and FOXO4 interact *in vitro* and *in vivo*. This interaction is sensitive to cellular stress and is dependent on a functional WW domain of Pin1 as well as phosphorylation of FOXO4.

Pin1 regulates peroxide-induced FOXO4 monoubiquitination and nuclear localization through inhibition of HAUSP/USP7

Regulation of FOXO activity is often mediated through a change in cellular distribution. For instance, signaling through PKB/Akt inactivates FOXO4 through phosphorylation, resulting in nuclear exclusion (3). Alternatively, increased oxidative stress as generated by hydrogen peroxide results in increased nuclear localization (17, 27). The observed Pin1-dependent decrease in FOXO4 transcriptional activity could therefore be the result of a changed nuclear-cytoplasmic FOXO4 localization. Immunofluorescence of FOXO4 in A14 cells shows that FOXO4 is distributed in both the cytoplasm and nucleus under normal growth conditions but is redistributed predominantly to the nucleus when stimulated with hydrogen peroxide, consistent with previous observations (Fig. 4a; refs. 17, 27). However, ectopic expression of Pin1 prevented the redistribution of FOXO4 to the nucleus in response to oxidative stress. This phenotype depends on the interaction of FOXO4 with Pin1, as cells coexpressing Pin1^{W34A} do not inhibit FOXO4 relocalization. Furthermore, the FOXO4-7S/TA mutant that is impaired in binding to Pin1 had a nuclear localization in normal serum-containing medium (Fig. 4a). Treatment of cells with hydrogen peroxide and Pin1 coexpression did not change FOXO4-7S/TA localization (data not shown), indicating that phosphorylation of these sites is required to retain FOXO4 in the cytoplasm. Taken together, these results indicate that Pin1 inhibits FOXO4-mediated transcriptional effects by inhibiting its nuclear localization.

The nuclear-cytoplasmic shuttling of FOXO factors by growth factor signaling is regulated through binding of 14-3-3 proteins, which leads to the export of FOXO in a Ran/Crm1-dependent manner to the cytoplasm (3). To uncover the mechanism by which Pin1 regulates FOXO4 localization, the interaction between 14-3-3 and FOXO4 was explored. FOXO4, but not a mutant in which all three PKB/Akt sites are mutated (FOXO4-A3), could bind 14-3-3 (Fig. 4b; ref. 3). In the presence of Pin1, however, the interaction of FOXO4 with 14-3-3 is unchanged. Altered 14-3-3 binding therefore, could not explain the Pin1-mediated effects observed on FOXO4 localization. Importantly, phosphorylation of FOXO4 on Thr28 which is mediated by PKB/Akt did not change upon Pin1 expression, indicating that Pin1 does not affect PKB-mediated FOXO4 signaling.

Recently, we have shown that FOXO4 can be monoubiquitinated in response to oxidative stress, a

Fig. 4: FOXO monoubiquitination and nuclear FOXO4 localization is inhibited by Pin1

a) Pin1 inhibits FOXO4 nuclear localization in response to hydrogen peroxide stress. A14 cells were transfected and left untreated or treated as indicated, after which cells were fixed and stained. Transfected cells were used to score the localization of FOXO4. *Columns,* mean of two independent experiments performed in triplicate; *bars*, SE. The significance of changes as compared with FOXO4 only was confirmed by *t* test (**, $P < 0.005$). **b)** Pin1 does not change 14-3-3 binding to FOXO4. HEK293T cells were transfected with HA-FOXO4 or HA-FOXO4-A3, a FOXO4 mutant that can no longer bind 14-3-3. Forty-eight hours after transfection, cells were treated as indicated, lysed, and subjected to coimmunoprecipitation. **c)** Pin1 negatively regulates FOXO4 monoubiquitination. HEK293T cells were transfected and treated as indicated with 50 μmol/L of H_2O_2 for 15 min, lysed, and analyzed for ubiquitinated FOXO4 (Ub-FOXO4). **d)** USP7-mediated deubiquitination of FOXO-7S/TA is reduced. HEK293T cells were transfected with His_6-Ubi, HA-FOXO4, or HA-FOXO4-7S/TA or Myc-USP7 and analyzed as in c).

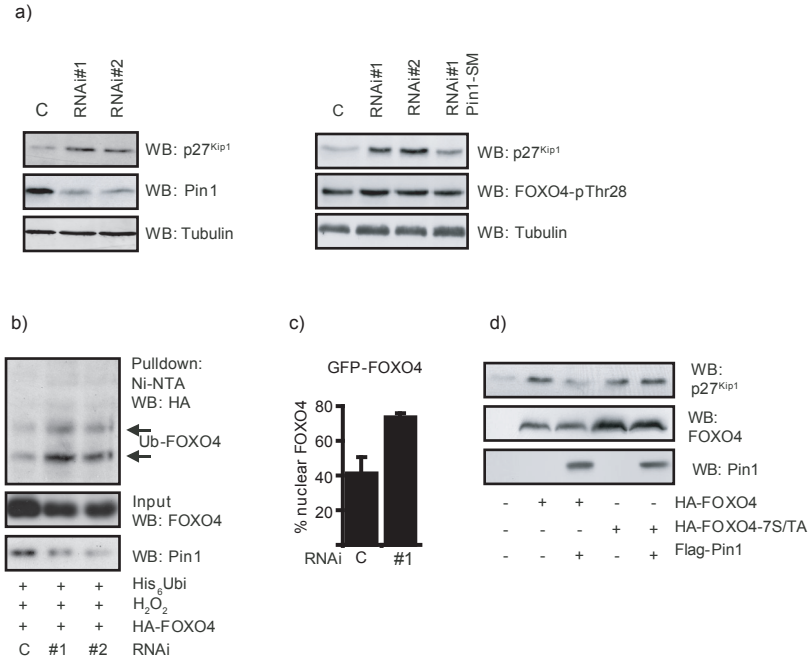

Fig. 5: Pin1 regulates p27^{kip1} through FOXO4
a) Pin1 depletion increases p27^{kip1} protein expression. HEK293T cells were transfected with either nontargeting RNAi oligonucleotides (C), or Pin1 RNAi oligonucleotides (#1 or #2) or in combination with pBabePuro, EV, or the Pin1 RNAi-silent mutant (Flag-Pin1-SM; right). Cell lysates were detected for p27^{kip1} and Pin1 expression by Western blotting. **b)** Knockdown of Pin1 increases FOXO4 monoubiquitination. HEK293T cells were treated with the indicated RNAi oligonucleotides and were subsequently cotransfected with His$_6$-Ubi and HA-FOXO4. A ubiquitination assay was performed. **c)** Knockdown of Pin1 increases FOXO4 nuclear localization. A14 cells were transfected with GFP-FOXO4 and control or Pin1 RNAi #1. Transfected cells were scored for FOXO4 localization. **d)** Pin1 inhibits p27^{kip1} expression through FOXO4. HEK293T cells were cotransfected with pBabePuro, EV, HA-FOXO4, HA-FOXO4-7S/TA, and/or Flag-Pin1. Cells were selected with puromycin for 48 h.

process that is reversed by the deubiquitinating enzyme HAUSP/USP7 (16). Monoubiquitination of proteins is a distinct modification leading to a change in cellular localization/signaling, clearly different from polyubiquitination that leads to proteasome-mediated degradation of protein substrates (28, 29). Indeed, monoubiquitinated FOXO4 leads to nuclear localization of FOXO4 independent of protein turnover (16). As shown in Fig. 4c, low amounts of hydrogen peroxide induce FOXO4 monoubiquitination. However, expression of Pin1 completely inhibits hydrogen peroxide–induced FOXO4 monoubiquitination. Because HAUSP/USP7 is the enzyme responsible for deubiquitinating FOXO4, we tested if the Pin1 binding impaired FOXO4-7S/TA mutant, could still be deubiquitinated by USP7. Surprisingly, whereas USP7 can efficiently and completely deubiquitinate FOXO4, deubiquitination is impaired for FOXO4-7S/ TA (Fig. 4d). This observation suggests that USP7-mediated deubiquitination on FOXO4 is in part dependent

	Low p27^{Kip1}	High p27^{Kip1}
N(100)	39	61
r	-0.41	-0.013
p	0.007*	0.81

Table I. p27^{Kip1} and Pin1 expression inversely correlate in low p27^{Kip1} expressing breast cancers.
* indicates significance, $p<0.01$ Spearman correlation test, 2-tailed.

of Pin1. USP7-mediated deubiquitination of FOXO4 involves binding of USP7 to FOXO4. Therefore, we tested whether FOXO4-7S/TA still binds to USP7. In coimmunoprecipitation assays, USP7 was found to interact equally well with both FOXO4 and FOXO4-7S/TA (Supplementary Fig. S4). Because FOXO4-7S/TA shows strongly impaired binding to Pin1, this result suggests that Pin1 enhances substrate, i.e. monoubiquitinated FOXO4 recognition of USP7. This also explains the small amount of FOXO4-7S/TA deubiquitination because USP7 still interacts with FOXO4. Together, these results show that Pin1 prevents hydrogen peroxide–induced FOXO4 nuclear localization and this likely results from the ability of Pin1 to inhibit hydrogen peroxide–induced FOXO4 monoubiquitination through stimulation of HAUSP/USP7-mediated FOXO4 deubiquitination.

Pin1 regulates p27^{kip1} through FOXO4

FOXOs are known regulators of p27^{kip1} expression, which as shown here, can be inhibited by Pin1. However, to our knowledge, Pin1 has not been described to affect p27^{kip1} expression. Therefore, we wished to address the role of endogenous Pin1 in regulating p27^{kip1} expression. To this end, we used small interfering RNA (siRNA) oligonucleotides against Pin1. Knockdown of Pin1 by two independent siRNAs increased the expression of p27^{kip1} protein, in agreement with the role of Pin1 in p27^{kip1} regulation and also in agreement with Pin1 acting as a negative regulator of FOXO (Fig. 5a). To exclude the possibility that the observed increase in p27^{kip1} expression is the result of off-target knockdown, we generated a siRNA-insensitive Pin1 construct by introducing a silent mutation in the RNAi recognition sequence of oligonucleotide no. 1 (Pin1-SM). Expression of this mutant in a Pin1 RNAi #1 background significantly rescued the RNAi-induced p27^{kip1} protein levels, indicating that the observed effects are specific for Pin1 (Fig. 5a, *right*). These results indicate that depletion of Pin1 increases p27^{kip1} expression *in vivo*. FOXO4-Thr28 phosphorylation did not change upon Pin1 depletion, consistent with the notion that Pin1 does not affect PKB-mediated FOXO4 signaling.

As shown above, Pin1 overexpression reduces FOXO monoubiquitination and consequent nuclear translocation. To further establish whether endogenous Pin1 can regulate FOXO monoubiquitination, we analyzed ubiquitination of FOXO4 after siRNA against Pin1. Indeed, knockdown of Pin1 increased FOXO4 monoubiquitination (Fig. 5b). Thus, p27^{kip1} regulation after Pin1 knockdown correlates with regulation of FOXO4 activity through monoubiquitination. Next, immunofluorescence experiments were performed to test if endogenous Pin1 also affects FOXO4 localization. Knockdown of Pin1 resulted in increased FOXO4 nuclear localization (Fig. 5c), consistent with the observations that FOXO4 monoubiquitination and transcriptional

activity are increased upon Pin1 depletion. Because of technical reasons, we were unable to perform double Pin1/FOXO knockdown experiments. Therefore, we used the FOXO4-7S/TA mutant defective in Pin1 binding to test if the effects of Pin1 on $p27^{kip1}$ are mediated through FOXO. FOXO4-7S/TA was able to induce $p27^{kip1}$ protein expression, indicating that these phosphorylation sites are not required for FOXO4 transcriptional activity per se (Fig. 5d). Importantly, whereas FOXO4-induced $p27^{kip1}$ expression is inhibited, Pin1 no longer inhibited FOXO4-7S/TA–induced $p27^{kip1}$ expression. Taken together, these results provide evidence that Pin1 regulates $p27^{kip1}$ expression through regulation of FOXO.

$p27^{kip1}$ expression inversely correlates with Pin1 expression in human breast cancers.
Many human cancers, particularly breast and prostate cancers, are characterized by high expression of Pin1 (30). Moreover, Pin1 contributes to oncogenic transformation as, for instance, Pin1 is essential for the transformation of mammary epithelial cells induced by Neu/Ras (31). Loss of $p27^{kip1}$ expression is also often found in many human cancers and correlates with poor survival (32, 33). This prompted us to determine if $p27^{kip1}$ expression correlates with Pin1 levels in human cancer. We had access to a panel of 100 human invasive ductal breast cancer tumors and stained them for $p27^{kip1}$ and Pin1. In agreement with previously published work, Pin1 was found to be overexpressed to various degrees in the majority of tumors (n = 77/100), as compared with normal ductal breast tissue, in which only weak staining of Pin1 was found (ref. 34 and Supplementary Fig. S5). We also stained for FOXO3 and FOXO4 and found no changes in expression staining, indicating that FOXO expression is not affected in our set of tumors (data not shown).
Expression of $p27^{kip1}$ was highly variable, varying from a complete loss to a staining comparable with normal breast tissue (Supplementary Fig. S5). Because loss of $p27^{kip1}$ is highly correlated with tumorigenesis and poor prognostic outcome, we divided the tumors into two distinct groups; one with normal $p27^{kip1}$ levels (defined as tumors with stronger staining than the statistical median, n = 61/100) and one with low $p27^{kip1}$ levels (n = 39/100). In the population with low $p27^{kip1}$ expression, a significant inverse correlation with Pin1 expression was found ($P < 0.01$, r = -0.42 Spearman correlation test, two-tailed). Importantly, this observation unlikely represents an artifact of Pin1 overexpression in the majority of tumors because we observed no correlation with the high $p27^{kip1}$–expressing group (Table 1). These results support the notion that Pin1 is important in $p27^{kip1}$ regulation and extends our observations to human breast cancer.

Discussion
Here, we provide evidence that the peptidyl-prolyl isomerase Pin1 acts as a negative regulator of FOXO transcriptional activity. Pin1, through its WW domain, binds FOXO directly in a phosphorylation-dependent manner. Furthermore, Pin1 regulation of FOXO requires, next to binding, the isomerase activity of Pin1. This indicates that Pin1-induced conformational changes within FOXO underlie the regulatory action of Pin1. Previously, it had been suggested that Pin1 catalyzes the dephosphorylation of the phosphorylated threonine/serine residues involved in Pin1 binding (14). Thus, Pin1-induced isomerization is thought to facilitate access of phosphatases like PP2A to the phosphorylated threonine/serine residues. Using phosphospecific antibodies against the PKB phosphorylation sites and phosphospecific antibodies against the sites identified in this study, we were unable to obtain any conclusive evidence that Pin1 binding to FOXO4 resulted in the dephosphorylation of these sites. In contrast, we observed that Pin1 binding to

FOXO4 regulates its monoubiquitination. Previous studies have indicated that Pin1 can modulate polyubiquitination and subsequent protein degradation of substrates with a short-lifetime, such as Myc, p53, p73, and β-catenin (reviewed in ref. 14). However, FOXOs are relatively stable proteins and monoubiquitination, in contrast, does not lead to proteasome-mediated degradation but instead often results in changes in signaling and subcellular localization (28, 29). Indeed, monoubiquitination of FOXO4 leads to nuclear localization and increased transcriptional activity but does not affect FOXO stability (16). Deubiquitination of FOXO4 is mediated by HAUSP/USP7 and similar to phosphatases, deubiquitinating enzymes such as USP7 require a cysteine within their catalytic activity to remove the ubiquitin moiety. We provide evidence that Pin1 increases USP7-mediated FOXO4 deubiquitination, which at least in part leads to decreased FOXO4 monoubiquitination. Thus, analogous to its role in regulating phosphatase activity towards its substrates, Pin1 may also regulate the activity of USP7 towards FOXO4. Nevertheless, Pin1-mediated attenuation of monoubiquitination represents, to the best of our knowledge, a previously uncharacterized mechanism of Pin1 substrate activity regulation.

Previously, we have shown that *p27^{kip1}* is a key transcriptional FOXO target gene in the regulation of cell cycle arrest and quiescence (10, 12), a notion that was recently supported by *in vivo* experiments (8). To maintain cell cycle arrest, FOXOs increase the cellular antioxidant capacity by up-regulating genes like MnSOD and catalase (9). In turn, increased cellular oxidative stress activates FOXOs, thereby creating a feedback loop that can prevent excessive cellular oxidative stress. Recent studies on conditional FoxO1/3/4 knockout mice underscore the critical importance of oxidative stress management by FOXOs in the hematopoietic stem cell compartment in keeping stem cells quiescent (35). Deletion of all three FOXO genes in hematopoietic stem cells results in stem cell depletion due to increased proliferation and concomitant increased intracellular levels of oxidative stress. Thus, FOXOs are important players in cellular oxidative stress management, and as such, it is of importance to understand how cellular oxidative stress impinges on FOXO. Under conditions of cellular stress, FOXO is phosphorylated, and here, we identified several novel sites of phosphorylation, which we show are involved in Pin1 binding after hydrogen peroxide treatment. Therefore, regulation of FOXO activity through Pin1 could present a novel mechanism of how cells re-enter the cell cycle from a quiescent state especially in response to cellular stress. In this respect, it is interesting to note that embryonic fibroblasts derived from Pin1 knockout mice show defective G0-G1 entry (36). In line with this, we observe that knockdown of Pin1 increases p27kip1 expression.

In contrast, overexpression of Pin1 inhibits FOXO4-induced p27^{kip1} expression. *p27^{kip1}* is a haploinsufficient tumor suppressor gene and decreased p27^{kip1} expression can result in loss of cell cycle control, a hallmark of carcinogenesis (37). Loss of p27^{kip1} is prevalent in human cancer and correlates with poor survival (33). Unlike loss of, for instance, the tumor suppressor p53, p27^{kip1} function is lost because of a transcriptional or posttranslational down-regulation rather than a genetic defect (11, 13). Moreover, it has been shown that Pin1 is overexpressed in numerous cancer tissues, notably in breast cancer and prostate cancer, and contributes to the malignant transformation of cancer cells (15). The significance of our data, in which Pin1 inhibits p27^{kip1} expression, is underscored by our findings in human breast cancers, where we find that loss of p27^{kip1} expression strongly correlates with high Pin1 expression. Thus, in some percentage of tumors, loss of p27^{kip1} may result from increased Pin1 expression inhibiting FOXO function. Reconstitution of nuclear FOXO activity has been shown to arrest both normal and transformed cells in G1, to inhibit soft agar growth, and to inhibit xenograft growth in nude

mice (38, 39). Disruption of the Pin1-FOXO interaction aimed at increasing the nuclear FOXO pool and restoring $p27^{kip1}$ transcription could therefore represent a potential therapeutic point for intervention.

Materials and Methods

Cell culture and transfection
HEK293T and A14 cells (3T3 fibroblasts stably expressing the insulin receptor) were maintained in DMEM (Cambrex), 10% FCS, penicillin/streptomycin and 0.05% glutamine.

Constructs and RNAi
pMT2-HA-FOXO4, pMT2-Flag-FOXO4, pMT2-GFP-FOXO4, CMV-$p27^{kip1}$, His_6-Ubi, and pBabe-puro have been described (16). pcDNA-His-Pin1, pcDNA-His-$Pin1^{W34A}$, pGEX-GST-Pin1, and pGEX-GST-$Pin1^{W34A}$ were gifts from Drs. C. Fila and P. van der Sluijs (Department of Cell Biology, University Medical Centre Utrecht, Utrecht, The Netherlands). Pin1 was Flag-tagged NH_2-terminally by PCR, using oligonucleotides sequences to Flag-tag Pin1 (forward oligonucleotide, 5'-CCGGATCCATGGACTACAAGGATGACGACGACAAGGCGG ACGAGGAGAAGCTG; reverse oligonucleotide, 5'-CGAATTCTCACTCAGTCGGAGGATGATG-3'). PCR products were digested with BamHI and EcoRI and cloned in pcDNA3.1. $Pin1^{K63A}$ was made by site-directed mutagenesis oligonucleotides sequences to make K63A mutant to Pin1 (forward, 5'-CGTGACTGGCTGTG CGCCACCAGCAGGTGCGA-3' and its reverse complement strand). Nontargeting RNAi duplex (C), RNAi oligonucleotides specific for Pin1 (Pin1 #1: sense, 5'-GCCAUUUGAAGACGCCUCGdTdT-3'; Pin1 #2: sense, 5'-CGUCCUGGCGGCAGGAGAAUUdTdT-3') were purchased from Dharmacon. RNAi was transfected with OligofectAMINE (Invitrogen). Additional cotransfections were performed 8 h after RNAi transfection.

Antibodies
The antibodies against FOXO4 (834) and HA (12CA5) have been described (10). The following antibodies were purchased: MPM2 (Upstate), FOXO4-phospho-Thr28 (Upstate), Pin1 (R&D systems), $p27^{Kip1}$ (BD Biosciences), FOXO4-N19, 14-3-3, and glutathione S-transferase (GST; Santa Cruz), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon), tubulin, and Flag-M2 (Sigma).

GST pulldown assays, coimmunoprecipitations, and Western blot analysis
GST proteins were coupled to glutathione agarose beads, washed twice with radioimmunoprecipitation assay buffer [RIPA; 20 mmol/L Tris-HCl (pH 8.0), 1% TX-100, 0.5% NaDoC, 5 mmol/L EDTA, 150 mmol/L NaCl, protease and phosphatase inhibitors]. Cells were lysed in RIPA, cleared and incubated with the glutathione agarose beads for 2 h at 4°C. Subsequently, beads were washed, boiled in Laemmli sample buffer, and analyzed by Western blotting. Lambda phosphatase was purchased from New England Biolabs. For coimmunoprecipitation studies, 50 μL of Protein-A Sepharose beads were precoupled to 1 μg of the indicated antibody. Cells were lysed in RIPA or, in the case of endogenous coimmunoprecipitation, in 20 mmol/L of Tris-HCl (pH 8.0), 1% NP40, 10% glycerol, 1 mmol/L of $MgCl_2$, 1 mmol/L of EDTA, 150 mmol/L of NaCl, protease and phosphatase inhibitors, and
incubated as described previously (17).

Mass spectrometry
Purified Flag-FOXO4 from hydrogen peroxide–treated HEK293T cells (200 μmol/L for 1 h) was digested with trypsin, subtilisin, and/or elastase (Roche). If required, samples were enriched for phosphorylated peptides using TiO_2 microcolumns, as described (18). Samples were subjected to nanoflow LC (Agilent 1100 series) coupled to a quadrupole time-of-flight tandem mass spectrometer (Micromass Waters). Data were processed and subjected to database searches using MASCOT software (Matrixscience). The identified peptides were confirmed by manual interpretation of the spectra.

Reverse transcriptase quantitative PCR
The expression of endogenous *$p27^{kip1}$* and *Gadd45a* genes in A14 cells was examined by reverse transcription of total RNA followed by real-time quantitative PCR (qPCR) on an ABI cycler using Sybr Green (ABI), with oligonucleotides specific to $p27^{kip1}$, Gadd45a and PBGD oligonucleotides specific for mouse $p27^{kip1}$ (forward, 5'-CTGGGTTAGCGGAGCACTGT-3'; reverse, 5'-GGAAAACAAAACGCTTCTTCTTAG-3'), mouse Gadd45a (

forward, 5'-AGACCGAAAGGATGGACACG-3'; reverse, 5'-TGACTCCGAGCCTTGCTGA-3'), mouse PBGD (forward, 5'-GGCAATGCGGCTGCAA-3'; reverse, 5'-GGGTACCCACGCGAATCAC-3').

Protein stability assay
HEK293T cells were transfected with a CMV driven p27^{kip1} construct and/or Pin1. Next, cells were treated with 10 µg/mL of cycloheximide for the indicated times. Protein levels were detected and corrected for GAPDH levels by Odyssey.

Immunohistochemistry
Paraffin blocks containing formaldehyde-fixed breast cancer tissues were sectioned, deparaffinized, and rehydrated and stained essentially as described (19) with the primary antibodies for Pin1, FOXO3a, FOXO4, and p27^{kip1}. These were detected using a poly-Hrp anti-Ms/Rb/Rt (ImmunoLogic) and developed with diaminobenzidine, followed by counterstaining with hematoxylin.

Ubiquitination assay
The monoubiquitination assay was performed as described (16). HEK293T cells were transfected with RNAi oligonucleotides and/or the indicated constructs. Forty-eight hours posttransfection, cells were treated as indicated and lysed in 8 mol/L of urea, 10 mmol/L of Tris-HCl (pH 8.0), 100 mmol/L of Na_2HPO_4/NaH_2PO_4, 0.2% TX-100, 5 mmol/L of NEM, and protease inhibitors. Ubiquitinated proteins were precipitated using Ni-NTA agarose beads and the experiment was analyzed by Western blotting.

Immunofluorescence
A14 cells were plated and transfected on coverslips. Immunostaining was performed 40 h after transfection (3). Paraformaldehyde-fixed cells were incubated with anti-FOXO4 (834) or anti-Pin1, followed by goat anti-mouse IgG conjugated to Alexa488 or goat anti-rabbit IgG conjugated to Alexa568. Nuclei were visualized with 4',6-diamidino-2-phenylindole. Fluorescence was captured using a Zeiss Axioskop microscope.

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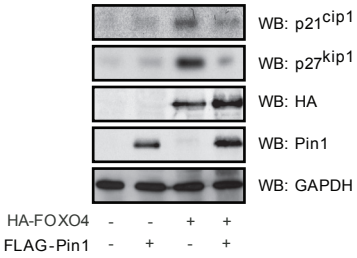

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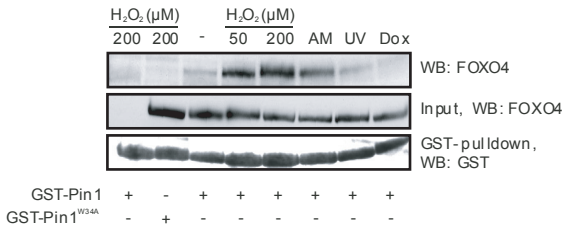

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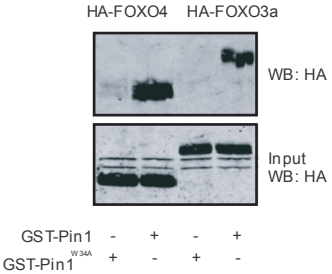

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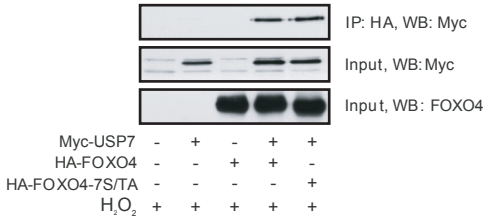

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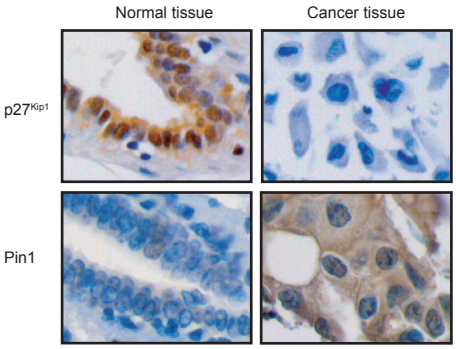

Fig. S1. Pin1 inhibits FOXO4-induced p21cip1 expression. HEK293T cells were transfected as indicated together with pBabePuro. Puromycin selected cells were analyzed by WB for p21cip1 expression. **Fig. S2.** Hydrogen peroxide and anisomycin induce FOXO4-Pin1 interaction. HEK293T cells were transfected and left untreated or treated 1 h. prior to lysis with H_2O_2. Lysates were subjected to GST-pulldown. (50 or 200μM), anisomycin (AM), doxorubicin (Dox) or exposed to UV light. **Fig. S3.** FOXO3a binds Pin1. HEK293T cells were transfected, treated with 200μM H_2O_2 for 1h. and subjected to GST-pulldown. **Fig. S4.** USP7 binds equally well to FOXO4 and FOXO4-7S/TA. HEK293T cells were transfected with HA-FOXO4, HA-FOXO47S/TA or Myc-Usp7. Prior to lysis, cells were treated with 200μM H_2O_2 for 1h. FOXO4 was co-immunoprecipitated with an HA antibody and the experiment was analyzed by WB for Myc reactivity. **Fig. S5.** Loss of p27kip1 correlates with high Pin1 expression. Breast cancer tissue sections were stained for p27kip1 and Pin1 and visualized with diaminobenzene. The left panels show normal mammary tissue, staining high for p27kip1 and low for Pin1 on ductal cells. The panels on the right show a representative human ductal breast cancer staining, with low p27kip1 and high Pin1 staining.

3

Mdm2 induces mono-ubiquitination of FOXO4

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Mdm2 induces mono-ubiquitination of FOXO4

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Background: **The Forkhead box O (FOXO) class of transcription factors are involved in the regulation of several cellular responses including cell cycle progression and apoptosis. Furthermore, in model organisms FOXOs act as tumor suppressors and affect aging. Previously, we noted that FOXOs and p53 are remarkably similar within their spectrum of regulatory proteins [1]. For example, the de-ubiquitinating enzyme USP7 removes ubiquitin from both FOXO and p53. However, Skp2 has been identified as E3 ligase for FOXO1, whereas Mdm2 is the prime E3 ligase for p53.**

Principal Findings/Methodology: **Here, we provide evidence that Mdm2 acts as an E3 ligase for FOXO as well. *In vitro* incubation of Mdm2 and FOXO results in ATP-dependent (multi)mono-ubiquitination of FOXO similar to p53. Furthermore, *in vivo* co-expression of Mdm2 and FOXO induces FOXO mono-ubiquitination and consistent with this result, siRNA mediated depletion of Mdm2 inhibits mono-ubiquitination of FOXO induced by hydrogen peroxide. Regulation of FOXO ubiquitination by Mdm2 is likely to be direct since Mdm2 and FOXO co-immunoprecipitate. In addition, Mdm2-mediated ubiquitination regulates FOXO transcriptional activity.**

Conclusions/Significance: **These data identify Mdm2 as a novel E3 ligase for FOXOs and extend the analogous mode of regulation between FOXO and p53.**

Introduction

Forkhead box O (FOXO) transcription factors have recently gained considerable attention because of their potentially critical role in aging [1,2]. The paradigm in this respect is the *C. elegans* FOXO ortholog DAF-16. Lifespan extension through a number of genetic and non-genetic interventions in these nematodes requires at least in part DAF-16 [2]. Especially, the effects of lowered insulin signaling critically depend on DAF-16 and DAF-16 acts downstream of the insulin signaling pathway consisting of the lipid kinase phosphoinositide-3 kinase (PI-3K) and the Serine/Threonine protein kinase B (PKB/AKT). PKB directly phosphorylates DAF-16/FOXO and this results in nuclear exclusion and therefore reduced DAF-16/FOXO transcriptional activity [3,4].
Aging may also result from the accumulating damage caused by reactive oxygen species (ROS)

[5]. In this respect regulation of cellular anti-oxidant capacity by DAF-16/FOXO provided rationale for its effect on lifespan. Interestingly, FOXO itself is also regulated by ROS and treatment of cells with hydrogen peroxide, which increases cellular oxidative stress, results in nuclear translocation of FOXO [6,7]. FOXOs are regulated through a multitude of post-translational modifications (PTMs) including phosphorylation, acetylation and ubiquitination (reviewed in [1]). Whereas PKB-mediated phosphorylation results in exclusion of FOXO from the nucleus, the mechanism and/or PTMs responsible for relocalization to the nucleus after increased cellular oxidative stress remain poorly understood. However, the enzymes responsible for adding these modifications are remarkably similar between p53 and FOXO (for a discussion see [1]). With respect to the regulation of ubiquitination we previously identified USP7 as a de-ubiquitinating enzyme for FOXO4 [8] and USP7 is also a de-ubiquitinating enzyme for p53 [9]. FOXOs are relatively stable proteins with a half-life of approximately 8–10 hrs in untransformed cells [8]. In transformed/oncogenic cells, especially cells transformed through activation of PI-3K signaling, FOXO protein half-life is shortened [10–12]. This is likely due to PI-3K/PKB mediated upregulation of Skp2 in these cells, as Skp2 has been identified as an ubiquitin E3 ligase responsible for FOXO poly-ubiquitination and degradation [10]. Consistent with Skp2 regulation by PKB and FOXO being degraded in a Skp2-dependent manner, several other PKB targets have been reported to be degraded in a Skp2-dependent manner as well [11,13]. Previously, we demonstrated that the signaling function of FOXO4 is regulated by mono-ubiquitination especially after increased cellular oxidative stress [8]. Mono-ubiquitination correlates with increased nuclear localization of FOXO and hence increased transcriptional activity. Consequently, USP7 expression inhibited FOXO4 transcriptional activity due to de-ubiquitination of FOXO4 and re-localization to the cytosol. To further understand the regulation of mono-ubiquitination of FOXOs we searched for ubiquitin E3 ligases that would ligate ubiquitin onto FOXO4. Here, we report the identification of Mdm2 as an E3 ligase for FOXO4 that mediates mono-ubiquitination of FOXO4 after increased cellular oxidative stress.

Results

In our attempt to identify potential E3-ligases for FOXOs we co-expressed several candidate E3 ligases with FOXO and noticed that Mdm2 co-expression resulted in an apparent reduction in FOXO4 expression (Fig. 1a). In transient expression experiments reduced protein expression can occur through various mechanisms including promoter squelching. However, as Mdm2 induces ubiquitin-mediated breakdown of target proteins we first analyzed whether Mdm2 could catalyze ubiquitin addition to FOXO4 *in vitro*. To this end we reconstituted a functional E1-E2-Mdm2 ubiquitin ligase system using purified proteins and added GST-FOXO4 as a substrate. Only in the presence of rATP this resulted in the addition of multiple ubiquitin moieties to GST-FOXO causing a laddering indicative for poly-ubiquitination or multiple mono-ubiquitination (Fig. 1b). Importantly, this *in vitro* reconstituted system displayed similar activity towards GST-p53, but not to GST alone, suggesting that in vitro FOXO is as good a substrate for Mdm2 as is p53. Mdm2 uses a C-terminal RING finger domain, critical to its function as an E3-ligase. Mdm2 mutant lacking this domain was tested and found unable to ubiquitinate FOXO4 (Fig. 1b), highlighting the specificity of Mdm2 and the dependency on its E3-ligase activity to mono-ubiquitinate FOXO4 *in vitro*. To further address whether the *in vitro* observed laddering represents poly-ubiquitination or multiple mono-ubiquitination, a number of ubiquitin mutants instead of wild-type ubiquitin were analyzed in the *in vitro* ubiquitination assay. Using ubiquitin-

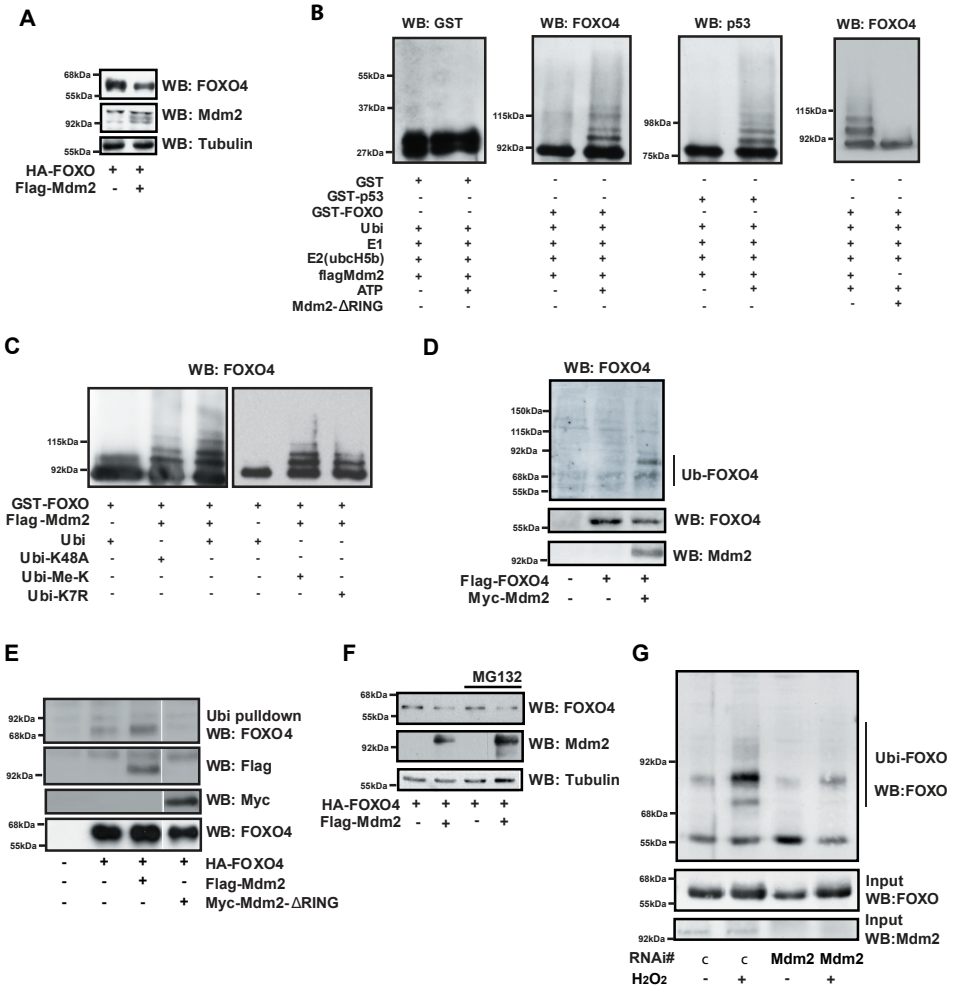

Fig. 1 a) Mdm2 co-expression decreases FOXO protein levels. FOXO4 and Mdm2 were co-expressed in HEK293T cells. Cell lysates were probed by western blot analysis as indicated. **b)** Mdm2 ubiquitinates FOXO4 and p53 *in vitro* with similar stoichiometry. Purified Mdm2 or Mdm2-delta-RING was incubated with GST-FOXO4, GST-p53 or GST alone, together with Ubiquitin and E1-E2-*in vitro* recombinant proteins. Ubiquitination was measured 2 h., after addition of rATP. **c)** FOXO4 is multi-mono-ubiquitinated by Mdm2. The experiment was performed as in (b), using ubiquitin proteins (Ubi-K48A, Methylated Ubiquitin and Ubi-K7R) that are unable to poly-ubiquitinate. **d)** Mdm2 ubiquitination of FOXO *in vivo*. HEK293T cells were transfected with myc-Mdm2, Flag-FOXO4 or control vector together with His-Ubiquitin. After 24 h, cells were treated with 50 μM H_2O_2 for 15 min, and subjected to a ubiquitination assay (see Methods). **e)** FOXO4 mono-ubiquitination *in vivo* depends on the Ring finger of Mdm2. HEK293T cells were transfected with indicated constructs and subjected to a ubiquitination assay. **f)** Mdm2 mediated FOXO4 downregulation is MG132 insensitive. MCF7 cells were transfected with the indicated constructs and treated with MG132 o/n. **g)** FOXO4 mono-ubiquitination is dependent on Mdm2. HEK293T cells were treated with either control (c) or human Mdm2 RNAi and subsequently transfected with His-Ubiquitin and HA-FOXO. Cells were treated with 50 μM H_2O_2 for 15 min and subjected to a ubiquitination assay.

K48A, defective in K48-mediated ubiquitin branching which targets proteins for the proteasome, and ubiquitin-K7R and methylubiquitin, both defective in mediating poly-ubiquitination, all resulted in same pattern of Mdm2-mediated GST-FOXO laddering (Fig. 1c). Taken together, these results clearly show that in an *in vitro* reconstituted system Mdm2 can act as an E3-ligase for FOXO4 and that in contrast to what has been reported for p53, Mdm2 catalyzes multiple mono-ubiquitination of GST-FOXO4 rather than poly-ubiquitination.

Next, we tested whether Mdm2 also can ubiquitinate FOXO4 *in vivo*. Co-expression of flag-FOXO4 and myc-Mdm2 induced mono-ubiquitination of FOXO4 (Fig. 1d). We did not observe substantial poly-ubiquitination, also not in the presence of the proteasome inhibitor MG132 (data not shown). Also, the delta-RING domain Mdm2 mutant did not induce FOXO4 mono-ubiquitination (Fig. 1e). Albeit consistent with our *in vitro* data this questions the mechanism underlying the reduced detection of FOXO4 protein by immunoblotting after overexpression of Mdm2. Reduced detection of FOXO4 concomitant with Mdm2 overexpression would normally be taken to indicate proteasomal degradation of FOXO4 and this should be reversed by MG132 treatment. However, we did not observe substantial rescue of FOXO4 protein expression after MG132 treatment, despite observing accumulation of auto-poly-ubiquitinated Mdm2 species, which indicates that the MG132 treatment did work (Fig. 1f). Again this is consistent with the observed lack of poly-ubiquitination and suggests that either FOXO4 is degraded through another pathway for example caspase-mediated breakdown, or alternatively, that (multiple)

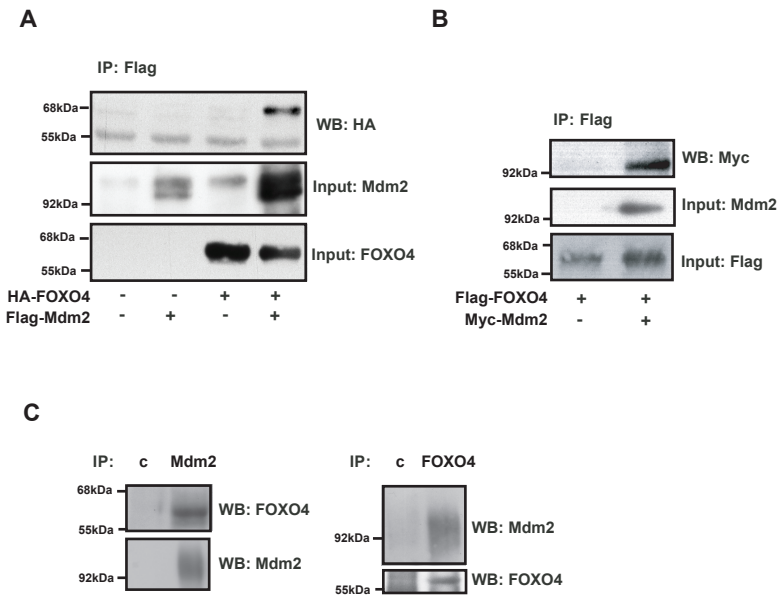

Fig. 2 a) HA-FOXO4 and Flag-Mdm2 were co-expressed in HEK293T cells, co-immunoprecipitated for Flag and probed as indicated. **b)** HEK293T cells were transfected with Flag-FOXO4 and Myc-Mdm2. Lysates were co-immunoprecipitated with Flag antibody and proteins were detected as indicated. **c)** FOXO4 and Mdm2 interact *in vivo*. HEK293T cells were immunoprecipitated for FOXO4, Mdm2 or isotype controls (c) and probed as indicated. Prior to Co-Ip, cells were treated for 15 min. with 200 μM hydrogen peroxide.

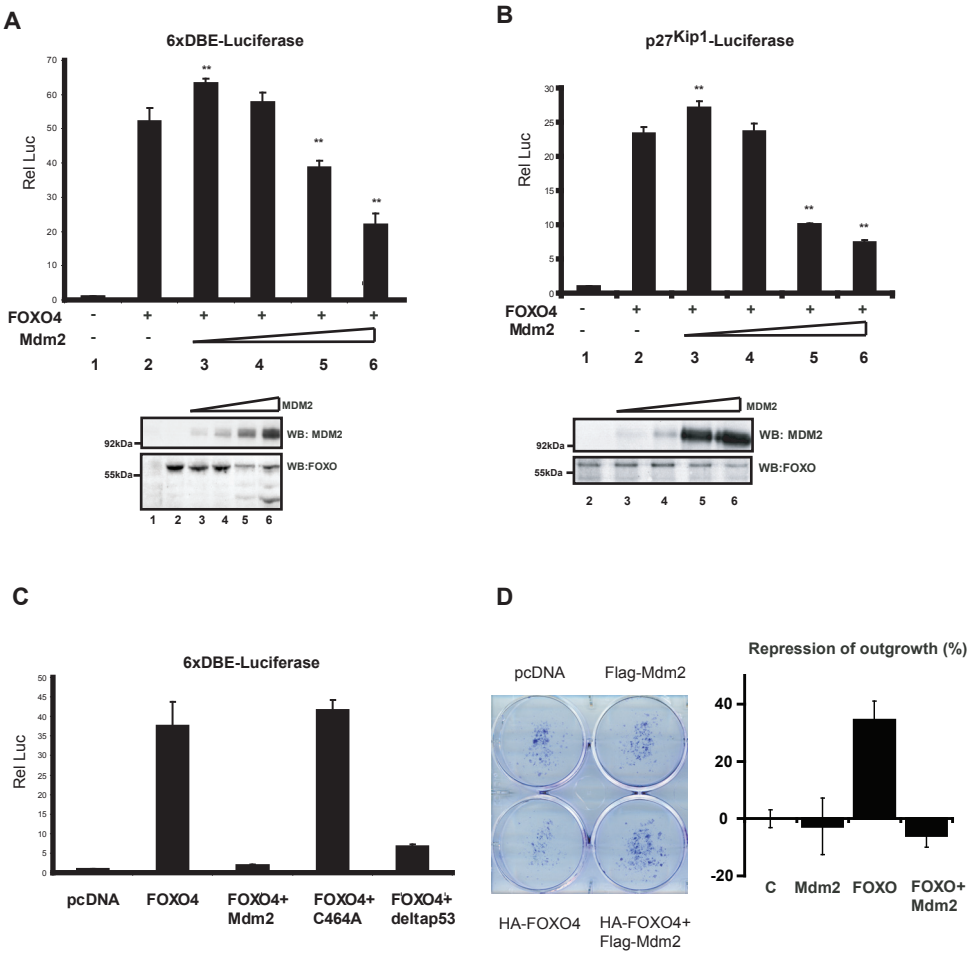

Fig. 3 a+b) Mdm2 regulates FOXO activity in a bell-shaped manner. MCF7 cells were transfected with indicated constructs, TK-Renilla and either a luciferase construct with 6 perfect FOXO4 DNA binding elements (6xDBE) (a) or a luciferase construct under the control of the endogenous $p27^{kip1}$ promoter (b). Representative data are shown as mean±s.d. of triplicates. The significance of changes in the lanes with Mdm2 as compared to wild-type FOXO (lane 2) was confirmed by t-test (**p<0.005). **c)** FOXO regulation of Mdm2 is independent of p53 but dependent on its RING finger activity. Luciferase activity was measured in MCF7 cells, 24 h after transfection with indicated constructs, TK-Renilla and (6xDBE). **d)** FOXO mediated cell cycle arrest is blocked by Mdm2. A14 cells were transfected by indicated constructs together with pbabePuro. Colony outgrowth of puromycin selected cells was monitored after 10 days.

mono-ubiquitinated FOXO4 is targeted to a cellular compartment for example PML bodies from which it is not efficiently extracted. To confirm our MG132 experiments, we performed FOXO4 half-life studies. Transfection of Mdm2 did not affect the half-life of co-transfected FOXO4 (Sup. fig. a). Taken together these results indicate that Mdm2 expression does not lead to FOXO4 degradation by means of regulating its protein stability through the proteasome. This finding is consistent with our MG132 experiments. Thus in all approaches we come to the conclusion that Mdm2 does not substantially affect FOXO4 protein half-life.

Finally, to further substantiate a role for endogenous Mdm2 in regulating the ubiquitin status of FOXO4 *in vivo*, we used siRNA against the human ortholog of Mdm2 (Sup. fig. b). As reported previously, increasing cellular oxidative stress by treating cells with hydrogen peroxide induced mono-ubiquitination of both FOXO4 and FOXO3a [8] and Sup. fig. c). Importantly, siRNA-mediated knockdown of Mdm2 significantly reduced hydrogen peroxide-induced mono-ubiquitination of FOXO4 (Fig. 1h). Together, these data provide compelling evidence that Mdm2 can mediate FOXO4 mono-ubiquitination *in vitro* and *in vivo*.

To test the possibility that Mdm2 could directly regulate FOXO we analyzed binding between Mdm2 and FOXO4. Upon coexpression of Flag-Mdm2 and HA-FOXO4, FOXO4 was co-immunoprecipitated with Mdm2, and *vice-versa* (Fig. 2a,b). Consistent with our *in vitro* data, this result suggests that Mdm2 directly binds and regulates FOXO4, rather than through regulating the activity of de-ubiquitinating enzymes such as USP7. Next, we tested binding between endogenous FOXO4 and Mdm2 proteins and observed reciprocal co-immunoprecipitation between endogenous FOXO4 and Mdm2 in HEK293T cells (Fig. 2c). Mono-ubiquitination of FOXO4 results in increased transcriptional activity of FOXO4 [8] and this is reversed by USP7-mediated de-ubiquitination. To see whether Mdm2 would also regulate FOXO4 transcriptional activity we performed FOXO reporter assays. Expression of increasing amounts of Mdm2 resulted in a bell-shaped regulation of FOXO4 transcriptional activity. Low amounts of Mdm2 transfected induced a reproducible increase in FOXO4 transcriptional activity on two different FOXO responsive reporters (Fig. 3a (6xDBE-luciferase) and Fig. 3b (p27-luciferase)), consistent with the notion that Mdm2 can induce mono-ubiquitination of FOXO4. In contrast, higher amounts of Mdm2 transfected resulted in reduced FOXO4 transcriptional activity. This suggests that the multiple mono-ubiquitination of FOXO4 induced by Mdm2 *in vitro* and possibly *in vivo* by high levels of Mdm2 represents inactivation of FOXO4. In effect this would be similar to poly-ubiquitination and degradation, but in contrast this leaves FOXO4 to be re-activated by de-ubiquitination by USP7 [8]. To assess the structural requirements of Mdm2 to regulate FOXO4 transcriptional activity we compared the effect of wild-type Mdm2 on FOXO4 transcriptional activity with that of Mdm2 mutated in its RING domain, and with that of Mdm2 mutated in its p53 interaction domain (Fig. 3c). The RING domain mutant of Mdm2 did not affect FOXO4 transcriptional activity indicating that Mdm2 regulated FOXO4 activity requires a functional E3 ligase domain. In contrast, p53 binding to Mdm2 appears not involved as Mdm2 defective in binding to p53 regulated FOXO4 in a manner identical to wild-type Mdm2. Ectopic expression of FOXO4 in cells induces cell cycle arrest and induction of apoptosis [1] and this can be monitored by a reduction in colony formation ([14] and Fig. 3d). Similar to decreased transcriptional activity at higher levels of Mdm2 co-expression Mdm2 represses the ability of FOXO4 to inhibit colony formation.

Discussion

Here, we provide evidence that Mdm2 is an E3-ligase that can ligate ubiquitin onto FOXO4 both *in vitro* and *in vivo*. Mono-ubiquitination of FOXO4 as induced by hydrogen peroxide treatment of cells requires endogenous Mdm2, because siRNA mediated knockdown of Mdm2 prevents mono-ubiquitination. This shows that Mdm2 is at least functional in regulating mono-ubiquitination of FOXO4. *In vitro*, Mdm2 induces a pattern of ubiquitination that normally is considered indicative of poly-ubiquitination. However, alternative to poly-ubiquitination, extensive mono-ubiquitination on multiple different lysine residues, 19 of which are present in FOXO4, can cause a similar characteristic laddering. This would be consistent with the current view that low mobility species of ubiquitinated p53 represent mono-ubiquitinated p53 (Fig. 1 and [15,16]). To further discriminate between these possibilities we used a number of ubiquitin mutants that are defective in poly-ubiquitination (K48A, K7R and methyl-ubiquitin).

When these mutants were used as only ubiquitin donor in the *in vitro* assay, Mdm2 catalyzed a highly similar, if not identical, pattern of laddering compared to including wild-type ubiquitin in this assay. This strongly suggests that *in vitro* Mdm2 catalyzes preferentially only (multi-)mono-ubiquitination of GST-FOXO4. Also *in vivo* overexpression or siRNA-mediated knockdown of Mdm2 resulted in the induction or loss of FOXO4 mono-ubiquitination respectively. Thus we conclude that Mdm2 regulates mono-ubiquitination of FOXO4. However, we observed an apparent reduction in protein expression of FOXO4, especially after high expression of Mdm2 and this would suggest protein degradation most likely through poly-ubiquitination-mediated proteasomal degradation. This would be at odds with the conclusion that Mdm2 catalyzes mono-ubiquitination. To establish whether proteasomal degradation is causal to the reduced detection of HA-FOXO4 after flag-Mdm2 overexpression we treated cells with MG132. Inhibition of proteasome-mediated degradation did not result in increased detection of HA-FOXO4 despite observed accumulation of Mdm2. Thus, this result does not implicate Mdm2 induced poly-ubiquitination of HA-FOXO4 and subsequent degradation through the proteasome and is consistent with the lack of effect of MG132 treatment on FOXO4 ubiquitination *in vivo* (Fig. 1, and B.M.T.B, unpublished data). Consistent with these observations, protein stability experiments indicate that Mdm2 does not affect FOXO4 protein stability. Consequently, reduced detection of FOXO4 after Mdm2 overexpression is likely due to other mechanism(s). This could be alternative mechanism(s) of degradation such as lysosomal degradation or protease-mediated degradation (e.g. caspase). Alternatively, mono-ubiquitination has been shown in several cases to regulate cellular localization of proteins and thus the apparent reduction in protein expression may equally represent a shift of target protein into a complex or towards a cellular location that results in inefficient extraction of protein and thereby reduced detection after immunoblotting. Indeed, recent developments have provided multiple examples in different signaling pathways that ubiquitination serves other purposes than merely targeting proteins for degradation [17–19]. This raises the interesting possibility that the initial function of (mono-)-ubiquitination is to provide a means to regulate protein function similar to for example phosphorylation. However, to terminate the signaling function of (mono-)ubiquitination a cell can choose between either de-ubiquitination or poly-ubiquitination. Depending on the urgency to terminate signaling, poly-ubiquitination and subsequent degradation, may be the preferred mode.

While our study was in progress Yang et al. also reported ubiquitination of FOXO3a by Mdm2 [20]. However, in contrast to our results presented here, their study suggests a role for Mdm2 mainly in the breakdown of FOXO3a. As discussed above, in our experiments only high expression of

Mdm2 may result in induced breakdown of FOXO4. Yang et al. also implicated a role for ERK in the regulation of FOXO3a by Mdm2 and provide evidence that FOXO3a phosphorylation by ERK through an unknown mechanism induces Mdm2 binding to FOXO3a [20]. Importantly, Yang et al. use EGF as stimulus whereas we use peroxide stress. It is of importance to note that the stimuli used by others (EGF, PDGF, insulin) all inhibit FOXO function whereas we and others have shown that oxidative stress (e.g. peroxide) as used here activates FOXOs [6]. This is an essential difference. In addition, for PDGF and insulin stimulation several previous studies have shown that Skp2 is involved in degradation of FOXO induced by these factors [10,11]. In addition, with respect to the issue here, several interesting observations within these studies were made. Firstly, FOXO half-life in 'normal' cells is around 8–10 hrs similar to our previous observations ([8] and the results in this study). Second, only in cells transformed through PI3K activation (v-Ha-RAS, active PI3K alleles) FOXO half-life is shortened [21], but again this is in these studies a PKB/AKT and Skp2 mediated process (and not ERK-Mdm2). We are tempted to speculate that Mdm2 induces FOXO mono-ubiquitination; this results in activation of FOXO. Activation can be terminated by USP7 de-ubiquitination or alternatively by Skp2-mediated poly-ubiquitination and degradation. The latter occurs as a result of oncogenic transformation through PI3K/PKB/AKT, but possibly also through ERK signalling. Thus if one considers the possibility of Mdm2 being a 'priming' E3-ligase for FOXO and Skp2 the branching E3-ligase, these different results can be reconciled. Clearly further studies are required to fully appreciate the role of ubiquitination in FOXO regulation in response to various cellular conditions. Mono-ubiquitination is observed and studied thus far, for proteins with a relative long half-life, such as PTEN [22], EGF receptor [23] and FOXOs (approximately 10 hrs in untransformed cells [8]). In contrast, mono-ubiquitination has not yet been considered for short lived proteins such as cell cycle regulators (cyclins) and oncogenes (myc, beta-catenin). However, establishing mono-ubiquitination for these short-lived proteins may just be a technical challenge. Indeed, recent results with p53 may provide basis for such a paradigm shift. Whereas initially p53 served as a classical example of a protein regulated through protein degradation, it is by now clear that mono-ubiquitination of p53 occurs and serves to provide a new signaling function to p53 [15,24]. Instead of acting as a transcription factor, monoubiquitination of p53 serves as a signal to relocate p53 from the nucleus to mitochondrial membrane [24]. Along the same line of reasoning, the role of Mdm2 in ubiquitination of p53 is now being discussed [17,25,26]. Thus, the possibility is being raised that endogenously expressed Mdm2 is actually preferentially involved in mono-ubiquitination of p53, whereas aberrant high expression of Mdm2 may result in poly-ubiquitination and degradation of p53. Essentially, our observations presented here indeed fully support a role for Mdm2, at endogenous level, in regulating monoubiquitination of in this case FOXO4. In summary, we have identified Mdm2 as an ubiquitin E3 ligase for FOXO4 which functions in oxidative stress-induced FOXO4 mono-ubiquitination. This extends the network of co-regulatory proteins of FOXO and p53 and therefore supports a model of coevolution of stress maintenance mechanisms.

Materials and Methods

Cell culture and Transfection

HEK293T, MCF7 and A14 cells (3T3 fibroblasts stably expressing the insulin receptor) were maintained in Dulbecco's Modified Eagle medium (Cambrex), supplemented with 10% fetal bovine serum, penicillin/streptomycin and 0.05% glutamine. Transient transfections were performed with FuGENE6 (Roche). Cycloheximide experiments

were performed as described [8]

Constructs, antibodies and RNAi
pMT2-HA-FOXO4 and pMT2-Flag-FOXO4, His-Ubiquitin, Flag-Mdm2, the inactive Mdm2 RING-finger mutant C464A, Myc-Mdm2-delta-RING domain, Myc-MDM2-delta-p53 mutants and Myc-Mdm2 have been described previously [8,27]. The luciferase constructs containing TK-Renilla, 6xDBE and $p27^{kip1}$ Luciferase have been described [6]. Non-targeting RNAi duplex (c), RNAi smartpool oligonucleotides specific for human Mdm2 were purchased from Dharmacon. Cells were transfected with 20 μM RNAi with Oligofectamine (Invitrogen) for RNAi oligonucleotides and FuGENE6 for DNA constructs. DNA constructs were transfected 8 h. after the last RNAi oligonucleotide transfection. The antibodies against FOXO4 (834) and HA (12CA5), have been described [4]. The following antibodies were purchased; Mdm2 (SMP-14, Santa Cruz), Tubulin and Flag-M2 (Sigma).

Co-immunoprecipitation assay
For co-immunoprecipitation studies, 50 μl Protein-A Sepharose beads were pre-coupled to 1 μg of the indicated antibody. Cells were lysed in Co-IP buffer (20 mM Tris-HCl pH 8.0, 1% NP-40, 10% glycerol, 1 mM MgCl2, 1 mM EDTA, 150 mM NaCl, protease and phosphatase inhibitors) and incubated as described previously [28]. For endogenous co-immunoprecipitations, cells were treated prior to lysis with hydrogen peroxide (200 μM, 15 min.).

Luciferase Reporter assay
Cells were transfected with the indicated constructs plus 20 ng TK-Renilla per condition as a transfection efficiency control. Lysates were measured after 24 h by the Promega Dual Luciferase reporter assay.

Ubiquitination assays
The *in vitro* ubiquitination assay was performed essentially as described [29]. Flag-Mdm2 was purified from HEK293T cells with Flag-M2 beads (Sigma). Precipitated protein was washed with RIPA, and eluted off with Flag peptide (Sigma). Eluted protein was dialysed o/n with a buffer containing (25 mM HEPES-pH 8.0, 150 mM NaCl, protease inhibitors). The in vitro ubiquitination assay was initiated with the addition of ATP (f.c.2.5 mM) and quenched after 2 h with Laemmli Sample Buffer. Purified GST and GST-FOXO4 were a kind gift from H. de Ruiter. Purified GST-p53, E1, E2 (UbcH5b) were kind gifts from Dr. K.W. Mulder. Ubiquitin, Ubiquitin-K7R (All lysines mutated to arginine), Ubiquitin-K48A (lysine 48 mutated to Alanine) and
methylated Ubiquitin (all lysines methylated) were purchased at Boston Biochem. *In vivo* ubiquitination assays were essentially performed as described [8]. In brief, HEK293T cells were transfected with the indicated constructs. 48 h. post transfection, cells were left untreated or treated as indicated, and lysed in lysis buffer (8 M Urea, 10 mM Tris-HCl pH 8.0, 100 mM Na_2HPO_4/NaH_2PO_4, 0.2% TX-100, 5 mM NEM, protease inhibitors). Ubiquitinated proteins were precipitated using Ni-NTA agarose beads and analysed by WB.

Colony outgrowth assay
Equal amounts of A14 cells were plated in triplicate in 6-well dishes and transfected with 2 μg of the indicated constructs in combination with 0.5 μg pbabe-puro. 24 hours post-transfection cells were placed under selection with 2 μg/ml Puromycin. Every two days the selection medium was refreshed. At 10 days post transfection cells were fixed for 10 minutes with ice-cold methanol and colonies were stained with 0.5% crystal violet, dissolved in 25% methanol. The plates were washed with dH_2O and dried overnight.

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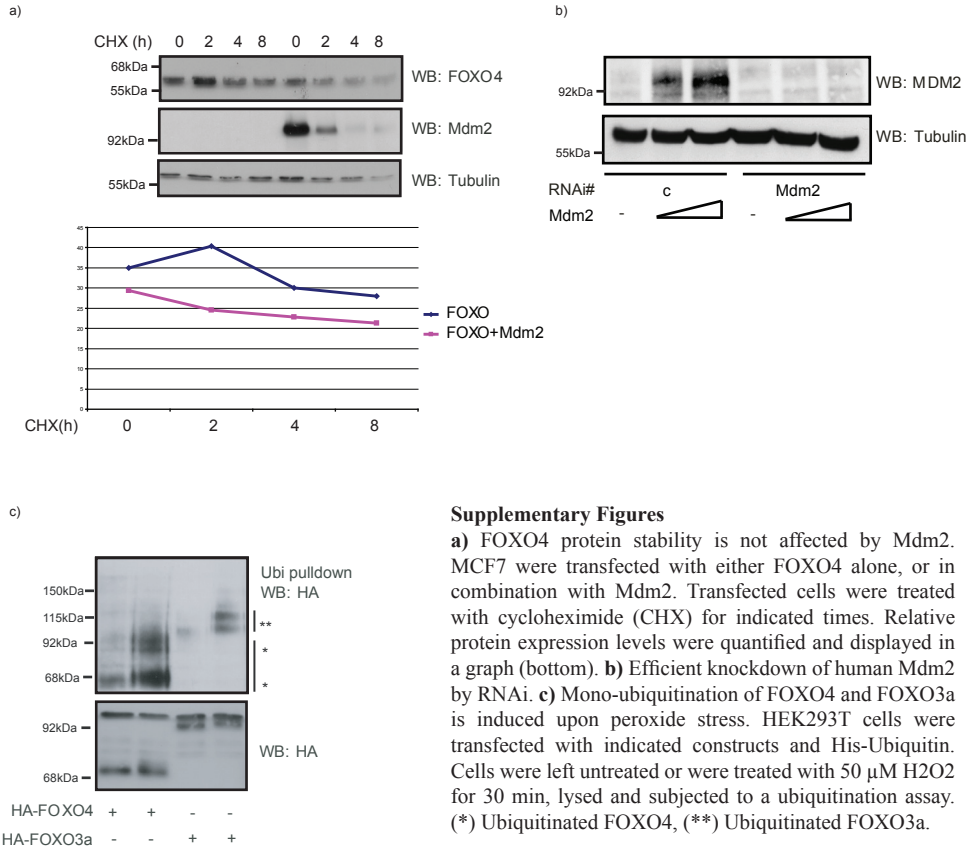

Supplementary Figures

a) FOXO4 protein stability is not affected by Mdm2. MCF7 were transfected with either FOXO4 alone, or in combination with Mdm2. Transfected cells were treated with cycloheximide (CHX) for indicated times. Relative protein expression levels were quantified and displayed in a graph (bottom). **b)** Efficient knockdown of human Mdm2 by RNAi. **c)** Mono-ubiquitination of FOXO4 and FOXO3a is induced upon peroxide stress. HEK293T cells were transfected with indicated constructs and His-Ubiquitin. Cells were left untreated or were treated with 50 μM H2O2 for 30 min, lysed and subjected to a ubiquitination assay. (*) Ubiquitinated FOXO4, (**) Ubiquitinated FOXO3a.

4

Forkhead box O (FOXO) tumor suppressors are mediators of oncogenic BRAF-induced senescence

Manuscript in submission

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Forkhead box O (FOXO) tumor suppressors are mediators of oncogenic BRAF-induced senescence

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Abstract

The potential of mutated oncogenes to drive tumorigenesis is restricted through induction of a permanent state of cellular arrest termed oncogene induced senescence (OIS). OIS is an important tumor suppressive mechanism, but it is thought to come at the cost of aging as senescent cells can no longer renew damaged tissue. Animal models have established the Forkhead Box O (FOXO) family of transcription factors as tumor suppressors. However, whether they are involved in OIS is elusive.

Here, we identify FOXOs as key mediators of $BRAF^{V600E}$-induced OIS through transcriptional regulation of $p21^{cip1}$. Furthermore, we show that these effects are mediated through a linear pathway involving MEK, chronic elevation in Reactive Oxygen Species (ROS) and subsequent phosphorylation of FOXO4 by the stress kinase JNK. FOXOs have thus far been associated with beneficial effects on longevity. Thus, these findings establish an antagonistic role of FOXOs in a pleiotropy between tumor suppression and aging.

Introduction

Oncogenic BRAF mutations are found in ~7% of all human tumors with high occurrence in thyroid carcinoma, colorectal cancer, ovarian cancer[1] and especially melanoma (~70%)[2]. Predominantly, BRAF is mutated at amino acid 600, where the hydrophobic Val is replaced with the acidic Glu (V600E). This causes release of an auto-inhibitory loop[3] and a significant increase in downstream signaling towards MEK[2]. Oncogenic mutation of BRAF initially induces an increase in proliferation. However after a few rounds of division cell cycle progression is arrested through induction of OIS[4,5]. The mechanisms through which $BRAF^{V600E}$ mediates OIS are still only partially unraveled.

ROS stimulate cellular proliferation through propagation of growth factor signaling and activation of the DNA synthesis machinery[6,7]. However, when ROS levels rise above a threshold, the cellular interior can be damaged, increasing the chance on tumorigenesis and accelerating the onset of organismal aging[8,9]. FOXO transcription factors are activated in the absence of growth factor signaling to induce cell cycle arrest[10]. Moreover, in the presence of growth factor

signaling, FOXOs can be activated by ROS, although it is unclear to which target genes[11].
FOXOs are genuine tumor suppressors as established by the observation that conditional knockout of *foxo1, 3a* and *4* in mice resulted in strong lymphoma development[12]. Importantly, individual knockout mice of *foxo3a* or *foxo4* are viable and do not lead to a significant tumor prone phenotype[13-15]. Also, conditional knockout combinations of two *foxo* genes only produced mild effects on lymphogenesis[12]. This identified FOXOs as functionally redundant tumor suppressors. Thus, FOXOs play a role in both tumor suppression and aging, two seemingly independent processes. The mechanisms through which FOXOs regulate tumor suppression are still unclear. In the present study, we identify a role for FOXOs in the OIS response by $BRAF^{V600E}$, thereby uncovering a mechanism through which FOXOs repress tumorigenesis.

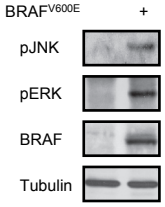
$BRAF^{V600E}$ induces phosphorylation of FOXO4 on JNK target sites

We set out to study the regulation of FOXOs by oncogenic $BRAF^{V600E}$. Interestingly, we observed that next to activation of MEK-ERK signaling ectopic expression of $BRAF^{V600E}$ resulted in activation of the c-Jun N-terminal Kinase JNK (Fig. 1a and [16]). By mutation analysis, we previously identified Thr447 and Thr451 as JNK phospho-acceptor sites in FOXO4, next to additional residues that were at that time unknown[11]. Thus we investigated if $BRAF^{V600E}$ could regulate FOXO signaling through JNK. Therefore, we first determined all possible JNK sites in FOXO4. To this end, we employed LC-MS/MS mass-spectrometry analysis upon *in vitro* phosphorylation of purified FOXO4 by JNK. For stability reasons we used a mutant that lacks the DNA binding domain, but does not contain any JNK phosphorylatable Ser/Thr-Pro patches. In addition to the previously characterized Thr447 and Thr451, we identified two novel residues, Thr223 and Ser226 as potential JNK target sites (Fig. 1b+c). To further investigate if these are genuine JNK target sites, we generated phosphospecific antisera against phospho-Thr223. As we failed to obtain specific antiserum against phospho-Ser226 we generated it against dual-phosphorylated Thr223/Ser226 instead. *In vitro* phosphorylation by JNK significantly increased detection of wildtype FOXO4 by the phospho-Thr223, Thr223/Ser226, Thr447 and Thr451 antibodies, whereas FOXO4-4A, in which these residues are mutated to Alanine was not detected (Fig. 1d). These data indicate that Thr223, Ser226, Thr447 and Thr451 are JNK target sites *in vitro*.
Given that $BRAF^{V600E}$ triggers JNK activation and JNK can phosphorylate FOXO4, we next analyzed whether $BRAF^{V600E}$ expression affects FOXO4 phosphorylation on the JNK acceptor sites *in vivo*. Indeed, $BRAF^{V600E}$ induced a significant increase in phosphorylation on all JNK sites Thr223, Ser226, Thr447 and Thr451, but not on the unrelated PKB/AKT site Thr28 (Fig. 1e). Thus, in parallel with enhanced JNK activation, $BRAF^{V600E}$ expression promotes phosphorylation of FOXO4 on the JNK sites *in vivo*.

$BRAF^{V600E}$ signaling elevates cellular ROS levels, which induce JNK-mediated phosphorylation of FOXO4

Next, we addressed how $BRAF^{V600E}$ signaling could result in JNK activation. Therefore, we determined whether $BRAF^{V600E}$ expression regulates cellular ROS levels by loading cells with the ROS detecting probe H_2DCFDA. $BRAF^{V600E}$ expression significantly increased the cellular levels of ROS as detected by the fluorescence signal of this probe (Fig. 2a). The $BRAF^{V600E}$-induced rise in cellular ROS could be further increased by treatment with H_2O_2 (45 minutes 200μM) and importantly it was impaired upon pre-incubation with the ROS scavenger N-Acetyl

a)



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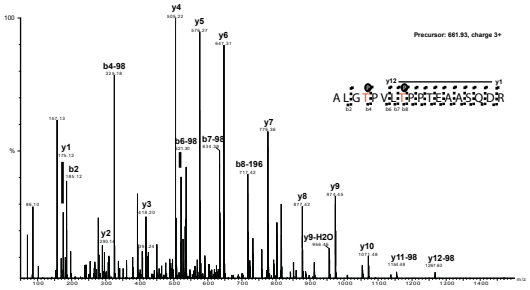
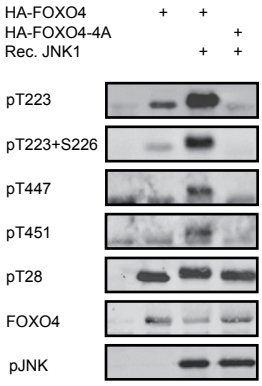


b)

Amino Acid Position	Peptide Sequence	Phosphorylated Residue	Peptide Charge State	Mascot Score
210-233	KKPSVLPAPPEGA<u>T</u>PT<u>S</u>PVGHFAK	Thr223/Ser226	4+	71
234-241	WSG<u>S</u>PCSR	Ser237	2+	53
444-461	ALG<u>T</u>PVLTPPTEAASQDR	Thr447	2+	62
438-449	SAPIPKALG<u>T</u>PV	Thr447	2+	42
444-461	ALG<u>T</u>PVL<u>T</u>PPTEAASQDR	Thr447/Thr451	3+	103

d)



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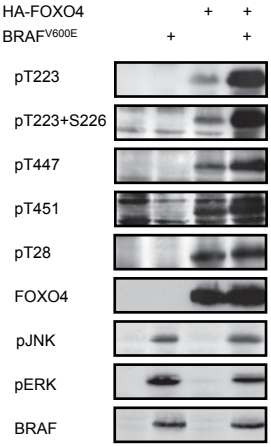


Fig. 1) BRAF^{V600E} promotes phosphorylation of FOXO4 on JNK target sites
a) BRAF^{V600E} expression results in JNK activation. Lysates of HEK293T cells expressing BRAF^{V600E} analyzed by immunoblotting for JNK and ERK activation. **b)** Potential *in vitro* JNK sites revealed by LC-MS/MS analysis. GST-FOXO4ΔDB (not containing Ser/Thr-Pro motifs) was precipitated and phosphorylated by recombinant JNK in an *in vitro* kinase assay and digested with different enzymes. The resulting peptides were subjected to tandem mass spectrometry (LC-MS/MS). Note that although Ser237 was also found phosphorylated, we could not confirm this to be a genuine JNK target site *in vitro* or *in vivo* (not shown). **c)** Representative spectrum of a dual-phosphorylated Thr447/451 FOXO4 peptide by mass spectrometry identified after *in vitro* phosphorylation by JNK. Sequence and

Cysteine (NAC). Downstream signaling through MEK is at least partially required for the increase in ROS levels, since pre-incubation with the MEK inhibitor U0126 reduced DCF fluorescence. These data indicate that ectopic $BRAF^{V600E}$ expression leads to the generation of cellular ROS through downstream MEK signaling.

Based on these results, we next investigated whether extracellular ROS can induce FOXO4 phosphorylation on the JNK target sites. Since the pT223 antibody systematically showed the highest quality of detection, the experiments shown from hereon are performed using this antibody, but essentially identical results were obtained for pT223/S226, pT447 and pT451. Treatment of cells with 200μM H_2O_2 resulted in a time-dependent increase in both JNK activation and Thr223 phosphorylation (Fig. 2b), indicating that elevated cellular ROS triggers phosphorylation of FOXO4 on the JNK target sites. Next to JNK, $BRAF^{V600E}$ signaling through MEK results in activation of ERK[4]. Importantly, external stimuli that activate JNK, but not ERK, promoted phosphorylation on Thr223 (Fig. 2c, sup fig. 1c and data not shown). Thus we conclude that activation of JNK, not ERK, directly promotes Thr223 phosphorylation of FOXO4 *in vivo*.

Finally, we determined the importance of JNK signaling for $BRAF^{V600E}$-induced phosphorylation of FOXO4. Thr223 phosphorylation and JNK auto-phosphorylation were reduced upon pretreatment with the JNK inhibitor SP600125. This indicates that in response to $BRAF^{V600E}$ signaling FOXO4 is indeed phosphorylated through JNK (Fig. 2d). Signaling of $BRAF^{V600E}$ towards MEK and the subsequent ROS production are required for these events to take place as both JNK activation and FOXO4 phosphorylation were reduced upon pretreatment with U0126 or NAC (Fig. 2e). Altogether these data point to a linear signaling pathway in which $BRAF^{V600E}$ induces FOXO4 phosphorylation by JNK through elevation of intracellular ROS.

$BRAF^{V600E}$ and FOXO4 can synergistically inhibit cell cycle progression in correlation with increased $p21^{cip1}$ transcription

To investigate the biological consequences of $BRAF^{V600E}$-induced phosphorylation of FOXO4 we analyzed how $BRAF^{V600E}$ and FOXO4 co-expression affects cell cycle progression. Ectopic FOXO4 expression promotes cell cycle arrest, which we could confirm (Fig. 3a and [17]). Notably, $BRAF^{V600E}$ enhanced the FOXO4 induced G1-arrest. This cooperative effect was also observed when assaying the ability of FOXO4 to inhibit colony formation, indicating that $BRAF^{V600E}$ enhances the repressive effect of FOXO4 on proliferation (Fig. 3b).

$p27^{kip1}$ is an important mediator of FOXO-induced G1 arrest[17], yet $p27^{kip1}$ is reported to be degraded in response to hyperactive BRAF signaling[18]. To understand these observations, which are seemingly contradictory to the results described here, we analyzed how cell cycle arrest occurred upon co-expression of $BRAF^{V600E}$ and FOXO4. Indeed, as expected from aforementioned studies, FOXO4 induced $p27^{kip1}$ expression, which was reduced in the presence

MS/MS spectrum of the Thr447/Thr451 double-phosphorylated peptide of FOXO4 (aa 444-461) as identified by MASCOT software (See experimental procedures) are shown. Identified b and y ions are indicated as well as their neutral losses. The location of the phosphorylations are indicated by a circled P and the Thr residues are indicated in red. **d)** Thr223, Ser226, Thr447 and Thr451 of FOXO4 are JNK target sites *in vitro*. Phosphorylation status of immunoprecipitated HA-FOXO4 or HA-FOXO4-4A expressed from HEK293T cells was determined upon *in vitro* phosphorylation by recombinant JNK1. **e)** $BRAF^{V600E}$ induces phosphorylation of FOXO4 on the JNK sites. FOXO4 phosphorylation status on all JNK sites determined upon $BRAF^{V600E}$ co-expression.

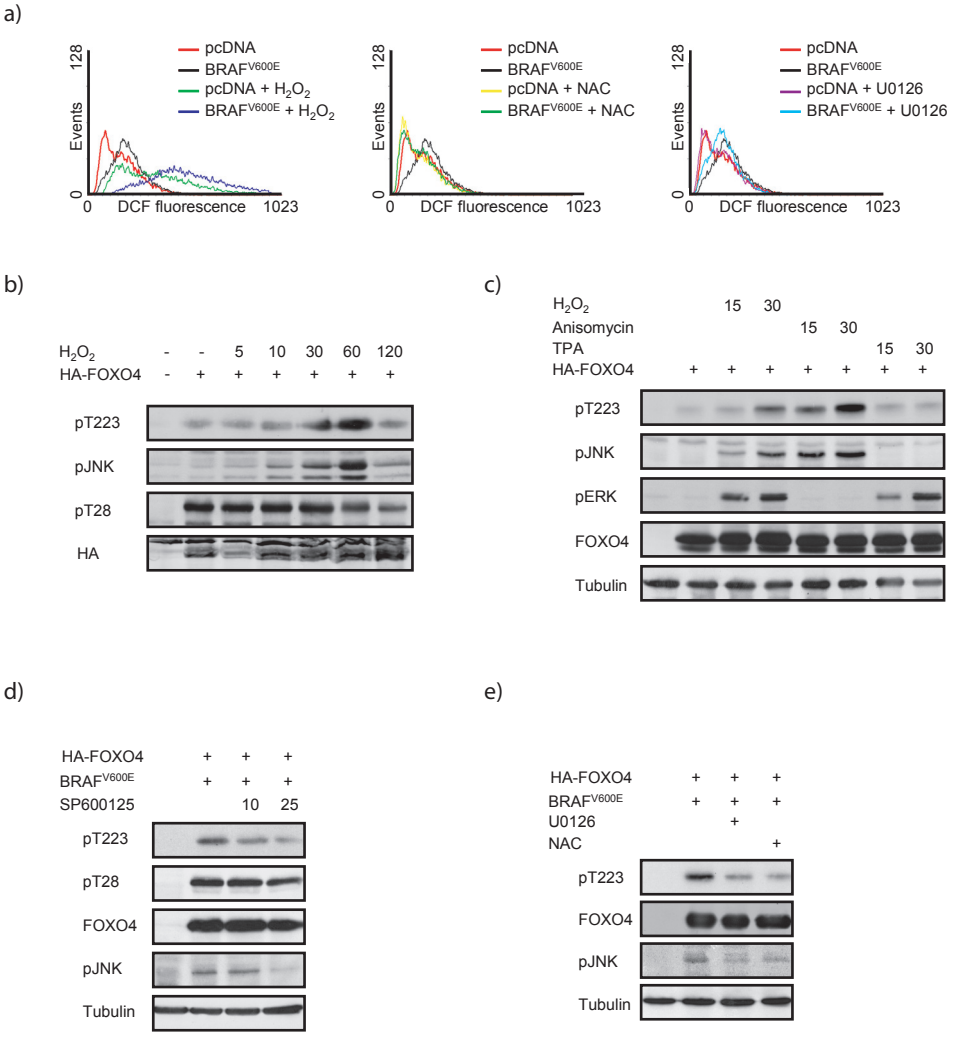
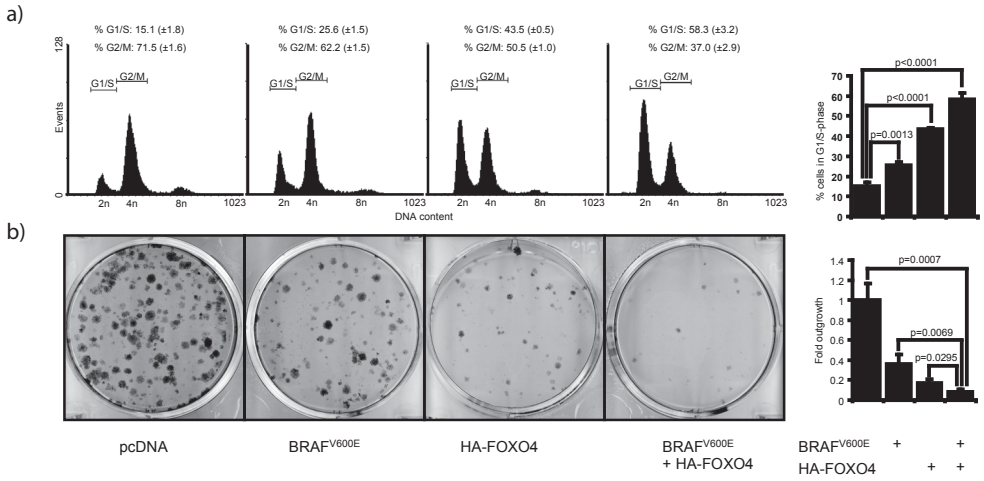
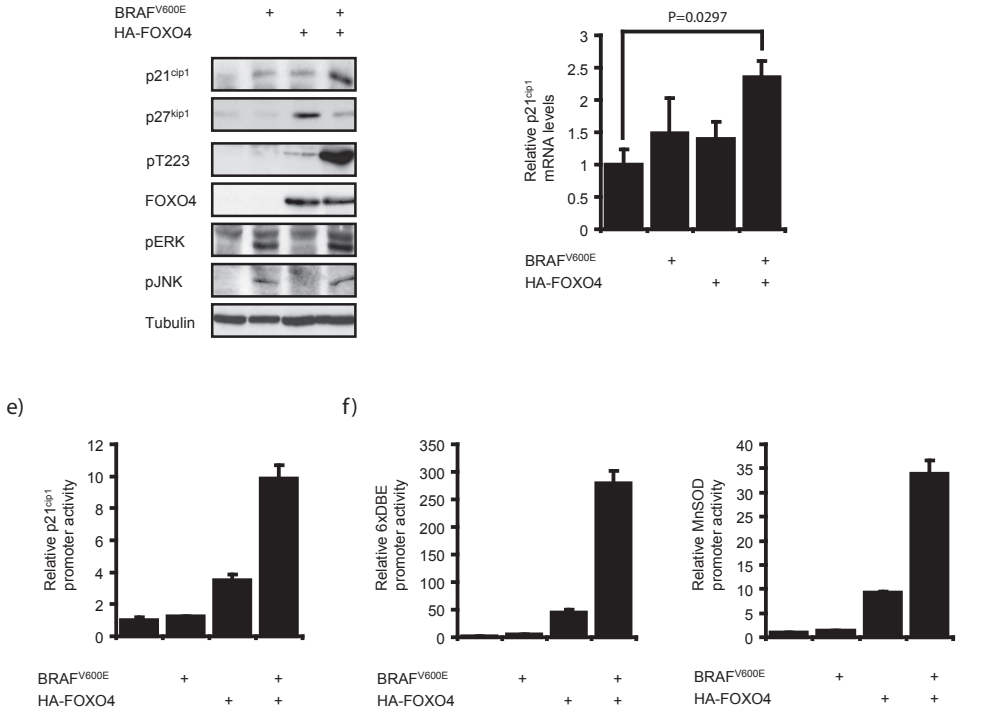


Fig. 2) BRAF^{V600E} signaling through MEK elevates cellular ROS levels which induce FOXO phosphorylation in a JNK-dependent manner

a) BRAF^{V600E} expression induces cellular ROS. BRAF^{V600E} expressing HEK293T cells were treated 24 hours with 4mM NAC, 10μM U0126 or 45 minutes with 200μM H_2O_2 and analyzed for DCF fluorescence by FACS to determine ROS production. **b)** Exogenous ROS (H_2O_2) activates JNK and induces phosphorylation of FOXO4 on the JNK target site Thr223. Phosphorylation status of HA-FOXO4 from untreated (-) or H_2O_2 treated HEK293T cells determined by immunoblotting. **c)** *In vivo* phosphorylation of FOXO4 on Thr223 correlates with JNK, but not ERK, activity. FOXO4 phosphorylation status determined as in d) upon treatment with the indicated stimuli. **d+e)** Interference with JNK activation inhibits BRAF^{V600E} induced FOXO4 phosphorylation on Thr223. Experiment as in Fig. 1e), but upon pretreatment for 10hrs with 20μM SP600125 **(d)** or 24hrs 20μM U0126 or 4mM NAC **(e).**


a)
% G1/S: 15.1 (±1.8)
% G2/M: 71.5 (±1.6)
% G1/S: 25.6 (±1.5)
% G2/M: 62.2 (±1.5)
% G1/S: 43.5 (±0.5)
% G2/M: 50.5 (±1.0)
% G1/S: 58.3 (±3.2)
% G2/M: 37.0 (±2.9)
G1/S
G2/M
Events
DNA content
2n
4n
8n
1023
p<0.0001
p<0.0001
p=0.0013
% cells in G1/S-phase
b)
p=0.0007
p=0.0069
p=0.0295
Fold outgrowth
pcDNA
BRAFV600E
HA-FOXO4
BRAFV600E + HA-FOXO4
BRAFV600E
HA-FOXO4


c)
BRAFV600E
HA-FOXO4
p21cip1
p27kip1
pT223
FOXO4
pERK
pJNK
Tubulin
d)
P=0.0297
Relative p21cip1 mRNA levels
BRAFV600E
HA-FOXO4
e)
Relative p21cip1 promoter activity
BRAFV600E
HA-FOXO4
f)
Relative 6xDBE promoter activity
Relative MnSOD promoter activity
BRAFV600E
HA-FOXO4

of BRAF^{V600E} (Fig. 3c). However, importantly, under these conditions p21^{cip1} protein expression was significantly elevated. Similar to the change in protein levels, p21^{cip1} mRNA expression, determined by quantitative real-time PCR, was enhanced by FOXO4 and BRAF^{V600E} (Fig. 3d). Also co-expression of BRAF^{V600E} showed synergistic activation of the p21^{cip1} promoter by FOXO4, as well as other FOXO responsive promoters (Fig. 3e+f and Sup. fig. 1b). Next to p21^{cip1}, induction of p16^{ink4a} expression has been observed in response to BRAF^{V600E} signaling[4]. However, in contrast to p21^{cip1}, we did not observe increased p16^{ink4a} promoter activity or protein expression by FOXO4 in response to BRAF^{V600E} signaling (not shown). Together, these data indicate that BRAF^{V600E} synergizes with FOXO4 to promote cell cycle arrest in correlation with increased p21^{cip1} transcription.

BRAF^{V600E}-induced cell cycle arrest through FOXOs is p21^{cip1}-dependent

Next we addressed whether the co-operative regulation of p21^{cip1} and cell cycle arrest by BRAF^{V600E} and FOXO4 results from parallel signaling, or direct signaling of BRAF^{V600E} towards FOXOs. Consistent with direct signaling from BRAF to FOXO4, incubation of cells with SP600125, NAC or U0126 reduced the ability of FOXO4 to promote p21^{cip1} expression upon BRAF^{V600E} co-expression (Fig. 4a+b).

In agreement with other reports, high expression of BRAF^{V600E} increased p21^{cip1} promoter activity ([19] and Fig. 4c). Notably, this induction was abrogated upon shRNA-mediated depletion of endogenous FOXO1, 3a and 4, while expression of a FOXO4 mutant insensitive to shRNA-mediated knockdown (FOXO4-SM) rescued BRAF^{V600E}-induced transactivation of the p21^{cip1} promoter (Sup. fig. 2a and Fig. 4c). Knockdown of FOXO4 by a different short hairpin produced a similar result (Sup. fig. 2b). Altogether, this shows that BRAF^{V600E} directly regulates endogenous FOXOs and that these are required for BRAF^{V600E} to promote p21^{cip1} transcription.

We subsequently determined the importance of p21^{cip1} regulation for the induction of cell cycle arrest through the combined activities of BRAF^{V600E} and FOXO4. Short hairpin mediated knockdown of p21^{cip1} (Sup. fig. 2c) alleviated the G1-arrest imposed by BRAF^{V600E} and FOXO4 co-expression (Fig. 4d). Thus, we conclude that BRAF^{V600E} activates FOXOs through JNK-mediated phosphorylation, which results in a switch from a p27^{kip1} to p21^{cip1} mediated cell cycle arrest.

<- Fig. 3) BRAF^{V600E} and FOXO4 synergistically induce p21^{cip1} transcription and cell cycle arrest

a, b) BRAF^{V600E} and FOXO4 co-operatively repress cell cycle progression and proliferation. **a)** HA-FOXO4 and/or BRAF^{V600E} expressing U2OS cells were selected with GFP-Spectrin and analyzed for cell cycle progression by FACS after 24hrs of nocodazole treatment. The experiment was performed in triplicate and quantified. **b)** HA-FOXO4 and/or BRAF^{V600E} expressing A14 cells were selected with puromycin and analyzed for colony formation at ten days post transfection. The experiment was performed in triplicate and quantified. Similar results were obtained in U2OS cells. **c)** BRAF^{V600E} co-expression diverts p27^{kip1} expression to p21^{cip1}. Total lysates of puromycin selected HEK293T cells expressing HA-FOXO4 and BRAF^{V600E} analyzed by immunoblotting. **d, e)** BRAF^{V600E} and FOXO4 co-operatively activate p21^{cip1} transcription. Quantitative real-time PCR for p21^{cip1} mRNA in HEK293T **(d)** and p21^{cip1}-luciferase assay on A14 cell lysates **(e)**, which transiently expressed HA-FOXO4 and BRAF^{V600E}. For the luciferase experiment similar effects were obtained in primary melanocytes **f)** BRAF^{V600E} and FOXO4 co-operatively induce activation of the MnSOD promoter and 6 optimal FOXO DNA binding elements. Luciferase assay on lysates of A14 cells, transfected with plasmids encoding HA-FOXO4 and BRAF^{V600E} in combination with MnSOD-firefly luciferase or 6xDBE-firefly luciferase.

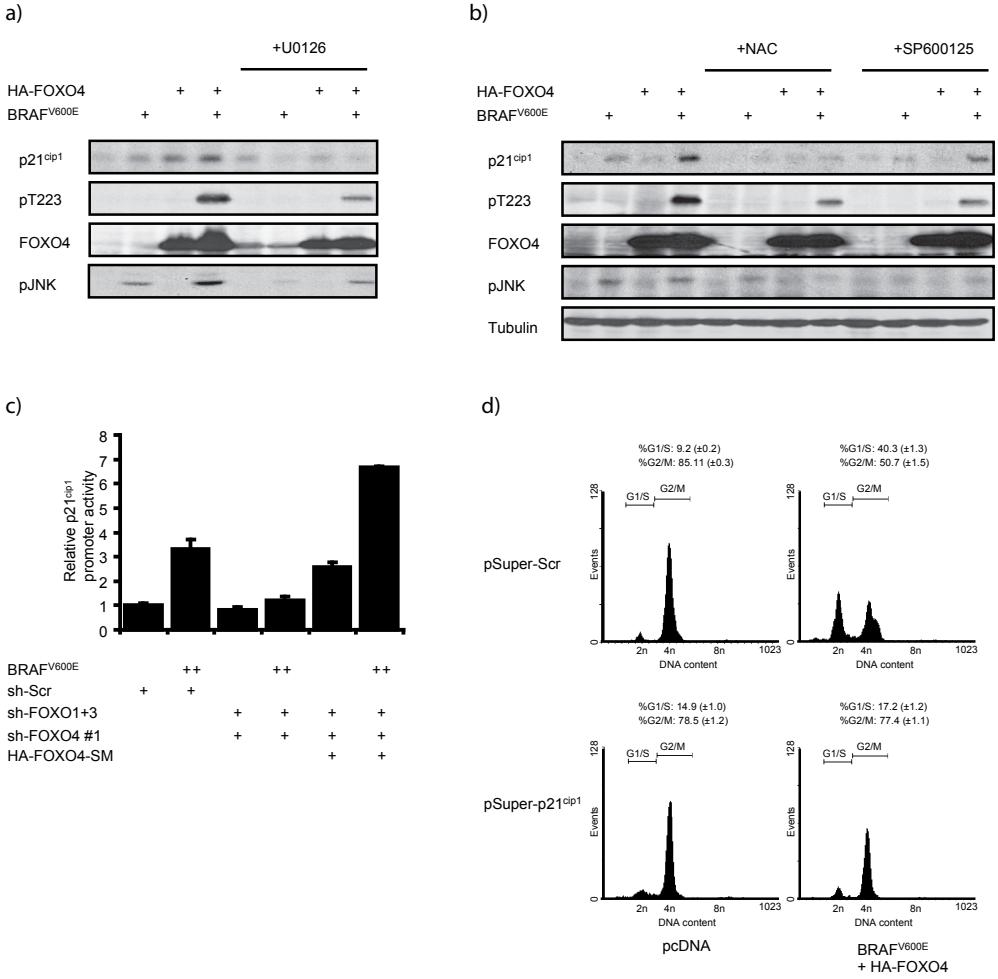


Fig. 4) BRAF^{V600E} induces cell cycle arrest through FOXO-mediated p21^{cip1} transcription
a+b) p21^{cip1} transcription induced by combined activities of BRAF^{V600E} and FOXO4 is dependent on MEK, ROS and JNK activation. Total lysates of puromycin selected HEK293T expressing HA-FOXO4 and/or BRAF^{V600E} were left untreated or treated for 24hrs with 20μM U0126 **(a)**, 4mM NAC or 20μM SP200126 **(b)** and analyzed by immunoblotting. **c)** Endogenous FOXOs are essential for BRAF^{V600E} to induce p21^{cip1} transcription. A14 cells were transfected with scrambled or FOXO1+3a and FOXO4 short-hairpins and subjected to a p21^{cip1}-luciferase assay. **d)** p21^{cip1} is essential for G1-arrest by FOXO4 and BRAF^{V600E}. U2OS cells were transfected and treated as in a) in combination with a plasmid encoding a short hairpin against p21^{cip1} or a scrambled control.

BRAF^{V600E} regulates p21^{cip1} expression endogenously through phosphorylation of FOXOs

To address the endogenous regulation of FOXOs by BRAF^{V600E}, we next investigated FOXO signaling in melanoma cell lines expressing either wildtype BRAF (CHL), or a hyperactive V600E or D mutant (Colo829 and WM266.4, respectively). In agreement to the effects we observed with ectopic expression of BRAF^{V600E}, ERK and JNK activity was significantly higher

in Colo829 and WM266.4 compared to CHL cells (Fig. 5a). Furthermore, opposite to CHL cells, DCF fluorescence in WM266.4 (and to a lesser extend Colo829) cells was barely responsive to induction by H_2O_2, while it was significantly reduced with NAC (Fig. 5b and data not shown). Combined with the observation that ectopic $BRAF^{V600E}$ expression generates ROS this suggests that in oncogenic BRAF expressing WM266.4 cells ROS levels are indeed higher than wt BRAF expressing CHL cells.
Expression of $p16^{ink4a}$ was not detectable in any of these three cell lines (Fig 5a and [20]). However, $p21^{cip1}$ expression levels were significantly elevated in WM266.4 cells, in contrast to $p27^{kip1}$ expression, which was the lowest in these cells. These observations suggest that in WM266.4 cells the $p21^{cip1}$ arrest has been bypassed while signaling downstream from $BRAF^{V600E}$ to induce $p21^{cip1}$ expression is active. Indeed, si-RNA-mediated knockdown of BRAF in WM226.4 cells reduced ERK and JNK activity and, importantly, resulted in diminished $p21^{cip1}$ expression (Fig. 5c). This indicates that the high $p21^{cip1}$ levels in WM266.4 cells are dependent on expression of $BRAF^{V600E}$ and consequent downstream signaling.
Treatment of WM266.4 with U0126 inhibited JNK activation, indicating that also in these cells MEK signaling is essential for JNK activation by endogenous $BRAF^{V600E}$ (Fig. 5d). Importantly, decreased JNK activation following U0126 treatment resulted in reduced phosphorylation of endogenous FOXO4 on the JNK sites Thr223+Ser226, as well as impaired $p21^{cip1}$ expression. Finally, siRNA mediated knockdown of endogenous FOXOs reduced $p21^{cip1}$ expression (Fig. 5e). U0126 further reduced $p21^{cip1}$ expression in this experiment, which probably reflects incomplete knockdown of FOXOs by these siRNAs. Together, these experiments indicate that in WM266.4 cells $BRAF^{V600E}$ regulates $p21^{cip1}$ expression through phosphorylation of FOXOs by JNK and this thereby confirms at an endogenous level our results obtained through overexpression of FOXO4 and $BRAF^{V600E}$.

Ectopic expression of FOXO4 in $BRAF^{V600E}$ expressing Colo829 melanoma cells induces $p21^{cip1}$-dependent senescence

In contrast to WM266.4, Colo829 cells express moderate levels of FOXO4 (Fig. 3a) and low levels of $p21^{cip1}$. Therefore we employed these melanoma cells to study the functional consequence(s) of FOXO activation and consequent $p21^{cip1}$ expression. Ectopic expression of FOXO4 in Colo289 cells significantly enhanced $p21^{cip1}$ promoter activity (Fig. 6a), whereas treatment of these cells with U0126 or NAC prevented this effect. These results demonstrate that in the response to functional $BRAF^{V600E}$ signaling FOXO4 promotes $p21^{cip1}$ transcription also in these cells.
Similar to our observations in U2OS and A14 cells, FOXO4 expression in Colo829 cells resulted in reduced colony formation (Fig. 6b). This was not due to FOXO4 expression inducing apoptosis, as FOXO4 expression did not increase TUNEL staining (Sup. fig 3a). The long term FOXO4 mediated repression in colony formation unlikely resulted from a G1 arrest followed by entry into quiescence as previously shown for colon carcinoma cells and which is dependent on FOXO mediated $p27^{kip1}$ transcription[21]. Rather, the FOXO4 induced arrest in Colo829 cells correlated with increased $p21^{cip1}$ expression and was associated with entry into senescence as indicated by positive senescence associated β-gal (SA-β-GAL) staining (Fig. 6b). To further establish entry into senescence, we also investigated the status of other senescence and proliferation markers. FOXO4 expression diminished PCNA and BrdU staining (Fig. 6c). Furthermore, FOXO4 expression resulted in formation of Senescence-Associated Heterochromatin Foci (SAHFs)

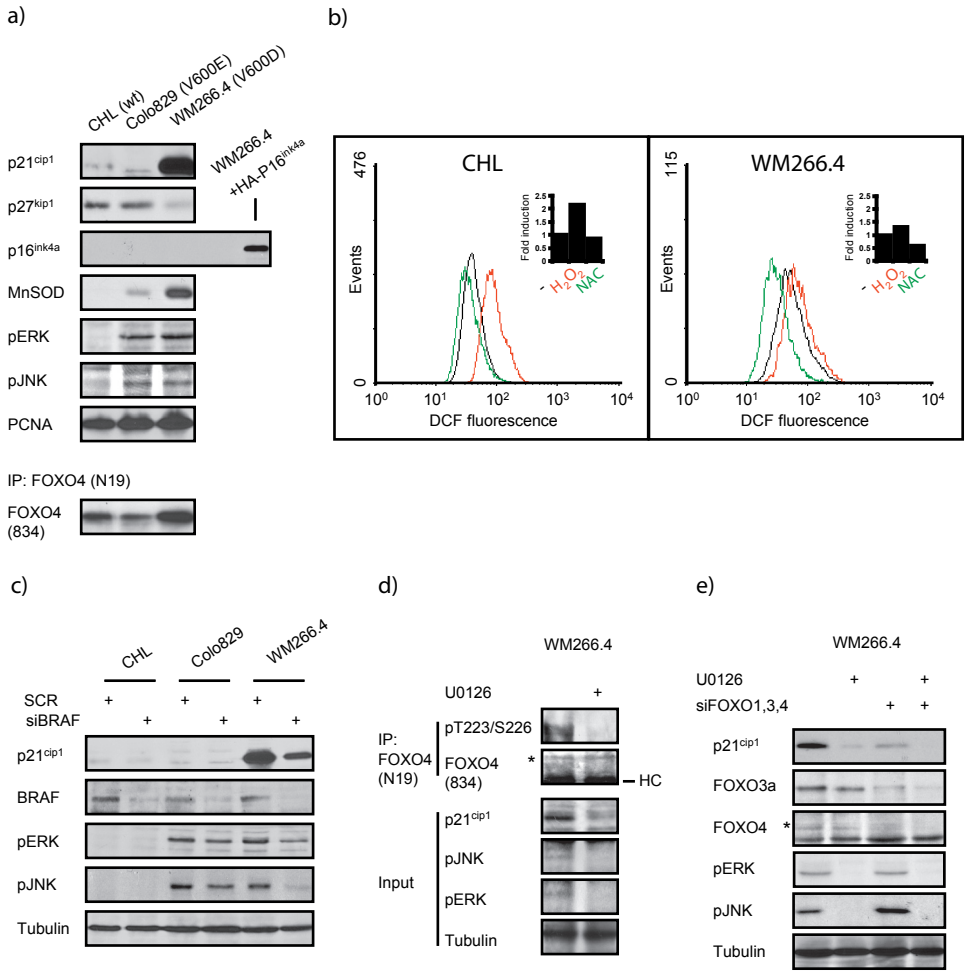
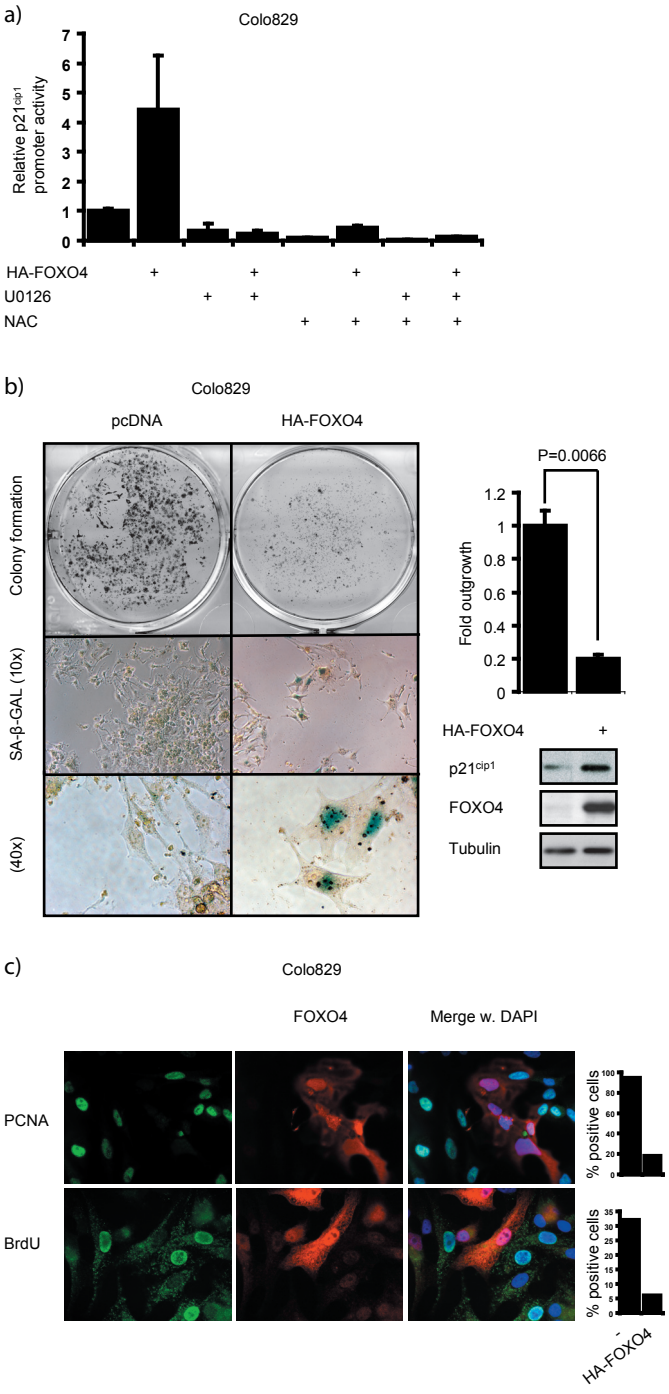


Fig. 5) Endogenous BRAF^{V600E} regulates p21^{cip1} transcription through FOXO4 phosphorylation
a) Expression of FOXO target genes is increased in BRAF^{V600E} mutated melanoma cells. CHL (wt BRAF), Colo829 (BRAF^{V600E}) and WM266.4 (BRAF^{V600D}) cells were lysed and analyzed by immunoblotting. Endogenous FOXO4 expression was determined after immunoprecipitation. **b)** Altered induction of DCF fluorescence in WM266.4 cells compared to CHL cells in response to H_2O_2 and NAC. CHL and WM266.4 cells were incubated with H_2DCFDA and analyzed as in Fig. 1e. **c)** Elevated p21^{cip1} expression in WM266.4 cells depends on BRAF^{V600E} expression. Lysates from CHL, Colo829 and WM266.4 cells transfected with scrambled or BRAF siRNA were analyzed by immunoblotting. **d)** U0126 abrogates JNK activation, phosphorylation of Thr223+Ser226 of endogenous FOXO4 and p21^{cip1} expression in WM266.4 cells. WM266.4 cells were untreated or treated for 24hrs with 10μM U0126 and analyzed as in a). The phosphorylation status of endogenous FOXO4 was determined after immunoprecipitation. (HC=Heavy Chain). **e)** Endogenous FOXOs are essential for p21^{cip1} expression in WM266.4 cells. Lysates of WM266.4 cells transfected with scrambled siRNA or siRNA against FOXO1,3a and 4 (siFOXO) and treated for 24 hours with 20μM U0126 or left untreated were analyzed by immunoblotting.


a)
Colo829
Relative p21cip1 promoter activity
7
6
5
4
3
2
1
0
HA-FOXO4 + + + +
U0126 + + + +
NAC + + + +
b)
Colo829
pcDNA
HA-FOXO4
Colony formation
SA-β-GAL (10x)
(40x)
P=0.0066
Fold outgrowth
1.2
1
0.8
0.6
0.4
0.2
0
HA-FOXO4 +
p21cip1
FOXO4
Tubulin
c)
Colo829
FOXO4
Merge w. DAPI
PCNA
BrdU
% positive cells
% positive cells
HA-FOXO4

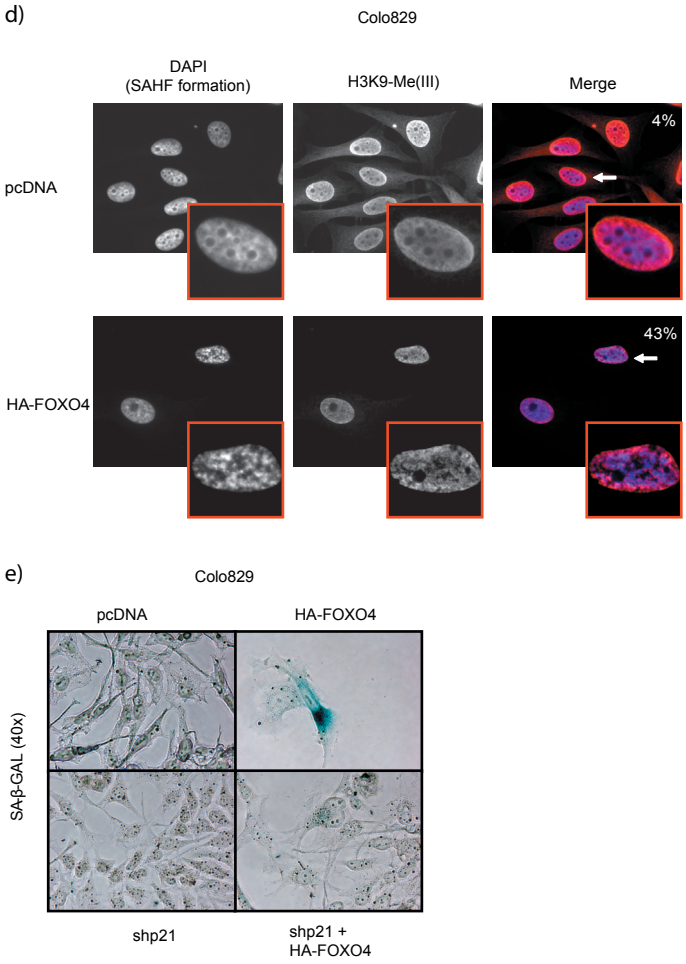


Fig. 6) Ectopic FOXO4 expression in Colo829 promotes p21^{cip1} expression and senescence
a) Ectopic expression of FOXO4 induces MEK/ROS dependent p21^{cip1} transcription in Colo829 cells. P21^{cip1} luciferase assay on lysates of Colo829 cells expressing HA-FOXO4 following 24h treatment with 10μM U0126 or 4mM NAC. **b)** Ectopic FOXO4 expression in Colo829 cells induces p21^{cip1} expression and senescence. Colo829 cells expressing HA-FOXO4 were selected with puromycin and stained for colony formation, or SA-β-GAL positivity after 10 days. Protein samples were obtained at 2.5 days post transfection and analysed by immunoblotting. **c)** FOXO4 expression in Colo829 cells reduces proliferation as determined by PCNA staining and BrdU incorporation. Colo829 cells expressing HA-FOXO4 and untransfected cells were stained at 2.5 days post transfection with anti-PCNA or analyzed for BrdU incorporation. 250 non-transfected and 50 transfected cells were quantified. **d)** FOXO4 expression in Colo829 cells induces SAHF formation and H3K9-trimethylation. Colo829 cells were transfected as in b) and at 5.5 days post transfection stained with DAPI for SAHF formation in parallel with anti-H3K9-Me(III). 100 cells were quantified and the percentage of positive cells indicated. **e)** P21^{cip1} expression is essential for FOXO4 to induce senescence in Colo829 cells. Colo829 cells were transfected and treated as in b) in combination with a plasmid encoding a scrambled short hairpin or a short hairpin against p21^{cip1}.

and H3K9-trimethylation (Fig. 6d), both of which are markers for senescence[5,22]. In contrast, expression of FOXO4 in wildtype BRAF expressing CHL cells did result in repression of colony formation, but not in SA-β-GAL staining (Sup. fig. 3b), indicating that these cells still undergo cell cycle arrest, independent of senescence. Together, these data indicate that in melanoma cells induction of $p21^{cip1}$ by FOXO4 in response to $BRAF^{V600E}$ signaling represses proliferation through induction of senescence.
Subsequently, we determined if phosphorylation of FOXO4 by $BRAF^{V600E}$ is important to promote senescence in Colo829 cells. Since prolonged treatment of these cells with U0126 induces apoptosis[23], we were not able to study the effect of this inhibitor on FOXO4 induced senescence. Therefore, we analyzed whether FOXO4-4A is able to induce senescence. In contrast to wild-type FOXO4, FOXO4-4A failed to induce senescence and did not repress colony formation (Sup. fig. 3c). This indicates that functional JNK sites are essential for the ability of FOXO4 to induce senescence in response to $BRAF^{V600E}$ signaling in Colo829 cells.
Inhibition of cell cycle progression and proliferation by $BRAF^{V600E}$ and FOXO4 requires $p21^{cip1}$ (Fig. 2h), and $p21^{cip1}$ expression was elevated in FOXO4 induced senescence in Colo829 cells (Fig. 6b). FOXO4 failed to induce SA-β–GAL staining in Colo829 cells upon $p21^{cip1}$ knockdown (Fig. 6e), indicating that $p21^{cip1}$ is also essential for FOXO4 induced senescence. Altogether, these data show that FOXO4 promotes senescence in $BRAF^{V600E}$ expressing Colo829 cells through $p21^{cip1}$.

Discussion

In this study we provide evidence for regulation of FOXO4 activity by oncogenic BRAF. $BRAF^{V600E}$ regulates FOXO4 through MEK-ROS-JNK-mediated phosphorylation to induce $p21^{cip1}$ expression and cellular senescence. Although we have analyzed the details of this mechanism for FOXO4, FOXOs are functionally redundant in this respect, as knockdown of FOXO1, 3a and 4 results in impaired $p21^{cip1}$ regulation. This is in agreement with studies in mice that show the same redundancy of FOXOs in their function as tumor suppressors[12]. Our study complements previous studies that underscored involvement of MEK-ERK signaling in JNK activation[24] and in $p21^{cip1}$ transcription by active BRAF[25]. Given that JNK activity is increased by oncogenic BRAF (Fig. 1a and[5]) our data in combination with these reports suggests a linear pathway of MEK-ROS-JNK signaling driven by oncogenic BRAF (Fig. 7). We also show that in contrast to activation of FOXO by reduced PI3K signaling, activation of FOXO by $BRAF^{V600E}$ diverts FOXO from a $p27^{kip1}$ to a $p21^{cip1}$ mediated arrest. Importantly, the biological consequence of this shift is the induction of senescence as opposed to quiescence. We emphasize that the induction of senescence by FOXOs is fully dependent on activation by oncogenic BRAF because in all oncogenic BRAF negative cells tested, in this study and previously, FOXOs do induce a cell cycle arrest, be it G1 or quiescence, without entry to senescence[17,21].

The results presented here have several implications. First, FOXOs have been identified as genuine tumor suppressors[12]. However, the mechanism behind these observations was elusive. We provide a novel mechanism on how FOXO signaling is involved in tumor suppression, i.e. through ROS-mediated induction of OIS. Second, $BRAF^{V600E}$ is an oncogene that is reported to promote JNK activation, albeit the functional consequences hereof have remained unclear. In more general terms oncogenic activation in melanocytes has recently been shown to link ROS to senescence[26]. The data presented here provide a potential mechanism for these observations

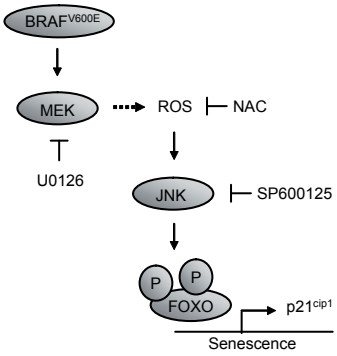


Fig. 7) Model for BRAF^{V600E} mediated p21^{cip1} transcription and senescence through phosphorylation of FOXOs by JNK
Upon oncogenic mutation of BRAF, downstream signaling through MEK is activated. Indirectly, cellular ROS levels are elevated, resulting in increased activation of JNK. JNK can subsequently induce phosphorylation of FOXO4 and thereby promote transcription of p21^{cip1} and trigger OIS.

as we indeed observed that BRAF^{V600E} expression generates ROS, which in turn activates JNK and FOXO to induce senescence. Third, the major FOXO target gene to induce cell cycle arrest in response to growth factor deprivation is p27^{kip1}. Importantly, no increase on p27^{kip1} levels have thus far been observed in response to ROS signaling, whereas FOXOs do mediate cell cycle arrest in this background[27] and also p21^{cip1} expression has been observed to be elevated[28]. Our data suggest that in response to elevated cellular ROS FOXOs induce a cell cycle arrest through p21^{cip1}, independent of p27^{kip1}. Finally, OIS has emerged as an important mechanism of tumor suppression, but in addition it is argued that cellular senescence is causative to organismal aging[29,30]. OIS therefore represents a trade-off between tumor suppression and lifespan. FOXOs are known to affect lifespan and key to this role may be their ability to regulate cellular oxidative stress. FOXOs are activated by cellular oxidative stress and in return may reduce cellular oxidative stress through increasing the expression of anti-oxidant genes such as MnSOD. By responding to oxidative stress FOXOs in this way protect cells by preventing build-up of excessive or damaging levels of ROS. Previously, we argued that this, in addition, requires a p27^{kip1}-mediated G1 cell cycle arrest and/or quiescence in order to also repair possible damage[31]. Recent evidence in mice shows this function of FOXO to operate in stem cells[32]. Here, we have shown that FOXOs ability to protect is yet more versatile, as in case of oncogenic BRAF, FOXOs also protect against the consequence of oncogenic stress i.e. tumorigenesis through senescence induction. Unlike the former, apparently this level of protection is not without cost. These findings clearly underline the pivotal role that FOXOs play in mediating the role of ROS in normal signaling as well as aging and it will be of interest to see whether for example age in return affects the ability of FOXO to mediate senescence.

Whereas we here show the importance of FOXO and p21^{cip1} in the onset of senescence it is clear that dependent on the setting, oncogenic activation of BRAF promotes transcription of p16^{ink4a} as well, and both CDK inhibitors are involved in BRAF^{V600E} induced OIS[4,33]. While BRAF^{V600E} can still induce OIS when either p21^{cip1} or p16^{ink4a} is inhibited individually[5,19,34], inactivation of both leads to a strong suppression of senescence[35,36]. Thus far, we have not obtained evidence for p16^{ink4a} regulation by FOXO but our results provide a molecular mechanism by which the p21^{cip1} arm of BRAF^{V600E} induced senescence is regulated through FOXO signaling.
Recent evidence suggests that besides activation of CDK inhibitors, secretion of a variety

of factors associated with inflammation and malignancy is required to establish a senescent phenotype, hence coined the senescence-associated secretory phenotype (SASP)[37]. SASP appears to be essential for cells that have undergone senescence in response to $BRAF^{V600E}$ and independent reports have shown activation of cytokine signaling, predominantly IL-6[38], and IGFBP signaling[39]. Whether FOXO signaling is involved in the initiation and/or maintenance of SASP remains to be elucidated. Irrespectively, based on our results, (re-)activation of FOXO signaling in $BRAF^{V600E}$ expressing melanomas could provide a novel strategy to therapeutic treatment of these tumors.

Materials and Methods

Cell culture and transfection

All cells were maintained in Dulbecco's Modified Eagle's Medium (Cambrex), 10% Fetal Calf Serum, penicillin/streptomycin and 0.05% glutamine. HEK293T, U2OS and A14 cells were transfected via the calcium-phosphate method. CHL, Colo829 and WM266.4 cells were transfected with Effectene according to the manufacturer's protocol (Qiagen).

Constructs and RNAi

The following constructs have been described before: pBabe-puro, pMT2-HA-FOXO4, pRP261-GST-FOXO4-ΔDB[40], 6xDBE-firefly luciferase, MnSOD-firefly luciferase and TK-renilla luciferase[31], pEFm-$BRAF^{V600E}$ [2], $p21^{cip1}$-luciferase[41]. pSuper-$p21^{cip1}$ was a kind gift from Mathijs Voorhoeve[42]. pMT2-HA-FOXO4-4A was generated by site-directed mutagenesis with the following oligo's: FOXO4-T223A-S226Afwd: 5' ccacccgaaggtgccgctccaacggcccctgtc 3' and FOXO4-T223A-S226Arev: 5' gacaggggccgttggagcggcaccttcgggtgg 3'. As a template we used pMT2-HA-FOXO4-T447/451A[43]. pSuperior-FOXO1/3 and pSuperior-FOXO4 (both against human and mouse) were generated according to the manufacturer's instructions using pSuperior-retro-puro as a template (OligoEngine) and the following oligo's:
FOXO4-1fwd: 5' gatcccggaaatcagtcatatgcagaattcaagagattctgcatatgactgatttccttttta 3',
FOXO4-1rev: 5' agcttaaaaaggaaatcagtcatatgcagaatctcttgaattctgcatatgactgatttccgg 3';
FOXO4-2fwd: 5' gatcccgttcatcaaggttcacaacttcaagagagttgtgaaccttgatgaacttttta 3', FOXO4-2rev: 5' agcttaaaaagttcatcaaggttcacaactctcttgaagttgtgaaccttgatgaacgg 3'; FOXO1/3-fwd: 5' gatcccgtgccctacttcaaggataagttcaagagacttatccttgaagtagggcacttttta 3'; FOXO1/3-rev: 5' agcttaaaaagtgccctacttcaaggataagtctcttgaacttatccttgaagtagggcacgg 3'. Smartpool oligo's against FOXO1,3a and 4, BRAF or scrambled oligo's (Dharmacon) were transfected at a final concentration of 100nM each (300nM for scrambled) using oligofectamine according to the manufacturer's protocol (Invitrogen).

Antibodies

The antibodies against FOXO4 (834), HA (12CA5), phospho-Thr447 and phosphoThr451 have been described before[11,44]. The following antibodies were purchased: phosphoThr183/Tyr185-JNK and phosphoThr202/Tyr204-ERK (Cell Signaling), FOXO4-phospho-Thr28 (Upstate), MnSOD (Stressgen) trimethyl-H3K9 and FOXO3a (Upstate), $p27^{kip1}$ and $p21^{cip1}$ (BD pharmingen), $p21^{cip1}$ (Ab-3) and p16ink4a (ab-2) (Neomarkers), BRAF (C19), FOXO4 (N19), FOXO1 (N18), PCNA (PC10) and p53 (DO-1) (Santa Cruz) and Tubulin (Sigma). Antibodies against phospho-Thr223 and Phospho-Tr223/Ser226 were generated by immunizing rabbits with the peptides CKAPKKKPSVLPAPPEGA-pT-PTSPVG and CKAPKKKPSVLPAPPEGA-pT-PT-pS-PVG, respectively, where pT and pS present phosphorylated Threonine and Serine (Covance).

Immunoprecipitation and *in vitro* JNK kinase assays

Immunoprecipitation and GST-pulldown assays were performed as described[44] in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 1% TX-100, 0.5% NaDoC, 5 mM EDTA, 150 mM NaCl, protease and phosphatase inhibitors. For *in vitro* JNK kinase assays HA-FOXO4 or GST-FOXO4ΔDB was precipitated and processed according to the manufacturer's protocol (Upstate).

Immunofluorescence, TUNEL staining and BrdU incorporation

Immunofluorescence was performed as described[44], using anti-FOXO4 (834), anti-HA (12CA5), PCNA (1:50),

H3K9-Me(III) (1:100). BrdU incorporation and TUNEL staining were performed according to the manufacturer's protocols (Roche)

Luciferase assays
Cells were transfected with 500ng -2.2kb $p21^{cip1}$-firefly luciferase, -3.34kb MnSOD-firefly luciferase, or a firefly luciferase construct bearing six canonical FOXO DNA binding elements (6xDBE-firefly luciferase) and 100ng TK-renilla luciferase. Experiments were performed in triplicate. Luciferase activity was analysed at 48 hours post transfection using a luminometer and dual luciferase assay kit according to the manufacturer's instructions (Promega).

Quantitative Real-time PCR
The expression of endogenous $p21^{cip1}$ mRNA in HEK293T cells was examined by reverse transcription of total RNA followed by real-time quantitative PCR (qPCR) on an ABI cycler using Sybr Green (ABI) as described[44], with the following oligonucleotides: $p21^{cip1}$fwd: 5' ccgaggcactcagaggag 3'; $p21^{cip1}$rev: 5' agctgctcgctgtccact 3' and PBGDfwd: 5'ggcaatgcggctgcaa3' PBGDrev: 5'gggtacccacgcgaatcac3', respectively.

Cellular ROS measurements with H_2DCFDA
HEK293T cells were plated in 6well plate dishes and transfected with pcDNA3 or a plasmid encoding $BRAF^{V600E}$ (2μg), in parallel with pbabe-puro (500ng). At 16hrs post transfection cells were selected with 2μg/ml puromycin for 36hrs before initiation of the measurements. Cells were left untreated or pretreated for 24hrs with 4mM NAC or 10μM U0126, washed once with warm, sterile PBS and incubated for 10min with 1ml 10μM H_2DCFDA (Invitrogen) in PBS. Following a recovery for 4 hours in medium with or without NAC or U0126 cells were pretreated with or without 45min 200μM H_2O_2 and collected by trypsinization. After centrifugation cells were incubated with 0.02mg/ml Propidium Iodide (PI) and PI negative cells were analyzed by FACS for DCF fluorescence. The experiments with CHL and WM266.4 were performed similarly, but without selection and PI treatment.

Cell cycle distribution by FACS
U2OS cells were transfected with the appropriate plasmids in combination with 250ng GFP-Spectrin. At 36hrs post transfection cells were treated for an additional 24hrs with 250ng/ml nocodazole and processed for FACS analysis as described[17] on a FACScalibur (ABI), using WinMDI v2.9 to analyse data.

Mass spectrometry
Input material was digested with trypsin, subtilisin, and/or elastase (Roche). Samples were enriched for phosphorylated peptides using TiO2 microcolumns, as described[44]. Samples were subjected to nanoflow LC (Agilent 1100 series) coupled to a quadrupole time-of-flight tandem mass spectrometer (Micromass Waters). Data were processed and subjected to database searches using MASCOT software (Matrix Science). The identified peptides were confirmed by manual interpretation of the spectra.

Colony Formation assay and SA-β-Gal staining
A14 or U2OS cells were plated in six well plates and transfected with the appropriate plasmids in combination with pbabe-puro (500ng). At 24 hours post transfection cells were subjected to puromycin selection (2μg/ml). Following 2.5 days of selection with 2μg/ml puromycin one set of cells was lysed and analyzed by immunoblotting for protein expression. Selection medium was refreshed every two days. At 10 days post transfection cells were fixed in methanol, stained with 0.5% crystal violet in 25% methanol and washed with water. The plates were dried overnight and colony formation was quantified by destaining in 10% acetic acid and measuring optical density at 560nm. CHL, Colo829 and WM266.4 cells were treated similarly, but transfected with 500ng FOXO4 and 250ng pbabe-puro. SA-β-GAL staining was performed at 9 days post transfection as described[45].

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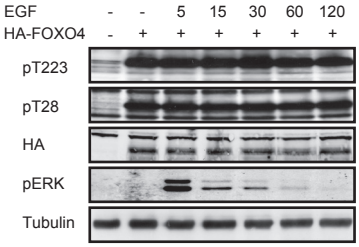
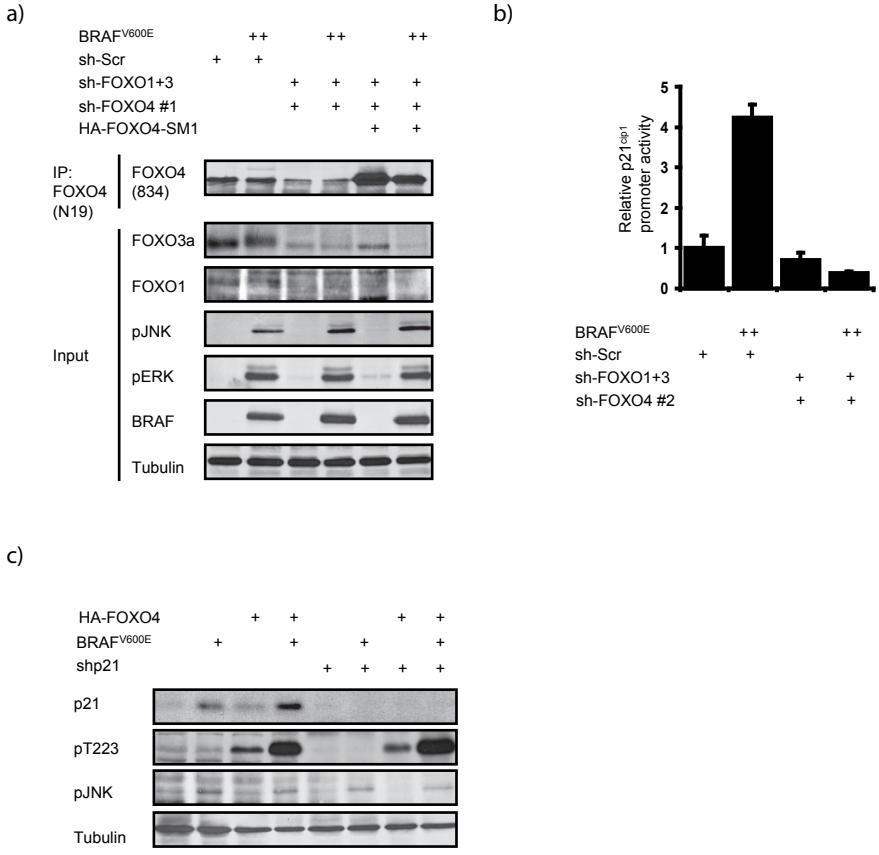
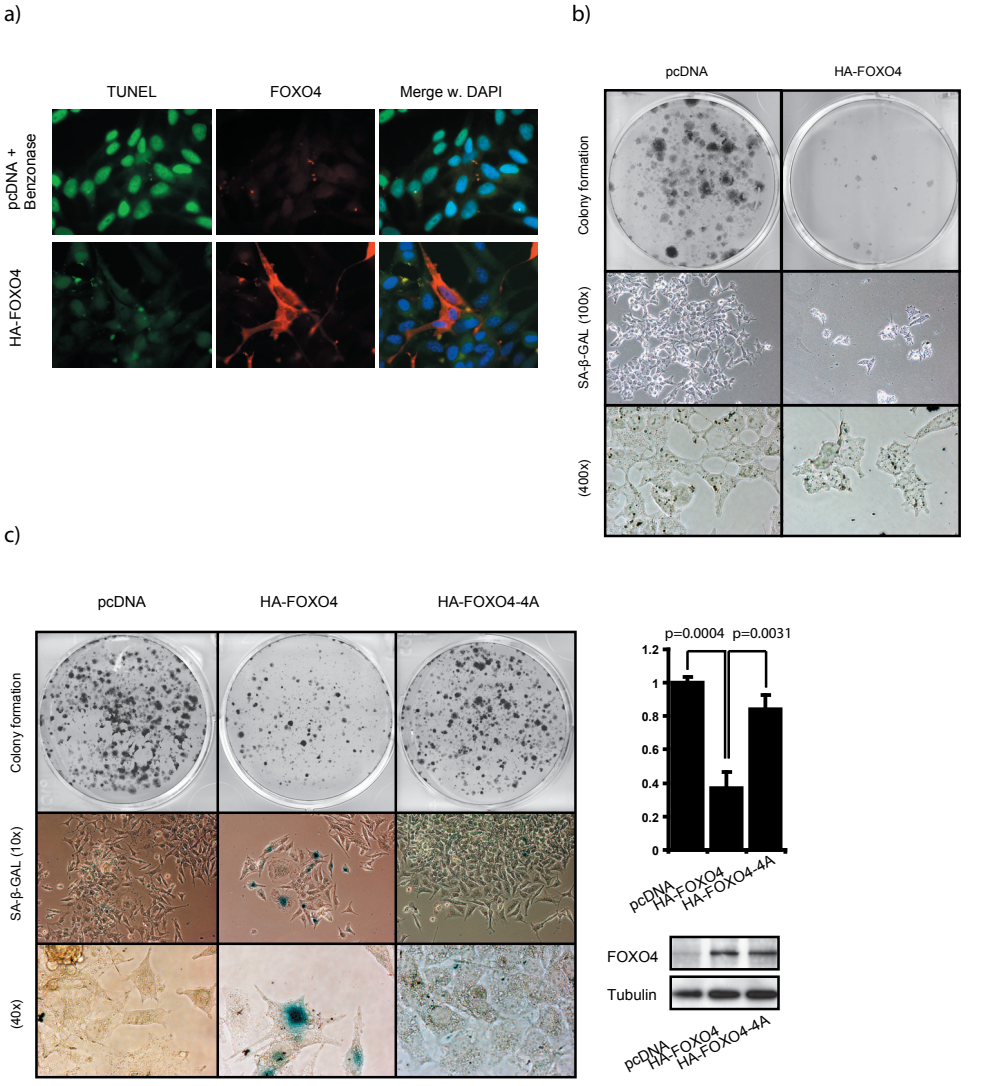
a)



Sup. fig. 1)
a) EGF stimulation does not induce Thr223 phosphorylation. HEK293T cells were transfected with or without a plasmid encoding HA-FOXO4. After 24hrs the cells were placed serum free for another 24hrs and left untreated (-) or treated with 20µg/ml EGF for 5, 15, 30, 60 or 120 mins. Lysates were analysed by immunoblotting using the indicated antibodies.



Sup. fig. 2)
a) Knockdown of endogenous FOXO1,3 and 4, but not the add-back mutant FOXO4-SM, by short hairpin oligo's. HEK293T cells were transfected with pbabe-puro and a scrambled short hairpin or short hairpins targeting human/mouse FOXO1, 3 and FOXO4 in combination with or without a plasmid encoding HA-FOXO4-SM bearing a silent mutation in the sequence targeted by the hairpin. Puromycin selected cells were lysed and expression of the indicated proteins determined by immunoblotting using the indicated antibodies. **b)** Knock-down of FOXO4 by a different short hairpin also impairs BRAF^{V600E} induced p21^{cip1} transcription. Luciferase assay on A14 cell lysates transfected with plasmids encoding BRAF^{V600E} and a scrambled short hairpin or short hairpins targeting FOXO1 and 3 or a different sequence in FOXO4. **c)** Successful knockdown of p21^{cip1} induced by FOXO4 and BRAF^{V600E}. HEK293T cells were transfected with pbabe-puro and plasmids encoding BRAF^{V600E} and HA-FOXO4 in combination with short hairpins targeting a scrambled sequence or p21^{cip1} analyzed as in Fig. 3f.



Sup. fig. 3)
a) Ectopic FOXO4 expression does not induce apoptosis in Colo829 cells. Colo829 cells were transfected with a plasmid encoding HA-FOXO4 or an empty vector and stained for TUNEL positivity at 2.5 days post transfection. As a positive control the pcDNA transfected cells were treated for 10 mins. with the DNase Benzonase to induce double strand breaks. **b)** Ectopic FOXO4 expression in CHL cells represses colony formation, but does not induce senescence. CHL cells transfected and treated as in Fig. 6b. **c)** Intact JNK sites are essential for FOXO4 to induce senescence in Colo829. Colo829 cells were transfected with a plasmid encoding HA-FOXO4, HA-FOXO4-4A or an empty vector control and treated as in Fig. 6b.

Addendum: FOXO4 induces senescence in BRAF^{V600E}, but not NRAS$^{Q61R/K}$, -mutated melanoma cells

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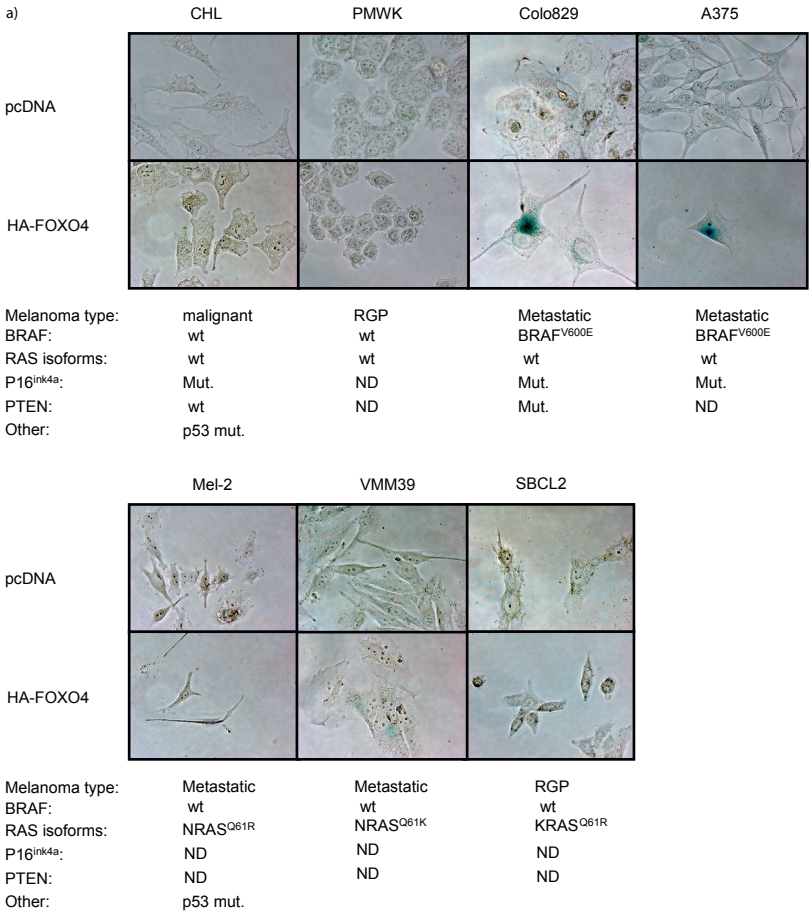
In chapter 4, we described in detail how FOXO signaling is modulated by oncogenic BRAF^{V600E}. Importantly, in BRAF^{V600E}-expressing Colo829 melanoma cells ectopic expression of FOXO4 resulted in cellular senescence, which was not observed in wt BRAF expressing melanoma CHL cells. We proposed a model in which FOXO signaling functions downstream of BRAF^{V600E} to contribute to oncogene induced senescence (OIS).

To further challenge this model, we determined if FOXO4 expression could induce senescence in other melanoma cells that express oncogenic RAF, RAS or are wildtype for both. Next to the already investigated Colo829 and CHL cells, we included a cell line that expresses wt BRAF, PMWK, and two additional cell lines that express BRAF^{V600E}, A375 and SK-Mel28[1]. FOXO4 expression induced a strong senescence staining in BRAF^{V600E} mutated Colo829 and A375 cells, but not in CHL and PMWK cells (Fig. 1a). Furthermore, in a different experiment FOXO4 also induced senescence in SK-Mel28 melanoma cells (BRAF^{V600E}), but not in primary melanocytes (data not shown). Together, these data suggest that FOXO4 induced OIS is specific for cells with a BRAF^{V600E} mutation.

p16^{ink4a} expression is associated with BRAF^{V600E}-induced senescence[2,3] (Check REF). Both Colo829 and A375 cells lack p16^{ink4a} expression[4]. Since FOXO expression induces senescence in these cells this further argues that BRAF^{V600E}-induced senescence through FOXO4 occurs independent of p16^{ink4a}. Although BRAF^{V600E} is the majority oncogenic mutation found in melanoma, RAS mutations (typically (N)RAS$^{Q61R/K}$) are also frequently observed (15-30%; Ref[5]). Therefore, we also addressed if FOXO4 can induce senescence in two melanoma cell lines that harbor this mutation in NRAS, Mel-2 (NRAF^{Q61R}) and WMM39 (NRAF^{Q61K}), or a similar mutation in KRAS, SBCL2 (KRAS^{Q61R}). Surprisingly, FOXO4 failed to induce senescence in both of these cell lines (Fig.1a). This suggests that FOXOs specifically induce senescence in response to oncogenic BRAF, rather than oncogenic RAS.

The finding that FOXO induces a senescence response in a background of oncogenic BRAF, but not in oncogenic RAS, was surprising since RAF and RAS show a high degree of similarity in their downstream signaling[2]. However, other recent results indicate dissimilarity in the mechanism of senescence induction between BRAF and RAS. For example, endoplasmic reticulum stress due to improper protein folding is involved in oncogenic RAS (H-, K- or N-RAS) but not BRAF^{V600E} induced cellular senescence[6]. Nonetheless, similar to BRAF^{V600E} oncogenic RAS has been shown to induce ROS and can induce p21^{cip1} expression (Chapter



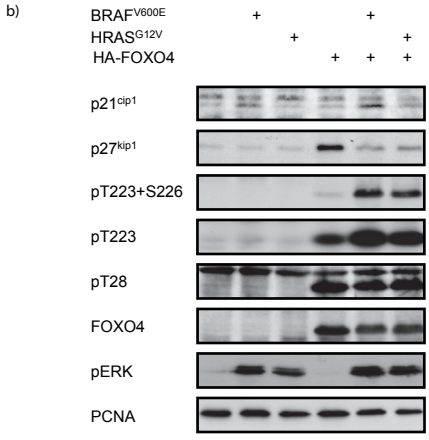


Fig. 1) FOXO4 induces senescence and p21^{cip1} expression in response to oncogenic BRAF, not N/H-RAS

a) Effect of ectopic FOXO4 expression in melanoma cells. CHL and PMWK (wt for BRAF and the RAS isoforms), Colo829, A375 (expressing BRAF^{V600E}), Mel-2, VMM39 (expressing NRAS$^{Q61R/K}$) and SBCL2 (KRAS^{Q61R}) were transfected with FOXO4 and after 10 days stained for the presence of senescent cells. **b)** FOXOs co-operate with oncogenic BRAF^{V600E}, but not HRAS^{G12V} to induce p21^{cip1} expression. Transfected HEK293T cells expressing the indicated proteins were selected with puromycin and analyzed by immunoblotting with the indicated antibodies.

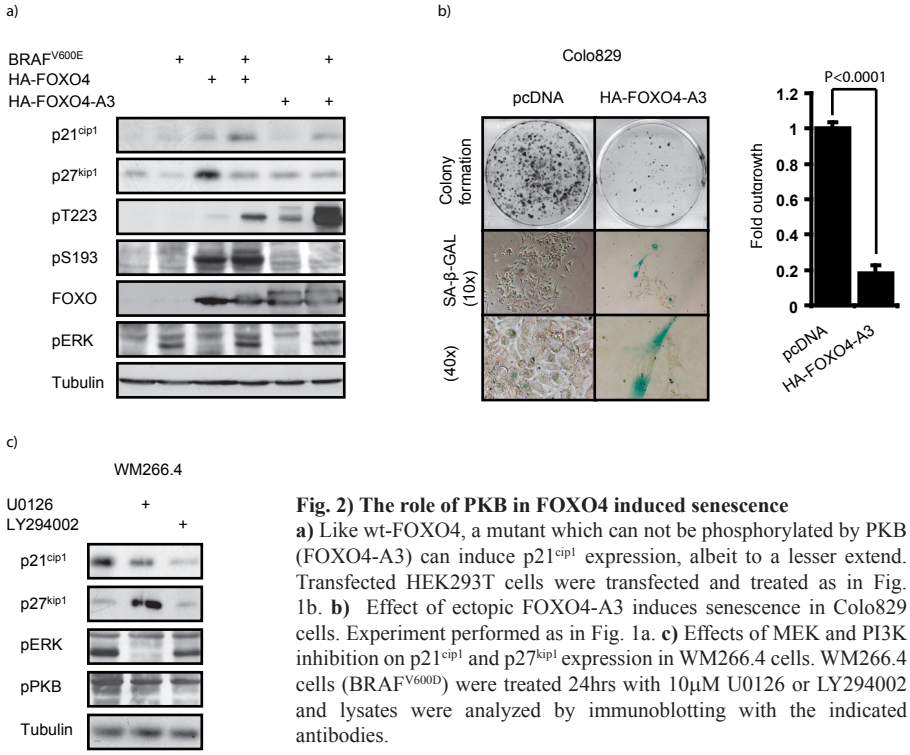


Fig. 2) The role of PKB in FOXO4 induced senescence
a) Like wt-FOXO4, a mutant which can not be phosphorylated by PKB (FOXO4-A3) can induce p21^{cip1} expression, albeit to a lesser extend. Transfected HEK293T cells were transfected and treated as in Fig. 1b. **b)** Effect of ectopic FOXO4-A3 induces senescence in Colo829 cells. Experiment performed as in Fig. 1a. **c)** Effects of MEK and PI3K inhibition on p21^{cip1} and p27^{kip1} expression in WM266.4 cells. WM266.4 cells (BRAF^{V600D}) were treated 24hrs with 10μM U0126 or LY294002 and lysates were analyzed by immunoblotting with the indicated antibodies.

4 and [7-10]). Therefore, we next addressed if FOXO4 is modified by oncogenic RAS. As we did not obtain a NRAS^{Q61R} construct, we used a HRAS^{G12V} construct instead, that to a large extend induces similar downstream signaling[11]. As observed for BRAF^{V600E}, co-expression of HRAS^{G12V} led to increased phosphorylation of the JNK target sites Thr223 and Thr223/Ser226 and decreased FOXO4 induced p27^{kip1} expression in HEK293T cells (Fig. 1b). Thus, this suggests that oncogenic RAS and RAF signaling does not differ at the level of FOXO4 phosphorylation and the regulation of p27^{kip1}.
Oncogenic RAS mediates downstream signaling towards ERK through CRAF rather than BRAF[4]. In contrast, in BRAF^{V600E} expressing Colo829 and A375 cells, signaling towards ERK is independent of CRAF[12]. However, ectopic expression of constitutive CRAF (CRAF-CAAX) also induced phosphorylation of Thr223 and co-operative p21^{cip1} induction with FOXO4 (Data not shown). Therefore, it is unlikely that the shift from BRAF to CRAF can explain why FOXO4 does not induce senescence in the NRAS mutated melanoma cells. Surprisingly, however, in contrast to BRAF^{V600E}, HRAS^{G12V} did not produce a strong induction in p21^{cip1} expression in these cells. Although further analysis is required, this may provide a possible explanation why FOXOs do not induce senescence in response to oncogenic RAS.

Next to activation of the RAF-MEK-ERK cascade, RAS also activates PI3K-PDK1-PKB

signaling[13]. FOXOs are classical PKB substrates in response to growth factor stimulation and PKB mediated phosphorylation induces their nuclear export[14]. PKB-mediated phosphorylation of wildtype FOXO4 may therefore account for its inability to induce senescence in response to oncogenic RAS. Albeit to a lesser extend, a mutant that is defective for PKB-mediated phosphorylation (FOXO4-A3) can co-operate with $BRAF^{V600E}$ to induce $p21^{cip1}$ expression (Fig. 2a). Furthermore, expression of this mutant induced senescence in Colo829 cells (Fig. 2b). To challenge this hypothesis it will therefore be interesting to determine if FOXO4-A3 can induce senescence in the oncogenic RAS expressing SBCL2, VMM39 and MEL-2 melanoma cells.
In contrast to repression of FOXO signaling, PKB can directly phosphorylate and stabilize $p21^{cip1}$. Furthermore, at least the WM266.4 cells used in Chapter 4 display a high level of p21cip1 and active PKB (data not shown). Notably, treatment of WM266.4 cells with the PI3K inhibitor LY294002 significantly reduced $p21^{cip1}$ expression (Fig. 2c). This argues against PKB activity to be an explanation as to why FOXOs fail to induce $p21^{cip1}$ expression and senescence expression in response to oncogenic RAS. Thus, it remains to be established why the ectopic FOXO4 induces $p21^{cip1}$ expression and senescence in response to oncogenic BRAF, but not RAS.

Materials and Methods

Cell culture and transfection

All cells were maintained in DMEM (Cambrex), except for SBCL2, which were maintained in RPMI. All media was supplemented with 10% Fetal Calf Serum, penicillin/streptomycin and 0.05% glutamine. HEK293T were transfected via the calcium-phosphate method. All melanoma cells were transfected with Effectene according to the manufacturer's protocol (Qiagen).

Constructs and RNAi

The following constructs have been described before: pBabe-puro, pMT2-HA-FOXO4, pMT2-HA-FOXO4-A3 (Chapter 2), pEFm-$BRAF^{V600E}$ (Chapter 4) and pMT2-HA-$HRAS^{G12V}$ (Ref[14]).

Antibodies and chemical inhibitors

The antibodies against FOXO4 (834), phospho-Thr223 and phospho-Thr223/Ser226, phospho-Thr28, phospho-Ser193 (all for FOXO4), $p21^{cip1}$, $p27^{kip1}$, PCNA and phospho-Thr202/Tyr204-ERK have been described in Chapter 4. The antibody against phospho-Ser473-PKB was purchased from Cell Signaling. The MEK inhibitor U0126 has been described in Chapter 4. The PI3K inhibitor LY294002 has been described elsewhere[15].

Immunofluorescence, colony formation and SA-β-GAL staining

All assays were essentially preformed as described in Chapter 4.

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5

Functional interaction between FOXO4 and p53 in response to oncogenic BRAF signaling

Manuscript to be submitted

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Functional interaction between FOXO4 and p53 in response to oncogenic BRAF signaling

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Abstract

Loss or mutation of p53 is strongly associated with increased susceptibility to develop cancer[1]. Conversely, activation of p53 can suppress tumorigenesis through induction of cell cycle arrest or apoptosis[2]. Recently, we established that FOXO transcription factors are phosphorylated and activated in response to oncogenic BRAF^{V600E} signaling to mediate a p21^{cip1}-dependent oncogene induced senescence (OIS) response[3]. p53 is also a well established regulator of p21^{cip1} expression and OIS[1,4]. However, whether there is a cross-talk between FOXO and p53 signaling in this process is unknown.

Here, we reveal a functional interplay between FOXO and p53 in response to BRAF^{V600E} signaling. First, we show that FOXO4 and p53 can physically interact, an effect that can be induced by BRAF^{V600E}. Furthermore, we show that besides phosphorylation, the mono-ubiquitination and subsequent nuclear localization of FOXO4 is increased by BRAF^{V600E}. These mono-ubiquitination events require the E3-ligase HDM2, a well known target and regulator of p53. Next to FOXO4, also p53 is modified by BRAF^{V600E} signaling through increased phosphorylation on Ser46, but not Ser15 and Ser20. Finally, we show that knockdown of either p53 or FOXOs in BRAF^{V600E} expressing Colo829 melanoma cells induces apoptosis. Based upon these results we propose a model, where regulation of the interplay between FOXOs and p53 determines cell fate in BRAF^{V600E} expressing cells.

Introduction

Following its characterization as a tumor suppressor[5], a vast amount of research has been conducted on the regulation and functioning of p53. p53 can be activated by genomic stress, which stabilizes its expression and allows transcription of its target genes[6]. Essentially, p53 can repress proliferation of damaged cells through induction of a cell cycle arrest to subsequently allow repair, or, in case of severe damage, apoptosis[2]. p53 can induce cell cycle arrest through transcriptional regulation of a multitude of target genes including the CDK inhibitor *p21^{cip1}* (Ref[4,7]). This p53-p21^{cip1} axis can be activated in response to oncogenic stress, thereby preventing tumorigenesis[8].

The FOXO family of transcription factors comprises four isoforms, FOXO1, FOXO3a, FOXO4 and FOXO6[9,10]. Like p53, FOXOs are *bona fide* tumor suppressors, a function in which they are redundant[11]. However, whereas p53 is primarily activated in response to genotoxic stress, FOXOs mainly regulate cell cycle arrest and apoptosis in response to growth factor deprivation or elevated levels of Reactive Oxygen Species (ROS)[12]. Recently, we showed that BRAF^{V600E}

signaling induces a chronic elevation in cellular ROS, thereby activating FOXO signaling[3]. In response to growth factor deprivation FOXOs arrest cell cycle progression primarily through induction of $p27^{kip1}$. However, in response to oncogenic $BRAF^{V600E}$ signaling this arrest is shifted to $p21^{cip1}$. This observation, combined with literature, suggested an interplay between FOXOs and p53, at least in *p21cip1* regulation. Indeed at present, evidence is accumulating that FOXO and p53 interact at various direct and indirect levels[12]. For example, interplay between FOXO and p53 signaling has been studied before with regard to nutrient deprivation, a background in which p53 signaling was repressed and the FOXO mediated quiescence response increased[13]. Conversely, in response to genotoxic damage the outcome is opposite[14]. Combined, these data prompted us to investigate whether p53 and FOXO4 interact in response to $BRAF^{V600E}$ signaling, thereby potentially regulating $p21^{cip1}$ transcription.

Results

p53 has been shown to interact with the FOXO4 homolog FOXO3a[15] and we could confirm this interaction for FOXO4 (data not shown and Fig. 1a). The interaction of FOXO3a with p53 could be increased by H_2O_2-treatment, which induces an acute elevation in cellular ROS[15]. Since $BRAF^{V600E}$ induces a chronic elevation in cellular ROS[3], we therefore determined if $BRAF^{V600E}$ affects the interaction with FOXO4 as well. Ectopic expression of p53 consistently resulted in decreased FOXO4 expression, similar as reported for FOXO3a (Fig. 1a, data not shown and[15]). $BRAF^{V600E}$ co-expression could rescue this reduction in FOXO4 levels and stimulated co-precipitation of FOXO4 by p53 (Fig. 1a). Next, we determined if $BRAF^{V600E}$ expression affects binding of ectopic FOXO4 to endogenous p53, thereby avoiding significant expression conflicts. Importantly, also under these conditions $BRAF^{V600E}$ induced the interaction of FOXO4 and p53 (Fig. 1b). Together, this indicates that in response to $BRAF^{V600E}$ signaling the interaction between p53 and FOXO4 is increased.

Although triggered by different stimuli, FOXOs and p53 can partially induce transcription of an overlapping set of target genes such as $p21^{cip1}$[12]. Notably, within the $p21^{cip1}$ promoter, the p53 and FOXO binding sites are located within close proximity, for FOXO at –1760bp and for p53 at –2251bp and –1344bp, respectively[4,16]. Therefore, the observed co-precipitation of FOXO4 and p53 could be the consequence of promoter binding, rather than direct interaction. To test this hypothesis, we determined if disruption of DNA through post-lysis treatment with the DNase Benzonase affected the ability of p53 to precipitate FOXO4. Furthermore, we also included Ethidium Bromide, which intercalates DNA, thereby competing for interacting proteins[17]. As expected, the Benzonase treatment resulted in complete digestion of a separate control plasmid (Fig. 1c). However, neither Benzonase, nor Ethidium Bromide decreased the interaction between FOXO4 and p53 (Fig, 1c), indicating that binding between FOXO4 and p53 is a protein-protein interaction, independent of DNA.

$BRAF^{V600E}$ signaling activates FOXO4 through activation of a linear cascade involving MEK-induced elevations in cellular ROS levels and subsequent JNK activation. Conversely, interference with MEK strongly represses FOXO4 phosphorylation and transcriptional activation by $BRAF^{V600E}$ (Ref[3]). Therefore, we addressed the effect of MEK inhibition with U0126 on the interaction with p53. Interestingly, U0126-treatment inhibited the $BRAF^{V600E}$-induced interaction between FOXO4 and p53 (Fig. 1d). Together, these data indicate that the protein-protein interaction between FOXO4 and p53 is enhanced by functional signaling downstream of $BRAF^{V600E}$.

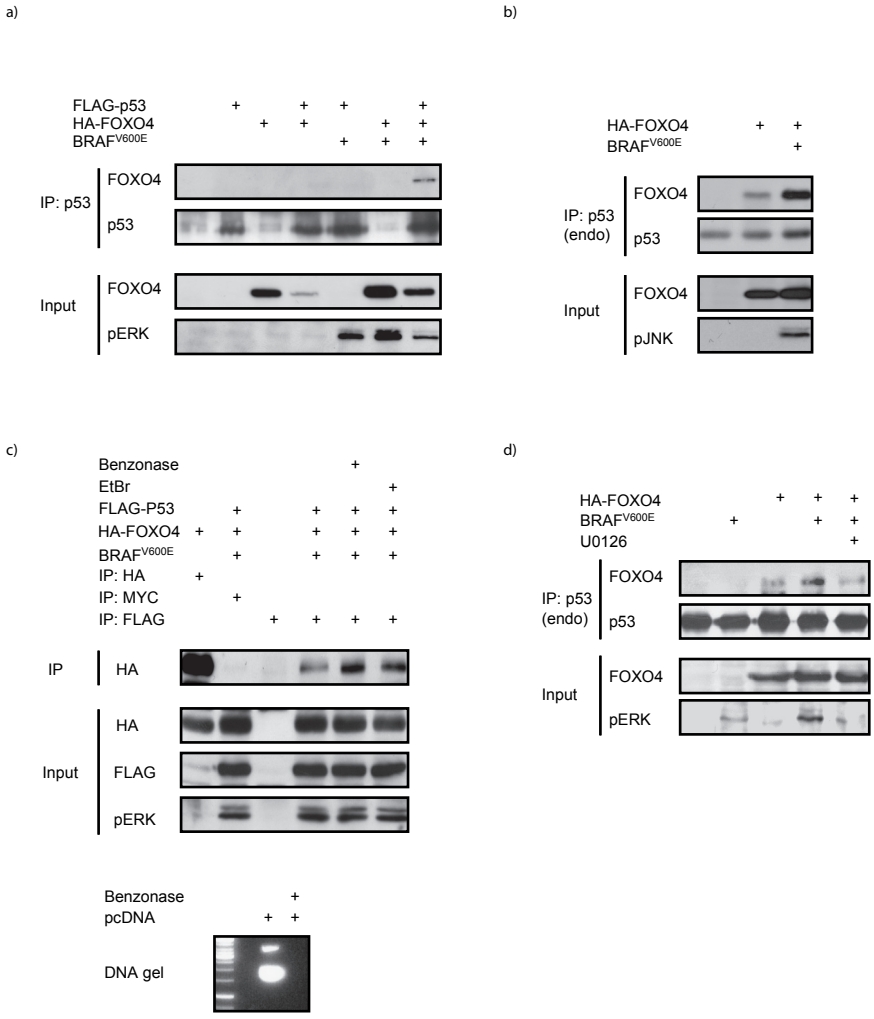

Fig. 1) In response to BRAF^{V600E} signaling FOXO and p53 interact to induce p21^{cip1} expression
a) BRAF^{V600E} inhibits the repressive effect of p53 on FOXO4 expression and induces their interaction. Lysates from HEK293T cells expressing FLAG-P53, HA-FOXO4 and BRAF^{V600E} were subjected to immunoprecipitation with anti-p53 and analyzed by immunoblotting. **b)** BRAF^{V600E} induces the interaction of FOXO4 with endogenous p53. Lysates from HEK293T cells expressing HA-FOXO4 and BRAF^{V600E} were subjected to immunoprecipitation with anti-p53 and analyzed by immunoblotting. **c)** The interaction of FOXO4 and P53 is independent of DNA. Lysates from HEK293T cells expressing HA-FOXO4 and BRAF^{V600E} were left untreated or treated with 15 minutes with 5units/ml Benzonase or 50μg/ml Etidium Bromide (EtBr) and subjected to immunoprecipitation and immunoblotting with the indicated antibodies. As a control, 1μg pcDNA vector was treated in parallel with benzonase to determine DNA fragmentation. **d)** Inhibition of MEK by U0126 supresses the BRAF^{V600E} induced interaction between FOXO4 and p53. Lysates from HEK293T cells expressing HA-FOXO4 and BRAF^{V600E} and left untreated or treated with 10μM U0126 were processed as in (b).

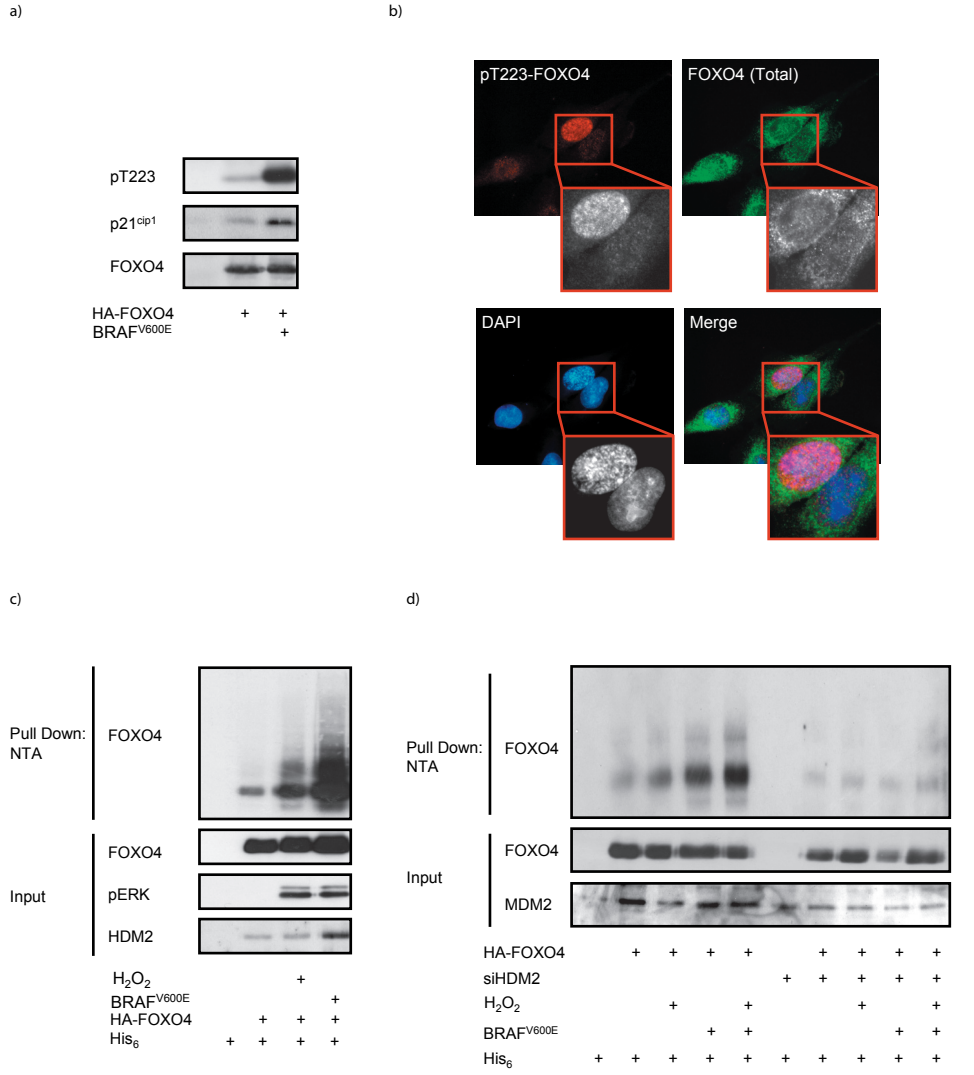

Fig. 2) BRAF^{V600E} induces FOXO nuclear localization and mono-ubiquitination through HDM2
a) BRAF^{V600E} induces Thr223 phosphorylation of FOXO4, correlative with p21^{cip1} expresison. HEK293T cells were transfected as indicated and analyzed by immunoblotting **b)** Thr223 phosphorylated FOXO4 localizes to the nucleus. A14 cells transiently expressing HA-FOXO4 were subjected to immunofluorescence with an antibody against phosho-Thr223. To detect total FOXO4 localization we used an antibody against HA. Similar results were obtained in U2OS cells, *p53-/-* H1299 cells and A375 melanoma cells. **c)** BRAF^{V600E} induces (multi-)mono-ubiquitination of FOXO4. HEK293T cells were transfected as indicated and left untreated or treated with 50 μM H_2O_2 for 15 min and the pool of ubiquitinated FOXO4 was determined after NTA precipitation and analysis by immunoblotting with the indicated antibodies. **d)** H_2O_2 and BRAF^{V600E} induced mono-ubiquitination of FOXO4 are HDM2 dependent. HEK293T cells were transfected and treated as in (c), in presence of cotranfected scrambled siRNA or siRNA against HDM2.

Next, we investigated how $BRAF^{V600E}$ signaling affects FOXO4 and p53 through post translational modification. Previously, we have shown that $BRAF^{V600E}$ signaling promotes JNK-mediated phosphorylation of FOXO4 on multiple residues, including Thr223 (Ref[3] and Fig. 2a). Acute elevations in cellular ROS levels by H_2O_2 induce similar phosphorylation events and result in nuclear import of FOXOs[18]. We therefore determined the subcellular distribution of FOXO4 once phosphorylated on this residue. Indeed, Thr223 phosphorylated FOXO4 localized constitutively to the nucleus (Fig. 2b). Besides phosphorylation, elevated ROS levels induce mono-ubiquitination of FOXOs[19]. Although the precise mechanism is still unclear, this promotes nuclear translocation of FOXOs and increases their transcriptional activity. Hence, the observed nuclear localization of Thr223-phosphorylated FOXO4 may correlate with increased mono-ubiquitination. Originally, the induction of FOXO4 mono-ubiquitination was observed with H_2O_2. Interestingly, as confirmed for H_2O_2 treatment, $BRAF^{V600E}$ expression strongly induced FOXO4 mono-ubiquitination (Fig. 2c). Ubiquitination of FOXO4 in response to H_2O_2 depends on the E3-ligase HDM2[20] and in parallel, we observed that knock-down of HDM2 also impaired $BRAF^{V600E}$ induced mono-ubiquitination of FOXO4 (Fig. 2d). These results indicate that next to JNK-mediated phosphorylation on Thr223, $BRAF^{V600E}$ can promote mono-ubiquitination and subsequent nuclear localization of FOXO4.

Following these observations for FOXO4, we addressed whether $BRAF^{V600E}$ signaling also results in post translational modification of p53. High levels of genotoxic stress can induce phosphorylation of p53 on Ser46, which in this background strongly correlates with apoptosis[21]. In contrast, Ser46 phosphorylation is also required for the induction of senescence in response to oncogenic signaling, e.g. through RAS[22-24] or PML-IV[25]. Therefore, we next examined if $BRAF^{V600E}$ signaling affects p53 phosphorylation on this residue. p53 can be phosphorylated by the ROS sensitive kinase p38 on Ser46[21] and in agreement with increased ROS production, $BRAF^{V600E}$ expression increased Ser46 phosphorylation (Fig. 3a+b). Whether this is p38-dependent remains to be determined.

Excessive levels of ROS may lead to DNA damage in response to which p53 is also phosphorylated on Ser15 and Ser20[26,27]. This has been reported to abolish HDM2 binding and ubiquitination of p53[26,27]. In contrast to Ser46, $BRAF^{V600E}$ did not induce phosphorylation of Ser15 and Ser20 (Fig. 3a+b). Furthermore, preliminary evidence suggests that $BRAF^{V600E}$ expression does not substantially increase γ-H2AX and Chk1/2 phosphorylation (Evi Paraskevi, pers. comm.), which are markers for DNA damage. Although further analysis is required, this suggests that $BRAF^{V600E}$ expression does not induce $p21^{cip1}$ expression through induction of a p53-mediated DNA damage response.

Subsequently, we investigated the biological relevance of the above mentioned effects. Therefore, we addressed the endogenous interplay between FOXOs and p53 in a subset of melanoma cells. Colo829 cells express oncogenic BRAF (V600E), but only harbor low levels of $p21^{cip1}$ compared to WM266.4 cells that also express oncogenic BRAF (V600D)[3]. Importantly, restoration of $p21^{cip1}$ expression by ectopic FOXO4 induces OIS in Colo829 cells. Therefore, we set out to determine if p53 is required for this process. Surprisingly, knockdown of p53 significantly reduced colony formation, independent of FOXO4 co-expression (Fig. 4a). Whereas FOXO4 also induced senescence in this experiment, no senescent cells were observed upon knockdown of p53 (data not shown). We therefore determined if the viability of these cells was affected. Knockdown of p53 induced a significant increase in TUNEL positivity in Colo829 cells (Fig. 4b). Thus, knockdown of p53 in Colo829 cells represses tumorigenesis through induction of

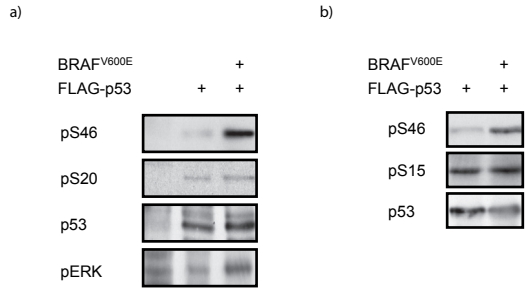

Fig. 3) BRAF^{V600E} induces phosphorylation of p53 on Ser46, but not Ser20 or Ser15
a) BRAF^{V600E} induces phosphorylation of P53 on Ser46, but not Ser20. HEK293T cells expressing FLAG-p53 and BRAF^{V600E} were analysed for p53 phosphorylation by immunoblotting. **b)** BRAF^{V600E} induces phosphorylation of P53 on Ser46, but not Ser15. HEK293T cells expressing FLAG-p53 and BRAF^{V600E} were analysed for p53 phosphorylation by immunoblotting.

apoptosis.
As mentioned, increased Ser46 phosphorylation in response to genotoxic damage strongly correlates with apoptosis rather than cell cycle arrest[28]. In this background FOXO signaling is inactivated[14]. In contrast, as we have shown, BRAF^{V600E} psignaling promotes activation of FOXO4 and triggers its interaction with p53. Prolonged interference with MEK activity, for instance by U0126, induces apoptosis in Colo839 cells[29]. Also, interference with MEK has been reported to induce apoptosis through p53 in a different set of melanoma cells with a BRAF^{V600E} mutation, but not in cells expressing oncogenic RAS or in which both RAS and RAF are wildtype[30]. U0126 impairs BRAF^{V600E}-induced FOXO4 activation[3] and as we have shown here inhibits the interaction with p53. As such, it could be that FOXOs repress apoptosis in response to oncogenic BRAF^{V600E} signaling in Colo829 cells.
To test this hypothesis, we addressed the viability of Colo829 cells when FOXO expression is depleted. Interestingly, knockdown of endogenous FOXOs (Fig. 4c) induced a significant increase in TUNEL staining in these cells. Next to Colo829 cells, also A375 melanoma cells express oncogenic BRAF^{V600E}, in contrast to CHL melanoma cells that express wildtype BRAF. Moreover, U0126 induces apoptosis in A375, but not CHL, cells[29]. We observed that FOXO knockdown also induced apoptosis in A375, but not in CHL cells (Fig. 4d). This suggests a conserved mechanism, where interference with FOXO activity in response to BRAF^{V600E} induces apoptosis through p53. Whether this is indeed p53 dependent remains to be determined. Together, these data indicate that interference with FOXO signaling in these BRAF^{V600E} expressing cells induces apoptosis, which may indicate that FOXOs repress p53-mediated apoptosis in response to oncogenic BRAF^{V600E} signaling.

Discussion
The interplay between FOXOs and p53 has been studied in response to nutrient deprivation and genotoxic stress, however how these proteins interact in response to oncogenic signaling was still unknown. Here, we showed that FOXO4 and p53 can physically interact. This interaction is independent of DNA and could be increased by MEK-dependent signaling downstream of BRAF^{V600E}. We had already shown that BRAF^{V600E} induces phosphorylation of FOXO4 on multiple residues, including Thr223[3]. Notably, this effect was dependent on cellular ROS, which is generated by BRAF^{V600E} expression. Cellular ROS has also been reported before to induce mono-ubiquitination and nuclear relocalization of FOXOs. We now showed that indeed Thr223 phosphorylated FOXO4 localizes constitutively to the nucleus. Furthermore, in parallel

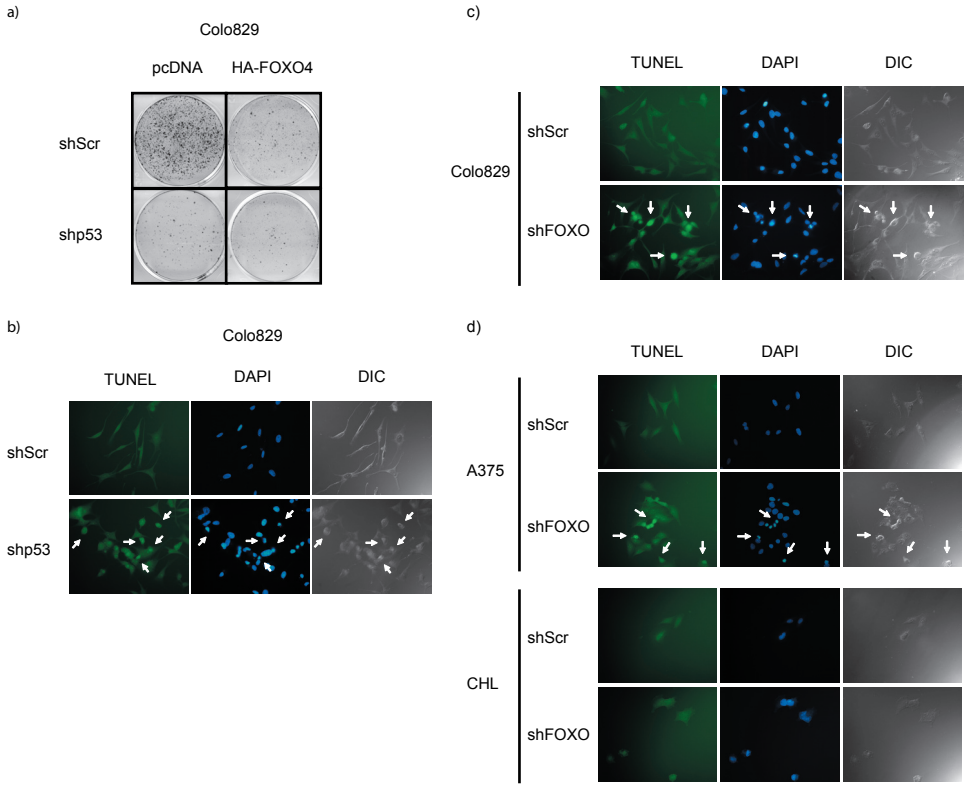

Fig. 4) Functional analysis of the endogenous FOXO-p53 interaction in Colo829, A375 and CHL melanoma cells
a) Knockdown of p53 reduces colony formation of Colo829 cells. Colo829 cells were transfected as indicated in the presence of pbabe-puro and following 10 days of puromycin selection (2μg/ml) analysed for colony formation. **b)** Knockdown of p53 induces apoptosis in Colo829 cells. Colo829, cells were transfected as indicated in the presence of pbabe-puro and following two days of puromycin selection (2μg/ml) analysed for TUNEL positivity. **c+d)** Knockdown of FOXOs induces apoptosis in Colo829 and A375, but not CHL cells. Colo829 (c), A375 and CHL cells (d) were transfected as indicated in the presence of pbabe-puro and following two days of puromycin selection (2μg/ml) analysed for TUNEL positivity.

to acute elevations in cellular ROS by H_2O_2-treatment, $BRAF^{V600E}$ induced FOXO4 mono-ubiquitination in a HDM2-dependent manner. Next to these post translational modifications on FOXO4, we observed that $BRAF^{V600E}$ induced p53 phosphorylation on Ser46, but not Ser15 and Ser20. Phosphorylation on this residue upon genotoxic stress strongly correlates with apoptosis, whereas in response to oncogenic signaling it correlates with senescence. We did not notably detect apoptosis upon ectopic $BRAF^{V600E}$ expression, nor in $BRAF^{V600E}$ expressing cell lines. This may indicate the p53-dependent apoptosis response is suppressed in favor of cell cycle arrest and senescence. Knockdown of endogenous FOXOs in $BRAF^{V600E}$ expressing

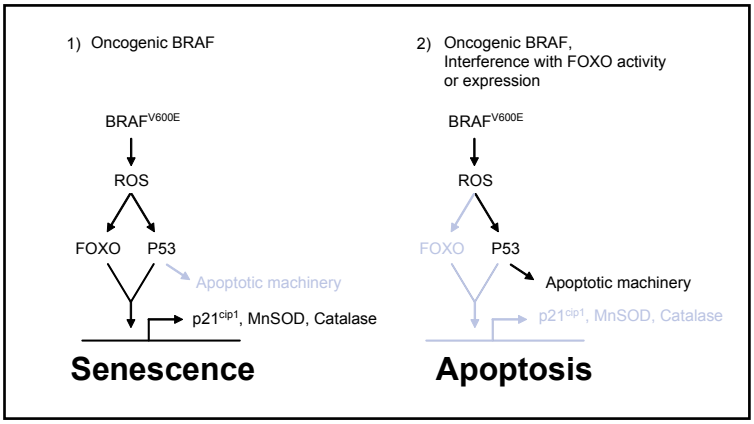

Fig. 5) Model that describes the interplay between FOXOs and p53 in response to oncogenic BRAF^{V600E} signaling.
a) Following oncogenic activation of BRAF, e.g. due to V600E mutation, cellular ROS levels rise. This induces activation of FOXOs and interaction with p53, which results in p21^{cip1}-dependent senescence. **b)** Interference with FOXO activity or expression in a background of oncogenic BRAF^{V600E} may allow p53 to induce activate an apoptosis response.

Colo829 melanoma cells did result in a significant induction in apoptosis. Similar results were observed in BRAF^{V600E} expressing A375 cells, but not wildtype BRAF expressing CHL cells. Interestingly however, also depletion of p53 induced apoptosis in Colo829 cells, which reduced colony formation independent of FOXO4-induced senescence. Since interference with BRAF^{V600E} signaling has been reported to do likewise, it together appears that the interaction between FOXOs and p53 is required for survival of these cells.
p21^{cip1} plays a dual role in tumor suppression. Whereas high expression of p21^{cip1} represses tumor progression trough induction of cell cycle arrest, cells that can bypass this arrest may misuse p21^{cip1} to prevent apoptosis[31]. Although further analysis is required, our results may indicate that interference with p21^{cip1} expression in BRAF^{V600E} expressing melanoma cells, either by interference with BRAF^{V600E} signaling (U0126), or knockdown of p53 or FOXOs, induces apoptosis.
In contrast to the previous studies that showed that FOXOs and p53 functionally compete in response to growth factor deprivation or genotoxic stress[13,14], our data add a new layer to their interplay by suggesting they may partially co-operate in response to ROS to induce senescence and prevent apoptosis (Fig. 5). Paradoxically, although p53 and FOXOs are tumor suppressors, interference with the activity or expression of either may be clinically relevant as a therapy to induce apoptosis in BRAF^{V600E} mutated tumors.

Materials and Methods
Cell culture and transfection
All cells were maintained in Dulbecco's Modified Eagle's Medium (Cambrex), 10% Fetal Calf Serum, penicillin/streptomycin and 0.05% glutamine. HEK293T, U2OS and A14 cells were transfected via the calcium-phosphate method. Colo829 cells were transfected with Effectene according to the manufacturer's protocol (Qiagen). The

following compounds were purchased: U0126 (Promega, Madison, WI) and Benzonase (Merck, New York, NY).

Constructs and RNAi
The following constructs have been described before: pBabe-puro, pMT2-HA-FOXO4, pEFm-BRAF^{V600E}, p21^{cip1}-luciferase, 6xDBE-firefly luciferase, TK-renilla luciferase and pSuper-p21^{cip1}, pSuperior-Scr, pSuperior-FOXO1/3 and pSuperior-FOXO4[32], pSuper-p53[33], and His$_6$-Ubi[18]. siRNA oligo-nucleotides against human MDM2 or a scrambled sequence (Dharmacon) were transfected at a final concentration of 100nM using oligofectamine according to the manufacturer's protocol (Invitrogen).

Antibodies
The antibodies against pT223-FOXO4, FOXO4 (834), HA (12CA5)[3] and MYC (9E10)[34]. Antibodies against the following proteins were purchased: phosphoThr183/Tyr185-JNK, phosphoThr202/Tyr204-ERK, phosphoSer15-P53, phosphoSer20-P53 and phosphoSer46-P53 (Cell Signaling), p21^{cip1} (BD pharmingen), MDM2 (SMP-14), p53 (DO-1) (Santa Cruz), FLAG-M2 and Tubulin (Sigma).

Immunoprecipitation
Immunoprecipitation was performed essentially as described[18] in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 1% TX-100, 0.5% NaDoC, 5 mM EDTA, 150 mM NaCl, protease and phosphatase inhibitors.

Immunofluorescence, TUNEL staining and BrdU incorporation
Immunofluorescence was performed as described[18], using anti-pT223 anti anti-HA. TUNEL staining was performed according to the manufacturer's protocols (Roche).

Ubiquitination assay
The monoubiquitination assay was performed as described[18]. HEK293T cells were transfected with RNAi oligonucleotides and/or the indicated constructs. Forty-eight hours posttransfection, cells were treated as indicated and lysed in 8 mol/L of urea, 10 mmol/L of Tris-HCl (pH 8.0), 100 mmol/L of Na2HPO4/NaH2PO4, 0.2% TX-100, 5 mmol/L of NEM, and protease inhibitors. Ubiquitinated proteins were precipitated using Ni-NTA agarose beads and the experiment was analyzed by immunoblotting.

Colony Formation assay and SA-β-Gal staining
Colo829 cells were plated in six well plates and transfected with the appropriate plasmids (1μg) in combination with pbabe-puro (250ng) and processed as described[3].

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Functional interaction between FOXM1 and Pin1 regulated by oncogenic BRAF

Manuscript to be submitted

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Functional interaction between FOXM1 and Pin1 regulated by oncogenic BRAF

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Abstract

The transcription factor FOXM1 is a key regulator of mitosis and is vital to prevent aneuploidy and genomic instability[1]. Furthermore, FOXM1 promotes cell cycle progression[2] and is frequently upregulated in tumors[3-5]. During G2/M transition FOXM1 is phosphorylated on numerous sites, including Ser/Thr-Pro sites that are targeted by Pro-directed kinases. Here, we show that the peptidyl-prolyl isomerase Pin1 interacts with FOXM1 and promotes expression of its target genes. Moreover, we identify FOXM1 as a c-Jun N-terminal Kinase (JNK) substrate *in vitro* and *in vivo*. JNK-mediated phosphorylation of FOXM1 induces the interaction with Pin1. Recently, we established that signaling by oncogenic $BRAF^{V600E}$ elevates the intracellular levels of Reactive Oxygen Species (ROS)[6] and as a consequence activates JNK. Consequently, we observed that $BRAF^{V600E}$ expression stimulates FOXM1 binding to Pin1. Interference with endogenous $BRAF^{V600E}$ signaling in melanoma cell lines with high FOXM1 expression, Colo829 and A375, impairs binding of Pin1 to FOXM1 and expression of FOXM1 target genes required for proliferation. Based on these and our previous results, we propose a model in which Pin1 binding to FOXM1 may contribute to a bypass of $BRAF^{V600E}$-induced senescence, by counteracting FOXO signaling.

Introduction

Forkhead box (FOX) transcription factors constitute a large family of transcription factors that share a homologous DNA binding domain, i.e. the Forkhead domain[7]. Of the various classes of Forkhead transcription factors the FOXO family and the FOXM1 family are involved in the regulation of cell cycle progression[8,9]. FOXM1 expression is restricted to cycling cells and correlates with ongoing cell cycle progression[2]. FOXM1 regulates cell cycle progression through activation of a number of target genes, including Cyclin-B1, CENP-F and Aurora-B, which promote mitotic entry and progression[10]. Moreover, FOXM1 can repress the G1/S checkpoint through upregulation of the E3 ubiquitin-ligase SKP2[11,12], which targets both FOXOs[13] and its transcriptional targets $p21^{cip1}$ and $p27^{kip1}$ for proteasomal degradation. Depletion of FOXM1 in mice results in developmental defects and embryonic lethality associated with decreased cell numbers[14]. Conversely, activation of FOXM1 results in accelerated cell cycle progression[10,15]. In agreement with the role of FOXM1 in cell cycle progression, FOXM1 levels are elevated in

many human carcinomas, including melanoma[3-5].

Mitotic defects not only increase the chance on tumor formation, but may also accelerate the onset of aging[16]. Notably, FOXM1 is one of the most significantly down-regulated genes in elderly people and patients that suffer from Progeria Syndrome (premature aging)[17]. Furthermore, expression of the FOXM1 target gene Cyclin-B1 is significantly downregulated in old versus young fibroblasts[18]. Although activation of FOXM1 signaling contributes to tumor progression and premature aging[9,19], it is not yet fully understood how FOXM1 itself is regulated in these processes.

Pin1 is a peptidyl-prolyl isomerase, that in contrast to other isomerases, specifically interacts with phosphorylated substrates[20]. Subsequently, Pin1 stimulates a *cis*-to-*trans* conversion of the peptide backbone thereby providing a unique mode of post-post translational modification[21,22]. Like FOXM1, Pin1 maintains genomic stability[23] and regulates proper execution of mitosis[24]. As we and others have shown, Pin1 is implicated in tumorigenesis and is overexpressed in human cancer tissues, including malignant melanoma and breast cancer[25-28]. However, although FOXM1 and Pin1 signaling affect similar processes, it is currently unclear if both pathways functionally interact.

Results

We set out to study if FOXM1 signaling is regulated by Pin1. Therefore, we first determined if FOXM1 can physically interact with Pin1. Immunoprecipitation of ectopically expressed FOXM1 showed co-precipitation of Pin1, whereas Pin1 was not precipitated in the absence of FOXM1 (Fig. 1a). Similarly, FOXM1 could be co-precipitated by Pin1. This indicates that Pin1 and FOXM1 can interact *in vivo*. Next, we determined if FOXM1 could interact with bacterially produced, recombinant GST-tagged Pin1. Pin1 can bind its substrates once they are phosphorylated on a Ser/Thr residue followed by a Pro through a domain that contains two critical Trp residues, the WW-domain. Therefore, we also produced a mutant in which one of these Trp residues is mutated to Ala, GST-Pin1^{W34A}. In a pull-down assay for these GST-tagged proteins, FOXM1 was specifically co-precipitated by wildtype GST-Pin1, but not the WW-domain mutant GST-Pin1^{W34A} (Fig. 1b). This indicates that Pin1 can interact with FOXM1 *in vitro* in a substrate-specific manner.

During cell cycle progression, FOXM1 is phosphorylated on multiple residues, thereby stabilizing the protein and increasing its transcriptional activity[29-31]. FOXM1 phosphorylation and activity peak at the G2/M boundary, where it is required to initiate mitotic entry[9]. The drug nocodazole can block cell cycle progression at this boundary through interference with microtubule polymerization. Synchronization of cells at the G2/M boundary by nocodazole treatment significantly increased the interaction of FOXM1 with wildtype GST-Pin1, but not the substrate-binding defective GST-Pin1^{W34A} (Fig. 1b). Since this assay was performed with recombinant GST-Pin1, this indicates that the increase in binding is due to modification of FOXM1, rather than Pin1. Thus, the substrate specific interaction between Pin1 and FOXM1 is increased at the G2/M boundary, when FOXM1 is multi-phosphorylated.

MEK dependent phosphorylation of FOXM1 in the G2-phase is essential for the transcriptional activity of FOXM1 and its ability to induce mitotic entry[32]. Notably, interference with MEK signaling by U0126 significantly reduced the nocodazole-induced interaction of FOXM1 with GST-Pin1 (Fig. 1c), suggesting that Pin1 can interact with FOXM1 once it is phosphorylated in a MEK-dependent manner.

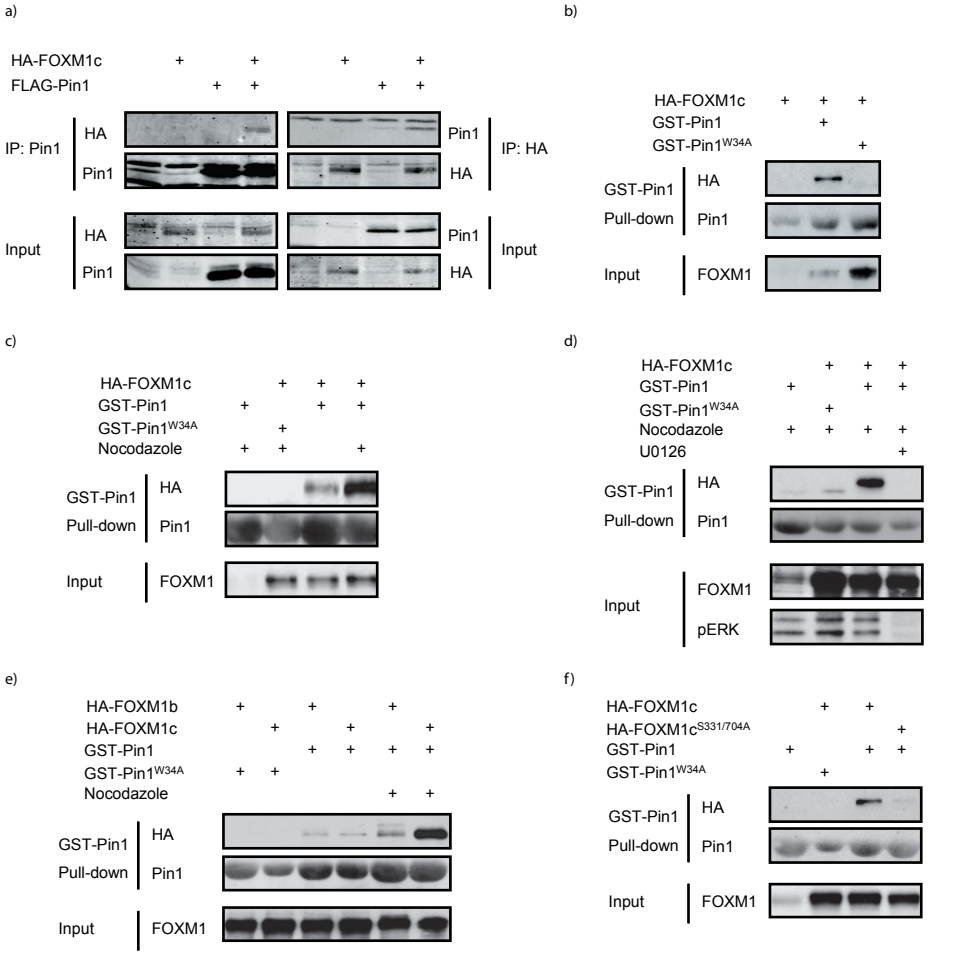

Fig. 1: Pin1 interacts with FOXM1 in response to MEK induced phosphorylation
a) Pin1 and FOXM1 physically interact *in vivo*. U2OS cells expressing FLAG-Pin1 and HA-FOXM1c were subjected to immunoprecipitation with anti-HA or anti-FLAG and analyzed by immunoblotting. The interaction was also observed in HEK293T cells. **b)** Pin1 interacts with FOXM1 through its WW-domain. U2OS cells expressing HA-FOXM1c were lysed and subjected to a pull-down with recombinant GST-Pin1 or the substrate binding defective GST-Pin1^{W34A} followed by immunoblotting. **c)** The interaction of Pin1 with FOXM1 is increased in the G2/M phase. U2OS cells expressing HA-FOXM1c were treated for 24hrs with 250ng/ml nocodazole and lysates were subjected to a pull-down with recombinant GST-Pin1 followed by immunoblotting. **d)** MEK-induced phosphorylation is essential for FOXM1 precipitation by Pin1. U2OS cells were transfected and treated as in (c) without or with 24hr pretreatment with 20μM U0126. **e)** Nocodazole strongly increases interaction between Pin1 and FOXM1c, but only marginally with FOXM1b. U2OS cells expressing FOXM1c or FOXM1b were processed as in (c). **f)** Mutation of Ser331 and Ser704 of FOXM1c reduces the nocodazole-induced interaction with Pin1. U2OS cells expressing wildtype FOXM1c or a mutant in which Ser331 and Ser704 are mutated to Ala (FOXM1$^{S331/704A}$) were processed as in (c).

Alternative splicing of FOXM1 mRNA generates two transcriptionally active isoforms FOXM1b and c, next to the inactive FOXM1a. MEK-dependent FOXM1 phosphorylation occurs on Ser331 and Thr704 in FOXM1c[32]. Whereas, both isoforms contain Ser704, FOXM1b differs from FOXM1c due to the lack of an intron that encodes Ser331. Next, we therefore determined if the difference in MEK-dependent phospho-sites between FOXM1b and FOXM1c affects the nocodazole induced binding of FOXM1 to Pin1. FOXM1b and c could both be co-precipitated equally well by GST-Pin1 under basal conditions (Fig. 1d). Intriguingly, nocodazole treatment greatly increased binding of FOXM1c to GST-Pin1, whereas it was only marginally increased for FOXM1b (Fig. 1d). To further address the relevance of these two residues for this interaction, we subsequently generated a construct that encodes FOXM1c in which Ser331 and Ser704 are mutated to Ala, FOXM1c$^{S331/704A}$. Whereas wildtype FOXM1c, isolated from nocodazole treated cells, could be precipitated by GST-Pin1, FOXM1c$^{S331/S704A}$ binding was significantly reduced (Fig. 1e). Individual mutation of these residues showed a partial decrease in binding (data not shown). Thus, both residues are involved in the interaction with Pin1. Altogether these data indicate that MEK dependent phosphorylation on the G2/M boundary increases the binding of FOXM1b and c to Pin1.

FOXM1 regulates entry into mitosis through transcriptional activation of Cyclin-B1 and the checkpoint proteins CENP-F and Aurora-B[10,33]. Expression of these proteins can be artificially induced by addition of 4-OHT to a cell line that stably expresses ER-tagged FOXM1. To test whether PIN1 binding to FOXM1 regulates FOXM function, we therefore determined if knockdown of endogenous Pin1 affects the induction of these proteins by FOXM1. First, we confirmed that 4-OHT increased expression and activation of FOXM1c and its target genes Cyclin-B1, CENP-F and Aurora-B (Fig. 2a). Importantly, knockdown of Pin1 impaired this induction, indicating that Pin1 is required for their full induction by FOXM1.

Upon initiation of the cell cycle program, FOXM1 is gradually stabilized[9], in parallel with increased transcriptional activity. To further investigate the biological effects of Pin1 on FOXM1 signaling, we serum deprived mouse embryonic fibroblast to abrogate FOXM1 expression and followed how serum re-addition affects expression of FOXM1 and its target genes. We also included a sample that was treated with nocodazole to ensure maximal FOXM1 expression and activity. We could confirm that serum addition to quiescent wildtype Mouse Embryonic Fibroblasts (MEFs) induced expression of FOXM1 itself and Cyclin-B1, CENP-F and Aurora-B expression in time, with optimal expression in nocodazole blocked cells (Fig. 2b+c). Although not abolished, expression of the FOXM1 target genes was delayed and reduced in *foxm1-/-* MEFs, indicating that FOXM1 is important but not essential for their expression (Fig. 2b). Interestingly, in *pin1-/-* MEFs expression of FOXM1 was delayed upon serum add-back and concomitantly expression of its target genes was reduced (Fig. 2c).

Following cytokinesis, FOXM1 expression is rapidly reduced through APC/C mediated degradation[34,35]. We also tested the effect of Pin1 on this reduction and observed that upon release of nocodazole block ectopic expression of Pin1 rapidly increased FOXM1 expression in time in U2OS cells, whereas the binding defective Pin1^{W34A} did not (data not shown). Together with the previous experiments, this suggested that FOXM1 stability is enhanced by Pin1. Next, we therefore determined if Pin is involved in FOXM1 stabilization by determining the half-life of FOXM1 in wildtype and *pin1-/-* MEFs. Blocking protein translation by addition of cycloheximide (CHX) to cells, resulted in a time-dependent decline in FOXM1 levels similar to previous reports (Fig. 2d and[34]). The half-life of endogenous FOXM1 was reduced in *pin1-/-*

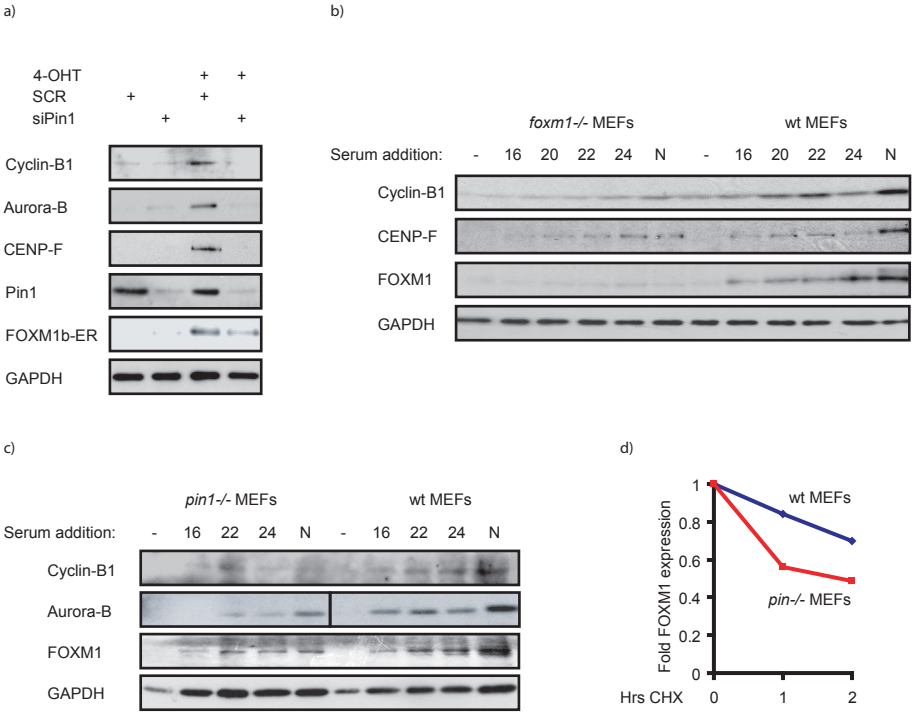

Fig. 2: Pin1 stabilizes FOXM1 and is required for expression of its target genes
a) Pin1 knockdown impairs FOXM1 mediated expression of Cyclin-B1, CENP-F and Aurora-B. U2OS cells stably expressing FOXM1-ER were transfected with scrambled siRNA oligo's (SCR) or oligo's against Pin1 (siPin1) and after 48hrs treated for an additional 24hrs with 2μM 4-OHT. Lysates were subsequently processed by immunoblotting. Similar results were obtained with 5 and 20μM 4-OHT. **b)** Serum addition to quiescent MEFs results in Cyclin-B1 and CENP-F expression which is reduced in *foxm1-/-* MEFs. *Foxm1-/-* or wt MEFs were forced into quiescence by 36hr serum starvation. Subsequently cell cycle progression was induced by addition of 10% FCS for the indicated time-points in absence or presence of 26hrs 250ng/ml Nocodazole (N) and lysates were analyzed by immunoblotting. **c)** Pin1 increases expression of FOXM1 and its target genes Cyclin-B1 and Aurora-B by serum addition to quiescent MEFs. *Pin1-/-* and wt MEFs were treated and analyzed as in (a). **d**) Pin1 depletion reduced FOXM1 stability. Wildtype MEFs and *pin1-/-* MEFs were treated with 10μg/ml cycloheximide (CHX) for the indicated time-points and analyzed by immunoblotting.

MEFs, indicating that Pin1 promotes stabilization of FOXM1. Altogether, these data indicate that Pin1 binding to FOXM1 results in stabilization of FOXM1 and correlates with expression of FOXM1 target genes.
Previously, we showed that $BRAF^{V600E}$ signaling through MEK results in elevated levels of cellular ROS. This leads to activation of FOXO4 through JNK mediated phosphorylation[6]. Similar to shown here for FOXM1, Pin1 can also bind to FOXO, but here Pin1-dependent isomerization counteracts activation of FOXO by ROS signaling[26]. We therefore wondered if FOXM1c signaling can also be modulated by $BRAF^{V600E}$ and whether Pin1 in this respect represents a pivot in switching between FOXO and FOXM1. As we have shown here, MEK

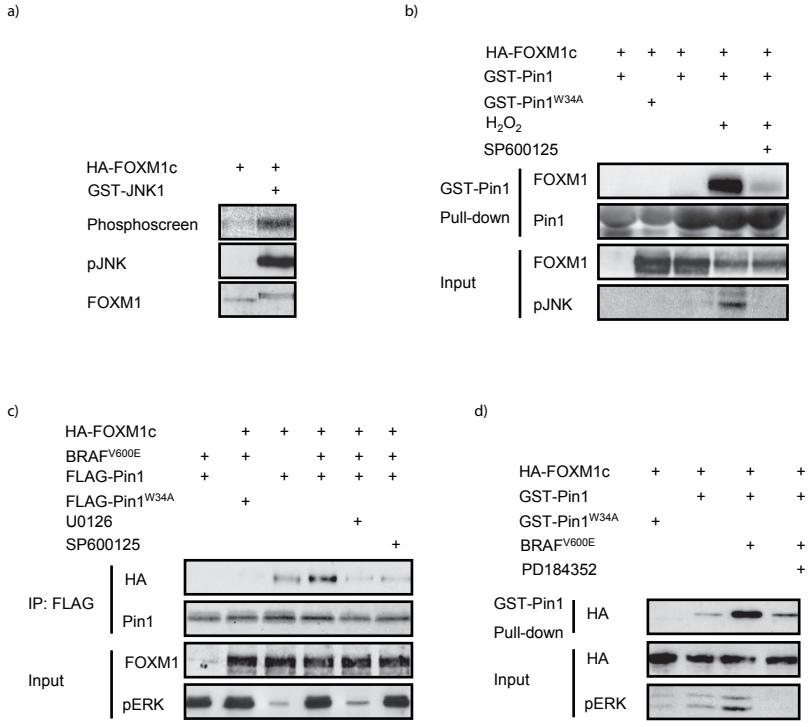

Fig. 3: Oncogenic BRAF^{V600E} signaling through MEK-ROS and JNK makes FOXM1c a Pin1 substrate
a) FOXM1c is a JNK substrate *in vitro*. HA-FOXM1c was immunoprecipitated from lysates of U2OS cells and subjected to an i*n vitro* kinase assay with recombinant JNK1 and $^{32}\gamma$-ATP. The mount of transferred radioactively labelled phosphate was determined by phosphoscreen detection. **b)** Exogenous ROS stimulates the interaction between FOXM1c and Pin1 in a JNK-dependent manner. U2OS cells transiently expressing HA-FOXM1c were left untreated or treated for 24hrs with 20μM SP600125, treated with or without 1hr 200μM H_2O_2 and subsequently lysates were subjected to GST-Pin1 pull-down followed by immunoblotting. **c)** BRAF^{V600E} stimulates the interaction between FOXM1c and Pin1 in a MEK- and JNK- dependent manner. U2OS cells transiently expressing BRAF^{V600E}, HA-FOXM1c and FLAG-Pin1 were left untreated or treated with for 16hrs with 10μM U0126 or 20μM SP600125 and subsequent immunoprecipitation was performed using anti-FLAG followed by immunoblotting. **d)** BRAF^{V600E} stimulates the interaction between FOXM1c and Pin1 through MEK-dependent modification of FOXM1c. U2OS cells transiently expressing HA-FOXM1c and BRAF^{V600E} were left untreated or treated for 24hrs with 20μM PD184352 and processed as in (b).

activity is required for the interaction between FOXM1 and Pin1. Since prolonged MEK activation by BRAF^{V600E} induces JNK activation, we determined if FOXM1 can be phosphorylated by JNK. Indeed, in an *in vitro* kinase assay, recombinant active JNK could transfer radioactively labeled phosphate to FOXM1c (Fig. 3a). Pin1 binding to FOXOs can be induced by addition of H_2O_2, which activates JNK. Furthermore, the Pin1 binding epitope in FOXO4 is at least partially generated through JNK mediated phosphorylation on multiple residues. Therefore, we next determined if JNK can facilitate the binding of FOXM1 and Pin1 in response to H_2O_2. In

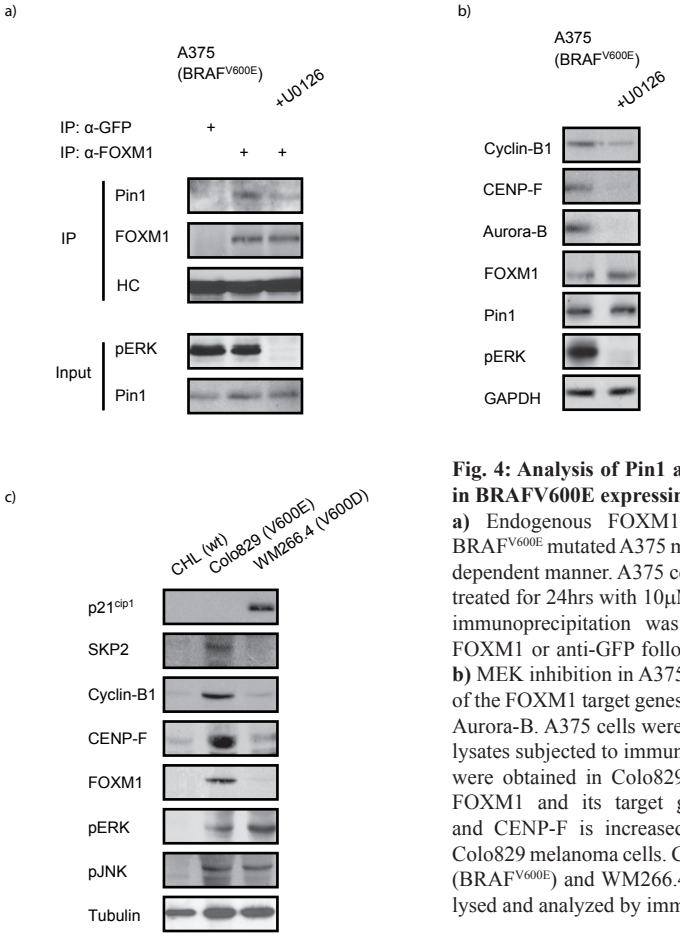

Fig. 4: Analysis of Pin1 and FOXM1 interaction in BRAFV600E expressing melanoma cells
a) Endogenous FOXM1 and Pin1 interact in $BRAF^{V600E}$ mutated A375 melanoma cells in a MEK-dependent manner. A375 cells were left untreated or treated for 24hrs with 10μM U0126 and subsequent immunoprecipitation was performed using anti-FOXM1 or anti-GFP followed by immunoblotting. **b)** MEK inhibition in A375 cells impairs expression of the FOXM1 target genes Cyclin-B1, CENP-F and Aurora-B. A375 cells were treated as in a) and their lysates subjected to immunoblotting. Similar results were obtained in Colo829 cells. **c)** Expression of FOXM1 and its target genes SKP2, Cyclin-B1 and CENP-F is increased in $BRAF^{V600E}$ mutated Colo829 melanoma cells. CHL (wt BRAF), Colo829 ($BRAF^{V600E}$) and WM266.4 ($BRAF^{V600D}$) cells were lysed and analyzed by immunoblotting.

agreement with JNK activation, H_2O_2 increased binding of FOXM1c to Pin1, which could be rescued by the JNK inhibitor SP600125 (Fig. 3b). This indicates that in the presence of an acute elevation in cellular ROS levels FOXM1c can be phosphorylated by JNK, thereby generating a Pin1 docking surface. Importantly, in this assay we used recombinant GST-Pin1, indicating that the observed increase in binding is due to modification of FOXM1c, rather than Pin1. Like H_2O_2 treatment, $BRAF^{V600E}$ induces cellular ROS, albeit of a chronic nature. Therefore, we next determined the effect of $BRAF^{V600E}$ expression on the interaction between FOXM1 and Pin1 *in vivo*. Furthermore, we also pretreated a subset of the cells with the MEK inhibitor, U0126, and the JNK inhibitor, SP600125. In agreement with $BRAF^{V600E}$ expression generating ROS in a MEK dependent manner and thereby activating JNK signaling, $BRAF^{V600E}$ expression *in vivo* enhanced Pin1 binding to FOXM1 (Fig. 3c). Importantly, downstream signaling from MEK to JNK is required for this induction, as it was impaired upon pretreatment with U0126,

or SP600125. The same repression was observed with the ROS scavenger N-Acetyl Cysteine (NAC), indicating that the $BRAF^{V600E}$ induced interaction is indeed ROS dependent (data not shown). Finally, the effects of U0126 are unlikely to occur independent of MEK as pretreatment with a different MEK inhibitor, PD184352 also resulted in decreased binding of FOXM1 to recombinant GST-Pin1 (Fig. 3d). Together these data indicate that, like FOXO4, FOXM1 is a downstream target of $BRAF^{V600E}$ through JNK mediated phosphorylation, thereby making it accessible for Pin1 binding.

Oncogenic BRAF mutations, such as V600E, are frequently observed in melanoma[36]. This mutation by itself is insufficient to induce tumorigenesis due to induction of an oncogene induced senescence (OIS) response[37,38]. OIS is regulated by at least two independent pathways, involving the CDK inhibitors $p16^{ink4a}$ and $p21^{cip1}$, which individually prevent pRb phosphorylation and inactivation[37,39]. In combination with additional genetic lesions, however, $BRAF^{V600E}$ has a strong transforming potential[37]. A375 cells are melanoma cells that express V600E mutated BRAF[40]. These cells do not express $p16^{ink4a}$ due to a frameshift mutation in the open reading frame. Together with moderate $p21^{cip1}$ expression (not shown) this may explain why the cells have bypassed OIS. Furthermore, we observed that A375 cells express high FOXM1 levels compared to non-$BRAF^{V600E}$ expressing CHL melanoma cells (not shown). Since we observed that FOXM1 can strongly interact with Pin1 in response to $BRAF^{V600E}$ signaling, we therefore investigated their endogenous interaction in these cells. Endogenous Pin1 could be co-precipitated with endogenous FOXM1, but not in a control situation where we used an antibody against GFP (Fig. 4a). Furthermore, in agreement with our overexpression data, interference with downstream $BRAF^{V600E}$ signaling by U0126 impaired the endogenous interaction. In parallel, this resulted in diminished FOXM1 target gene expression (Fig. 4b). These results indicate that in an endogenous setting $BRAF^{V600E}$ stimulates the interaction of Pin1 with FOXM1, thereby contributing to increased FOXM1 target gene expression.

The role of FOXOs in $BRAF^{V600E}$ signaling was initially identified using three melanoma cell lines that express either wildtype BRAF (CHL), or a hyperactive V600E or D mutant (Colo829 and WM266.4, respectively)[6]. Since FOXM1 regulation by $BRAF^{V600E}$ and Pin1 is similar to FOXO4, we therefore determined the expression levels of FOXM1 and its target genes in these cells as well. Strikingly, FOXM1, Cyclin-B1 and CENP-F were highly expressed in Colo829 cells compared to CHL and WM266.4 cells (Fig. 4c). As shown before, WM266.4 cells show high $p21^{cip1}$ expression, whereas it is virtually absent in Colo829 (Fig. 4c and[6]). FOXM1 inhibits $p21^{cip1}$ expression, but not at the transcriptional level[41]. Rather, $p21^{cip1}$ stability is regulated by the E3-ligase SKP2, a protein, which gene is under control of FOXM1[11]. Interestingly, SKP2 levels are elevated in Colo829 cells, which may explain why these cells do not express significant amounts of $p21^{cip1}$ (Fig. 4c). Combined with the lack of functional $p16^{ink4a}$ in these cells (Ref[6,40]), this provides a plausible explanation why these cells have bypassed OIS. Preliminary data suggest that Pin1 and FOXM1 depletion reduces SKP2 expression in these cells (data not shown). However, whether this rescues the $p21^{cip1}$-dependent OIS response remains to be determined.

Discussion

Pin1 and FOXM1 expression have both been associated with tumor progression. However, whether there is a direct interplay between these proteins in cancer was unknown. Here, we showed that Pin1 and FOXM1 can physically interact. Pin1 interacts with phosphorylated Ser/

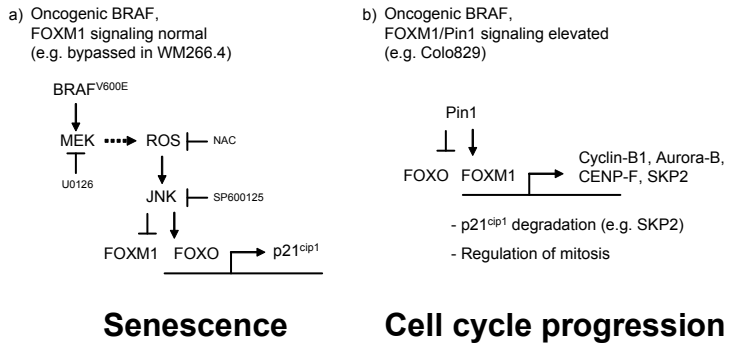

Fig. 5: Model to describe the biological consequences of oncogenic BRAF activation on FOXO and FOXM1 signaling

a) Upon oncogenic BRAF activation cellular ROS levels increase in a MEK dependent manner. This results in JNK activation which can subsequently induce phosphorylation and transactivation of FOXOs. As a result cells will undergo OIS. **b)** In case of additional genetic lesions this may allow for increased expression of FOXM1. This then may induce expression of SKP2 to inhibit the FOXO-regulated $p21^{cip1}$ expression and OIS response. Furthermore, expression of other FOXM1 target genes as Cyclin-B1, Aurora-B and CENP-F may allow accelerated cell cycle progression and drive tumorigenesis.

Thr-Pro motifs through its WW-binding domain. The interaction of Pin1 with FOXM1 was lost when we mutated a critical residue in the WW-domain of Pin1, suggesting that FOXM1 is indeed a genuine Pin1 substrate. FOXM1 activity in the G2/M phase of the cell cycle is required for proper entry into mitosis. Interestingly, the interaction with Pin1 was enhanced when cells were pretreated with nocodazole which induces a proliferation block in the G2/M phase of the cell cycle. The transcriptional activity of FOXM1 is regulated during G2/M by MEK dependent phosphorylation[32] and we showed that interference with MEK signaling inhibited the binding of Pin1 to FOXM1. Biologically, we observed that Pin1 expression stabilizes FOXM1 allowing increased expression of FOXM1 target genes. Together this suggests that following MEK-dependent phosphorylation Pin1 can interact with FOXM1 to increase its stability and facilitate expression of FOXM1 target genes.

Additionally, to this mode of regulation in normal cell cycle progression, we established that FOXM1 is also regulated by oncogenic signaling of $BRAF^{V600E}$. $BRAF^{V600E}$ expression induces JNK activation through a linear pathway involving MEK, ROS and JNK[6] and through various experiments we observed that FOXM1 is a downstream target of this pathway. First of all, JNK can phosphorylate FOXM1 *in vitro*. Second, acute elevations in cellular ROS could induce the interaction with Pin1 in a JNK dependent manner. And finally, $BRAF^{V600E}$, which induces a chronic increase in cellular ROS[6], also increased this interaction in a MEK-, ROS- and JNK-dependent manner.

At this stage it is not clear what biological consequences elevated ROS has on FOXM1 signaling. We speculate that since ROS induces a cell cycle arrest at least in part by activating FOXO tumor suppressors and increased $p21^{cip1}$ transcription, that FOXM1 activity initially will be repressed. However, Pin1 binding can repress FOXO activity, whereas it can activate FOXM1 signaling to induce cell cycle progression again. Thus, in this context Pin1 binding provides a mechanism to

resume cell cycle progression after oxidative stress has been resolved.
We obtained some evidence in support of the latter. For instance, interference with endogenous $BRAF^{V600E}$ signaling in A375 melanoma cells impairs the interaction of Pin1 with FOXM1 and expression of FOXM1 target genes. This may indicate that the interaction of Pin1 to FOXM1 indeed contributes to cell cycle progression of these cells. Furthermore, Colo829 melanoma cells express high levels of FOXM1 and of its target genes SKP2, Cyclin-B1 and CENP-F. SKP2 represses $p21^{cip1}$ expression through proteasomal degradation, which also adds to the proliferative phenotype of these cells. We showed before that ectopic expression of FOXO4 could restore $p21^{cip1}$ levels and induce OIS. This would mean that FOXO and FOXM1 signaling actively compete with each other in response to $BRAF^{V600E}$ expression. It will therefore be interesting to see how $BRAF^{V600E}$ expression affects the transcription of FOXM1 target genes, for instance in the U2OS cell line that stably expresses FOXM1-ER.
Based on the findings presented here and in our previous study[6], we propose a model where the balance between FOXM1 and FOXO activity determines whether cells will proliferate or undergo senescence (Fig. 5). When BRAF is activated through an oncogenic mutation, a pathway of MEK-ROS-JNK signaling activates FOXOs, which promotes senescence onset through $p21^{cip1}$ transcription. In contrast, additional genetic lesions that allow increased FOXM1 expression can promote a bypass of senescence through upregulation of SKP2 and increased expression of FOXM1 target proteins required for proliferation such as Cyclin-B1, CENP-F and Aurora-B. Interestingly, SKP2 is not only reported to downregulate transcriptional targets of FOXO involved in cell cycle arrest i.e. $p27^{kip1}$ and $p21^{cip1}$, but is also reported to regulate FOXO degradation as well[13]. Hence, SKP2 expression may not only repress FOXO mediated OIS through inhibition of FOXO transcriptional targets, but also at the level of FOXO itself.
Additional experiments will be required to explore the consequences of the results presented here. For instance, we have shown that in a subset of breast cancer samples high Pin1 levels correlate with low $p27^{kip1}$ expression, which may reflect impaired FOXO activity[26]. Furthermore, FOXM1 inversely correlates with $p27^{kip1}$ and $p21^{cip1}$ in various cancer tissues[3,42]. In this respect it would be interesting to see if in the same samples expression of FOXM1 or its target genes is elevated, which may add to our understanding and possible treatment of these tumors.
Finally, it will be interesting to see how Pin1 is regulated itself in these situations. The binding of Pin1 to its substrates is inhibited by PKA mediated phosphorylation in its WW-domain[43]. cAMP levels have been reported to drop in G2/M[44] and indeed Pin1 is active in mitosis. Conversely to Pin1 inhibition, cAMP stimulates FOXO activity[45]. Combined with the data reported here, this may indicate that while cAMP levels are high FOXO represses proliferation. When cAMP levels drop Pin1 is activated, thereby inhibiting FOXO and activating FOXM1 to allow ongoing cell cycle progression. Recently, it was found that cAMP repressed proliferation of $BRAF^{V600E}$ mutated thyroid carcinoma cells[46]. This involved $p21^{cip1}$ expression and inhibition of CDK4 phosphorylation, which thereby resulted in hypo-phosphorylation of the pRb protein. Thus it may be interesting to investigate the effect of cAMP activation FOXO and FOXM1 activity in $BRAF^{V600E}$ mutated melanoma cells in which $p21^{cip1}$ levels are low, such as Colo829 cells.

Materials and Methods

Cell culture and transfection

All cells were maintained in Dulbecco's Modified Eagle's Medium (Cambrex), 10% Fetal Calf Serum, penicillin/streptomycin and 0.05% glutamine. U2OS stably expressing FOXM1-ER cells were furthermore cultured with 0.2 µg/ml puromycin. *foxm1-/-* MEFs and wt MEFs of littermate controls have been described[10]. *pin-/-* MEFs were a

kind gift of Dr. P. van der Sluijs. HEK293T and U2OS cells were transfected via the calcium-phosphate method. Colo829 cells were transfected with Effectene according to the manufacturer's protocol (Qiagen). The following compounds were purchased: Cycloheximide and Nocodazole (both Sigma, St Louis, MO), U0126 and SP600125 (Promega, Madison, WI). PD184352 was a kind gift of dr. Philip Cohen.

Constructs and RNAi

The following constructs have been described before: pcDNA3, pBabe-puro, pEFm-BRAF^{V600E} and psuperior-shScrambled[6], pcDNA3-FLAG-Pin1, pCDNA3-FLAG-Pin1^{W34A}, pGEX-GST-Pin1, pGEX-GST-Pin1^{W34A} (Ref[26]), mouse pcDNA3-HA-FOXM1b and pSuper-FOXM1[10]. pSuperior-Pin1was generated according to the manufacturer's instructions using pSuperior-retro-puro as a template (OligoEngine) and the following oligo's: shPin1-fwd: 5'gatcccccgtcctggcggcaggagaattcaagagattctcctgccgccaggacgttttta3' and shPin1-rev: 5'agcttaaaaacgtcc tggcggcaggagaatctcttgaattctcctgccgccaggacgggg3'. pcDNA3-HA-FOXM1c was generated by ligation of an HA-FOXM1c fragment obtained by PCR using oligo-nucleotides EcoRI-HA-FOXM1fwd: 5'ccgggatccatgtacccatacg atgttccagattacgctcttgccgaggcgcctcaggtgg3' and EcoRI-FOXM1rev: 5'ccggaattcctactgtagctcaggaataaactg3' into a EcoRI (New England Biolabs) digested pcDNA3 vector (Invitrogen). Correct orientation was verified by automated sequencing. HA-FOXM1c$^{S331/S704A}$ was created by two rounds of site-directed mutagenesis using the following oligo-nucleotides S331Afwd: 5'ccactggacccaggggctccacaattgcccg3' S331Arev: 5'cgggcaattgtggagcccctgggtccagtgg3', S704Afwd: 5'gtccccaagccaggcgccccggagccacagg3', S704Arev: 5'cctgtggctccggggcgcctggcttggggac3'). siRNA oligo-nucleotides against Pin1 or a scrambled sequence (Dharmacon) have been described[26] and were transfected at a final concentration of 100nM using oligofectamine according to the manufacturer's protocol (Invitrogen).

Antibodies

The antibody against HA (12CA5), has been described[6]. The following antibodies were purchased: phosphoThr183/Tyr185-JNK and phosphoThr202/Tyr204-ERK (Cell Signaling), Aurora-B, p21^{cip1} (BD pharmingen), p21^{cip1} (Ab-3) (Neomarkers), BRAF (C19), Cyclin-B1 (GNS1; sc-245), FOXM1 (MPP2; C-20) (Santa Cruz), Pin1 (R&D systems), FLAG-M2, Tubulin (Sigma), CENP-F (Abcam) and GAPDH (Chemicon).

Immunoprecipitation, GST Pull-down and *in vitro* JNK kinase assay

Immunoprecipitations and GST-pulldown assays were performed as described[26] in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 1% TX-100, 0.5% NaDoC, 5 mM EDTA, 150 mM NaCl, protease and phosphatase inhibitors. For *in vitro* JNK kinase assays HA-FOXM1c was precipitated and processed according to the manufacturer's protocol (Upstate) with $^{32}\gamma$-ATP.

Colony Formation assay

Colo829 cells were plated in six well plates and transfected with the indicated plasmids (2µg) in combination with pbabe-puro (250ng) and processed as described[6].

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General Discussion

Excessive cellular ROS contributes to premature aging and increases the chance on tumorigenesis[1,2]. Cells can actively respond to excessive ROS levels by undergoing a temporal cell cycle arrest, senescence or apoptosis, all of which are inactive or bypassed in tumor cells[3]. In this thesis, we focused on the regulation of FOXO signaling in response to acute and chronic elevations in cellular ROS levels. Our results increase the fundamental understanding on how FOXOs regulate cell cycle progression and further elucidates their role in tumor suppression. Next, we will discuss their possible implications.

FOXO-induced G1 arrest: Differential regulation of $p27^{kip1}$ and $p21^{cip1}$?

To arrest cell cycle progression, FOXOs can transactivate transcription of $p27^{kip1}$ (Ref[4]) and $p21^{cip1}$ (Ref[5,6]). However whether activation of FOXOs always result in transcription of both, or whether there are specific conditions under which they are transcribed individually was not yet clear. $p27^{kip1}$ is the classical FOXO target responsible for the cell cycle arrest in response to growth factor deprivation[4,7]. In contrast, no stimulus other than TGFβ has been reported yet that can trigger $p21^{cip1}$ transcription by FOXOs[5,6,8].

H_2O_2 can induce a FOXO-dependent cell cycle arrest, albeit through an unknown mechanism[9]. This is in agreement with earlier observations that cellular ROS activate FOXO signaling by inducing nuclear translocation and transcriptional activity on an artificial promoter designed for FOXO binding (6xDBE, see also Chapter 4)[10]. Seemingly in contrast, no major increase in FOXO-induced $p27^{kip1}$ expression is observed in response to H_2O_2 treatment ([11,12] and data not shown), suggesting that in response to elevated cellular ROS, FOXOs induce cell cycle arrest through another cell cycle inhibitor. Interestingly, it has been reported that as a consequence of treatment with H_2O_2 cells can undergo an arrest in cell cycle progression in G1/S or G2/M through upregulation of $p21^{cip1}$ (Ref[12]). Furthermore, other reports have shown that H_2O_2 can induce $p21^{cip1}$ expression[13], which we could confirm (data not shown). This increase is JNK-dependent, which in combination with our data, may point to a regulatory role for FOXOs.

As described in Chapter 4, oncogenic $BRAF^{V600E}$ signaling generates cellular ROS (Chapter 4). Subsequently, the FOXO-induced expression of $p27^{kip1}$ is shifted to $p21^{cip1}$. This shift from $p27^{kip1}$ to $p21^{cip1}$ is in part explained by the observation that $BRAF^{V600E}$ induces degradation of $p27^{kip1}$ (Ref[14]). However, effects on cell cycle progression usually occur at multiple levels, e.g. next to protein stability also on transcription. For instance PKB activation in response to growth factor stimulation reduces $p27^{kip1}$ expression through inhibition of FOXO-mediated transcription[4] and through direct phosphorylation of the protein, which targets it for proteasomal degradation[15-18].

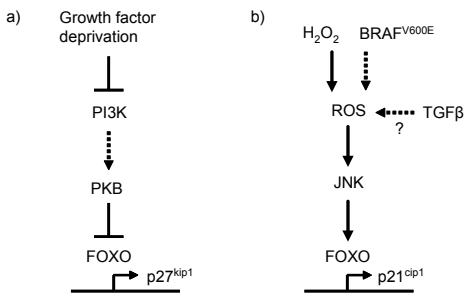

Fig. 1: Stimulus-specific $p27^{kip1}$ or $p21^{cip1}$ transcription by FOXOs

a) Deprivation of growth factors prevents activation of PI3Ksignaling towards PKB. As a consequence FOXOs are not phosphorylated by PBK and accumulate in the nucleus where they can induce $p27^{kip1}$ transcription. **b)** Even in the presence of growth factors, FOXOs can be activated by Reactive Oxygen Species (ROS), for instance induced by H_2O_2-treatment (acute elevation), or $BRAF^{V600E}$ signaling (chronic elevation). This also leads to increased nuclear localization, but importantly, may result in transcription of a different CKI, $p21^{cip1}$.

Likewise, also the $BRAF^{V600E}$ induced shift from $p27^{kip1}$ to $p21^{cip1}$ may be regulated at multiple levels, e.g. reduced $p27^{kip1}$ transcription, in contrast to increased $p21^{cip1}$ transcription. This may indicate that $p21^{cip1}$, rather than $p27^{kip1}$, is the cell cycle arrest target of FOXOs in response to elevated cellular ROS (Fig 1). Although the effect of FOXO depletion still has to be addressed, H_2O_2 can induce a concentration dependent $p21^{cip1}$-promoter activation in DLD-1 colon carcinoma cells, which is significantly enhanced by FOXO4 co-expression (A.B. Brenkman, data not shown). Thus, it will be interesting to determine if and how in response to elevated ROS promoter binding by FOXO is diverted from *p27kip1* to *p21cip1*.

The role of FOXO-induced $p21^{cip1}$ expression $BRAF^{V600E}$-induced OIS and survival

Although oncogenic mutation of BRAF is frequently observed in melanoma, tumorigenesis of mutated melanocytes initially prevented through a series of possible responses. One mechanism to prevent progression into melanoma is upregulation of the CDK inhibitor $p16^{ink4a}$, which allows cell cycle arrest and OIS[19] (Fig. 2a). $P16^{ink4a}$ plays an important role in the suppression of melanoma progression *in vivo* as for instance observed for a number families that are predisposed to develop melanoma and were found to show homozygous loss of $p16^{ink4a}$ (Ref[20]). $p16^{ink4a}$ is frequently lost in melanoma, however, this appears especially the case for the more malignant types[21]. This suggests that $p16^{ink4a}$ loss is not sufficient to initiate tumorigenesis of melanocytes. This notion is supported by other observations. First, 16^{ink4a} is not essential for the OIS response by $BRAF^{V600E}$ (Ref[22]). Second, in ~80% of all human and mouse naevi, which are thought to represent an example of cellular senescence *in vivo*, oncogenic BRAF mutations are found, whereas $p16^{ink4a}$ is only expressed in a mosaic pattern in naevi[23,24].

Together this points to the existence of an alternative and/or redundant mechanism to

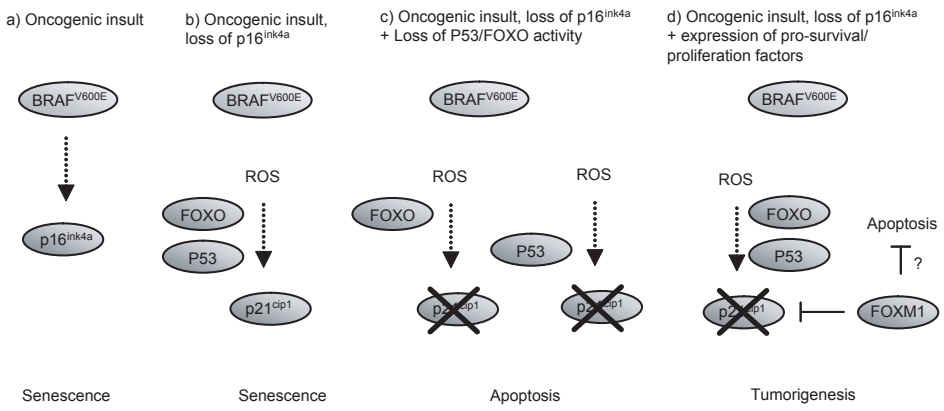

Fig. 2: Putative mechanisms to prevent tumorigenesis upon oncogenic BRAF mutation

a) Following oncogenic BRAF mutation, the typical response is increased $p16^{ink4a}$. As described in Fig. 2 of the general introduction, $p16^{ink4a}$ inhibits CDK2 activation and thereby induces a G1-arrest. **b)** When $p16^{ink4a}$ expression is compromised, e.g. in patients with a genetic predisposition, cell cycle arrest can still be induced through the ROS-JNK-FOXO-$p21^{cip1}$ pathway described in this thesis. **c)** When either FOXO or p53 activity is lost, cells will still not become tumorigenic through induction of apoptosis. **d)** Only when these mechanisms are prevented or bypassed, such as in cells with high FOXM1 expression, cells will proliferate and remain viable, thus becoming tumorigenic.

compensate for loss $p16^{ink4a}$. $p21^{cip1}$ can induce senescence in response to various stimuli, but its relevance to $BRAF^{V600E}$-induced senescence is not clear as $p21^{cip1}$ expression is also observed in melanomas[21]. In contrast to $p16^{ink4a}$, which does not regulate apoptosis, $p21^{cip1}$ fulfills a dual, antagonistic role in tumor suppression. On one hand $p21^{cip1}$ can repress G1/S transition and arrest proliferation, whereas on the other hand it inhibits apoptosis[25]. Oncogenically mutated cells that have bypassed the cell cycle arrest arm of $p21^{cip1}$ may therefore be prone to progress into a state of malignant growth.

Our data indicates that forced expression of $p21^{cip1}$ by ectopic FOXO4 induces OIS in $BRAF^{V600E}$ expressing melanoma cells that are mutated for $p16^{ink4a}$ (Chapter 4). This indicates that next to $p16^{ink4a}$ tumorigenesis of melanocytes can be arrested by $p21^{cip1}$ (Fig. 2b). Interference with $p21^{cip1}$ expression either through knockdown of FOXOs or p53 would not only prevent the anti-proliferative arm of $p21^{cip1}$, but also its pro-survival arm. Interference with this pathway will still inhibit tumorigenesis, albeit not through senescence, but apoptosis (Fig. 2c). Melanoma cells that have fully lost $p21^{cip1}$ expression will therefore be hard to find, simply because they need additional mutations in the apoptosis machinery to survive. In contrast, melanoma cells may exploit low levels of $p21^{cip1}$ to remain viable and proliferate. In Chapter 5, we showed some data to support this model. In Colo829 melanoma cells $p21^{cip1}$ expression is low, but not completely absent. Interference with signaling downstream of $BRAF^{V600E}$ either by U0126, or through knockdown of endogenous p53 or FOXO in all cases induced apoptosis (Chapter 5 and Ref[26]). This may indicate that $p21^{cip1}$ is required for survival of these cells. How then do melanocytes progress into melanoma without undergoing apoptosis? A possible explanation is given in Chapter 6. The same Colo829 cells express high levels of FOXM1. We observed that knockdown of FOXM1 in Colo829 cells significantly reduced cell viability (Data not shown). Also chemical interference with FOXM1 activity (see below) resulted in a similar effect. This suggests that FOXM1 can keep $p21^{cip1}$ expression low through SKP2 mediated proteasomal degradation and at the same time ensure survival, thereby allowing melanoma progression (Fig. 2d). Interestingly, FOXM1 is indeed highly expressed in numerous melanoma cell lines, including the A375 cells used here, and melanoma tissue ([27,28] and data not shown) which further supports this model.

A role for FOXOs in the antagonistic pleiotropy between tumor suppression and accelerated aging

Aging is at least partially the result of life-long build-up of cellular damage, for instance induced by ROS[29]. FOXOs induce transcription of ROS scavenging proteins as MnSOD and Catalase, and thereby FOXOs counteract the negative effects of ROS on accelerated aging[30,31]. Also, in *C. elegans* the FOXO ortholog DAF-16 is essential for extended lifespan in response to reduced insulin signaling, suggesting that FOXO activity is beneficial for longevity[32]. Polymorphisms in the *foxo3a* gene have been observed in people of exceptional old age, centenarians[33,34], furthermore underscoring its importance in this process. If these polymorphisms are beneficial for longevity, why then are they not present in the majority of the human population? A possible answer can be found in what is defined as the antagonistic pleiotropy of aging. This states that in terms of natural selection genes that allow organisms to survive until the reproductive stage are favored even if activation of these comes at a cost later in life, i.e. accelerated aging[35]. Since ROS produces damage over a lifelong period activation of MnSOD and Catalase by FOXOs are hardly required in the early stages of life, i.e. before the reproductive phase. Therefore there

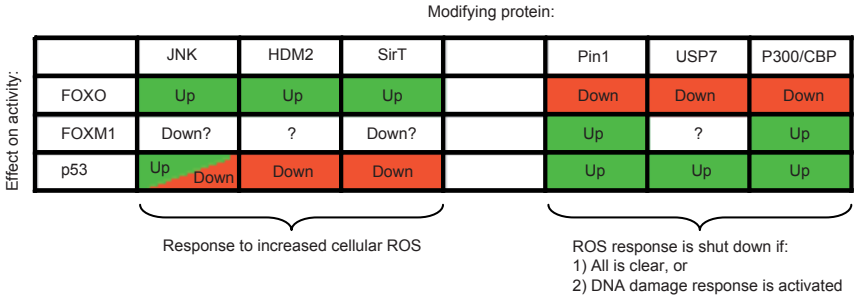

Fig. 3: Effects on the activity of FOXOs, FOXM1 and p53 by post-translational modification-inducing proteins
Schematic overview of the regulation of FOXOs, FOXM1 and p53 by JNK, HDM2 and SIRT1 vs. Pin1, USP7 and P300. This may point to a general mode of substrate regulation specific for ROS signaling.

simply is no selection pressure for gain-of-function mutations or extra copies of the *foxo* genes. Rather, there would be selection for mild activity enough to ensure tumor suppression, but not too much to result in developmental defects. In this respect, it is interesting to draw a parallel with p53 again. Loss of p53 does result in tumor formation and is therefore evolutionarily not favored. Interestingly, however, mice expressing extra copies of *p53*, super-p53 mice, are more resistant to tumor formation, show increased DNA damage response and, importantly, develop and age normally[36]. It will therefore be interesting to see how mice with extra copies of the *foxo* genes develop.
Tumor suppression upon mutational activation of oncogenes can be facilitated through induction of OIS or apoptosis. Opposite to the benefits of FOXO on longevity and delayed aging, we have shown here (Chapter 4) that FOXOs are essential for the OIS response by oncogenic $BRAF^{V600E}$. In contrast to p53 or $p21^{cip1}$, FOXO signaling has not yet been shown to mediate this dual response upon ROS signaling, i.e. delayed aging through increased ROS scavenging in contrast to accelerated aging through its function in OIS. Thus, these data point to an antagonistic role for FOXO signaling in the pleiotropy between tumor suppression and aging.

	FOXOs	FOXM1	P53
JNK	Thesis + [10,37]	Thesis	[38-40]
HDM2	Thesis + [41]	Unknown	[42]
SIRT1	[11]	Unknown	[43,44]
Pin1	Thesis	Thesis	[45-47]
USP7	Thesis + [48]	Unknown	[49-51]
P300/CBP	[11,52,53]	[54]	[45,55,56]

Table 1: summary of references that describe the regulation of FOXOs, FOXM1 and p53 through post translational modification by JNK, HDM2, SirT, Pin1, USP7 and P300/CBP.

Regulation of FOXOs, FOXM1 and p53 by elevated cellular ROS levels: A systematic biological response?

As described in this thesis (Chapters 2-5) and elsewhere (Table 1 and Fig. 3), FOXOs can be regulated in response to excessive ROS through a host of post translational modifications. Moreover, we established that also the activity of FOXM1 and p53 is regulated in response to ROS signaling (Chapters 5+6). Although mostly described for other stimuli, also p53 and FOXM1 can at least partially be modified by the same enzymes as described for FOXOs. This may point to an orchestrated mode of regulation where excessive ROS modulates the activity of groups of transcription factors through similar patches of post translational modifications induced by clusters of modifying enzymes.

In case of FOXO, ROS signaling triggers activation through JNK-mediated phosphorylation, HDM2-mediated mono-ubiquitination and SIRT1 mediated de-acetylation. We described here that FOXM1 can be phosphorylated by JNK *in vitro* and *in vivo*. Whether FOXM1 is a target of SIRT1 and HDM2 is still unknown. However, since P300 activity is required for FOXM1 transcriptional activity it is likely that SIRT1 represses FOXM1 signaling. p53 can be phosphorylated by JNK with different reported outcomes. HDM2 and SIRT1 can repress p53, however whether they regulate p53 in response to ROS is unclear. On a cellular level, protein modification by these enzymes may ensure that proliferation is repressed through inhibition of FOXM1, activation of FOXO and differential regulation of p53. This thereby leads to a "ROS-response".

In case ROS levels are reduced enough to allow proliferation again, or, in case the amount of excessive ROS results in genotoxic stress, the ROS response will be turned off. This can be facilitated through systematic activation of a second set of enzymes, e.g. Pin1, p300 and USP7 that modulate or reverse the former set of modifications. All of these enzymes inhibit ROS-induced FOXO activity towards cell cycle arrest and ROS scavenging. Conversely, we showed

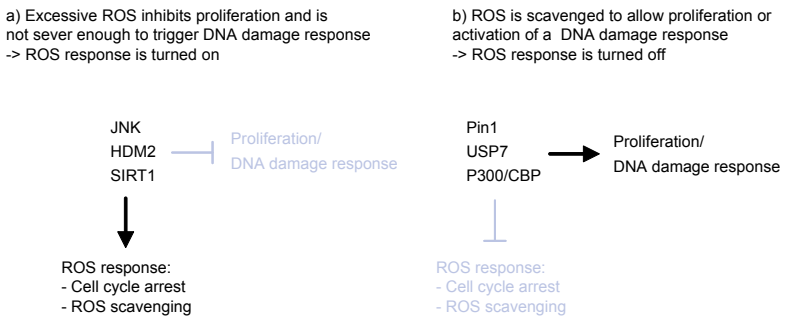

Fig. 4: Model for a systematic response to turn on or off signaling by elevated ROS levels and hence mediate a "ROS-response".

a) When cellular ROS levels increase cells may respond by systematic activation of a cluster of enzymes, i.e. JNK, HDM2 and SIRT1 to mediate post translational modification on groups of transcription factors to facilitate a cell cycle arrest and ROS scavenging. **b)** When this ROS response needs to be turned off, e.g. to allow proliferation or a shift to the DNA damage response, a different cluster of enzymes may be activated that reverse or modulate the induced post translation activations.

here that Pin1 activates FOXM1 signaling and a similar effect has been reported for CBP/P300. Whether USP7 regulates FOXM1 is unknown. Also p53 activity can activated by these three enzymes, which may induce a DNA damage response, including apoptosis. Thus, there may be two independent sets of enzymes that orchestrate opposite effects in response to elevated ROS. The first set would comprise JNK, HDM2 and SIRT and stimulate a cellular ROS response, whereas the second set, comprising Pin1, USP7 and P300/CBP represses it (Fig. 4).

Future Perspectives:

Effects of FOXO signaling on the Senescence Associated Secretory Phenotype

Based on the results described in this thesis, several lines of further investigation may be interesting to continue on. FOXO4-induced senescence in Colo829 cells is $p21^{cip1}$ dependent, however while $p21^{cip1}$ expression alone does result in cell cycle arrest in these cells, it is insufficient to induce senescence (data not shown). This indicates that other factors than $p21^{cip1}$ are required to regulate the senescence phenotype induced by FOXO4.

Recently, the Senescence Associated Secretory Phenotype (SASP) has gained much attention with regard to $BRAF^{V600E}$ and RAS^{G12V}-induced senescence[24,37,38]. During SASP, a variety of chemokines and cytokines are secreted, which are required for and induce OIS. Out of the chemokines and cytokines that are secreted in response to $BRAF^{V600E}$ signaling, IL6 and IGFBP7 have recently been best studied. IGFBP7 secretion results in apoptosis of $BRAF^{V600E}$, but not NRAS mutated melanoma cells or cells in which both proteins are wildtype[38]. FOXO4 expression did not result in apoptosis in Colo829 cells (see Chapter 4). Therefore, it seems unlikely that induction of IGFBP7 is the secreted factor responsible for the FOXO4-induced repression in proliferation of these cells.

In contrast IL-6 would be an interesting candidate to mediate FOXO-induced senescence. A high percentage of individuals with age-related diseases exhibit a chronic inflammatory state associated with increased circulatory levels of cytokines, such as IL-6[39]. Furthermore, ROS can induce chemokine production[39,40] and, as we have shown here extensively, ROS can regulate FOXO signaling. Since FOXO-mediated OIS is dependent on $BRAF^{V600E}$-induced cellular ROS (Chapter 4 and data not shown), this may partially be due to increased IL-6 production. IL-6 can induce an cell-autonomous rather than paracrine-induced senescence response[24]. In agreement with this, we observed that exogenous IL-6 treatment did not induce senescence, nor did blocking antibodies repress FOXO4 mediated senescence in Colo829 cells (data not shown). Interestingly however, preliminary data indicate that FOXO4 expression can induce IL-6 expression (data not shown). It will be interesting to see whether knockdown of IL-6 blocks FOXO4 induced senescence in oncogenic BRAF expressing melanoma cells. This would suggest that $p21^{cip1}$ is responsible for the effects on cell cycle arrest, whereas IL-6 may be required for the actual senescence response.

Interplay between FOXO and MITF signaling in melanocytes

An additional transcription factor that may be of interest to FOXO signaling in $BRAF^{V600E}$-induced OIS is the Microphtalmia-Induced Transcription Factor, MITF. MITF is a key regulator of melanocyte differentiation[41]. Activation of the RAF-MEK-ERK signaling cascade results in activation of MITF activity[42], but can also target MITF for proteasomal degradation[43]. These

observations suggest a correlation between MITF activity and MITF degradation. Oncogenic $BRAF^{V600E}$ signaling stimulates MITF transcription, yet reduces cellular MITF protein expression in melanocytes[44]. Because of the corollary between degradation and activity this may indicate $BRAF^{V600E}$ induces MITF activity[44].
Importantly, MITF expression is reduced in spontaneously transformed melanocytes[45] and low MITF expression correlates with poor prognosis in melanoma[46]. This suggests that MITF expression is required to maintain a state of cell cycle arrest in response to oncogenic insults. Forced expression of MITF in melanoma cells indeed impairs proliferation[44], and interestingly, MITF induced cell cycle arrest was found to be $p21^{cip1}$-dependent[47]. Recently, expression of a micro-RNA, miR182, was shown to be highly upregulated in melanoma[48]. Overexpression of miR182 promoted migration and survival of melanoma cells in correlation with repressed FOXO3a and MITF activity, suggesting a possible interplay between their FOXO and MITF signaling. Furthermore, interference with MITF expression lowers $p21^{cip1}$ expression in WM266.4 melanoma cells[49] and we have shown here (Chapter 4) that knockdown of endogenous FOXOs does likewise. Thus, it will be interesting to determine if and how FOXOs interplay with MITF to induce $p21^{cip1}$ transcription in response to oncogenic BRAF signaling.

Putative new lines of treatment for $BRAF^{V600E}$ mutated melanomas
Besides putative new directions for fundamental research, our data also identified several nodes in signaling that can represent targets for clinical treatment for a subset of $BRAF^{V600E}$ mutated melanomas. For instance, inhibition of FOXO signaling results in apoptosis in at least two melanoma cell lines that express elevated FOXM1 levels, Colo829 and A375 cells (Chapter 5 and data not shown). Also FOXM1 knockdown in these cell lines resulted in a strong decrease in cell viability, possibly due to increased apoptosis (Chapter 6). Thiostrepton is an antibiotic that can induce apoptosis in, at least, MCF-7 breast cancer cells through repression of FOXM1 activity. Interestingly, in line with reduced FOXM1 activity, we observed that Thiostrepton also significantly decreased cell viability of Colo829 cells (data not shown). The effect of Thiostrepton on healthy melanocytes and melanoma cells with low FOXM1 expression is not yet clear, however as FOXM1 appears primarily important for proliferating cells Thiostrepton may provide a suitable treatment specifically for FOXM1 positive melanomas with a $BRAF^{V600E}$ mutation.
A second putative drug for melanoma treatment is Tamoxiphen (not to be confused with 4-hydroxy-tamoxifen (4-OHT) used to activate ER-fused proteins, such as used in Chapter 6). Also in MCF-7 breast cancer cells, FOXO3a and ER have been shown to functionally interact, resulting in repression of ER activity[50]. Furthermore, Tamoxiphen induced cell death has been linked to inhibition of the IGFR-PI3K-PKB pathway and may therefore affect FOXO activity[51]. Tamoxiphen as a melanoma treatment has been studied in randomized clinical trails with disappointing results and is therefore controversial as a treatment[52]. However, in these studies the genetic background of the tumors was not defined. Interestingly, Tamoxiphen significantly repressed cell viability of Colo829 cells (data not shown). It may therefore be worthwhile to investigate in which cells Tamoxiphen specifically induces cell death in melanomas with a $BRAF^{V600E}$ mutation.

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Summary

Elevated levels of cellular ROS are damaging to the interior of the cell and inherently accelerate the onset of aging and increase the risk on carcinogenesis. FOXO transcription factors counteract the excess in cellular ROS by transactivating transcription of the ROS scavenging enzymes MnSOD and Catalase. As such, FOXO activity is associated with longevity in model organisms and humans. In turn, FOXO activation can be triggered by ROS-induced signaling towards FOXOs inducing a feed-back loop through which FOXO activation is negatively regulated. In the past, the results of this signaling have been studied with regard to post translational modification of FOXOs (phosphorylation, acetylation and ubiquitination) and the interaction with other proteins (e.g. β-Catenin and p53). Nonetheless, how FOXO activity is controlled under conditions of elevated ROS is still only partially unraveled. In this thesis, we have explored the molecular mechanisms behind FOXO signaling in response to elevated cellular ROS, predominantly with regard to tumor suppression.

Modulation of FOXOs through acetylation and ubiquitination is reversible and thereby FOXO4 activity can be tightly controlled. It has been unclear, however, how ROS-induced phosphorylation can be counteracted. In **Chapter 2**, we showed that following ROS-induced phosphorylation of FOXO4 provides a docking surface for the peptidyl-prolyl isomerase Pin1. ROS-induced phosphorylation enhances FOXO activity and subsequent cell cycle arrest, however, we observed that Pin1 binding could counteract this effect. ROS-induced phosphorylation strongly correlates with mono-ubiquitination and nuclear import of FOXO4, an effect that is reversed by the de-ubiquitinating enzyme USP7/HAUSP. In line with reduced transcriptional activity, we showed that Pin1 promotes de-ubiquitination of FOXO4 by USP7. Pin1 induces a *cis*-to-*trans* conformational change in the peptide backbone of its substrates once phosphorylated on Ser/Thr residues followed by a Pro. Indeed, this enzymatic activity of Pin1 is essential for its ability to repress FOXO signaling. Importantly, a mutant of FOXO4 in which the Pin1 docking sites are mutated is strongly ubiquitinated, localizes constitutively to the nucleus and is no longer repressed by USP7 or Pin1 expression. Thus, Pin1 counteracts ROS-induced nuclear localization of FOXO4 by promoting their de-ubiquitination through USP7.

De-ubiquitination of FOXOs by USP7 has been well studied. However, through which mechanism FOXOs are ubiquitinated in the first place was still unclear. In **Chapters 3 and 5**, we observed that the E3-ubiquitin ligase HDM2 can induce FOXO4 ubiquitination in response to elevated cellular ROS. Importantly, we found that, in contrast to poly-ubiquitination, which consists of multiple ubiquitin moieties attached to one another, FOXOs are mono-ubiquitination on multiple residues instead. When expressed at low levels, HDM2 expression stimulates the transcriptional activity of FOXO4, whereas higher expression of HDM2 decreases FOXO4 levels and impairs the repressive effects of FOXO4 on cell cycle progression and proliferation. Mono-ubiquitination of FOXOs in response to elevated cellular ROS increases their activity. WE observed that depletion of HDM2 prevents FOXO mono-ubiquitination in this background, which suggests that endogenous HDM2 is required for FOXO activation in response to elevated ROS levels.

Most studies that addressed FOXO signaling in response to elevated cellular ROS used H_2O_2 to induce an acute increase in cellar ROS levels. In **Chapter 4,** we showed that signaling by oncogenic $BRAF^{V600E}$ induces a chronic increase in cellular ROS levels. H_2O_2 has been shown to signal towards FOXO4 by JNK-mediated phosphorylation on partially unraveled residues.

We identified the undiscovered JNK sites to be Thr223 and Ser226 and showed that $BRAF^{V600E}$ induced FOXO4 phosphorylation on all JNK sites through a linear pathway downstream of $BRAF^{V600E}$ through MEK, elevated cellular ROS and finally JNK. Importantly, modification of FOXO4 by this pathway resulted in a $p21^{cip1}$-dependent cell cycle arrest, independent of $p27^{kip1}$, suggesting ROS-specific shift in the cell cycle arrest response by FOXOs from $p27^{kip1}$ to $p21^{cip1}$. Oncogenic mutation of BRAF frequently occurs in melanocytes and results in oncogene induced senescence (OIS), at least in part through $p21^{cip1}$. We showed that signaling from oncogenic BRAF to $p21^{cip1}$ transcription is also endogenously regulated by this linear cascade towards FOXOs. Importantly, in a cell-line that expresses $BRAF^{V600E}$, but only showed low levels of $p21^{cip1}$ (Colo829) ectopic FOXO expression restored $p21^{cip1}$ levels and subsequently induced a state of senescence. Thus, for the first time and in mechanistical detail, these data characterized a role for FOXOs in the antagonistic pleiotropy between tumor suppression and aging.

Next to FOXOs, a well known activator of $p21^{cip1}$ transcription is the tumor suppressor p53. In **Chapter 5**, we showed that like FOXO4, $BRAF^{V600E}$ signaling induces phosphorylation of p53 on Ser46 and in parallel increases the interaction with FOXO4. Ser46 phosphorylation is associated with apoptosis in case of DNA damage and with senescence in case of oncogenic signaling, suggesting that FOXO and p53 co-operate to facilitate OIS by $BRAF^{V600E}$. Although $p21^{cip1}$ activation clearly represses tumor progression trough induction of cell cycle arrest, cells that can bypass this arrest may misuse $p21^{cip1}$ to prevent apoptosis. Knockdown of FOXOs or p53 resulted in a significant increase in apoptosis. This may indicate that in response to oncogenic signals high expression of $p21^{cip1}$ induces senescence, whereas repression of $p21^{cip1}$ induces apoptosis, providing to fundamentally distinct mechanisms of tumor suppression.

In contrast to FOXO signaling, FOXM1 activation promotes cell cycle progression and is abundantly expressed in multiple types of cancer, such as melanoma. In **Chapter 6**, we showed that also FOXM1 is a downstream target of $BRAF^{V600E}$ signaling and that, in contrast to what we showed in Chapter 2 for FOXOs, FOXM1 is activated by Pin1. Indeed, FOXM1 and Pin1 interact endogenously in a $BRAF^{V600E}$-expressing melanoma cell line that expresses high levels of FOXM1. Furthermore, interference with signaling downstream of $BRAF^{V600E}$ inhibits this interaction and the expression of FOXM1 target genes. FOXM1 is known to overrule G1/S arrest through transcriptional activation of the E3-ligase SKP2, which promotes proteasomal degradation of for instance $p21^{cip1}$. Interestingly, we now observed that FOXM1 levels are highly upregulated in Colo829 melanoma cells, providing a possible explanation for the low $p21^{cip1}$ expression in these cells. This suggests that differential regulation of FOXO4 and FOXM1 activity in response to $BRAF^{V600E}$ signaling determines the fate of cell cycle progression, an outcome that may be opposed by Pin1.

Nederlandse samenvatting (voor de leek)

In dit proefschrift is gekeken naar de regulatie van de tumor suppressor FOXO door Reactieve Zuurstof Soorten. Samengevat beschrijven wij het volgende:

Het menselijk lichaam is samengesteld uit cellen, die elk een specifieke taak vervullen. In volwassen mensen bevinden verreweg de meeste cellen in het lichaam zich in een rusttoestand waarin ze hun taken uitvoeren, maar niet delen. Gezonde cellen kunnen worden gestimuleerd om te delen door groeifactoren. Deze zorgen er voor dat binnen in de cel processen in gang worden gezet die leiden tot het verdubbelen van het DNA van de cel en de eerlijke verdeling hiervan over twee nieuwe dochtercellen. Dit proces is omschreven als celcyclus progressie.
De celcyclus is onderverdeeld in vier verschillende hoofdfasen: G1, S, G2 en M. De eerste groeifase (G1) is een voorbereidingsfase waarin de cel klaargemaakt wordt voor het verdubbelen van de hoeveelheid DNA. Deze verdubbeling vindt uiteindelijk plaats in de daarop volgende synthese fase (S). Wanneer de S-fase is afgerond, komt de cel in een nieuwe groeifase (G2) terecht, waarin de verdeling van het DNA over twee dochtercellen wordt voorbereid. Dit gebeurt uiteindelijk in mitose, oftewel de M-fase. In mijn proefschrift heb ik voornamelijk gekeken naar processen die betrokken zijn bij de overgang van de G1 naar S-fase.
Wanneer de celcyclus eenmaal vanuit de voorbereidende G1 naar de S-fase is overgegaan is er geen weg meer terug en zal de cel hoe dan ook doorgaan met het verdubbelen van het DNA. Het is dus belangrijk dat condities optimaal zijn voordat dit gebeurd. Er zijn verschillende redenen waarom celcyclus progressie tijdelijk stilgelegd kan worden, iets wat celcyclus arrest wordt genoemd. Een oorzaak die celcyclus arrest tot gevolg heeft, is het afwezig zijn van groeifactoren. In tegenstelling tot gezonde cellen zijn kankercellen ongevoelig voor het afwezig zijn van groeifactoren en blijven delen in afwezigheid ervan. Dit leidt tot ongeremde groei, tumorgenese.
Reactieve Zuurstof Soorten, in het engels Reactive Oxygen Species (ROS), komen onder andere vrij als bijproduct van energieproductie van een cel. ROS is tevens nodig voor cellen om te kunnen delen, doordat ze in de cel groeisignalen doorgeven. Echter, teveel ROS is schadelijk voor de cel doordat eiwitten, lipiden en DNA erdoor permanent veranderd kunnen worden. Hoge hoeveelheden ROS versnellen zo het ouderdomsproces en verhogen de kans op tumorgenese. Het wegvangen van het teveel aan ROS draagt bij aan het tegengaan van ouderdom en als indirect gevolg een langere levensduur.
Signalering in de cel windt veelal plaats via eiwitten. Een specifieke klasse eiwitten zijn de transcriptiefactoren, die als taak hebben het aflezen van bepaalde genen (transcriptie) te bevorderen of juist te remmen. De eiwitten die centraal staan in het onderzoek beschreven in mijn proefschrift zijn de transcriptiefactoren FOXO (Forkhead BOX O). Recentelijk is aangetoond dat FOXOs “tumor suppressors” zijn, wat wil zeggen dat ze tumorgenese tegengaan en gebrek eraan leidt tot verhoogde kans op kanker.
Wanneer er teveel ROS aanwezig is in een cel wordt FOXO signalering aangezet, met als direct gevolg transcriptie van eiwitten die het teveel aan ROS omzetten in water. Tevens zorgen FOXOs in deze situatie voor een celcyclus arrest, zodat de enzymen die voor deze omzetting zorgen ook de tijd krijgen om dit te doen. Wanneer de overmaat aan ROS weggevangen is zal de celcyclus weer verdergaan. Het arrest is dus reversibel, iets wat “quiescence” wordt genoemd.

In het verleden is er door onze vakgroep en anderen gekeken naar het moleculaire mechanisme waardoor FOXOs worden geactiveerd door ROS. Hieruit is gebleken dat er aan FOXOs een kleine verandering in de structuur aangebracht kan worden door er andere moleculen aan te koppelen, waardoor de chemische eigenschappen ervan veranderen. Dit kan bijvoorbeeld door een kleine fosfaat groep (fosforylering) of door een geheel ander eiwit, ubiquitine (ubiquitinering). Fosforylering en ubiquitinering van FOXOs zijn beide geassocieerd met import van FOXOs naar de celkern. Transcriptie vindt plaats in de celkern en inderdaad leidt deze translocatie van FOXOs tot verhoogde activiteit. Hoe ubiquitinering en fosforylering van FOXOs gereguleerd worden en hoe deze effecten weer ongedaan gemaakt kunnen worden als het teveel aan ROS weggevangen is, is slechte beperkt duidelijk. De regulatie van FOXOs door ROS en met name het effect hiervan op tumor suppressie, vormt de rode draad van dit proefschrift.

Voordat er met het hier beschreven werk werd begonnen was het onduidelijk hoe ROS-geïnduceerde activering van FOXOs door fosforylering weer geremd kon worden. Fosforylering en ubiquitinering van FOXOs zijn nauw verbonden met elkaar en zijn geassocieerd met lokalisatie van FOXOs in de kern, waar ze transcriptie reguleren. In **hoofdstuk 2** laten we zien dat het eiwit Pin1 kan binden aan FOXOs die als gevolg van verhoogde ROS gefosforyleerd zijn. Vervolgens zorgt Pin1 door een verandering in de structuur van FOXOs er voor dat de ubiquitine-groepen op FOXO makkelijker verwijderd kunnen worden. Dit heeft tot gevolg dat FOXO uit de celkern naar het cytoplasma worden geëxporteerd, waar ze geen invloed meer op transcriptie uitoefenen. Hierdoor remt Pin1 de activiteit van FOXOs en wordt het celcyclus arrest opgeheven.
Hoe Pin1 zelf gereguleerd wordt is nog niet geheel duidelijk. Wel is bekend dat Pin1 zeer hoog tot expressie komt in een deel van alle borstkanker soorten. Met betrekking hiertoe laten wij tenslotte zien dat er in tumoren met hoge Pin1 expressie een omgekeerde correlatie bestaat met $p27^{kip1}$, een eiwit waarvan de transcriptie door FOXOs aangezet wordt, met celcyclus arrest tot gevolg. Dit suggereert dat als gevolg van remming van FOXOs door Pin1, $p27^{kip1}$ niet meer tot expressie komt, waardoor er geen celcyclus arrest meer optreedt en deze cellen zijn uitgegroeid tot borstkanker. Deze observaties kunnen daardoor bijdragen aan de behandeling van de ziekte.

In **hoofdstuk 3 (en 5)** zijn we verder ingegaan op de regulatie van ubiquitinering van FOXOs als gevolg van verhoogde ROS niveaus. Hoewel het bekend was dat het eiwit USP7 de ubiquitine-groepen van FOXOs kon verwijderen, was het nog niet duidelijk hoe FOXOs überhaupt in eerste instantie ge-ubiquitineerd worden. Wij laten zien dat HDM2 hiervoor verantwoordelijk is.
Wanneer meedere ubiquitine-eiwitten aan elkaar worden gekoppeld (poly-ubiquitinering) kan dit leiden tot afbraak van het eiwit waar ze aan vast zitten. Van HDM2 was al bekend dat het poly-ubiquitinering van p53 induceerde en daarmee de afbraak ervan. Wij laten zien dat FOXO4 door HDM2 worden ge-ubiquitineerd op verschillende plaatsen, maar slechte met één ubiquitine-groep per keer (mono-ubiquitinering). Bij lage hoeveelheden leidt dit tot verhoging van de activiteit van FOXO4, in plaats van afbraak. Bij hoge hoeveelheden leidt ook HDM2 tot verminderde expressie van FOXO4. Het mechanisme hierachter is nog niet geheel duidelijk. Desalniettemin, laten we in hoofdstuk 5 zien dat HDM2 inderdaad essentieel is voor mono-ubiquitinering van FOXO4, en daarmee zeer hoogstwaarschijnlijk voor de ROS-geïnduceerd activiteit van FOXO4.

In **hoofdstuk 4** gaan we dieper in op de rol van FOXOs als tumor suppressors en wel in melanoma (een vorm van huidkanker). Een eiwit dat gemuteerd is in maar liefst in ~70% van alle gevallen van melanoma is BRAF, veelal op positie 600 van Valine naar Glutamine (V600E). Mutatie van BRAF alleen is echter niet genoeg voor tumorgenese doordat cellen een beschermingsmechanisme in werking zetten waardoor ze in een soort permanente winterslaap gaan, "senescence" geheten. In tegenstelling tot de bovengenoemde quiescence, wat een reversibele arrest is, kunnen deze cellen vele jaren in deze toestand blijven zonder dat ze nog in staat zijn te delen. Moedervlekken zijn een voorbeeld waarvan de cellen allemaal in senescence zijn, zonder dat deze direct gevaarlijk voor ons zijn. Wij laten zien dat gemuteerd $BRAF^{V600E}$ voor een chronische verhoging in ROS zorgt, dat vervolgens FOXOs aanzet via fosforylering door de stress-kinase JNK. Bovendien laten we zien dat activering van FOXOs op deze manier essentieel is voor senescence respons door gemuteerd $BRAF^{V600E}$ middels transcriptie van de cel cyclus remmer $p21^{cip1}$. In melanoma cellen waarin gemuteerd $BRAF^{V600E}$ voorkomt en dit mechanisme van senescence verstoord is kunnen we toch weer $p21^{cip1}$ niveaus verhogen en senescence induceren door FOXO in te brengen. In het addendum op dit hoofdstuk laten we zien dat dit specifiek lijkt te zijn voor melanoma cellen waarin BRAF gemuteerd is en niet in cellen met een andere mutatie, NRAR, wat in ~15% van alle melanoma gevallen gemuteerd is. Samen dragen onze resultaten bij aan nieuwe inzichten voor de bestrijding van melanoma.

Naast regulatie door FOXOs, is een welbekende transcriptiefactor die $p21^{cip1}$ transcriptie kan reguleren p53. Tot nu toe was er alleen bekend dat signalering van FOXO en p53 tegen elkaar in werkt. In **hoofdstuk 5** laten wij zien dat in de achtergrond van verhoogde cellulaire ROS, bijvoorbeeld door gemuteerd $BRAF^{V600E}$, FOXOs en p53 juist samenwerken voor $p21^{cip1}$ transcriptie. Inhibitie van deze effecten leidt tot geprogrammeerde celdood (apoptose) in melanoma cellen waarin BRAF gemuteerd is, maar niet in cellen waarin "normaal" BRAF tot expressie komt.

Als laatste hebben we gekeken naar een eiwit dat verwant is aan FOXOs, namelijk FOXM1. In tegenstelling tot FOXOs bevordert FOXM1 juist celcyclus progressie. Op zijn beurt wordt FOXM1 alleen aangemaakt in cellen die delen. In verschillende soorten kanker, zoals huidkanker, is FOXM1 in verhoogde niveaus aanwezig en remming van de activiteit van FOXM1 remt tumor progressie. In **hoofdstuk 6** laten we zien dat het eiwit uit hoofdstuk 2, Pin1, niet alleen FOXO, maar ook FOXM1 reguleert. We beschrijven hoe Pin1 nodig is voor de activiteit van FOXM1 en de effecten op cel cyclus progressie. Bovendien reguleert Pin1 ook FOXM1 activiteit in aanwezigheid van verhoogde niveaus ROS. Hierdoor stellen wij een model voor waarin ROS signalering door FOXO aanzet en FOXM1 uitzet met als gevolg cel cyclus arrest. Pin1 doet vervolgens het tegenovergestelde en zorgt ervoor dat het arrest opgeheven wordt.

Samengevat zijn de belangrijkste bevindingen van dit proefschrift ten eerste dat signalering van gemuteerd BRAF door middel van chronische verhoging van de hoeveelheid cellulaire ROS FOXOs aanzet, waardoor deze cellen senescent worden en tumorgenese wordt geremd. Ten tweede laten we zien dat FOXO signalering geremd kan worden door Pin1. En tenslotte dat Pin1 in tegenstelling tot remming van FOXOs FOXM1 signalering activeert. Deze resultaten zijn bediscussieerd in **hoofdstuk 7**.

Curriculum vitae

Peter de Keizer werd geboren op 21 maart 1981 te Gorinchem. In 1999 behaalde hij het diploma van het Gymnasium Camphusianum te Gorinchem en in datzelfde jaar begon hij met de opleiding Biologie aan de Universiteit Utrecht. In 2002 vervolgde hij de opleiding met het Master-programma Biomolecular Sciences en gedurende deze fase werd onderzoekservaring opgedaan bij de afdeling Moleculaire en Cellulaire biologie onder begeleiding van Dr. E.E. Klapisz-Regan en Dr. P.M.P. van Bergen en Henegouwen. In 2003 was hij vervolgens voor een buitenlandse stage werkzaam aan het Massachusetts General Hospital / Harvard Medical School in Boston, MA, U.S.A. onder begeleiding van Dr. K. Shah en Prof. Dr. X.O. Breakefield. Ter afronding behaalde hij in 2004 het doctoraalexamen en begon in datzelfde jaar met het promotie-onderzoek beschreven in dit proefschrift bij de afdeling Fysiologische Chemie van het Universitair Medisch Centrum Utrecht onder begeleiding van Dr. A.B. Brenkman en Prof. Dr. Ir. B.M.T. Burgering.

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To be submitted.

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Selected Color Figures

Fig. 2a)

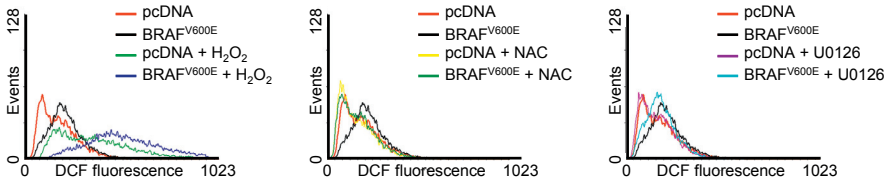
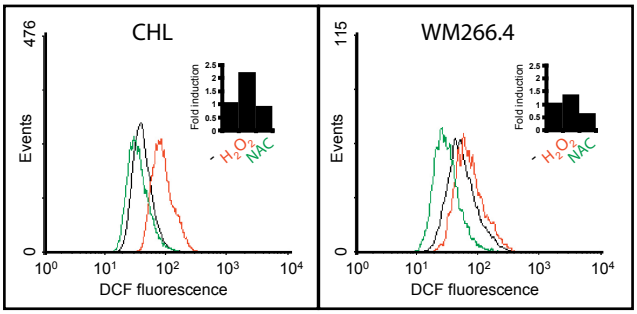


Fig. 5b)



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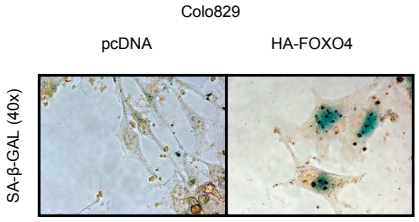
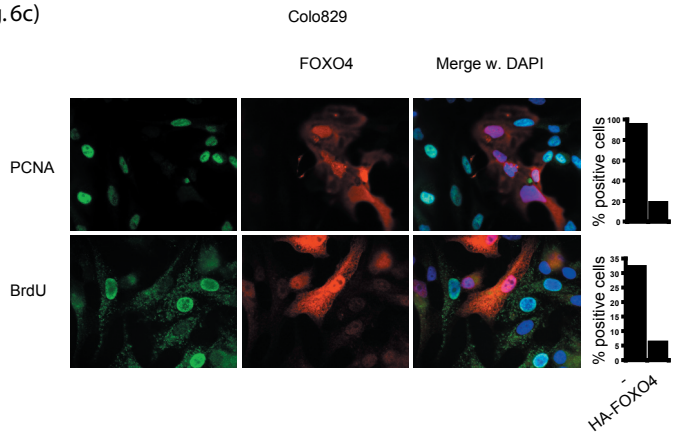
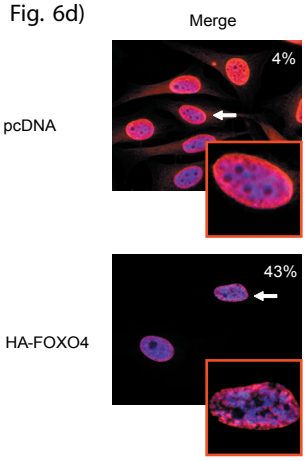


Fig. 6c)

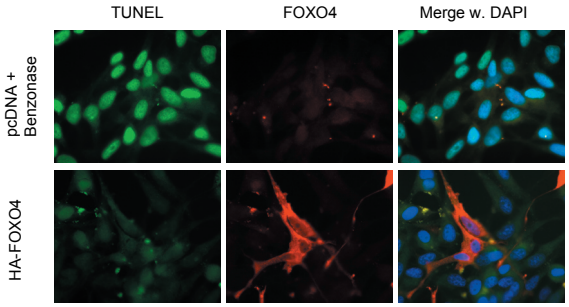


Chapter 4

Fig. 6d)



Sup. fig 3a)



Chapter 5

Fig. 2b)

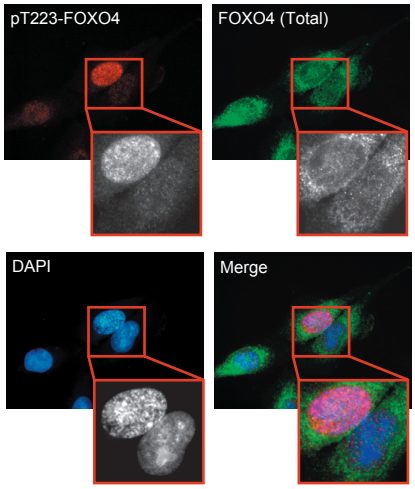


Fig. 4b)

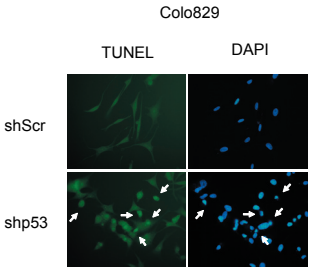


Fig. 4c)

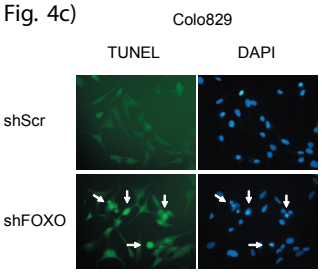


Fig. 4d)