The multifaceted role of FOXOs in the maintenance of genomic and metabolic homeostasis

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The multifaceted role of FOXOs in the maintenance of genomic and metabolic homeostasis

De veelzijdig rol van FOXO in het onderhoud van genomische en metabolische homeostase

(met een samenvatting in het Nederlands)

Proefschrift

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door

Paraskevi Charitou

geboren op 31 mei 1981

te Thessaloniki, Griekenland

Promotor: Prof. dr.ir. B.M.T. Burgering

To my parents, Achilleas &Elisabeth

To my brother, Grigoris

To my husband, Christos

Στους γονείς μου, Αχιλλέα&Ελισάβετ

Στον αδερφό μου, Γρηγόρη

Στο σύζυγό μου, Χρήστο

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Chapter 1

General Introduction

FOXOs in control of ROS and genomic stability to ensure healthy lifespan

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Abstract

Transcription factors of the Forkhead box O class (FOXOs) are associated with lifespan and play a role in age-related diseases. FOXOs therefore serve as a paradigm for developing an understanding as to how age-related diseases such as cancer and diabetes interconnect with lifespan. Understanding the regulatory inputs on FOXO may reveal how changes in these regulatory signalling pathways affect disease and lifespan. Numerous regulators of FOXO have now been described and a clear and evolutionary conserved role has emerged for phosphoinositide-3 kinase (PI3K)/ protein kinase B (PKB, also known as c-Akt or AKT) signalling and c-jun N-terminal kinase (JNK) signalling. Analysis of FOXO function in the context of these signalling pathways has shown the importance of FOXO-mediated transcriptional regulation on cell cycle progression and other cell fates, such as cell metabolism, stress resistance and apoptosis in mediating disease and lifespan. Persistent DNA damage is also tightly linked to disease and aging, yet data on a possible link between DNA damage and FOXO have been limited. Here, we discuss possible connections between FOXO and the DNA damage response in the context of the broader role of connecting lifespan and disease.

Introduction

During the past decades average life expectancy has steadily increased. However, advanced aging has remained a major risk factor for the development of chronic diseases and degenerative pathologies, including diabetes, cardiovascular disease and cancer. To maximize the population's healthy lifespan, it is crucial to obtain a better understanding of the molecular mechanisms underpinning the process of aging itself and how these processes affect the onset of age-related disease. Aging includes a multitude of processes influenced by a number of genetic and environmental factors that interact with each other in a complex manner. Numerous theories have been proposed to explain the aging phenomenon, two of which, the "free radical" and the "DNA damage" theory of aging, we will further elaborate on.

Free radical theory of aging

The "free radical theory of aging", as formulated by Denham Har man proposes that reactive oxygen species (ROS), generated due to oxygen metabolism, are the driving force behind aging and age-related diseases (1). Reactive oxygen species include the free radicals oxygen singlet ($^1O_2$), superoxide anion (O_2•–) and hydroxyl radical (OH•–) as well as the non radical H_2O_2. ROS are normal by-products of metabolic processes, such as oxidative phosphorylation in mitochondria, or they are generated by growth factors and cytokines through the action of NADPH-oxidases (reviewed in (2)). Cellular homeostasis of ROS is maintained by the function of certain detoxification systems, namely the superoxide dismutases, catalase, the glutathione/ glutathione peroxidase and the thioredoxin/ peroxiredoxin system. However, intracellular free radicals increase with age due to accumulation of dysfunctional mitochondria and functional decline of the cellular antioxidant defences. Eventually, ROS react with cellular constituents and cause oxidative damage; it is proposed that this accumulation of ROS-induced damage over time drives the aging process **(Figure 1)**.

In support of the "free radical theory of aging", lifespan in most animals inversely correlates with their metabolic rates and therefore the rates of ROS generation (3). Moreover, mouse models with impaired antioxidant defences, and thereby increased intracellular ROS, show increased prevalence of age-related diseases. For example superoxide dismutase (SOD1) null mice exhibit shortened lifespan and age-related

degeneration (4, 5). However, the reverse, a mere increase in expression of ROS detoxifying enzymes does not always result in increased longevity. Mice engineered to overexpress catalase targeted to the nucleus or the peroxisomes do not show any change in their median or maximal lifespan. Rather, targeted

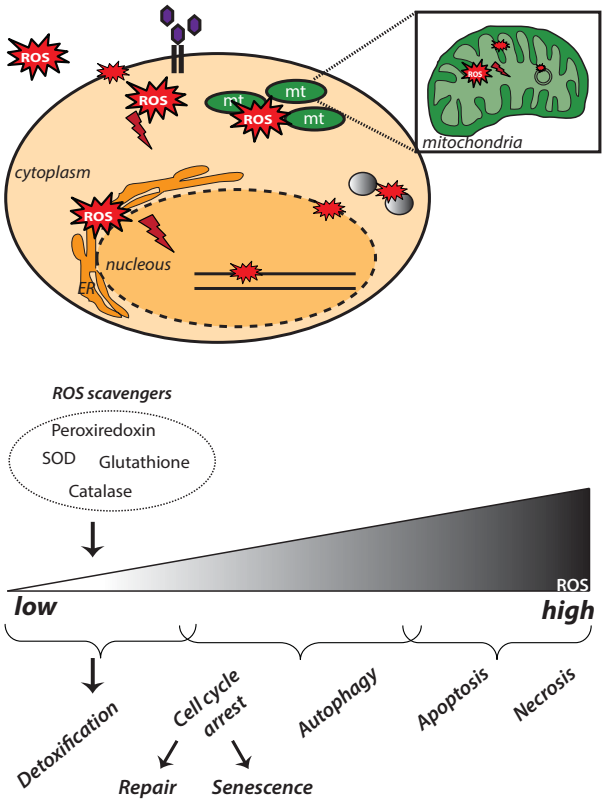

Figure 1. Intracellular ROS accrual and downstream effects. ROS are produced by several normal physiological processes, including mitochondrial oxidative phosphorylation or generated downstream of growth factors and cytokines, as well as by endoplasmatic reticulum stress etc. Eventually, ROS build up in various cellular compartments, including the cytoplasm and mitochondria, where they react with and induce changes in lipids, proteins and the DNA. Several cellular defence mechanisms maintain intracellular ROS levels, including the enzymatic functions of superoxide dismutases, catalase, the glutathione/glutathione peroxidase and the thioredoxin/peroxiredoxin system. The final cellular fate depends on the degree of ROS accumulation, ranging from growth arrest and damage repair in cases of low intracellular ROS, to senescence and apoptosis or even necrosis in cases of excessive ROS.

overexpression of catalase in the mitochondria resulted in increased lifespan of these mice compared to their wild type littermates, accompanied by attenuation of aging associated pathologies (6). Interestingly, SOD2-/- mice are short lived (7, 8) but this is in apparent contrast with recent studies in *C. elegans* that show SOD2 lacking worms to have extended lifespan, despite increased oxidative damage (9). At the same time, overexpression of SOD1 in *C.elegans* increases lifespan, but this is not attributable to improved ROS scavenging (10). Moreover, depletion of all nematode superoxide dismutases does not appear to affect lifespan (11). Overall, despite the fact that data from studies in *C.elegans* appear to challenge the free radical theory of aging, one could argue that they actually reflect the complicated balance of SOD function(s) in maintaining optimal cellular redox. This is particularly important in the light of the essential and non-damaging roles of ROS in normal signalling. In addition, when comparing these apparent discrepancies in

lifespan determination between nematodes and mice, it should be taken into account that there are crucial differences in the physiology of animals having mainly post-mitotic somatic cells (*C.elegans*) versus animals in which somatic cells do still divide.

Overall, even though the 'free-radical theory' provides a framework for understanding lifespan and aging, the biology of the aging process appears to be more complicated.

ROS and DNA damage

ROS can induce a variety of DNA lesions, including base modifications and DNA breaks (12). Base modifications (for example 8-oxo-guanine, 8-oxo-G) have a high mutagenic potential by generating mismatches, whereas DNA breaks are predominantly cytostatic/cytotoxic lesions, inducing growth arrest and apoptosis (13) **(Figure 2)**. To avoid the accumulation of ROS-induced DNA mutations, cells are equipped with several systems dedicated to the detection and clearance of modified nucleotides, collectively referred to as Base Excision Repair (BER). After the induction of DNA breaks specialized signalling cascades are initiated (14); recognition of DNA breaks activates the PI3 kinases-related kinases family (PIKKs), that subsequently orchestrate the cellular response for DNA repair. DNA breaks are repaired by two main pathways, namely non-homologous end-joining (NHEJ) and homologous recombination (HR) (14). NHEJ occurs throughout the cell cycle and is a very rapid mechanism for DNA repair, as it is based on bridging together the broken DNA ends. This mechanism of DNA repair however is error-prone, as it may lead to the loss or gain of bases as well as to chromosomal translocations and eventually genomic instability. In contrast to NHEJ, HR utilizes the genetic information of the intact sister chromatid for the faithful repair of the lesion and thus is considered an error-free mechanism of repair; HR occurs exclusively in the late S and G2 phase of the cell cycle, when the sister chromatid is available.

When damage is too severe or not properly repaired, other protection mechanisms are activated. Cell cycle arrest, which is normally induced by the DNA damage response signalling to facilitate DNA repair, can become irreversible, a phenomenon termed senescence. Moreover, cells experiencing severe DNA damage can commit to programmed cell death (apoptosis).

The DNA damage theory of aging

The aging process and aging-related pathologies have been linked to the accrual of DNA damage (12, 15, 16). This accrual may derive from the increased ROS load in aged tissues, as well as the functional decline of DNA repair mechanisms. Ultimately, accumulated DNA lesions are associated with increased prevalence of mutations and blockage of physiological processes, including replication and transcription. Responses that are activated eventually result in the induction of senescence and apoptosis, processes that are both linked to the functional decline observed in aging. Furthermore, it is important to consider that, whereas other damaged cellular macromolecules (lipids, proteins) can be efficiently removed and replaced by new undamaged molecules, this is not the case for the genetic material. Damaged DNA persists for the cell's lifetime, while carrying the information for the generation of all other cellular components. Therefore, accumulation of genomic instability over time results, among others, in the production of defective cellular components and is thereby connected to the cellular deterioration associated with aging (DNA damage theory of aging, reviewed in (17)).

In agreement with the above, human genetic disorders with disruptions in certain DNA repair pathways are

characterized by early onset of aging associated diseases **(Table 1)**. Some typical examples are the Werner syndrome and Ataxia Telangiectasia (A-T), both with mutations in proteins associated with double strand breaks repair, as well as syndromes associated with defects in transcription-coupled repair. Interestingly, most of these genetic disorders are also characterized by increased intracellular ROS (Table 1). These observations beg the question what is cause and consequence with respect to the aging phenotype and whether impaired DNA repair or increased ROS load, is the cause of premature aging observed in these disorders.

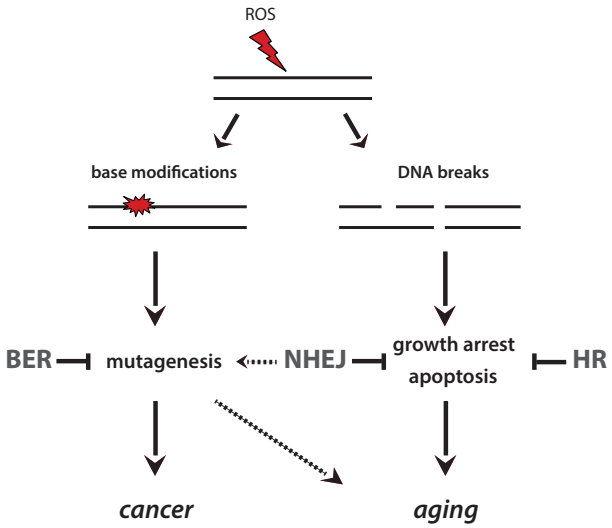

Figure 2. Oxidative DNA damage and repair pathways. ROS induce a variety of DNA lesions, including base modifications and DNA breaks. Oxidative modifications of DNA bases can result in mutagenesis and oncogenesis. BER is the pathway responsible for the removal and repair of oxidized bases. On the other hand, DNA breaks are cytostatic lesions, inducing responses leading to deterioration and aging phenotypes. DNA breaks are mainly repaired by NHEJ, an error-prone mechanism that can potentially lead to genomic instability, and HR, a high fidelity repair mechanism for the maintenance of genomic stability.

Nonetheless, whether the "free radical" and "DNA damage theory" are complementary or not is still under debate. Indeed, mice engineered with proof-reading deficient mitochondrial DNA (mtDNA) polymerase exhibit reduced lifespan and early onset of age-related disorders (18). However, no evidence for increased oxidative mtDNA damage was observed in this model. Moreover, as mentioned, targeted overexpression of catalase in the nucleus does not have an impact on lifespan, suggesting that nuclear oxidative damage may not be a cause of aging. Regarding the "DNA damage theory", the main criticism is raised on the grounds that, whereas reduced DNA repair is associated to premature aging, the opposite, an increase in lifespan due to enhanced efficiency of DNA repair has not been observed (19).

Aging and cellular homeostasis

The theories of aging discussed above, although attractive to explain certain aspects of aging and age-associated diseases, certainly do not provide all answers and have been challenged by experimental data. Irrespectively, these theories share the perspective that the aging process is driven by progressively disturbed organismal homeostasis. Under normal conditions there is an established balance between cell removal and regeneration in tissues; damaged and transformed cells are constantly removed and replaced

by new functional cells. However, during aging homeostasis is disturbed **(Figure 3)**. When clearance of dysfunctional cellular components is impaired, these damaged components (lipids, protein and organelles) will accumulate and cause cellular dysfunction (e.g. cellular senescence). If not eliminated, this will maintain tissue mass but also contribute to aging (discussed later). Alternatively, when eliminated cells are no longer replaced, tissues are progressively becoming more atrophic. Also, tissue regeneration upon trauma is not as efficient and different organs are more labile to malfunction; all the above constitute hallmarks of aging.

The insulin-dependent regulation of the FOXO transcription factors serves as a paradigm for our understanding as to how disturbed homeostasis interconnects with age-related diseases and lifespan. Insulin regulates FOXO activity through the PI3K/PKB pathway (20, 21). In human cancer the PI3K/PKB pathway is often deregulated. Activating mutations in PI3K and PKB occur at moderate frequency in various types of cancer, while deletion or loss of function mutations within phosphatase/tensin homolog deleted on chromosome 10 (PTEN), the lipid phosphatase that counteracts PI3K function, occur at relative high frequency (for review see (22)). Furthermore, several receptor tyrosine kinases (e.g. EGF receptor) are also frequently overexpressed in certain types of human cancer and in these settings efficiently activate PI3K signalling. Thus, on average over 50% of human tumours express somehow hyperactive PI3K/PKB signalling. On the other hand, reduced PI3K/PKB signalling is a hallmark of diabetes (23). The importance of the PI3K/PKB pathway in insulin action towards metabolic endpoints such as glucose regulation is illustrated by the observation that within a family showing autosomal dominant inheritance of severe insulin resistance and diabetes mellitus a mutation in the PKB/AKT2 gene was identified and that expression of the resulting mutant kinase in cultured cells disrupted insulin signalling to metabolic end points (24). Thus, either gain- or loss-of-function of PI3K/PKB signalling contributes significantly to two important age-related diseases i.e. cancer and diabetes **(Figure 4)**. The relevance of the PI3K/PKB pathway towards FOXO to age-associated disorders is ultimately supported by the fact that this pathway has an evolutionary conserved role in lifespan determination. In 1993 Kenyon *et*

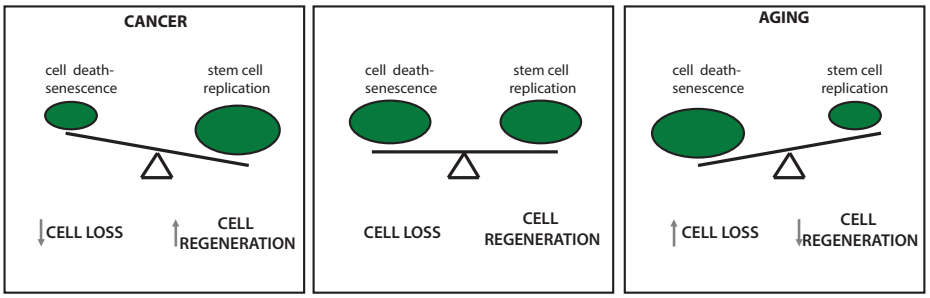

Figure 3. Aging as a result of disturbed homeostasis. Under normal conditions there is a balance between cell removal (by senescence and apoptosis) and regeneration (by the function of tissue specific stem cells) in tissues. In cases that the balance shifts to higher rates of cell removal than regeneration, the end result is aging. Contrary, increased rates of cell proliferation with reduced cell death results in cancer.

al reported that a mutation in *daf-2*, the homologue of the insulin and IGF receptor in *C. elegans*, increases lifespan by two fold and this is dependent on daf-16, the FOXO homologue (25). Likewise, in *Drosophila melanogaster*, overexpression of the dFOXO protein was shown to increase lifespan (26). Importantly, FOXOs appear to regulate lifespan also in mammals, including humans; genome-wide association studies using centenarian cohorts, reveal polymorphisms in FOXO1 and FOXO3a loci that associate with increased longevity (27-29).

Table 1. Progeria disorders associated with DNA repair defects and the respective intracellular ROS accrual.

Disorder	Gene mutated	DNA repair pathway affected	Aging-associated phenotype	Intracellular ROS
Ataxia telangiectasia	ATM	DSB response/ repair	Progressive cerebellar degeneration, ataxia, dilated blood vessels (telangiectasia), immunologic defects, increased cancer predisposition, type II diabetes	Increased ROS in cerebellum (70, 206-208)
Nijmegen breakage syndrome	NBS1	DSB response/ repair	Immunodeficiency, increased cancer predisposition, growth retardation	ND
Bloom syndrome	BLM	DNA recombination	Growth retardation, immune deficiency, increased cancer predisposition, type II diabetes	Increased ROS (207, 209)
Werner syndrome	WRN	DNA recombination	Atrophic skin, thin gray hair, osteoporosis, cataracts, arteriosclerosis, increased cancer predisposition, type II diabetes	Increased ROS (206, 207, 210)
Fanconi anaimia	FA complementation groups A-L	DNA crosslink repair	Pancytopenia, bone marrow failure, renal dysfunction, abnormal pigmentation, increased cancer predisposition	Increased ROS (150, 206, 207)
Rothmund-Thomson	RECQL4	DSB repair and Base excision repair	Growth deficiency, gray hair, skin and skeletal abnormalities, increased cancer predisposition	ND
Trichothiodystrophy	XPB, XPD, TTDA	Nucleotide excision repair	Neurologic and skeletal degeneration, cachexia, brittle hair	ND
Cockayne syndrome	CSA, CSB	Nucleotide excision repair	Neurologic degeneration, cachexia	Increased ROS (211)

FOXOs are integral components in the responses to ROS and DNA damage

FOXOs regulate cell fate decisions, including cell proliferation, cell death and cell metabolism. To provide cellular homeostasis towards stress signals that disturb homeostasis, the FOXO-induced response should combine and fine-tune these distinct processes into one.

FOXO activity is regulated by a number of signalling cascades activated in response to increased intracellular ROS (30), as well as by direct ROS-induced changes (cysteine oxidation (31)). FOXOs in turn act as homeostatic regulators because they do not only respond to oxidative stress but also protect cells from oxidative stress

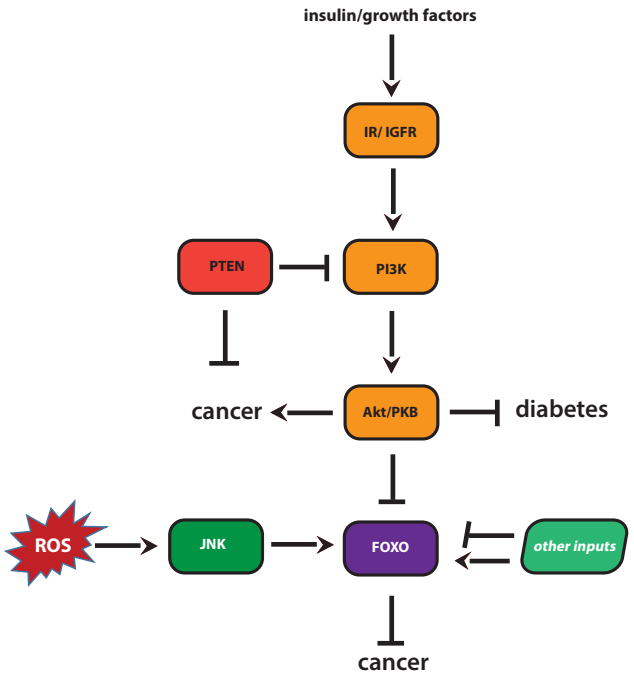

Figure 4. The PI3K/PKB pathway is central in cancer and disease. PKB is activated downstream of PI3K in response to insulin or growth factors and in turn phosphorylates downstream substrates, including the FOXO transcription factors. FOXO phosphorylation by PKB results in their nuclear exclusion and inhibition of their function as transcription activators. Abnormal activation of PKB is associated with cancer progression, whereas the opposite, PKB inhibition, results in diabetes. FOXOs on the other hand act as tumour suppressors. In response to endogenous and exogenous cues, additional signalling pathways, besides PI3K/PKB, also regulate FOXO function both in a positive and a negative manner.

through the transcription of ROS scavenger genes, including superoxide dismutase 2 (SOD2 or MnSOD), catalase, peroxiredoxin 3 and members of the sestrin family (32-35). Mice engineered with inducible deletion of the three predominant FOXO members (FOXO1, FOXO3a and FOXO4, thereafter called FOXO TKO mice) display increased intracellular ROS in the stem cell compartment, associated with decreased expression of certain ROS detoxification enzymes. The associated effects, including hematopoietic stem cell depletion, were alleviated by concomitant treatment with antioxidant agents (36, 37).

A growing body of evidence suggest that FOXOs also facilitate DNA repair, by regulating defined transcriptional programs and executing transcription-independent functions. Induction of UV-damage activates FOXO transcriptional activity towards Gadd45a (38), a protein implicated in DNA damage checkpoint maintenance (39, 40) as well as in certain types of DNA repair (41-43). However, how GADD45a partakes in DNA repair has remained fully enigmatic. A role for GADD45 in DNA demethylation has been suggested (44), but remains debated (see e.g. (45)). In fact, there is still no consensus as to how active enzymatic DNA demethylation is achieved in mammalian cells, but recent studies implicate BER in genome-wide DNA demethylation in germ cells and early embryos. Thus, GADD45a may somehow interact with, or be part of the BER machinery. Considering that UV irradiation induces, *inter alia*, oxidative damage that requires BER for its removal, this may account for the observed increase in DNA repair following FOXO activation reported by Tran et al. (38). However FOXOs may also have additional functions in genotoxic stress protection. Cells deficient for PTEN or

expressing hyperactive PKB show increased genomic instability due to inhibition of HR (46-49). Considering that FOXOs are important downstream components of PI3K/PKB signalling, one could speculate that FOXOs could also play a role in faithful DNA repair via HR and thereby contribute to genomic stability. Furthermore, FOXO3 was recently reported to physically interact with ataxia-telangiectasia mutated (ATM) kinase and this interaction was suggested to be required for full activation of ATM in response to DNA damage and the subsequent DNA damage checkpoint activation (50). Importantly, this physical association is in agreement with the observed FOXO1 phosphorylation by ATM, reported in a study by Matsuoka et al (51). In addition, FOXOs were also reported to regulate the transcription of ATM in hematopoetic stem cells (52).

Under conditions of genotoxic stress FOXOs have been shown to regulate the expression of the CDK inhibitors $p27^{kip1}$ and $p21^{waf1/cip1}$ to induce cell cycle arrest (53-55). Importantly, sustained $p21^{waf1/cip1}$ expression is associated with senescence induction and recent studies on oncogene-induced senescence suggest FOXO activation to mediate senescence (56) at least in part through regulation of $p21^{waf1/cip1}$. Under conditions of excessive cellular damage FOXOs were shown to regulate induction of apoptosis, by initiating the transcription of the Bcl-2 family proteins Bim and PUMA (57, 58). Interestingly, FOXO-mediated apoptosis appears to have practical implications in cancer therapies. In particular, FOXOs appear to sensitize cancer cells to chemotherapy that acts through genotoxic stress, such as doxorubicin and cisplatin, thereby reducing the effective dosage of these drugs and accordingly the risk of side effects (59-62).

Overall, FOXOs appear to contribute to longevity by regulating processes that are both anti-aging (ROS scavenging, DNA repair) as well as pro-aging (inducing senescence and apoptosis). Below we will discuss how these FOXO roles relate to the main cellular events associated with excessive cellular elimination - and therefore disturbed homeostasis - during aging, which are increased genomic instability, telomere attrition and accumulation of damaged and dysfunctional macromolecules and organelles. Furthermore, we will elaborate on the association between ATM and FOXO because of their corresponding functions in aging and cancer and the increasing evidence for reciprocal regulation of FOXOs and ATM.

Tumour suppression and aging; FOXOs and ATM are *bona fide* tumour suppressors

Aged tissues accumulate unrepaired (or not properly repaired) DNA lesions caused by both excessive ROS and exogenous insults (e.g. UV irradiation) (63-65). The accrual of DNA mutations increases the potential for cellular transformation and cancer. Paradoxically, the aging process is characterized by a gradual functional decline attributed mostly to cell loss, whereas cancer, a major age-related disease, is characterized by the opposite; that is an uncontrolled increase in cell numbers. On the other hand, responses activated to prevent cancer and limit cell number are inducing aging phenotypes.

Recognition of DNA lesions by the cellular surveillance mechanisms initiates signalling cascades for cell cycle arrest and DNA repair or, in cases of severe and persistent damage, senescence and apoptosis (14). Senescence is the irreversible withdrawal from the cell cycle and it constitutes a prominent tumour suppressor mechanism since it leads to the elimination of aberrantly proliferating cells. What determines entry into senescence or apoptosis is still unclear; however the cell type appears to be important. It is hypothesized that senescence occurs more often in less proliferative tissues and tissues in which the physical presence of the cell is crucial for their integrity, for instance the skin. Contrary to senescence, apoptosis is more often induced in highly proliferative tissues, with the exception of neuronal cells that although post-mitotic commit to apoptosis in response to damage accumulation. Importantly, although senescence is employed as a mechanism for tumour suppression, senescent cells produce and secrete inflammatory

cytokines that have been shown to stimulate tumourigenesis in neighbouring cells (66). Both senescent and apoptotic cells are cleared by the immune system, providing efficient mechanisms to prevent disease. However, the efficiency by which the immune system clears senescent cells appears to become less during aging, thereby contributing to aging-associated diseases (67). In line with this, a recent study showed that improved removal of senescent cells in a mouse model of premature aging alleviated the aging process (68). Senescence and apoptosis are typical responses activated downstream of the DNA damage response. ATM is a central kinase in the DNA damage response and it regulates the pathway for the induction of both senescence and apoptosis, mainly through activation of the p53 tumour suppressor. In fact, deletion or inhibition of ATM kinase activity *in vitro* and *in vivo* abolishes both of these processes, resulting in uncontrolled proliferation of damaged cells and carcinogenesis (69). Moreover, A-T patients suffer from cancer predisposition, with increased prevalence of lymphomas and lymphoid leukaemias (70). Also, after induction of replication stress due to oncogene activation, ATM and Rad3 related (ATR) kinase regulates similar responses, again through p53 (71, 72). Early research on FOXO function already implied a potential role for FOXOs as tumour suppressors, because FOXOs regulate growth inhibition and cell death in response to cellular stress (73). Importantly, FOXO TKO mice suffer from thymic lymphomas and haemangiomas (37) and this established FOXOs as bona fide tumour suppressors. FOXOs inhibit cancer progression by affecting several processes, including inhibition of tumour vascularization (for review see (73)). At the cellular level their main contribution is by inducing cell cycle arrest (also senescence) and apoptosis in response to oxidative stress and other genotoxic insults (73, 74).

DNA damage-induced transcription block and progeria

Accumulated DNA lesions are not only deleterious with respect to oncogenic transformation but are also responsible for cellular dysfunction and induction of cytostatic events. Transcription blocking lesions (75, 76) cause defects in protein production and subsequent performance of vital cellular functions. Seminal work by Hoeijmakers and associates revealed that genetic disruption of transcription-coupled repair components results in premature aging and age-related pathologies, associated with defects in transcription progression (77-80).

Interestingly, transcriptome analysis of progeroid mice with DNA repair dysfunctions revealed a suppression of the somatotropic axis (growth hormone, GH and IGF-1) (78, 80). This is quite paradoxical, considering that previous studies linked reduced IIS signalling to increased longevity in *C. elegans* and mice (25, 81, 82). However, in cases of chronic DNA damage due to repair defects, attenuation of the somatotropic axis can be considered as an adaptive response that shifts the natural resources from growth to maintenance. By attempting to reduce metabolic rates, this response adds to the reduction of intracellular free radicals and the ensuing genomic instability. At the same time, increased transcription of ROS detoxification enzymes is also observed, as a mechanism to further reduce ROS-induced damage. Importantly, these mice also exhibit increased transcripts of pro-apoptotic proteins, probably due to the inhibition of the pro-survival IIS axis (78).

The role of FOXOs has not been studied yet in this genetic system. However, since FOXOs are expected to be active due to loss of IIS signalling, these mouse models would be particularly informative to study FOXO function under conditions of increased genomic instability.

Telomere attrition in aging

Telomeres constitute the chromosome ends and are protected by specialized structures, to prevent their attrition and fusion (83-85). Each DNA replication round results in gradual shortening of the telomeres and, in the absence of adequate telomerase activity, telomeres gradually shorten until they reach a critical length (86). At this point telomeres lose their protective capping and are sensed by the DNA damage surveillance system as DNA breaks (83, 85). Eventually, the DNA damage response, orchestrated by ATM and ATR, results in senescence induction (replicative senescence) or, in cases that DNA repair with NHEJ is activated, chromosome fusions occur and the ensuing genomic instability leads to carcinogenesis.

Telomere erosion occurs during DNA replication, but is also modulated by other factors. Telomeres are repetitive sequences rich in G and C nucleotides,,which have been shown to be particularly sensitive to oxidative damage (87), and telomere shortening occurs with higher rates under oxidative stress conditions (88-91). Importantly, treatment with antioxidants or increased expression of dismutases under oxidative stress conditions appeared to rescue the telomeric length and delay the subsequent replicative senescence (92). Considering the role of FOXOs in regulating the expression of ROS detoxifying enzymes, it is tempting to speculate that another contribution of FOXOs in extending lifespan is by indirectly regulating telomere length. Irrespective, other potential functions of FOXOs in the setting of uncapped and dysfunctional telomeres would be equally interesting to determine.

Aging and mitochondrial dysfunction; FOXOs and ATM in mitochondria protection

An important mediator of the increased ROS accrual in aged tissues is mitochondrial dysfunction (89, 93). MtDNA is especially sensitive to ROS and consequent accumulation of damage as it is directly exposed to free radicals leaking from the respiratory chain. Moreover, mtDNA is not protected by histones and the mitochondria resident repair mechanisms are less sophisticated, compared to the repair systems operating within the nucleous (94). Importantly, mtDNA regulates the expression of proteins involved in mitochondria assembly and function. As a result, “aged” mitochondria, with increased load of mtDNA mutations, function sub-optimally and release more free radicals (95, 96). Neurodegeneration associated with age-related disorders, such as Parkinson’s and Alzheimer’s disease, is connected to an increase in ROS due to accumulation of defective mitochondria within post-mitotic neurons (97). Mitochondrial dysfunction is also observed in metabolic syndrome, a pathology associated with insulin resistance and eventually development of diabetes and cardiovascular disorders (98). Additionally, age-associated dysfunctions in the cardiovascular system have been connected to increased mtDNA mutations and increased ROS leakage (98-100).

Cells can mount certain protective responses to mitochondrial dysfunction and thus mitochondria-induced ROS accumulation. One cellular strategy is mitophagy, an autophagic process (see below) that results in the specific removal of dysfunctional mitochondria (101, 102). Whereas mitophagy is still not fully understood, certain mediators of the process have been identified. An important mediator of mitophagy is the PTEN-induced putative kinase-1 (PINK1), which regulates the accumulation of the protein parkin in damaged mitochondria, thereby regulating mitophagy initiation (103, 104). Importantly, when dysfunctional mitochondria are not efficiently cleared and the intracellular free radical load increases significantly, cell death is induced (93).

FOXO proteins are important regulators of oxidative stress resistance and FOXO TKO mice were reported to show mitochondria dysfunction (105). FOXOs regulate the expression of free radical detoxifying enzymes, including the mitochondrial resident MnSOD, thereby regulating mtDNA integrity and ultimately mitochondria

function. FOXOs were recently reported in two independent studies to be activated in hypoxic tissues and antagonize c-Myc function (106, 107). As a result, at least in the setting of hypoxia, FOXOs contribute to the maintenance of low mitochondria number and the reduction of intracellular ROS by inhibiting transcription of mitochondrial genes. Moreover, FOXOs were reported to regulate the transcription of PINK1, suggesting a possible involvement of FOXOs in clearance of damaged mitochondria (108). Importantly, transcription of PINK1 by FOXO was found associated with protection from apoptosis, possibly by inducing mitophagy. Furthermore recently in *Drosophila*, dFOXO was shown to rescue the mitochondria dysfunction stemming from PINK deletion, suggesting PINK-independent functions as well (109). In fact, FOXOs were reported to regulate the expression of CITED2 (110) that in turn regulates the levels of Bmi1 (111). Interestingly, mice deficient for Bmi1 show increased stress characterized by the accumulation of dysfunctional mitochondria and increased $p16^{INK4}$ expression (112), an increase also observed in FOXO TKO mice (36).

Considering the role of dysfunctional mitochondria in aging related pathologies, it is conceivable that other aging associated components and signalling pathways will also be involved in mitochondria integrity. Indeed, impaired mitophagy was recently reported in mice deficient for the ATM kinase (113). In fact, it was proposed that this deficiency causes the increased free radical accumulation in A-T patients' tissues and could account for cerebellar degeneration, a common A-T symptom (70).

Autophagy in aging; functions for FOXOs and ATM in regulating autophagy

Autophagy is an evolutionary conserved process involving the controlled degradation of cytoplasmic contents, through the lysosomic pathway. More specifically, it involves the engulfment of parts of the cytoplasm in double membraned vesicles termed "autophagosomes" and subsequent fusion of these vesicles with lysosomes (for more detailed review see (114)). Eventually, the macromolecules are dismantled to smaller "building blocks" that become available for new cellular anabolic processes. Autophagy is the main mechanism for the turnover of organelles (e.g. mitophagy, see above) and it is activated in cases of nutrient starvation or in stress conditions. More specifically, oxidative stress, as mentioned, induces irreversible modifications in macromolecules (e.g. proteins), as well as whole organelles (e.g. mitochondria) affecting their function. Autophagic removal of these defective molecules/ organelles maintains cellular homeostasis, whilst facilitating the production of new, undamaged molecules.

Autophagy is a process with important implications in aging. It has been observed that expression of central autophagy components, as well as autophagy efficiency *in toto* is impaired with aging (114). The importance of autophagy in the aging process in multicellular organisms was primarily demonstrated by studies in *C. elegans*, where deletion of genes important for autophagy results in dauer defects and reduced lifespan (115-117). Most importantly, the lifespan extension observed in nematodes due to reduced insulin/IGF-1 signalling or by dietary restriction is not only dependent on DAF-16, the FOXO homologue in *C. elegans*, but also on functional autophagy (116). Moreover, in *Drosophila melanogaster* defective autophagy is associated with shortened lifespan (118) and in mice tissue specific knockdown of certain autophagic genes results in aging-associated phenotypes (114). Defective autophagy is also associated with age-associated disorders in humans; the main aetiology of Alzheimer's disease is the accumulation of protein aggregates (beta amyloid plaques and tau protein tangles) and the subsequent neuronal death, whereas accumulation of protein aggregates in neuronal cells is also observed in Parkinson's and Huntington's disease (119-121).

FOXO proteins are involved in autophagy, both by regulating certain transcriptional programs, as well as mediating transcription independent functions. FOXOs were reported to regulate the transcription of

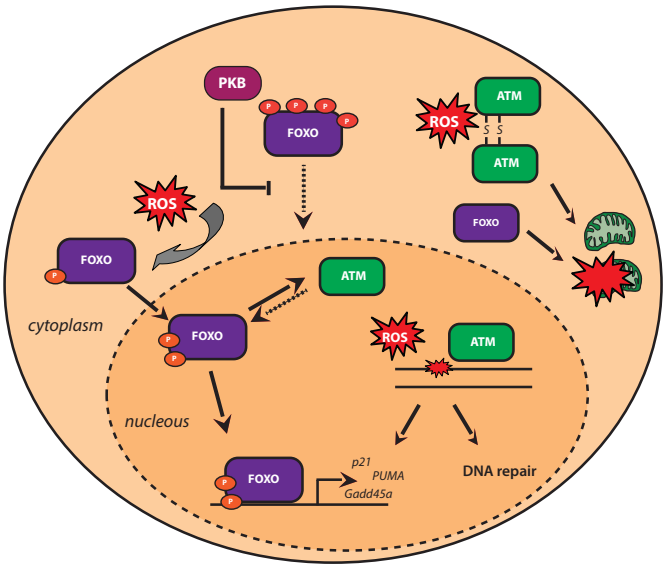

Figure 5. FOXOs and ATM in the responses to increased intracellular ROS. Under normal growth conditions FOXOs are kept cytoplasmic due to inhibitory phosphorylation. ATM is predominantly located in the nucleus and, to a lesser extent, the cytoplasm. Both FOXOs and ATM in the cytosol regulate autophagy and mitophagy in response to increased free radical or nutrient deprivation. In cases of increased oxidative stress the nuclear exclusion of FOXOs is alleviated. Once in the nucleus FOXOs activate transcription of genes for ROS detoxification, cell cycle arrest and DNA repair. Also, by the interaction of FOXO3 with ATM, ATM is activated to mediate the regulation of the DNA damage response towards DNA repair and growth arrest. At the same time ATM regulates FOXOs by phosphorylating them, although the functional relevance of this phosphorylation is not known. Increased redox potential leads to the formation of disulfide bridges between two ATM molecules. These homodimers are active kinases phosphorylating substrates and modulating ROS load.

important autophagy regulators, including Atg8/LC3b, Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3 (Bnip3), GABA(A) receptor-associated protein like 1 (Gabarapl1), Beclin, and Atg12 (reviewed in (122)). In agreement with this, in neuronal cells, JNK deficiency was shown to activate FOXO transcriptional activity towards *Bnip3*, *Atg8/LC3b* and *Atg12*, thereby inducing autophagy and neuronal cell survival (123). Autophagy induction has been linked however to both cytoprotection and cytotoxicity. The latter is better illustrated in a recent report by Zhao et al, in which cytosolic FOXO1 was shown to mediate autophagy, independently of its ability to regulate transcription (124). In particular, cytoplasmic FOXO1 was shown to be acetylated in response to oxidative stress or nutrient deprivation and subsequently interacting with Atg7, an autophagy mediator, thereby regulating autophagy and cell death of neoplastic cells.

In agreement with the reported function of ATM in mitophagy, ATM is also associated with autophagy regulation. In response to oxidative stress, cytosolic resident ATM was shown to activate AMP-dependent kinase (AMPK), thereby activating tuberous sclerosis protein 2 (TSC2) (125). TSC2 is found in complex with TSC1 protein, regulating the function of the target-of-rapamycin kinase (TOR), via the inactivation of the small GTPase Rheb (126). TOR is an inhibitor of autophagy (114); therefore ATM activity, via AMPK, results in inhibition of TOR and subsequent enhancement of autophagy, in response to oxidative stress.

Overall, apart from their association in response to DNA damage, FOXOs and ATM appear to regulate similar functions in protecting cells from diverse stress insults. It is therefore important that future research addresses whether these proteins function in distinct or the same signalling pathways in order to confer

protection against ROS **(Figure 5)**.

Aging and cell regeneration

Aging, as previously mentioned, can be considered the result of disturbed homeostasis, which in part can be due to a reduced capacity to replenish damaged and dysfunctional cells. Tissue regeneration is achieved by tissue specific adult stem cells (SCs), which are resident in most tissues and are characterized by their unique abilities to self-renew, in order to replenish the SC pool and to differentiate to other types of cells (multipotency). Considering the importance of the SCs for the lifetime maintenance of tissue homeostasis, it is conceivable that these cells should be equipped with very strictly controlled mechanisms for their protection against exogenous and endogenous insults.

Free radicals and DNA damage responses in SCs

Recent studies on the intestinal SCs characterized by LGR5 expression have challenged the concept that all adult SCs are maintained in a slow cycling or quiescent state for most of their lifetime (127). To reconcile most of the experimental data, a model has been put forward proposing that for most tissues basically two adult stem cell types exist (128); one is maintained in quiescent state and contributes to the maintenance of the SC pool while the other type comprises the highly proliferative SCs whose function contributes to cell replenishment and tissue regeneration.

Quiescence is a state of reversible growth arrest also referred to as G0; cells can accumulate in the G0 phase of the cell cycle following an arrest in G1; however upon mitogenic stimuli they can resume cycling. Contrary to cycling cells, which have to devote the majority of their metabolism towards synthesis of cellular constituents (DNA, proteins and lipids) in order to replicate, quiescent cells lack this demand and thus can decrease and/or divert their metabolic rates towards different requirements. Important in the context of aging is the accompanying ability of quiescent cells to shift metabolism towards increased stress resistance. In agreement, numerous studies have reported that quiescent cells are reduced in size, show lower rates of nucleotide synthesis, increased stress resistance, increased autophagy to recycle cellular components, and increased glycolytic flux and hence reduced oxidative phosphorylation. However, the latter paradigm stems largely from studies using lymphocytes and it may not be in fact a general hallmark of quiescence, since a recent report showed a similar glycolytic flux in quiescent as well as proliferating human primary fibroblasts (129). Irrespectively, quiescent SCs are considered to be less metabolically active and experience less mitochondrial respiration and thereby ROS accumulation (130). Moreover, by remaining in the G0 phase of the cycle, SCs are not subjected to erroneous DNA replication. At the same time, antioxidant defences are enhanced in SCs, thereby minimizing the risk of ROS-induced damage. The requirement for ROS modulation in the establishment of quiescence is further illustrated by the fact that fibroblasts from MnSOD-/- mice exhibit increased superoxide steady state levels and failure to exit the proliferative cycle (131). Therefore, in general quiescent SCs are expected to be less exposed to genotoxic insults and accumulate less DNA damage. Nevertheless, DNA damage was found to accumulate in SCs with age, as measured by the accrual of γH2AX foci, a marker of DNA damage response, suggesting lack of mechanisms for faithful DNA repair in these cells (65, 132). Importantly, SCs are largely dependent on their microenvironment for their maintenance and fate determination. Excreted factors, including cytokines, from cells comprising the SC niche were shown to induce free radical accumulation and DNA damage in hematopoetic SCs (HSCs), leading to SC transformation or dysfunction (133).

DNA repair is significantly impaired in quiescent SCs, in which cell cycle checkpoints as well as certain types of repair are inactive (134, 135). Importantly, in case that quiescent SCs exposed to genotoxic stress re-enter the cycle, they will first proceed to the G1 phase. As previously mentioned, in G1 DSBs are repaired by the error-prone NHEJ, resulting in the accumulation of mutations or genomic insertions, deletions and translocations that can be subsequently transmitted to the daughter cells. Indeed, it has been experimentally demonstrated that the quiescent state and the prevalence of NHEJ as the major DNA repair mechanism, are associated with DNA damage accumulation in HSCs during aging (136, 137). The high fidelity HR is largely inhibited in SCs and only in the case that the cells were mobilized prior to damage infliction, they were found to preferentially employ HR for DNA repair (136, 138). Therefore quiescence for the SCs can be considered as a "double-edged sword", keeping damaging insults to the minimum on the one hand, while elevating genomic instability due to inefficient repair on the other.

Accumulated genomic instability in SCs elicits certain responses depending on the nature and extend of the damage. The majority of the cycling SCs (for instance intestinal SCs) as well as quiescent SCs re-entering cell cycle after their exposure to genotoxic insults, are eliminated by apoptosis (135). Another response to accumulated genomic instability in SCs is the induction of cellular senescence (135). The tumour suppressor p16INK4 is strongly associated with senescence induction in transformed cells. Expression of the INK4a/ARF locus (encoding for $p16^{INK4}$ and ARF) is repressed early in life, mainly by function of the Polycomb repressor complex; however the repression is alleviated during aging (139). Importantly, overexpression of $p16^{INK4a}$ has been experimentally linked to functional decline of SCs, whereas $p16^{INK4a}$-deficient mice show improved SC function with age, compared to their wild type littermates (140). Interestingly, $p16^{INK4}$ was suggested to drive apoptosis with stress in SCs. Genomic damage accrual in SCs is further associated with increased SC differentiation. An elegant study by Wang et al, suggests that DNA damage in HSCs results in the preferential differentiation of HSCs to the myeloid lineage instead of the lymphoid (141), a skewing widely observed in aged HSCs as well. This skewed differentiation towards the lymphoid lineage is associated with the aging-associated decline in the adaptive immune system. Importantly, whereas the gradual removal of dysfunctional SCs is a potent tumour suppressor mechanism, it eventually results in the exhaustion of SC reserves. Ultimately, the reduced capacity of SCs for tissue regeneration upon injury and the tissue atrophy- both hallmarks of aging- are stemming from SC exhaustion.

Longevity factors in SC maintenance

The importance of SCs in the long-term maintenance of adult tissues and subsequently to the aging process is well illustrated by the observation that genetic factors associated with aging have crucial functions in stem cell maintenance. The FOXO transcription factors, as well as DNA repair components have all been shown to regulate SC long-term survival and repopulation capacity.

As argued above, maintenance of a low ROS load is crucial for SC function, since intracellular ROS accrual was shown to result in increased genomic instability and subsequently SCs differentiation (142). Recent studies in the FOXO TKO mice revealed a vital role of FOXOs in HSC maintenance via the regulation of intracellular ROS and cell cycle progression (36, 143). HSCs from the FOXO TKO animals suffered from increased ROS due to impaired expression of several ROS detoxifying enzymes. Importantly, administration of the ROS scavenger N-acetyl-cysteine (NAC) in these animals alleviated several of the stem cell defects stemming from FOXO deficiency. The importance of FOXOs in SCs ROS modulation is further illustrated by observations in PKBa/b deficient mice that display reduced levels of free radicals and increased quiescence (144). Interestingly,

similar phenotypes to FOXO depletion were observed in mice with ATM deletion. More specifically, ATM-/- mice exhibit depletion of their HSC pool and bone marrow failure due to elevated ROS, whereas the HSC defects are relieved with concomitant administration of NAC (145). Interestingly, in both FOXO TKO and ATM-/- mice elevated ROS are associated with increased p38MAPK activity and $p16^{INK4}$ levels. Recent studies support a direct involvement of ATM in regulation of intracellular ROS (146). ATM was described to act as a ROS sensor by the formation of intermolecular disulfide bridges (147), similar to FOXO proteins (31). However, the exact mechanism of ROS regulation by ATM is not clear still, with some reports suggesting this to be via regulation of the pentose phosphate pathway (146, 148). In addition to ATM, FANCD2, another DNA repair protein, was also recently associated with FOXOs and free radical regulation in HSCs. FANCD2, a component of the Fanconi anaemia group of proteins, was found to interact with FOXO3 to regulate the expression of ROS scavenger genes, including MnSOD and catalase (149). Importantly, Fanconi anaemia patients show bone marrow failure as a result of increased intracellular ROS in HSCs and HSC pool depletion (150).

In addition to their function in ROS regulation, FOXOs appear to regulate other processes related to SC maintenance as well. FOXOs are integral factors for the quiescent state of SCs, regulating the expression of $p21^{cip1/waf1}$ and $p27^{kip1}$. Indeed, in FOXO TKO mice, HSCs were depleted due to increased cell cycle mobilization and subsequent differentiation, at the expense of self-renewal (143). Interestingly, HSC differentiation in these mice was skewed towards the myeloid lineage, at the expense *of* the lymphoid lineage, as is observed in aging. Remarkably similar phenotypes were observed in PTEN conditional knock-out mice (151, 152), in which PKB is expected to be overly active and thereby inactivate FOXOs. PTEN deficiency in the HSCs resulted in increased HSC mobilization and eventually SC depletion, while an increased differentiation towards the myeloid lineage was also observed (153, 154). Moreover, similar effects were observed in the neuronal SCs (NSCs) of the FOXO TKO mice. These mice exhibited increased proliferation of NSCs early in life, followed by depletion of the NSC pool later in life, thereby contributing to reduced long-term neurogenesis (155). In another SC compartment, the spermatogonial SCs (SSC), FOXOs appear to regulate SSC self-renewal capacity and differentiation (156), whereas FOXO3 also suppresses ovarian follicle activation, since FOXO3a-/- mice exhibit oocyte exhaustion and infertility (157). ATM, in accordance to its established role in cell cycle regulation, also regulates the cycle status of SCs. In particular, ATM was recently reported to regulate the quiescence status of HSCs, via regulation of BID and intracellular ROS (158) and in ATM-/- mice the SSC population is progressively depleted by increased quiescence exit and cell cycle re-entry (159).

Regulation of SC homeostasis is crucial for their long-term survival and re-population capacity. Eventually, maintenance of SC pools is of utmost importance for tissue regeneration in aging. Longevity factors, including FOXOs and DNA repair components such as ATM, play important roles in SC maintenance. The major impact of these factors in SCs is in the regulation of the intracellular free radical load, thereby contributing to genomic stability in these cells. Moreover, by retaining SCs in quiescence, they contribute to their protection by severe damage insults. Importantly however, whereas the processes they mediate are clear, the regulation of these factors in SCs is not fully elucidated. It would be particularly important to expand our understanding of protein interacting modules as well as post-translational modifications (PTMs) regulating FOXOs function in the SCs, as this holds promise for relevant interventions, and thus will be valuable for regenerative medicine.

FOXO and ATM in diabetes, the "other" age-associated disorder

Diabetes is a condition stemming either from decreased insulin secretion from the pancreatic β-cells, or

decreased insulin perception in peripheral tissues (insulin resistance). As mentioned, reduced PI3K/PKB signalling plays an important role in cancer, but also in diabetes, another typical age-related disease. In agreement with FOXOs being important downstream components of PI3K/PKB, a role for FOXOs has been established in diabetes, both in peripheral insulin resistance as well as in β-cell function (for more elaborate reviews see e.g.(160, 161)). Intriguingly, a role for ATM in diabetes has also been documented.

Patients with ataxia telangiectasia display metabolic abnormalities, such as poor growth, insulin resistance and increased risk of developing diabetes mellitus, which at least in part are due to lack of ATM activity (70). However, how ATM controls metabolic function with respect to glucose handling and insulin signaling remains unclear. Interestingly, oxidative stress appears to be an important determinant for the pathology of both diabetes and metabolic syndrome, a condition with increased risk of developing insulin resistance. As already mentioned, loss of ATM results in increased ROS and, similar to FOXO, free radicals have recently been shown to directly control ATM activity (147). Moreover, combined ATM and apolipoprotein E deficiency results in increased JNK activity and ATM has been proposed to reduce JNK activity (162) thereby relieving JNK feedback inhibition on insulin signaling (163). Furthermore, ATM appears to be activated by insulin and to subsequently contribute to the full activation of PKB, thereby regulating the translocation of the cell surface glucose transporter 4 (GLUT4) in response to insulin (164). Thus, whereas FOXOs are inhibited by insulin signalling, ATM appears activated. This may underlie the different mode of involvement of FOXO versus ATM in peripheral insulin resistance, in which loss of FOXO improves insulin function (160), whereas loss of ATM provokes insulin resistance (70). The role of ATM is further illustrated by the observation that genetic variants of ATM correlate with treatment success of metformin, the most commonly used drug to treat type 2 diabetes (165).

With respect to pancreatic β-cells, ATM and FOXO appear to be both important factors for their survival and functionality. In β-cells FOXO1, the major FOXO member in the pancreas, is continuously kept cytoplasmic, due to the constant production of insulin. However in conditions of increased oxidative stress, such as induced by hyperglycemia, FOXO translocates to the nucleus. Nuclear FOXO1 was shown to protect β-cells and to induce the transcription of NeuroD and MafA, transcription factors that regulate insulin production (166). Importantly however, prolonged FOXO1 activation also induces cell growth attenuation in β-cells of mice with disturbed insulin signalling, leading to β-cell dysfunction. This effect of FOXO is likely through induction of $p27^{kip1}$ and reduction of cyclinD, as it has been shown that $p27^{kip1}$-/- mice are protected from diabetes (167). ATM was also found to be active in β-cells to ensure insulin secretion (168). These functions of ATM described are important determinants for the prevalence of an aging associated disorder such as diabetes and appear independent of its established role in DNA repair. Nonetheless, we can envision additional functions for both FOXO and ATM in ROS modulation and genomic stability in β-cells.

FOXOs balancing cancer and disease - signals from above

Tumour suppressors have been long categorized in two main groups, namely the caretakers and the gatekeepers (169). The caretakers are proteins involved in maintenance of genomic stability, thereby minimizing the potential threats for neoplastic transformation. Typically components of DNA repair pathways and proteins otherwise ensuring survival and genomic stability are categorized in this group. Gatekeepers on the other hand are proteins such as p16INK4, inhibiting oncogenesis by inducing the removal of the transformed cells via senescence and apoptosis. FOXOs are bona fide tumour suppressors however their categorization to one of the above groups appears to be more complicated. Indeed, FOXOs regulate transcriptional programs for

ROS detoxification and DNA repair yet at the same time regulate senescence and apoptosis, typical pro-aging responses. These functions appear to be contradictory when considering the well-established role of FOXOs as positive regulators of lifespan extension. Thus, one may speculate on a differential regulation of cellular fate by FOXOs, depending on the input signals, that determines FOXOs to be pro- or anti-aging. Under low stress conditions ("young" cells), FOXOs promote stress protection and survival thus acting as anti-aging factors; in cases when the ensuing instability is of great threat for tissue functionality ("old" cells) then they promote cell clearance and aging.

But are the signalling inputs indeed tipping the balance in the regulation of these contradictory programs by FOXOs? A differential balance in input signals due to differential regulation of upstream components could affect FOXO post-translational modifications and subsequent association with binding partners, eventually shifting the output effect from stress resistance to cell death. Importantly, intracellular ROS accumulate with age and upstream components regulating FOXO function, including PTEN phosphatase, share redox-sensitive cysteines that oxidize, thereby affecting enzyme functionality with aging. In fact ROS regulation of FOXOs comprises an intricate and complex network of proteins with differing sensitivities and modes of ROS regulation (reviewed in e.g. (170)). To increase complexity, FOXOs are central nodes in ROS signalling being both regulated by, as well as regulators of intracellular ROS load. Eventually, the balance in regulation may be found in the details of redox regulation.

FOXO regulation by stress

FOXOs activity is regulated through a variety of PTMs, such as phosphorylation, methylation, acetylation etc. These PTMs regulate FOXO subcellular localization, protein stability and protein-protein interactions. As for other transcription factors, the variety of PTMs identified for FOXOs led to the suggestion that combinatorial codes of PTMs regulate the function as well as the binding of co-factors under various conditions, towards specific functional outputs (30). Although an attractive hypothesis, there is at present little evidence for the existence of such codes and in this respect it is evident that detailed studies are required to fully understand the role of all PTMs and their interconnections, for FOXO function. Here we will describe PTMs identified for FOXOs in response to oxidative and genotoxic stress, as well as to growth factors and cellular nutrients.

FOXO regulation in response to ROS

Phosphorylation. In mammalian cells oxidative stress activates JNK via the small GTPase Ral and subsequently JNK phosphorylates FOXO4 on multiple residues, resulting in its nuclear accumulation (171). Studies in *C. elegans* and *Drosophila* further demonstrated by genetic means that JNK activates DAF-16 and dFOXO respectively to mediate lifespan changes and stress resistance (172, 173). JNK was also demonstrated to phosphorylate FOXO3a in response to the cytotoxic agent Paclitaxel. However, thus far FOXO1 appears not to be a substrate for JNK, at least *in vitro* (174). Mammalian Sterile 20-like kinase 1 (MST1) is a serine/threonine kinase activated by cellular stress and MST1 was shown to play important role in apoptosis induction by a variety of stresses. Following apoptotic stimulation, MST1 activation regulates a number of downstream targets including JNK/p38, histone H2B and FOXO. MST1 has been shown to phosphorylate FOXO1 *in vitro* on Ser212 (175). In cells MST1 phosphorylation of FOXO has been shown to induce loss of 14-3-3 binding to FOXO and consequent nuclear translocation. Also, whereas MST1 is primarily involved in apoptotic signaling (e.g. neuronal cells (176)) the opposite has been reported as well, where the Mst1-FoxO signaling pathway plays a crucial role in survival, but not apoptosis, of naïve T cells (177). Nemo-like kinase (NLK) (178, 179)

was also demonstrated to phosphorylate FOXO4 in response to oxidative stress and inhibit FOXO-dependent transcription, albeit with an undefined mechanism still.

Acetylation/de-acetylation. In response to oxidative stress FOXOs were found to be acetylated by the histone acetyl-transferases (HATs) p300, CREB-binding protein (CBP) and p300/CBP-associated factor (PCAF) (reviewed in (180)). The result of FOXO acetylation on its transcriptional activity is still debated, as deciphering the FOXO specific effects from the effects of histone acetylation remains tedious (reviewed in (181)). FOXO acetylation is removed by the action of Sir2/Sirt family de-acetylases (182, 183).

Ubiquitination/de-ubiquitination. FOXOs were also found to be regulated by mono- and poly-ubiquitination. In response to oxidative stress the E3 ligase MDM2 was shown to mono-ubiquitinate FOXO4, resulting in its nuclear translocation and transcriptional activation, whereas the de-ubiquitinating enzyme USP7/HAUSP removes these marks (184, 185).

FOXO regulation in response to genotoxic stress

Phosphorylation. Cyclin-dependent kinases (CDKs) are key regulators of cell cycle progression and, through phosphorylation, they control many essential cell cycle components. Both CDK1 and CDK2 have been shown to phosphorylate FOXO1 on Ser249 *in vitro* as well as *in vivo* (186, 187). In proliferating cells, this phosphorylation event was shown to inhibit FOXO1 activity, via its cytoplasmic retention. Importantly, genotoxic stress activates signalling cascades eventually inhibiting the CDKs. Indeed, it was shown that under DNA damage conditions the negative FOXO1 regulation by CDKs is alleviated, resulting in FOXO1 nuclear re-localization. Interestingly, in post-mitotic neurons FOXO1 phosphorylation by CDKs appeared to activate FOXO-dependent transcription (188). Whether this reflects a cell type specific difference or experimental differences is still unclear. FOXO3 was recently reported to be phosphorylated by the MAP kinase MK5 (189). This phosphorylation results in FOXO nuclear translocation and transcriptional activation, towards miR-34, to antagonise overproliferation induced by Myc oncogene. More importantly, previous work from the same group suggested that MK5 regulates the expression of miR-34 in response to genotoxic stress, suggesting DNA damage signalling to regulate FOXO towards different endpoints. ATM together with ATR and DNA-dependent protein kinase (DNA-PK) belong to the PIKKs. ATM, ATR and DNA-PK are activated by genotoxic stress and phosphorylate a sequence motif within proteins defined as Sp/TpQ. A proteome wide analysis of proteins phosphorylated by the PIKKs in response to DNA damage revealed many potential ATM substrates, including FOXO1 (51). Interestingly, all FOXO members possess this Sp/TpQ motif, suggesting this to be a potential common regulatory mechanism. Whether this phosphorylation has a functional relevance in the DNA damage response however remains to be determined.

FOXO regulation by growth factors and nutrients

Phosphorylation. PKB has been shown to phosphorylate three evolutionary conserved residues within FOXO members, which for FOXO1 are Thr24, Ser256 and Ser319. Phosphorylation of proteins by PKB results in 14-3-3 binding, but full binding of 14-3-3 to FOXO requires only the first two PKB phosphorylation sites. The third PKB site is involved as a gatekeeper in further phosphorylation of FOXO by casein kinase 1 (CK1). CK1 phosphorylates Ser322 and Ser325 in FOXO1, following the third PKB site. Together with DYRK1a mediated phosphorylation at Ser329, this generates a patch of negative charges involved in interaction with the nuclear export/import machinery (190). Moreover, phosphorylation of FOXOs by PKB inhibits their DNA binding potential, as the second phosphorylation site (Ser256 in FOXO1) introduces a negative charge within

the DNA binding domain, thereby inducing repulsive forces. Serum and Glucocorticod induced Kinases (SGK) is distantly related to PKB, and its activation is also dependent on the action of PI3K through PDK1. The consensus sequence for SGK phosphorylation is highly similar to that of PKB. Thus SGK can also phosphorylate the PKB sites of FOXOs. However, comparison of FOXO3a phosphorylation by PKB and SGK suggested the third PKB site (Ser315 in FOXO3a) to be preferentially phosphorylated by SGK, whereas the others are preferentially phosphorylated by PKB (191). SGK was also identified as a kinase for the PKB sites of FOXOs. Interestingly, studies in *C. elegans* suggest that depending on the experimental condition either PKB or SGK is the main mediator of DAF-16 dependent lifespan, and biochemical analysis suggests that PKB and SGK are components of the same protein complex (192). This would indicate that PKB and SGK are largely redundant with respect to FOXO regulation. Interestingly Leucine-rich repeat kinase 2 (LRRK2) and cyclic guanosine monophosphate (cGMP)-dependent kinase II (cGKII) have been shown to also phosphorylate the third PKB/SGK site in dFOXO. However, unlike PKB/SGK, both LRRK2 and cGKII activate dFOXO transcriptional activity (193, 194). A mechanism that can possibly unify this differential regulation is at present hard to envision. Following the notion that in human primary tumours FOXO cytosolic localization did not completely correlate with high PKB activity, Hu *et al.* identified IkB-kinase (IKK) as a novel negative regulator of FOXO3a (195). IKK phosphorylates FOXO3a at Ser644 a site not conserved within the other FOXO members. IKK-mediated FOXO3a phosphorylation has been shown to result in poly-ubiquitination and proteosomal degradation of FOXO3a. The MAPkinases p38 (196) and ERK (197) are implicated as well in FOXO regulation. Knowledge on the functional significance of these phosphorylation events is limited; however initial studies suggest that ERK mediated phosphorylation of FOXO3 induces its poly-ubiquitination and proteasomal degradation.

FOXOs are also positively regulated by phosphorylation, independently of oxidative stress. AMPK is a heterotrimeric serine/threonine kinase that performs a central role in cellular energy homeostasis. Under low energy conditions AMPK activation controls cell growth and energy expenditure by phosphorylating a number of substrates, including FOXOs. *In vitro* phosphorylation of FOXO3a by AMPK identified 6 potential AMPK phosphor-acceptor sites. Subsequent analysis of a FOXO3a mutant lacking all identified AMPK sites suggested that AMPK phosphorylation channels FOXO activity towards activation of alternative energy sources and stress resistance (198). In agreement, it was found that a similar pathway operating in *C. elegans* confers lifespan extension when using an appropriate regimen for caloric restriction (199). Thus, regulation of FOXO by AMPK appears an evolutionary conserved mechanism for homeostasis of energy metabolism. In addition, this indicates FOXOs to be a nodal point for cross-talk between the AMPK pathway and the insulin-PI3K-PKB pathway.

GlcNAcylation. O-linked β-N-acetylglucosamine (GlcNAc) addition, similar to phosphorylation, occurs on serine and threonine residues of proteins and this notion led to the hypothesis that O-GlcNAc modifications could directly oppose phosphorylation. FOXOs are also GlcNAcylated and this correlates with FOXO activation (200, 201). O-GlcNAc modified residues were identified by mass-spectrometry, but did not correspond to any of the known FOXO phosphorylation sites. Therefore the mechanism of activation remains to be identified.

Methylation. FOXOs are targeted for arginine methylation by the protein arginine methyltransferase-1 (PRMT1) (202). Methylated residues identified in FOXO1 are Arg248 and Arg250, which are part of the consensus PKB phosphorylation site for Ser256 phosphorylation. Methylation of these residues has been shown to inhibit PKB-mediated FOXO1 phosphorylation. In this way, PRMT1-mediated FOXO methylation appears to induce protein stabilization, nuclear localization and transcriptional activation.

Ubiquitination. The E3 ligase MDM2 was also shown to induce the poly-ubiquitination of FOXOs. Mdm2-dependent poly-ubiquitination was reported in response to ERK phosphorylation (203). In response to PKB

phosphorylation, the Skp2 E3 ligase regulates FOXOs' poly-ubiquitination and degradation (204), whereas IKK phosphorylation of FOXO3 results in the formation of a βTrCP "degron" and the subsequent FOXO3 poly-ubiquitination (205). Clearly, detailed analysis has to reveal how E3 regulation is interconnected and/or whether different E3 ligases partake in mediating a specific signal towards FOXOs.

Concluding remarks

Aging includes a multitude of processes, both in cellular and organismal level, that interconnect and influence each other in a complex manner. The accumulation of damaged molecules and dysfunctional organelles due to endogenous and exogenous stresses appears to drive the aging process by disrupting the

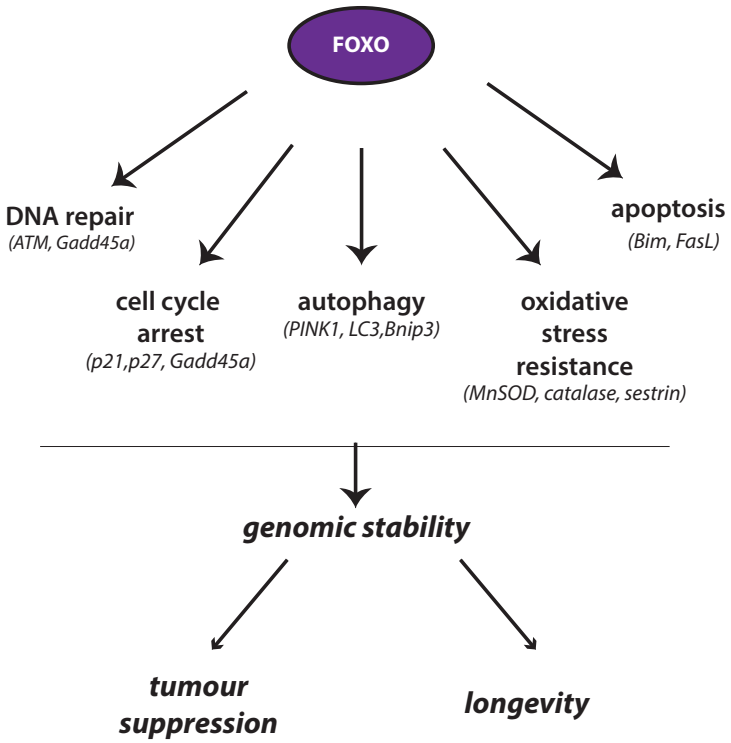

Figure 6. FOXO functions associated with genomic stability. FOXOs regulate a variety of responses in cellular stress conditions, including autophagy, ROS detoxification and DNA repair, as well as growth arrest and apoptosis. All these responses ensure genomic stability, a prerequisite for cancer prevention and healthy aging.

cellular homeostasis, however aging *in toto* is challenging to fully comprehend. Nonetheless, certain factors that are known to influence aging and related disorders can provide useful answers, both on the molecular determinants of the process itself, as well as on potential interventions to ensure healthy lifespan.

FOXOs are proteins with evolutionary conserved roles in lifespan determination and age-related disorders, including cancer and diabetes. The functions that FOXOs exert appear to be rather contradictory, in respect to their end effect on the aging process. On one hand, by transcriptional regulation of senescence and

apoptosis, FOXOs can be seen as pro-aging factors; on the other hand, by regulating stress resistance, they function as anti-aging factors. This apparent discrepancy however can be better understood, if seen under the prism of genomic stability. Indeed, all the processes regulated by FOXOs eventually serve in maintaining cellular genomic stability and in turn, genomic stability ensures both increased longevity and tumour free survival **(Figure 6)**. Important in this respect is also the notion that reducing oxidative stress by FOXOs not only adds in preventing genetic damage and consequent disease, but also reduces non-genetic contributions to disease, such as the contribution of redox misbalance to protein-folding diseases and diabetes. In fact, the emerging close association between FOXOs and ATM may suggest that FOXOs should have more direct implications in maintaining genomic stability. Eventually, progress on understanding FOXO regulation in the context of individual cell fates sets a basis for our understanding of FOXO regulation in the context of aging, which is the ultimate challenge.

Scope of the thesis

FOXO transcription factors are emerging as important homeostatic regulators. In **Chapter 1** we provided a comprehensive review on the mechanisms by which FOXOs maintain cellular homeostasis in response to genotoxic and oxidative stress. We further discussed the FOXO contribution to proteostasis by autophagy and to metabolic adaptations to nutrient availability. In this thesis, we aimed to broaden the picture of FOXOs involvement in homeostasis and we chose to focus on genomic and metabolic homeostasis.

In **Chapter 2** we demonstrate a novel role of FOXOs in maintaining DNA homeostasis. We show that FOXOs mediate the error-free repair of DNA double-strand breaks through a mechanism that "tips the balance" of the repair pathway choice towards homologous recombination. In **Addendum** we describe a global gene expression analysis in HeLa cells after acute FOXO depletion. We identify genes whose expression depends on endogenous FOXO and suggest the potential involvement of FOXOs in novel processes, including RNA metabolism and transmembrane transport, via their identified target genes. The analysis further identifies the isocitrated dehydrogenase 1 (IDH1) and several mitochondria-associated factors, as novel FOXO transcriptional targets. In **Chapter 3** we describe the contribution of FOXO in the metabolic crosstalk between cytosol and mitochondria by the regulation of IDH1 levels and further demonstrate IDH1-depedent FOXO functions in tumor suppression and tumor promotion. In **Chapter 4** we report on the multilayered mechanisms by which FOXOs contribute to mitochondrial homeostasis.

The findings described in this thesis provide novel understanding on the multifaceted roles of FOXOs in tumor suppression and tumor promotion. Our findings are discussed in **Chapter 5** in the context of cellular homeostasis.

References

1. Harman D (1956) Aging: A Theory Based on Free Radical and Radiation Chemistry. *Journal of Gerontology* 11(3):298-300.
2. Dworakowski R, Anilkumar N, Zhang M, & Shah AM (2006) Redox signalling involving NADPH oxidase-derived reactive oxygen species. *Biochem Soc Trans* 34(Pt 5):960-964.
3. Hulbert AJ, Pamplona R, Buffenstein R, & Buttemer WA (2007) Life and Death: Metabolic Rate, Membrane Composition, and Life Span of Animals. *Physiological Reviews* 87(4):1175-1213.
4. Elchuri S*, et al.* (2004) CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life. *Oncogene* 24(3):367-380.
5. Muller FL, Lustgarten MS, Jang Y, Richardson A, & Van Remmen H (2007) Trends in oxidative aging theories. *Free Radical Biology and Medicine* 43(4):477-503.
6. Schriner SE*, et al.* (2005) Extension of Murine Life Span by Overexpression of Catalase Targeted to Mitochondria. *Science* 308(5730):1909-1911.
7. Li Y*, et al.* (1995) Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet* 11(4):376-381.
8. Lebovitz RM*, et al.* (1996) Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. *Proceedings of the National Academy of Sciences* 93(18):9782-9787.
9. Van Raamsdonk JM & Hekimi S (2009) Deletion of the Mitochondrial Superoxide Dismutase *sod-2* Extends Lifespan in *Caenorhabditis elegans*. *PLoS Genet* 5(2):e1000361.
10. Cabreiro F*, et al.* (2011) Increased life span from overexpression of superoxide dismutase in Caenorhabditis elegans is not caused by decreased oxidative damage. *Free Radical Biology and Medicine* 51(8):1575-1582.
11. Van Raamsdonk JM & Hekimi S (2012) Superoxide dismutase is dispensable for normal animal lifespan. *Proceedings of the National Academy of Sciences*.
12. Sedelnikova OA*, et al.* (2010) Role of oxidatively induced DNA lesions in human pathogenesis. *Mutation Research/ Reviews in Mutation Research* 704(1â€“3):152-159.
13. Hoeijmakers JHJ (2009) DNA Damage, Aging, and Cancer. *New England Journal of Medicine* 361(15):1475-1485.
14. Ciccia A & Elledge SJ (2010) The DNA Damage Response: Making It Safe to Play with Knives. *Molecular Cell* 40(2):179-204.
15. Barzilai A & Yamamoto KI (2004) DNA damage responses to oxidative stress. *DNA Repair* 3(8-9):1109-1115.
16. Hamilton ML*, et al.* (2001) Does oxidative damage to DNA increase with age? *Proceedings of the National Academy of Sciences* 98(18):10469-10474.
17. Freitas AA & de Magalhaes JP (2011) A review and appraisal of the DNA damage theory of aging. *Mutation Research/ Reviews in Mutation Research* 728(1â€“2):12-22.
18. Trifunovic A*, et al.* (2004) Premature aging in mice expressing defective mitochondrial DNA polymerase. *Nature* 429(6990):417-423.
19. de Magalhaes JP (2005) Open-minded scepticism: inferring the causal mechanisms of human aging from genetic perturbations. *Aging Research Reviews* 4(1):1-22.
20. Kops GJPL*, et al.* (1999) Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature* 398(6728):630-634.
21. Brunet A*, et al.* (1999) Akt Promotes Cell Survival by Phosphorylating and Inhibiting a Forkhead Transcription Factor. *Cell* 96(6):857-868.
22. Liu P, Cheng H, Roberts TM, & Zhao JJ (2009) Targeting the phosphoinositide 3-kinase pathway in cancer. *Nat Rev Drug Discov* 8(8):627-644.
23. Taniguchi CM, Emanuelli B, & Kahn CR (2006) Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* 7(2):85-96.
24. George S*, et al.* (2004) A family with severe insulin resistance and diabetes due to a mutation in AKT2. *Science* 304(5675):1325-1328.
25. Kenyon C, Chang J, Gensch E, Rudner A, & Tabtiang R (1993) A C. elegans mutant that lives twice as long as wild type. *Nature* 366(6454):461-464.
26. Giannakou ME*, et al.* (2007) Dynamics of the action of dFOXO on adult mortality in Drosophila. *Aging Cell* 6(4):429-438.
27. Flachsbart F*, et al.* (2009) Association of FOXO3A variation with human longevity confirmed in German centenarians. *Proceedings of the National Academy of Sciences* 106(8):2700-2705.
28. Kuningas M*, et al.* (2007) Haplotypes in the human Foxo1a and Foxo3a genes; impact on disease and mortality at old age. *Eur J Hum Genet* 15(3):294-301.
29. Willcox BJ*, et al.* (2008) FOXO3A genotype is strongly associated with human longevity. *Proceedings of the National Academy of Sciences* 105(37):13987-13992.
30. Calnan DR & Brunet A (2008) The FoxO code. *Oncogene* 27(16):2276-2288.
31. Dansen TB*, et al.* (2009) Redox-sensitive cysteines bridge p300/CBP-mediated acetylation and FoxO4 activity. *Nat Chem Biol* 5(9):664-672.
32. Chiribau CB*, et al.* (2008) FOXO3A Regulates Peroxiredoxin III Expression in Human Cardiac Fibroblasts. *J. Biol. Chem.* 283(13):8211-8217.
33. Kops GJPL*, et al.* (2002) Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. *Nature*

419(6904):316-321.
34.Nemoto S & Finkel T (2002) Redox Regulation of Forkhead Proteins Through a p66shc-Dependent Signaling Pathway. *Science* 295(5564):2450-2452.
35.Chen C-C, *et al.* (2010) FoxOs Inhibit mTORC1 and Activate Akt by Inducing the Expression of Sestrin3 and Rictor. *Developmental Cell* 18(4):592-604.
36.Tothova Z, *et al.* (2007) FoxOs Are Critical Mediators of Hematopoietic Stem Cell Resistance to Physiologic Oxidative Stress. *Cell* 128(2):325-339.
37.Paik J-H, *et al.* (2007) FoxOs Are Lineage-Restricted Redundant Tumor Suppressors and Regulate Endothelial Cell Homeostasis. *Cell* 128(2):309-323.
38.Tran H, *et al.* (2002) DNA Repair Pathway Stimulated by the Forkhead Transcription Factor FOXO3a Through the Gadd45 Protein. *Science* 296(5567):530-534.
39.Wang XW, *et al.* (1999) GADD45 induction of a G2/M cell cycle checkpoint. *Proceedings of the National Academy of Sciences* 96(7):3706-3711.
40.Reinhardt HC, *et al.* (2010) DNA Damage Activates a Spatially Distinct Late Cytoplasmic Cell-Cycle Checkpoint Network Controlled by MK2-Mediated RNA Stabilization. *Molecular Cell* 40(1):34-49.
41.Jung HJ, *et al.* (2007) Base excision DNA repair defect in Gadd45a-deficient cells. *Oncogene* 26(54):7517-7525.
42.Smith ML, *et al.* (2000) p53-Mediated DNA Repair Responses to UV Radiation: Studies of Mouse Cells Lacking p53, p21, and/orgadd45 Genes. *Molecular and Cellular Biology* 20(10):3705-3714.
43.Smith ML, *et al.* (1996) Antisense GADD45 expression results in decreased DNA repair and sensitizes cells to u.v.-irradiation or cisplatin. *Oncogene* 13(10):2255-2263.
44.Barreto G, *et al.* (2007) Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation. *Nature* 445(7128):671-675.
45.Le May N, Egly JM, & Coin F (2010) True lies: the double life of the nucleotide excision repair factors in transcription and DNA repair. *J Nucleic Acids* 2010.
46.Dedes KJ, *et al.* (2010) PTEN Deficiency in Endometrioid Endometrial Adenocarcinomas Predicts Sensitivity to PARP Inhibitors. *Science Translational Medicine* 2(53):53ra75.
47.Plo I, *et al.* (2008) AKT1 Inhibits Homologous Recombination by Inducing Cytoplasmic Retention of BRCA1 and RAD51. *Cancer Research* 68(22):9404-9412.
48.Xu N, *et al.* (2010) Akt/PKB suppresses DNA damage processing and checkpoint activation in late G2. *The Journal of Cell Biology* 190(3):297-305.
49.Mendes-Pereira AM, *et al.* (2009) Synthetic lethal targeting of PTEN mutant cells with PARP inhibitors. *EMBO Molecular Medicine* 1(6-7):315-322.
50.Tsai W-B, Chung YM, Takahashi Y, Xu Z, & Hu MCT (2008) Functional interaction between FOXO3a and ATM regulates DNA damage response. *Nat Cell Biol* 10(4):460-467.
51.Matsuoka S, *et al.* (2007) ATM and ATR Substrate Analysis Reveals Extensive Protein Networks Responsive to DNA Damage. *Science* 316(5828):1160-1166.
52.Yalcin S, *et al.* (2008) Foxo3 Is Essential for the Regulation of Ataxia Telangiectasia Mutated and Oxidative Stress-mediated Homeostasis of Hematopoietic Stem Cells. *Journal of Biological Chemistry* 283(37):25692-25705.
53.Medema RH, Kops GJPL, Bos JL, & Burgering BMT (2000) AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature* 404(6779):782-787.
54.Lei H & Quelle FW (2009) FOXO Transcription Factors Enforce Cell Cycle Checkpoints and Promote Survival of Hematopoietic Cells after DNA Damage. *Molecular Cancer Research* 7(8):1294-1303.
55.Klagge A, *et al.* (2011) The role of FOXO3 in DNA damage response in thyrocytes. *Endocrine-Related Cancer* 18(5):555-564.
56.de Keizer PLJ, *et al.* (2010) Activation of Forkhead Box O Transcription Factors by Oncogenic BRAF Promotes p21cip1-Dependent Senescence. *Cancer Research* 70(21):8526-8536.
57.Luo X, Puig O, Hyun J, Bohmann D, & Jasper H (2007) Foxo and Fos regulate the decision between cell death and survival in response to UV irradiation. *EMBO J* 26(2):380-390.
58.You H, *et al.* (2006) FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. *The Journal of Experimental Medicine* 203(7):1657-1663.
59.Gomes AR, Brosens JJ, & Lam EWF (2008) Resist or die: FOXO transcription factors determine the cellular response to chemotherapy. *Cell Cycle* 7(20):3133-3136.
60.Shiota M, *et al.* (2010) Foxo3a expression and acetylation regulate cancer cell growth and sensitivity to cisplatin. *Cancer Science* 101(5):1177-1185.
61.Sunters A, *et al.* (2003) FoxO3a Transcriptional Regulation of Bim Controls Apoptosis in Paclitaxel-treated Breast Cancer Cell Lines. *Journal of Biological Chemistry* 278(50):49795-49805.
62.Dudgeon C, *et al.* (2010) PUMA Induction by FoxO3a Mediates the Anticancer Activities of the Broad-Range Kinase Inhibitor UCN-01. *Molecular Cancer Therapeutics* 9(11):2893-2902.
63.Wang C, *et al.* (2009) DNA damage response and cellular senescence in tissues of aging mice. *Aging Cell* 8(3):311-323.
64.Sedelnikova OA, *et al.* (2004) Senescing human cells and aging mice accumulate DNA lesions with unrepairable double-strand breaks. *Nat Cell Biol* 6(2):168-170.
65.Rube CE, *et al.* (2011) Accumulation of DNA Damage in Hematopoietic Stem and Progenitor Cells during Human Aging. *PLoS ONE* 6(3):e17487.

66.Davalos A, Coppe J-P, Campisi J, & Desprez P-Y (2010) Senescent cells as a source of inflammatory factors for tumor progression. *Cancer and Metastasis Reviews* 29(2):273-283.
67.Burton D (2009) Cellular senescence, aging and disease. *AGE* 31(1):1-9.
68.Baker DJ*, et al.* (2011) Clearance of p16Ink4a-positive senescent cells delays aging-associated disorders. *Nature* 479(7372):232-236.
69.Shiloh Y (2003) ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer* 3(3):155-168.
70.Shiloh Y (1997) ATAXIA-TELANGIECTASIA AND THE NIJMEGEN BREAKAGE SYNDROME:Related Disorders But Genes Apart. *Annual Review of Genetics* 31(1):635-662.
71.Bartek J, Bartkova J, & Lukas J (2007) DNA damage signalling guards against activated oncogenes and tumour progression. *Oncogene* 26(56):7773-7779.
72.Halazonetis TD, Gorgoulis VG, & Bartek J (2008) An Oncogene-Induced DNA Damage Model for Cancer Development. *Science* 319(5868):1352-1355.
73.Dansen TB & Burgering BMT (2008) Unravelling the tumor-suppressive functions of FOXO proteins. *Trends in Cell Biology* 18(9):421-429.
74.Greer EL & Brunet A (2005) FOXO transcription factors at the interface between longevity and tumor suppression. *Oncogene* 24(50):7410-7425.
75.Garinis GA*, et al.* (2009) Persistent transcription-blocking DNA lesions trigger somatic growth attenuation associated with longevity. *Nat Cell Biol* 11(5):604-615.
76.Charlet-Berguerand N*, et al.* (2006) RNA polymerase II bypass of oxidative DNA damage is regulated by transcription elongation factors. *EMBO J* 25(23):5481-5491.
77.Schumacher Br, Hoeijmakers JH, & Garinis GA (2009) Sealing the gap between nuclear DNA damage and longevity. *Molecular and Cellular Endocrinology* 299(1):112-117.
78.Niedernhofer LJ*, et al.* (2006) A new progeroid syndrome reveals that genotoxic stress suppresses the somatotroph axis. *Nature* 444(7122):1038-1043.
79.Andressoo J-O*, et al.* (2009) An Xpb Mouse Model for Combined Xeroderma Pigmentosum and Cockayne Syndrome Reveals Progeroid Features upon Further Attenuation of DNA Repair. *Molecular and Cellular Biology* 29(5):1276-1290.
80.van der Pluijm I*, et al.* (2006) Impaired Genome Maintenance Suppresses the Growth Hormone-Insulin-Like Growth Factor 1 Axis in Mice with Cockayne Syndrome. *PLoS Biol* 5(1):e2.
81.Bartke A & Brown-Borg H (2004) Life Extension in the Dwarf Mouse. *Current Topics in Developmental Biology*, (Academic Press), Vol Volume 63, pp 189-225.
82.Holzenberger M*, et al.* (2003) IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature* 421(6919):182-187.
83.Palm W & de Lange T (2008) How Shelterin Protects Mammalian Telomeres. *Annual Review of Genetics* 42(1):301-334.
84.Cech TR (2004) Beginning to Understand the End of the Chromosome. *Cell* 116(2):273-279.
85.de Lange T (2005) Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes & Development* 19(18):2100-2110.
86.Linskens MH, Harley CB, West MD, Campisi J, & Hayflick L (1995) Replicative senescence and cell death. *Science* 267(5194):17.
87.Henle ES*, et al.* (1999) Sequence-specific DNA Cleavage by Fe2+-mediated Fenton Reactions Has Possible Biological Implications. *Journal of Biological Chemistry* 274(2):962-971.
88.Gorman S*, et al.* (2009) Radiation and chemotherapy bystander effects induce early genomic instability events: Telomere shortening and bridge formation coupled with mitochondrial dysfunction. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 669(1â€“2):131-138.
89.Passos JoF & von Zglinicki T (2005) Mitochondria, telomeres and cell senescence. *Experimental Gerontology* 40(6):466-472.
90.von Zglinicki T (2002) Oxidative stress shortens telomeres. *Trends in Biochemical Sciences* 27(7):339-344.
91.Richter T & Proctor C (2007) The role of intracellular peroxide levels on the development and maintenance of telomere-dependent senescence. *Experimental Gerontology* 42(11):1043-1052.
92.Serra V, von Zglinicki T, Lorenz M, & Saretzki G (2003) Extracellular Superoxide Dismutase Is a Major Antioxidant in Human Fibroblasts and Slows Telomere Shortening. *Journal of Biological Chemistry* 278(9):6824-6830.
93.Mammucari C & Rizzuto R (2010) Signaling pathways in mitochondrial dysfunction and aging. *Mechanisms of Aging and Development* 131(7â€“8):536-543.
94.Larsen NB, Rasmussen M, & Rasmussen LJ (2005) Nuclear and mitochondrial DNA repair: similar pathways? *Mitochondrion* 5(2):89-108.
95.Short KR*, et al.* (2005) Decline in skeletal muscle mitochondrial function with aging in humans. *Proceedings of the National Academy of Sciences of the United States of America* 102(15):5618-5623.
96.Hagen TM*, et al.* (1997) Mitochondrial decay in hepatocytes from old rats: Membrane potential declines, heterogeneity and oxidants increase. *Proceedings of the National Academy of Sciences of the United States of America* 94(7):3064-3069.
97.Correia SC*, et al.* (2012) Mitochondrial Importance in Alzheimer's, Huntington's and Parkinson's Diseases_ Neurodegenerative Diseases. Advances in Experimental Medicine and Biology, (Springer US), Vol 724, pp 205-221.
98.Mercer JR*, et al.* (2010) DNA Damage Links Mitochondrial Dysfunction to Atherosclerosis and the Metabolic Syndrome / Novelty and Significance. *Circulation Research* 107(8):1021-1031.

99.Tsutsui H, Kinugawa S, & Matsushima S (2011) Oxidative stress and heart failure. *American Journal of Physiology - Heart and Circulatory Physiology* 301(6):H2181-H2190.
100.Wojtovich AP, Nadtochiy SM, Brookes PS, & Nehrke K (2011) Ischemic preconditioning: The role of mitochondria and aging. *Experimental Gerontology* 47(1):1-7.
101.Lee J, Giordano S, & Zhang JH (2012) Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling. *Biochem. J.* 441:523-540.
102.Wang K & Klionsky DJ (2011) Mitochondria removal by autophagy. *Autophagy* 7(3):297-300.
103.Kawajiri S, Saiki S, Sato S, & Hattori N (2011) Genetic mutations and functions of PINK1. *Trends in Pharmacological Sciences* 32(10):573-580.
104.Narendra DP & Youle RJ (2011) Targeting Mitochondrial Dysfunction: Role for PINK1 and Parkin in Mitochondrial Quality Control. *Antioxid. Redox Signal.* 14(10):1929-1938.
105.Sahin E & DePinho RA (2010) Linking functional decline of telomeres, mitochondria and stem cells during aging. *Nature* 464(7288):520-528.
106.Ferber EC*, et al.* (2011) FOXO3a regulates reactive oxygen metabolism by inhibiting mitochondrial gene expression. *Cell Death Differ*.
107.Jensen KS*, et al.* (2011) FoxO3A promotes metabolic adaptation to hypoxia by antagonizing Myc function. *EMBO J* 30(22):4554-4570.
108.Mei Y*, et al.* (2009) FOXO3a-dependent regulation of Pink1 (Park6) mediates survival signaling in response to cytokine deprivation. *Proceedings of the National Academy of Sciences* 106(13):5153-5158.
109.Koh H*, et al.* (2012) Silent Information Regulator 2 (Sir2) and Forkhead Box O (FOXO) Complement Mitochondrial Dysfunction and Dopaminergic Neuron Loss in Drosophila PTEN-induced Kinase 1 (PINK1) Null Mutant. *Journal of Biological Chemistry* 287(16):12750-12758.
110.Bakker WJ, Harris IS, & Mak TW (2007) FOXO3a Is Activated in Response to Hypoxic Stress and Inhibits HIF1-Induced Apoptosis via Regulation of CITED2. *Molecular Cell* 28(6):941-953.
111.Kranc KR*, et al.* (2003) Transcriptional Coactivator Cited2 Induces Bmi1 and Mel18 and Controls Fibroblast Proliferation via Ink4a/ARF. *Molecular and Cellular Biology* 23(21):7658-7666.
112.Liu J*, et al.* (2009) Bmi1 regulates mitochondrial function and the DNA damage response pathway. *Nature* 459(7245):387-392.
113.Valentin-Vega YA*, et al.* (2012) Mitochondrial dysfunction in ataxia-telangiectasia. *Blood* 119(6):1490-1500.
114.Rubinsztein David C, Marino G, & Kroemer G (2011) Autophagy and Aging. *Cell* 146(5):682-695.
115.Hansen M*, et al.* (2008) A Role for Autophagy in the Extension of Lifespan by Dietary Restriction in C. elegans. *PLoS Genet* 4(2):e24.
116.Melendez A*, et al.* (2003) Autophagy genes are essential for dauer development and life-span extension in C. elegans. *Science* 301(5638):1387-1391.
117.Toth ML*, et al.* (2008) Longevity pathways converge on autophagy genes to regulate life span in *Caenorhabditis elegans*. *Autophagy* 4(3):330-338.
118.Simonsen A*, et al.* (2008) Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistance in adult Drosophila. *Autophagy* 4(2):176-184.
119.Cheung ZH & Ip NY (2011) Autophagy deregulation in neurodegenerative diseases – recent advances and future perspectives. *Journal of Neurochemistry* 118(3):317-325.
120.Hara T*, et al.* (2006) Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* 441(7095):885-889.
121.Komatsu M*, et al.* (2006) Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* 441(7095):880-884.
122.van der Vos KE & Coffer PJ (2011) The Extending Network of FOXO Transcriptional Target Genes *Antioxid. Redox Signal.* 14(4):579-592.
123.Xu P, Das M, Reilly J, & Davis RJ (2011) JNK regulates FoxO-dependent autophagy in neurons. *Genes & Development* 25(4):310-322.
124.Zhao Y*, et al.* (2010) Cytosolic FoxO1 is essential for the induction of autophagy and tumour suppressor activity. *Nat Cell Biol* 12(7):665-675.
125.Alexander A*, et al.* (2010) ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS. *Proceedings of the National Academy of Sciences* 107(9):4153-4158.
126.Martin DE & Hall MN (2005) The expanding TOR signaling network. *Current Opinion in Cell Biology* 17(2):158-166.
127.Barker N*, et al.* (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 449(7165):1003-1007.
128.Li L & Clevers H (2010) Coexistence of quiescent and active adult stem cells in mammals. *Science* 327(5965):542-545.
129.Lemons JM*, et al.* (2010) Quiescent fibroblasts exhibit high metabolic activity. *PLoS Biol* 8(10):e1000514.
130.Li L & Bhatia R (2011) Stem Cell Quiescence. *Clinical Cancer Research*.
131.Sarsour EH, Venkataraman S, Kalen AL, Oberley LW, & Goswami PC (2008) Manganese superoxide dismutase activity regulates transitions between quiescent and proliferative growth. *Aging Cell* 7(3):405-417.
132.Yahata T*, et al.* (2011) Accumulation of oxidative DNA damage restricts the self-renewal capacity of human hematopoietic stem cells. *Blood* 118(11):2941-2950.
133.Bonafè M, Storci G, & Franceschi C (2011) Inflamm-aging of the stem cell niche: Breast cancer as a paradigmatic

example. *BioEssays* 34(1):40-49.
134.Mandal PK, Blanpain C, & Rossi DJ (2011) DNA damage response in adult stem cells: pathways and consequences. *Nat Rev Mol Cell Biol* 12(3):198-202.
135.Blanpain C, Mohrin M, Sotiropoulou PA, & Passegué E (2011) DNA-Damage Response in Tissue-Specific and Cancer Stem Cells. *Cell stem cell* 8(1):16-29.
136.Mohrin M*, et al.* (2010) Hematopoietic Stem Cell Quiescence Promotes Error-Prone DNA Repair and Mutagenesis. *Cell stem cell* 7(2):174-185.
137.Rossi DJ*, et al.* (2007) Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature* 447(7145):725-729.
138.Tichy ED*, et al.* (2010) Mouse Embryonic Stem Cells, but Not Somatic Cells, Predominantly Use Homologous Recombination to Repair Double-Strand DNA Breaks. *Stem Cells and Development* 19(11):1699-1711.
139.Bracken AP*, et al.* (2007) The Polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. *Genes & Development* 21(5):525-530.
140.Janzen V*, et al.* (2006) Stem-cell aging modified by the cyclin-dependent kinase inhibitor p16INK4a. *Nature* 443(7110):421-426.
141.Wang J*, et al.* (2012) A Differentiation Checkpoint Limits Hematopoietic Stem Cell Self-Renewal in Response to DNA Damage. *Cell* 148(5):1001-1014.
142.Naka K, Muraguchi T, Hoshii T, & Hirao A (2008) Regulation of reactive oxygen species and genomic stability in hematopoietic stem cells. *Antioxid Redox Signal* 10(11):1883-1894.
143.Tothova Z & Gilliland DG (2007) FoxO Transcription Factors and Stem Cell Homeostasis: Insights from the Hematopoietic System. *Cell stem cell* 1(2):140-152.
144.Juntilla MM*, et al.* (2010) AKT1 and AKT2 maintain hematopoietic stem cell function by regulating reactive oxygen species. *Blood* 115(20):4030-4038.
145.Ito K*, et al.* (2004) Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. *Nature* 431(7011):997-1002.
146.Ditch S & Paull TT (2012) The ATM protein kinase and cellular redox signaling: beyond the DNA damage response. *Trends in Biochemical Sciences* 37(1):15-22.
147.Guo Z, Kozlov S, Lavin MF, Person MD, & Paull TT (2010) ATM Activation by Oxidative Stress. *Science* 330(6003):517-521.
148.Cosentino C, Grieco D, & Costanzo V (2010) ATM activates the pentose phosphate pathway promoting anti-oxidant defence and DNA repair. *EMBO J* 30(3):546-555.
149.Li J, Du W, Maynard S, Andreassen PR, & Pang Q (2010) Oxidative stress specific interaction between FANCD2 and FOXO3a. *Blood* 115(8):1545-1548.
150.Du W, Adam Z, Rani R, Zhang X, & Pang Q (2008) Oxidative Stress in Fanconi Anemia Hematopoiesis and Disease Progression *Antioxid. Redox Signal.* 10(11):1909-1921.
151.Groszer M*, et al.* (2006) PTEN negatively regulates neural stem cell self-renewal by modulating G0-G1 cell cycle entry. *Proceedings of the National Academy of Sciences of the United States of America* 103(1):111-116.
152.Groszer M*, et al.* (2001) Negative Regulation of Neural Stem/Progenitor Cell Proliferation by the Pten Tumor Suppressor Gene in Vivo. *Science* 294(5549):2186-2189.
153.Zhang J*, et al.* (2006) PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. *Nature* 441(7092):518-522.
154.Yilmaz ÄmH*, et al.* (2006) Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. *Nature* 441(7092):475-482.
155.Paik J-h*, et al.* (2009) FoxOs Cooperatively Regulate Diverse Pathways Governing Neural Stem Cell Homeostasis. *Cell stem cell* 5(5):540-553.
156.Goertz MJ, Wu Z, Gallardo TD, Hamra FK, & Castrillon DH (2011) Foxo1 is required in mouse spermatogonial stem cells for their maintenance and the initiation of spermatogenesis. *The Journal of Clinical Investigation* 121(9):3456-3466.
157.Castrillon DH, Miao L, Kollipara R, Horner JW, & DePinho RA (2003) Suppression of Ovarian Follicle Activation in Mice by the Transcription Factor Foxo3a. *Science* 301(5630):215-218.
158.Maryanovich M*, et al.* (2012) The ATM-BID pathway regulates quiescence and survival of haematopoietic stem cells. *Nat Cell Biol* 14(5):535-541.
159.Takubo K*, et al.* (2008) Stem Cell Defects in ATM-Deficient Undifferentiated Spermatogonia through DNA Damage-Induced Cell-Cycle Arrest. *Cell stem cell* 2(2):170-182.
160.Accili D & Arden KC (2004) FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. *Cell* 117(4):421-426.
161.Gross DN, van den Heuvel AP, & Birnbaum MJ (2008) The role of FoxO in the regulation of metabolism. *Oncogene* 27(16):2320-2336.
162.Schneider JG*, et al.* (2006) ATM-dependent suppression of stress signaling reduces vascular disease in metabolic syndrome. *Cell Metab* 4(5):377-389.
163.Yang R & Trevillyan JM (2008) c-Jun N-terminal kinase pathways in diabetes. *Int J Biochem Cell Biol* 40(12):2702-2706.
164.Halaby M-J, Hibma JC, He J, & Yang D-Q (2008) ATM protein kinase mediates full activation of Akt and regulates glucose transporter 4 translocation by insulin in muscle cells. *Cellular Signalling* 20(8):1555-1563.
165.Zhou K*, et al.* (2011) Common variants near ATM are associated with glycemic response to metformin in type 2

diabetes. *Nat Genet* 43(2):117-120.
166.Kitamura YI*, et al.* (2005) FoxO1 protects against pancreatic Î² cell failure through NeuroD and MafA induction. *Cell Metabolism* 2(3):153-163.
167.Uchida T*, et al.* (2005) Deletion of Cdkn1b ameliorates hyperglycemia by maintaining compensatory hyperinsulinemia in diabetic mice. *Nat Med* 11(2):175-182.
168.Miles PD, Treuner K, Latronica M, Olefsky JM, & Barlow C (2007) Impaired insulin secretion in a mouse model of ataxia telangiectasia. *American Journal of Physiology - Endocrinology And Metabolism* 293(1):E70-E74.
169.Campisi J (2005) Senescent Cells, Tumor Suppression, and Organismal Aging: Good Citizens, Bad Neighbors. *Cell* 120(4):513-522.
170.Szypowska AA & Burgering BM (2011) The peroxide dilemma: opposing and mediating insulin action. *Antioxid Redox Signal* 15(1):219-232.
171.Essers MAG*, et al.* (2004) FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. *EMBO J* 23(24):4802-4812.
172.Oh SW*, et al.* (2005) JNK regulates lifespan in Caenorhabditis elegans by modulating nuclear translocation of forkhead transcription factor/DAF-16. *Proc Natl Acad Sci U S A* 102(12):4494-4499.
173.Wang MC, Bohmann D, & Jasper H (2003) JNK signaling confers tolerance to oxidative stress and extends lifespan in Drosophila. *Dev Cell* 5(5):811-816.
174.Asada S*, et al.* (2007) Mitogen-activated protein kinases, Erk and p38, phosphorylate and regulate Foxo1. *Cell Signal* 19(3):519-527.
175.Lehtinen MK*, et al.* (2006) A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span. *Cell* 125(5):987-1001.
176.Yuan Z*, et al.* (2009) Regulation of neuronal cell death by MST1-FOXO1 signaling. *J Biol Chem* 284(17):11285-11292.
177.Choi J*, et al.* (2009) Mst1-FoxO signaling protects Naive T lymphocytes from cellular oxidative stress in mice. *PLoS One* 4(11):e8011.
178.Kim S, Kim Y, Lee J, & Chung J (2010) Regulation of FOXO1 by TAK1-Nemo-like kinase pathway. *J Biol Chem* 285(11):8122-8129.
179.Szypowska AA, de Ruiter H, Meijer LA, Smits LM, & Burgering BM (2011) Oxidative stress-dependent regulation of Forkhead box O4 activity by nemo-like kinase. *Antioxid Redox Signal* 14(4):563-578.
180.van der Heide LP & Smidt MP (2005) Regulation of FoxO activity by CBP/p300-mediated acetylation. *Trends in Biochemical Sciences* 30(2):81-86.
181.van der Horst A & Burgering BMT (2007) Stressing the role of FoxO proteins in lifespan and disease. *Nat Rev Mol Cell Biol* 8(6):440-450.
182.van der Horst A*, et al.* (2004) FOXO4 Is Acetylated upon Peroxide Stress and Deacetylated by the Longevity Protein hSir2SIRT1. *Journal of Biological Chemistry* 279(28):28873-28879.
183.Brunet A*, et al.* (2004) Stress-Dependent Regulation of FOXO Transcription Factors by the SIRT1 Deacetylase. *Science* 303(5666):2011-2015.
184.Brenkman AB, de Keizer PLJ, van den Broek NJF, Jochemsen AG, & Burgering BMT (2008) Mdm2 Induces Mono-Ubiquitination of FOXO4. *PLoS ONE* 3(7):e2819.
185.van der Horst A*, et al.* (2006) FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP. *Nat Cell Biol* 8(10):1064-1073.
186.Huang H, Regan KM, Lou Z, Chen J, & Tindall DJ (2006) CDK2-Dependent Phosphorylation of FOXO1 as an Apoptotic Response to DNA Damage. *Science* 314(5797):294-297.
187.Liu P, Kao TP, & Huang H (2008) CDK1 promotes cell proliferation and survival via phosphorylation and inhibition of FOXO1 transcription factor. *Oncogene* 27(34):4733-4744.
188.Yuan Z*, et al.* (2008) Activation of FOXO1 by Cdk1 in Cycling Cells and Postmitotic Neurons. *Science* 319(5870):1665-1668.
189.Kress TR*, et al.* (2011) The MK5/PRAK kinase and Myc form a negative feedback loop that is disrupted during colorectal tumorigenesis. *Mol Cell* 41(4):445-457.
190.Rena G*, et al.* (2002) Two novel phosphorylation sites on FKHR that are critical for its nuclear exclusion. *EMBO J* 21(9):2263-2271.
191.Brunet A*, et al.* (2001) Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). *Mol Cell Biol* 21(3):952-965.
192.Hertweck M, Gobel C, & Baumeister R (2004) C. elegans SGK-1 is the critical component in the Akt/PKB kinase complex to control stress response and life span. *Dev Cell* 6(4):577-588.
193.Kanao T*, et al.* (2010) Activation of FoxO by LRRK2 induces expression of proapoptotic proteins and alters survival of postmitotic dopaminergic neuron in Drosophila. *Human Molecular Genetics* 19(19):3747-3758.
194.Kanao T*, et al.* (2012) The Nitric Oxide-Cyclic GMP Pathway Regulates FoxO and Alters Dopaminergic Neuron Survival in *Drosophila*. *PLoS ONE* 7(2):e30958.
195.Hu MC*, et al.* (2004) IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. *Cell* 117(2):225-237.
196.Ho KK*, et al.* (2012) Phosphorylation of FOXO3a on Ser-7 by p38 promotes its nuclear localization in response to doxorubicin. *J Biol Chem* 287(2):1545-1555.

197.Yang JY, *et al.* (2008) ERK promotes tumorigenesis by inhibiting FOXO3a via MDM2-mediated degradation. *Nat Cell Biol* 10(2):138-148.
198.Greer EL, *et al.* (2007) The Energy Sensor AMP-activated Protein Kinase Directly Regulates the Mammalian FOXO3 Transcription Factor. *Journal of Biological Chemistry* 282(41):30107-30119.
199.Greer EL, *et al.* (2007) An AMPK-FOXO Pathway Mediates Longevity Induced by a Novel Method of Dietary Restriction in C. elegans. *Current biology : CB* 17(19):1646-1656.
200.Housley MP, *et al.* (2008) O-GlcNAc Regulates FoxO Activation in Response to Glucose. *Journal of Biological Chemistry* 283(24):16283-16292.
201.Kuo M, Zilberfarb V, Gangneux N, Christeff Nvn, & Issad T (2008) O-glycosylation of FoxO1 increases its transcriptional activity towards the glucose 6-phosphatase gene. *FEBS Letters* 582(5):829-834.
202.Yamagata K, *et al.* (2008) Arginine Methylation of FOXO Transcription Factors Inhibits Their Phosphorylation by Akt. *Molecular Cell* 32(2):221-231.
203.Yang J-Y, *et al.* (2008) ERK promotes tumorigenesis by inhibiting FOXO3a via MDM2-mediated degradation. *Nat Cell Biol* 10(2):138-148.
204.Huang H, *et al.* (2005) Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation. *Proceedings of the National Academy of Sciences of the United States of America* 102(5):1649-1654.
205.Tsai W-B, *et al.* (2010) Inhibition of FOXO3 Tumor Suppressor Function by βTrCP1 through Ubiquitin-Mediated Degradation in a Tumor Mouse Model. *PLoS ONE* 5(7):e11171.
206.Pallardó F, *et al.* (2010) Mitochondrial dysfunction in some oxidative stress-related genetic diseases: Ataxia-Telangiectasia, Down Syndrome, Fanconi Anaemia and Werner Syndrome. *Biogerontology* 11(4):401-419.
207.Lloret A, *et al.* (2008) Different patterns of in vivo pro-oxidant states in a set of cancer- or aging-related genetic diseases. *Free Radical Biology and Medicine* 44(4):495-503.
208.Barlow C, *et al.* (1999) Loss of the ataxia–telangiectasia gene product causes oxidative damage in target organs. *Proceedings of the National Academy of Sciences* 96(17):9915-9919.
209.Nicotera T, Thusu K, & Dandona P (1993) Elevated Production of Active Oxygen in Bloom's Syndrome Cell Lines. *Cancer Research* 53(21):5104-5107.
210.Szekely AM, *et al.* (2005) Werner Protein Protects Nonproliferating Cells from Oxidative DNA Damage. *Molecular and Cellular Biology* 25(23):10492-10506.
211.Pascucci B, *et al.* (2012) An altered redox balance mediates the hypersensitivity of Cockayne syndrome primary fibroblasts to oxidative stress. *Aging Cell* 11(3):520-529.

Chapter 2

FOXO transcription factors maintain genomic stability by regulating histone acetylation around DSBs to promote DNA-end resection and homologous recombination repair

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Under Review

Abstract

Members of the Forkhead box O (FOXO) family of transcription factors regulate cellular processes that ultimately determine cell survival, proliferation and death. FOXOs act as bona fide tumor suppressors and control lifespan; both processes intrinsically related to genomic stability. Here we demonstrate that FOXOs are substrates of the ataxia telangiectasia mutated (ATM) kinase in response to the induction of DNA double strand breaks. FOXOs accumulate on sites of DNA damage, via association with the MRN (MRE11-Rad50-NBS1) complex. Herewith FOXOs regulate the recruitment to the sites of damage of TRRAP in complex with a HAT to induce the acetylation of H4K16. Thereby FOXOs negatively regulate DNA-end occupancy by 53BP1 and promote DNA-end resection and initiation of homologous recombination. In agreement, we show FOXOs to promote faithful DNA repair by homologous recombination in a 53BP1-dependent manner and independent of their established role as transcription factors. Our findings reveal a novel function of FOXOs in the maintenance of genomic stability.

Introduction

Cells are constantly exposed to endogenous and exogenous insults, such as free radicals, ionizing radiation (IR) and ultraviolet irradiation (1), which can damage the genetic material in various ways (e.g. base modifications or breakage of the DNA phosphate backbone). To ensure genomic stability, cells have evolved a number of tightly regulated mechanisms for surveillance, detection and repair of DNA lesions, collectively referred to as the DNA damage response (DDR) (1, 2). Important mediators of the DDR are the PI3 kinases-related kinases (PIKKs) ATM, ATM and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK), which orchestrate responses downstream of DNA breaks and replication stress. One of the most deleterious DNA lesions are double strand breaks (DSBs); DSBs are repaired by two main pathways, namely homologous recombination (HR) and non-homologous end-joining (NHEJ) (3). HR is associated with the maintenance of genomic integrity, as the intact sister chromatid is used for the “error free” repair of the DNA lesion. On the other hand, NHEJ is a pathway leading to rapid resolution of DNA lesions, although at the expense of fidelity, as it may result in deletions or translocations of parts of the genetic material. Accrual of unrepaired or not properly repaired DNA lesions is associated with cancer and aging (1, 4). In agreement, human genetic disorders with disruptions in DNA repair components are characterized by increased tumorigenesis and/or premature aging (1, 5).

FOXO transcription factors are members of the winged-helix-winged family of transcription factors, with four members in mammals, denoted FOXO1, FOXO3a, FOXO4 and FOXO6. FOXOs play a central role in cell fate determination, by regulating the expression of genes involved in metabolism, proliferation and cell death (6). The FOXO proteins are evolutionary conserved; their homologues in the nematode *C. elegans* (DAF-16) and the fruit fly *Drosophila melanogaster* (dFOXO) are associated with lifespan, while studies on the mouse Foxos have established them as bona fide tumor suppressors (reviewed in (7)). A recent phosphoproteomic study suggested that FOXO1 may be phosphorylated by ATM or ATR in response to IR (8). We reasoned that FOXO involvement in tumor suppression and lifespan extension could rely in part to their ability to maintain genomic stability, in agreement also with previous studies (9-12).

Here we address the involvement of FOXOs in the error-free repair of DSBs. We provide evidence that FOXOs are responsive to the induction of DSBs by acquiring ATM-mediated phosphorylation and they are recruited to the sites of DNA damage by interacting with NBS1, a component of the MRN complex (13). We further

demonstrate that FOXOs co-recruit a TRRAP-containing histone acetyltransferase (HAT) complex at the sites of DNA damage to regulate histone 4 lysine 16 acetylation (H4K16ac) and thereby 53BP1 binding to the sites of damage. Consequently, we demonstrate a role for FOXOs in DNA end resection and HR. These data provide new insights in the cell protective functions of FOXOs and their involvement in longevity and tumor suppression.

RESULTS

FOXOs are substrates for phosphorylation by ATM

The PIKKs recognize and phosphorylate a minimal consensus sequence motif i.e. serine/threonine followed by glutamine (S/TQ) within their substrates (14, 15). A number of such S/TQ motifs are present in all FOXO members. To investigate whether FOXOs are PIKKs substrates following DNA damage, we treated human retinal pigment epithelium (RPE) cells with 5 Gy of IR and subsequently checked the phosphorylation of FOXO1, FOXO3a and FOXO4 on the S/TQ motif, using a generic antiserum recognizing this phosphorylated motif **(Figure 1A)**. We found all FOXOs to be recognized by this antiserum under non-damaging conditions and this recognition was further enhanced upon DNA damage, albeit to variable extend. Similar results were obtained in the cancer cell line U2-OS and in HEK293T cells **(Supplementary Figure 1A)**. It should be noted that detection by generic antisera such as the phosphorS/TQ antiserum is context dependent and the various S/TQ sites are thus differentially recognized. Indeed, Matsuoka et al. found, similar to our results, that the induction of phosphorylation of FOXO1 by ATM following DNA damage is only clearly recognized by an antiserum raised against ATM-phosphorylated BRCA1 (pSer1423) (8). Therefore the induction of signal of the different FOXOs, as detected by the phosphorS/TQ antiserum, is not a measure whether any of the FOXOs respond stronger to DNA damage in terms of ATM-mediated phosphorylation. Irrespective, because the dynamic range using this antiserum appeared to work out the best for FOXO4, we focused on this FOXO member for our further analysis. We verified that the recognition by the phosphorS/TQ antiserum indeed reflects a phosphorylation event, as the signal disappeared upon phosphatase treatment, and it is PIKKs dependent, as pre-treatment of cells with caffeine, a general PIKKs inhibitor (16) significantly reduced FOXO4 phosphorylation **(Figure 1B)**.

To identify the specific residue(s) phosphorylated on FOXO4, we mutated each of the potential serines/threonines to alanine. S/TQ phosphorylation of FOXO4 induced by IR was severely reduced in the Ser462 to Ala mutant, indicating that Ser462 is the major PIKKs-dependent phosphorylation site on FOXO4 **(Figure 1C)**. In agreement with these results, rabbit antiserum that specifically recognizes FOXO4 phosphorylated on Ser462, detected increased phosphorylation following DNA damage **(Supplementary Figure 1B)**. Cytoplasmic-nuclear fractionation further revealed that FOXO4 phosphorylated on Ser462 resides predominantly in the nucleus **(Supplementary Figure 1C)**.

To identify the kinase responsible for FOXO4 Ser462 phosphorylation, we used specific inhibitors of the major kinases involved in the DNA damage response. Inhibition of ATM kinase activity with the specific inhibitor Ku-55933 (17) significantly reduced the IR-induced FOXO4 phosphorylation **(Figure 1D)**. Similar results were obtained with ATM knockdown **(Supplementary Figure 1D)**. Moreover, in an *in vitro* kinase assay, ATM directly phosphorylated Ser462 of FOXO4 **(Figure 1E)**. Together, these results suggest ATM to predominantly mediate DNA damage-induced FOXO4 phosphorylation on Ser462. ATM also phosphorylated FOXO1 and FOXO3 in an *in vitro* kinase assay **(Supplementary Figure 1E)**, suggesting that all FOXO members are direct substrates of ATM kinase.

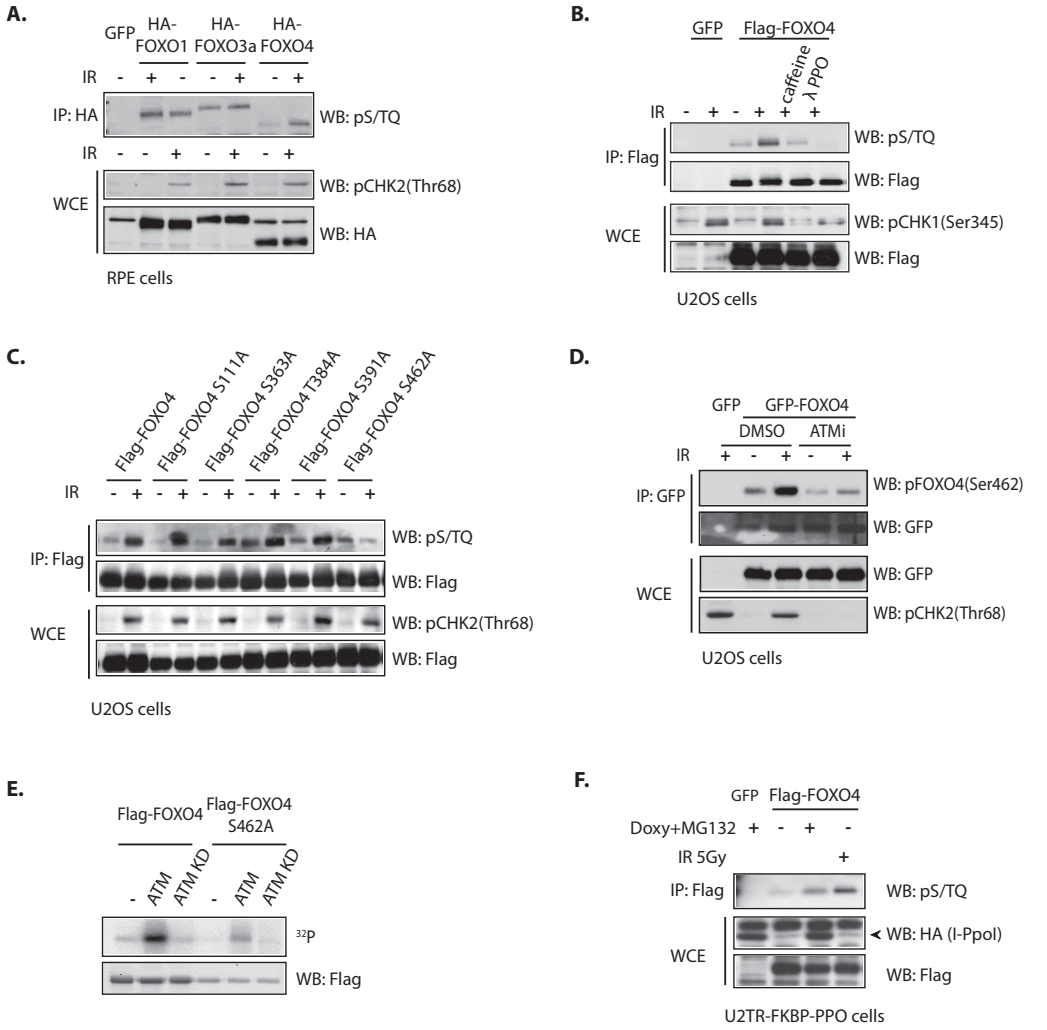

Figure 1. FOXOs are phosphorylated in response to double strand breaks. (A) RPE cells show increased phosphorylation of HA-FOXO1, HA-FOXO3a and HA-FOXO4 on S/TQ motifs in response to IR (5 Gy) **(B)** The FOXO4 pS/TQ signal is reduced by caffeine pre-treatment (PIKKs inhibition) and disappears after λ-phosphatase treatment **(C)** Identification of Ser462 as the S/TQ phosphorylation site on FOXO4. Wild type and mutant forms of Flag-FOXO4 were subjected to IR (5 Gy) and tested for S/TQ phosphorylation **(D)** Ser462 phosphorylation is reduced in cells pre-treated with ATM inhibitor (Ku-55933) for 1 hr prior to IR **(E)** ATM and ATM KD expressed in HEK293T cells were isolated and utilized for in vitro kinase assay with FOXO4 and FOXO4 S462A as substrates **(F)** Ser462 phosphorylation is induced in U2TR-FKBP PPO cells, in which expression of FKBP-I-PpoI was induced by doxycycline for 16hrs and MG132 for 2 hours. Arrowhead indicates height of HA-FKBP-I-PpoI.

FOXO phosphorylation is induced by double-strand breaks

ATM is known to be activated in response to DSBs, however other stimuli are also reported to regulate ATM kinase activity (18-20). To investigate whether other stimuli besides IR activate ATM-mediated FOXO phosphorylation, we assessed FOXO4 Ser462 phosphorylation following treatment with agents that induce different types of DNA damage. We observed a correlation between treatment with agents that

predominantly induce DSBs under the specific conditions employed (IR, neocarzinostatin, etoposide and doxorubicin) and increased FOXO4 phosphorylation **(Supplementary Figure 1F)**. Phosphorylation on Ser462 was also increased upon treatment with hydrogen peroxide (H2O2). Although H2O2 can induce several types of DNA lesions, including DSBs, it was recently demonstrated that ROS can also activate ATM independent of the DNA damage signaling (20). To discriminate between ROS- and DSB-triggered ATM-mediated phosphorylation, we exposed cells pre-treated with N-acetyl cysteine (NAC), a ROS scavenger, to IR or H2O2 and examined FOXO4 phosphorylation. NAC pre-treatment inhibited FOXO4 Ser462 phosphorylation in response to excessive cellular ROS; however IR-induced phosphorylation did not change after NAC pre-treatment **(Supplementary Figure 1G)**. This indicates that IR-induced FOXO phosphorylation proceeds through DSBs signaling, independently of ROS-dependent changes on ATM.

To further assess the link between DSBs and FOXO phosphorylation in a more defined manner, we used a system employing the homing endonuclease I-PpoI (U2TR-FKBP-PPO cells). I-PpoI was described to generate approximately 30 DSBs at defined sites in human cells, resulting in a detectable DDR with ATM activation (21). Induction of I-PpoI increased the phosphorylation of Ser462 on FOXO4, which is in agreement with DSBs being the major type of DNA damage that triggers ATM-dependent FOXO4 phosphorylation **(Figure 1F)**.

FOXOs are recruited to sites of DNA damage

Recruitment to sites of DNA damage is commonly observed for proteins involved in DNA damage signaling and repair (2, 22, 23). To investigate a possible involvement of FOXOs in the DDR, we tested whether FOXOs accumulate on sites of DNA damage. First we utilized the I-PpoI system that allows studying the recruitment of proteins to a defined single DSB. To this end, we established a cell line (HeLashFOXO-PPO cells) that stably expresses a doxycyline inducible shRNA for FOXOs and the I-PpoI endonuclease fused to the ER domain to induce DSBs by 4OHT treatment, as was previously described (21). By employing chromatin immumoprecipitation (ChIP) in these cells, we found endogenous FOXO3a to be recruited to DSBs induced by I-PpoI; more specifically, as shown in **Figure 2A**, FOXO3a accumulates at the proximal area around the single DSB induced by I-PpoI in Chromosome 1. When endogenous FOXOs were depleted by doxycycline application, no signal was detected, validating FOXO antibody specificity.

The lack of a ChIP-grade antibody for endogenous FOXO4 precluded a similar analysis for FOXO4 however, in agreement with a previous study showing by immunofluorescence microscopy FOXO3a to reside at the sites of DNA damage (24), we could also observe FOXO4 foci to be readily formed after IR treatment **(Supplementary Figure 2A)**. Importantly, the FOXO IR-induced foci (IRIF) co-localized to a large extent with other proteins phosphorylated on S/TQ (an established marker of the DDR (2)), further verifying that FOXOs localize to sites of DNA damage.

To study the kinetics of FOXO recruitment to DNA lesions, we analyzed the real-time assembly of GFP-FOXO fusion proteins to sites of DNA damage induced by laser micro-irradiation. As shown in **Figure 2B**, all FOXO proteins (GFP-FOXO1, GFP-FOXO3a and GFP-FOXO4) accumulate at the sites of DNA damage. Recruitment of GFP-FOXO4 was observed within 15 sec and reached equilibrium 4 min after laser micro-irradiation **(Figure 2C)**. Similar kinetics was also observed for FOXO1 and FOXO3a **(Supplementary Figure 2B)**. The accumulation of FOXOs on sites of DNA damage followed similar kinetics as MDC1, a known mediator of the DDR (25), and it was specific, as GFP alone was not recruited under the conditions employed **(Supplementary Figure 2C)**.

To measure the mobility of FOXOs on the sites of DNA damage we employed fluorescence recovery after photobleaching (FRAP). We bleached the fluorophore of the FOXO4 fusion accumulated on the sites of

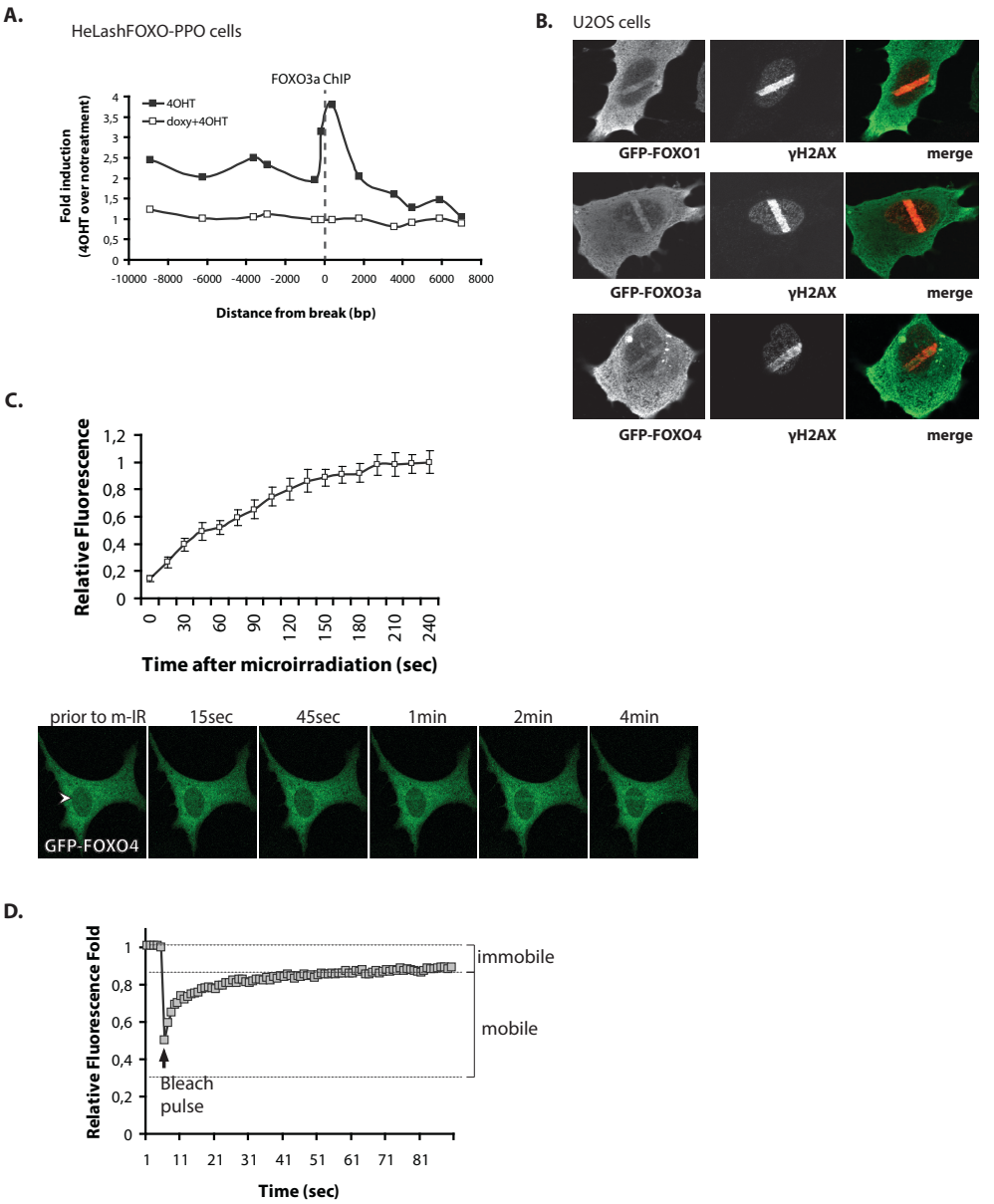

Figure 2. FOXOs are recruited to sites of DNA damage. (A) FOXO3a distribution around a single DSB. ChIP of endogenous FOXO3a around the single cut site of I-PpoI in Chromosome 1. HeLashFOXO-PPO cells were cultured in the presence or absence of doxycycline for 72hrs before I-PPO was induced by 4-OHT for 16hrs. Cells were used for ChIP against FOXO3 and Q-PCR was performed with oligonucleotide primer pairs surrounding the single chromosome 1 I-PpoI cleavage site. Data are represented as fold induction over no treatment. Results represent the mean of two independent experiments **(B)** GFP-FOXO1, GFP-FOXO3a and GFP-FOXO4 are recruited to sites of damage by laser micro-irradiation. Cells were fixed 30 min post irradiation and stained with anti-γH2AX and anti-GFP antibodies **(C)** Kinetics of GFP-FOXO4 recruitment; U2OS cells transfected with GFP-FOXO4 were subjected to laser micro-irradiation. GFP fluorescence intensity was measured over a period of 5 min. Measurements are mean ±SD from 100 cells. Arrowhead indicates site of laser micro-irradiation **(D)** FRAP of GFP-FOXO4 accumulated to laser micro-irradiation sites. U2OS cells transfected with GFP-FOXO4 were subjected to laser micro-irradiation and 10 minutes post-irradiation FRAP was performed on a small region within the micro-irradiated area. GFP fluorescence intensity was measured every 1 sec. Measurements are mean from 50 cells.

damage induced by laser micro-irradiation and measured its fluorescence recovery over time. FOXO4-GFP fluorescence recovered fast after bleaching, indicating that FOXO molecules rapidly exchange at the sites of DNA damage **(Figure 2D)**. However, the fluorescence recovery was not complete; approximately 20% of the initial fluorescence intensity was not recovered after equilibrium was reached, indicating that a population of FOXO molecules is retained on the sites of DNA damage.

FOXOs recruitment to sites of DNA damage is dependent on ATM-mediated phosphorylation and their interaction with NBS1

To better understand the mechanistic details of FOXO recruitment and retention to the sites of DNA damage we studied the real-time assembly of FOXO4 mutants to sites of laser-induced damage. A specific mutation in FOXOs (H156R in FOXO4) within the conserved third helix of the Forkhead DNA binding domain abolishes binding to DNA (26). The H156R mutant accrued to the laser micro-irradiation sites with similar kinetics as the wild type FOXO4 **(Supplementary Figure 3A)**, suggesting that the accumulation of FOXOs on sites of DNA damage is mediated by their association with other components accumulating at the damaged chromatin, rather than their direct binding to DNA.

Next, we assessed whether ATM-mediated FOXO phosphorylation is required for FOXO recruitment to sites of damage, by measuring the kinetics of the S462A mutant. We observed the recruitment of this mutant to be significantly impaired compared to the wild type protein **(Figure 3A)**, suggesting that Ser462 phosphorylation promotes the interaction of FOXO4 with certain DDR factors, thereby inducing its accumulation to the sites of DNA damage. We then tested the contribution of DDR components to FOXO recruitment at the sites of damage. We chose DDR components based on two criteria: (i) proteins that participate in the initial steps of the DDR, since FOXOs respond rapidly to the infliction of DNA damage, (ii) proteins bearing BRCA1 C Terminus (BRCT) domain for interaction with substrates phosphorylated on Ser residues (27, 28). These include NBS1, MDC1 and PARP1 (2). We observed that FOXO recruitment was significantly impaired following siRNA-mediated NBS1 depletion **(Figure 3B)** and similar results were obtained by knocking down MRE11, another component of the MRN complex **(Supplementary Figures 3B and 3D)**. PARP1 activity was described to be required for the early association of NBS1 to the sites of DNA damage (29) and MDC1 is required for NBS1 to stably associate with the sites of DNA damage (25); accordingly, both MDC1 knockdown and PARP1 inhibition resulted in a moderate impairment in FOXO recruitment, however the observed differences were not significant **(Supplementary Figures 3B, 3C and 3D)**. No defect in FOXO4 recruitment was observed in cells depleted of proteins involved in later steps of the DDR, such as RNF8 and 53BP1 **(Supplementary Figures 3C and 3D)**. Thus, NBS1 appears to have an indispensable role in the recruitment of FOXO to the sites of DNA damage. In agreement, ChIP analysis in the HeLashFOXOs-PPO cells showed impaired association of endogenous FOXO3a with the sites of DNA damage when NBS1 was silenced **(Figure 3C and Supplementary Figure 3E)**.

The dependency on NBS1 for FOXO recruitment to the sites of DNA damage prompted us to look into the potential interaction between FOXO and NBS1 in response to genotoxic stress. As shown in **Figure 3D**, FOXO4 and NBS1 co-immunoprecipitate under basal conditions and their interaction is further enhanced upon DNA damage. In contrast, the S462A mutant does not show enhanced interaction with NBS1 after IR, suggesting that the association of these proteins in response to DNA damage requires ATM-mediated FOXO phosphorylation. Similarly, FOXO1 and FOXO3 showed increased binding to NBS1 in response to IR and this induction was lost in cells pre-treated with ATM inhibitor **(Supplementary Figure 3F)**. The requirement for

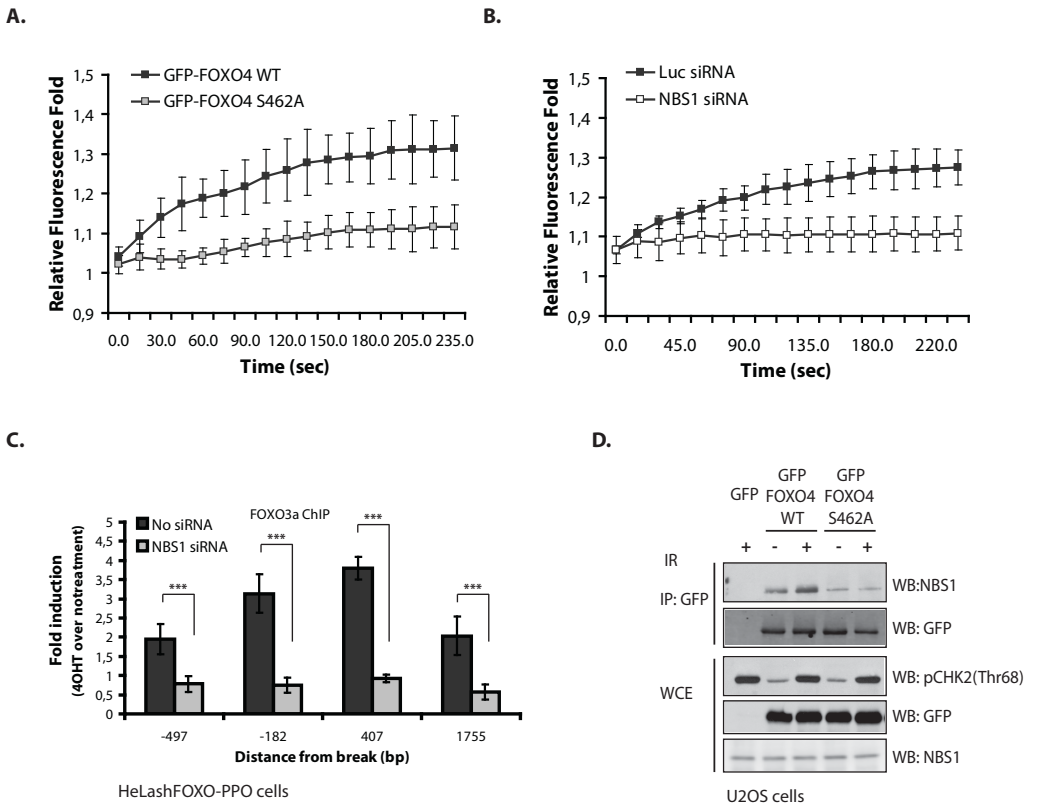

Figure 3. FOXOs phosphorylated on S/Q interact with NBS1 and this interaction facilitates their re-cruitment to sites of damage. (A) Kinetics of GFP-FOXO4 S462A recruitment to laser micro-irradiation sites. Measurements are mean ±SD from 100 cells **(B)** Kinetics of GFP-FOXO4 recruitment in the absence of NBS1; U2OS cells transfected with siRNAs against luciferase or NBS1 for 72hrs, were transfected with GFP-FOXO4 and 24 hrs later were subjected to laser micro-irradiation. Measurements are mean ±SD from 60 cells **(C)** Defective recruitment of endogenous FOXO3a to the single cut site in Chromosome1 in NBS1 depleted cells. HeLashFOXO-PPO cells were either untransfected or transfected with NBS1 siRNA for 72hrs before I-PpoI was induced with 4-OHT for 16hrs. ChIP analysis was performed as in (Figure 2A). Results represent the mean ±SD from the three PCR replicates of one representative experiment **(D)** Interaction between GFP-FOXO4 wild type and S462A with NBS1 in response to IR (5Gy, 30 min). ***P<0.001

ATM-mediated phosphorylation in the interaction of FOXO with NBS1 is further supported by analysis of FOXO4 binding to NBS1 mutants disturbed in the FHA domain (R28A) or the BRCT tandem domain (K160M) (30); NBS1 with mutated BRCT domain exhibited impaired binding to FOXO4 **(Supplementary Figure 3G)**. We could further observe partial co-localization of FOXO with MRE11 in response to IR **(Supplementary Figure 3H)**.

FOXOs bind to and induce the recruitment of TRRAP to the sites of DNA damage

Considering that FOXOs do not possess an intrinsic enzymatic activity that would facilitate DNA repair, their association with sites of DNA damage suggested a potential "adaptor/mediator" role (31). A number of FOXO binding partners have already been described, several of which could potentially mediate a role of FOXO in the DDR. To obtain a comprehensive picture of FOXO interacting proteins that are not chromatin

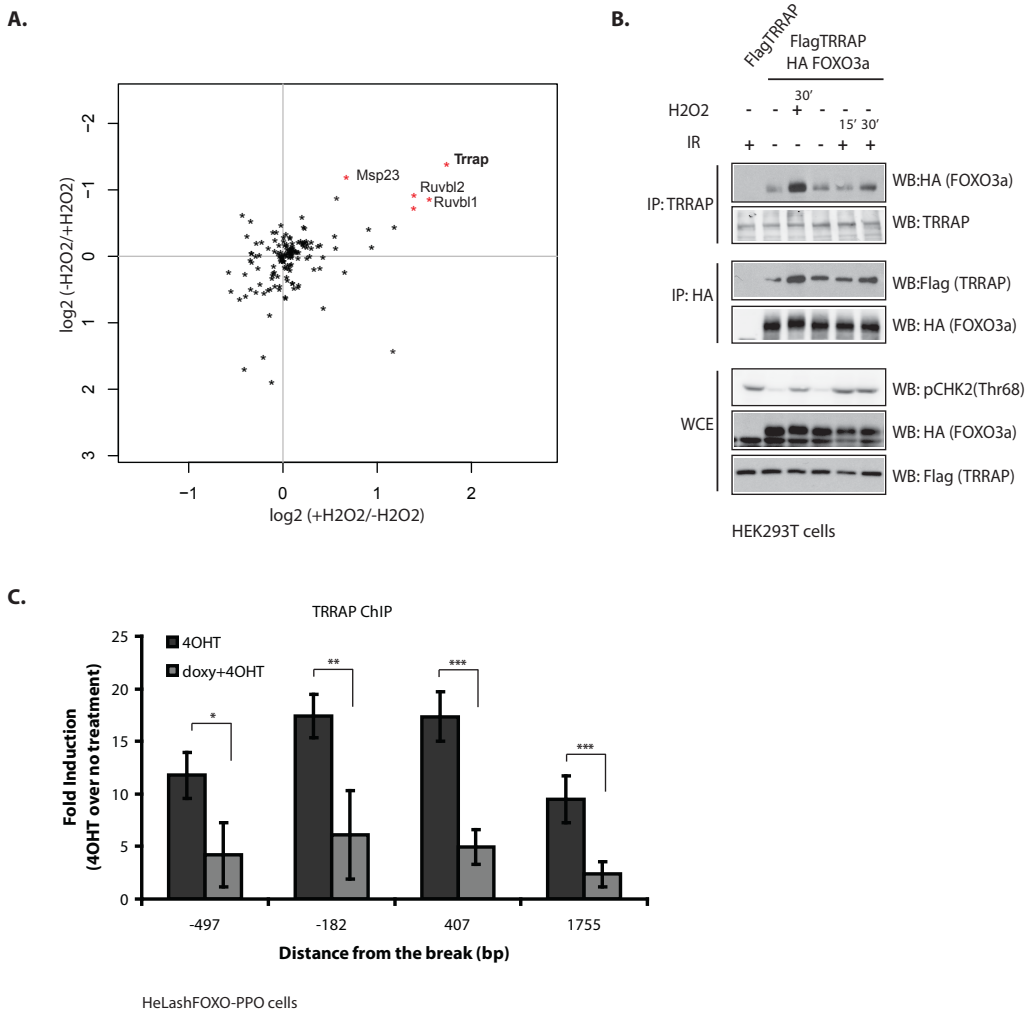

Figure 4. FOXOs bind to and induce the recruitment of TRRAP to the sites of DNA damage (A) Scatterplot of GFP pulldown showing the identified FOXO4 interactors after H2O2 treatment. Specific interactors have a high H/L forward ratio and low H/L reverse ratio and will appear in the right upper quadrant of the scatterplot **(B)** Interaction between FOXO3a and TRRAP after genotoxic stress. HEK293T cells were transfected with Flag-TRRAP and HA-FOXO3a and treated with 200μM H2O2 for 30 min or 5Gy IR for 15 and 30 min. Cell lysates were immunoprecipitated with either anti-TRRAP or anti-HA and the complexes were analyzed by western blot **(C)** HeLashFOXO-PPO cells were cultured in the presence or absence of doxycycline for 72hrs before I-PPO was induced by 4-OHT for 16 hrs. Cells were used for ChIP against TRRAP and Q-PCR was performed with oligonucleotide primer pairs surrounding the single chromosome 1 I-PpoI cleavage site. Data are represented as fold induction over no treatment. Results represent the mean±SD of two independent experiments *P<0.05, **P<0.01, ***P<0.001

associated and interact with FOXOs in response to DNA damage, we employed Stable Isotope Labeling by Amino acids in Cell culture (SILAC) to compare the FOXO binding partners in cells that were either untreated or treated with H2O2 to induce genotoxic stress.

Our SILAC approach identified TRansformation/tRanscription domain-Associated Protein (TRRAP) as a FOXO interacting protein in response to ROS **(Figure 4A)**. We validated this interaction by co-immunoprecipitation and observed basal binding between TRRAP and FOXO1, FOXO3 or FOXO4 that was further enhanced in

response to genotoxic stress **(Figure 4B and Supplementary Figures 4A and 4B)**. Previous studies showed TRRAP to be recruited to the sites of DNA damage (32, 33), so we looked into the FOXO dependency for this recruitment. To this end, we performed ChIP analysis at the area surrounding the single DSB in Chromosome 1 in the HeLashFOXOs-PPO cells. We found an increase in TRRAP recruitment proximal to the DSB, which was significantly diminished when FOXOs were depleted **(Figure 4C)**. Together, these results indicate that the binding of TRRAP to FOXOs results in TRRAP's recruitment to the sites of DNA damage.

FOXOs regulate the levels of H4K16 acetylation around DSBs.

TRRAP belongs to the same family as ATM, the PIKKs, however it does not possess any kinase activity. In fact TRRAP was shown to function as an adaptor protein for the recruitment of several HAT complexes to the chromatin (34). In the case of DNA damage, TRRAP was shown to facilitate the recruitment of the HAT Tip60 to the sites of damage, where it regulates DNA repair (32). The histone mark deposited by HATs and associated with DNA repair is H4K16ac (35). ChIP analysis at the area surrounding the single DSB in Chromosome 1 in the I-PpoI system revealed an increase in H4K16ac levels proximal to the DSB, as was previously reported (35), and this was significantly impaired in FOXO-depleted cells **(Figure 5A)**. Thus, in agreement with the FOXO-mediated recruitment of TRRAP and the proposed role of TRRAP in DDR, we observed FOXO-dependent H4K16 acetylation, suggesting FOXOs to regulate chromatin dynamics around the DSBs.

The regulation of H4K16 acetylation by FOXOs results in the reduced recruitment of 53BP1 to DSBs

The chromatin status around DSBs is known to affect 53BP1 association to the sites of DNA damage. In particular, increased histone ubiquitination and exposure of dimethylated histone 4 lysine 20 (H4K20me2) around the DSB (36-40) facilitate 53BP1 recruitment, whereas H4K16ac prevents 53BP1 binding to the damaged chromatin (35, 41). We hypothesized that a possible effect of FOXO regulation of H4K16 acetylation around DSBs is to affect DNA end occupancy by 53BP1. To this end, we looked into the association of 53BP1 with the sites of DNA damage by studying the 53BP1 subcellular localization and how this is affected by FOXOs. In cells with FOXOs knockdown, the fluorescence intensity of 53BP1 foci after IR was higher when compared to control cells, without a concomitant increase in the 53BP1 protein levels **(Figure 5B and Supplementary Figure 5A)**. Moreover, cells overexpressing FOXO4 showed more diffuse 53BP1 localization compared to non-transfected cells **(Figure 5C)**. These results suggest a role for FOXOs in the exclusion of 53BP1 from DSBs ends, which appears to be H4K16ac dependent, as no differences in H4K20me2 or ubiquitination were observed in our system after FOXOs depletion **(Supplementary Figure 5B and C)**.

Reduced 53BP1 recruitment to DSBs in a FOXO-dependent manner results in the induction of DNA end resection

Recent work on BRCA1-/- tumors suggested that 53BP1 functions in regulating the pathway choice for DSBs repair, by regulating DNA end resection (42, 43). DNA end resection is one of the initial steps in HR, followed by wrapping of the ssDNA that is produced by RPA, a ssDNA binding protein. Subsequently, the recombinase Rad51 displaces RPA from ssDNA and invades the sister chromatid for homology search. The genetic code from the intact chromatid is then used as a template for the repair of the lesion (44).

In cells depleted of FOXOs, RPA2 phosphorylation on Ser4 and Ser8 (45) in response to IR **(Figure 6A)**, as

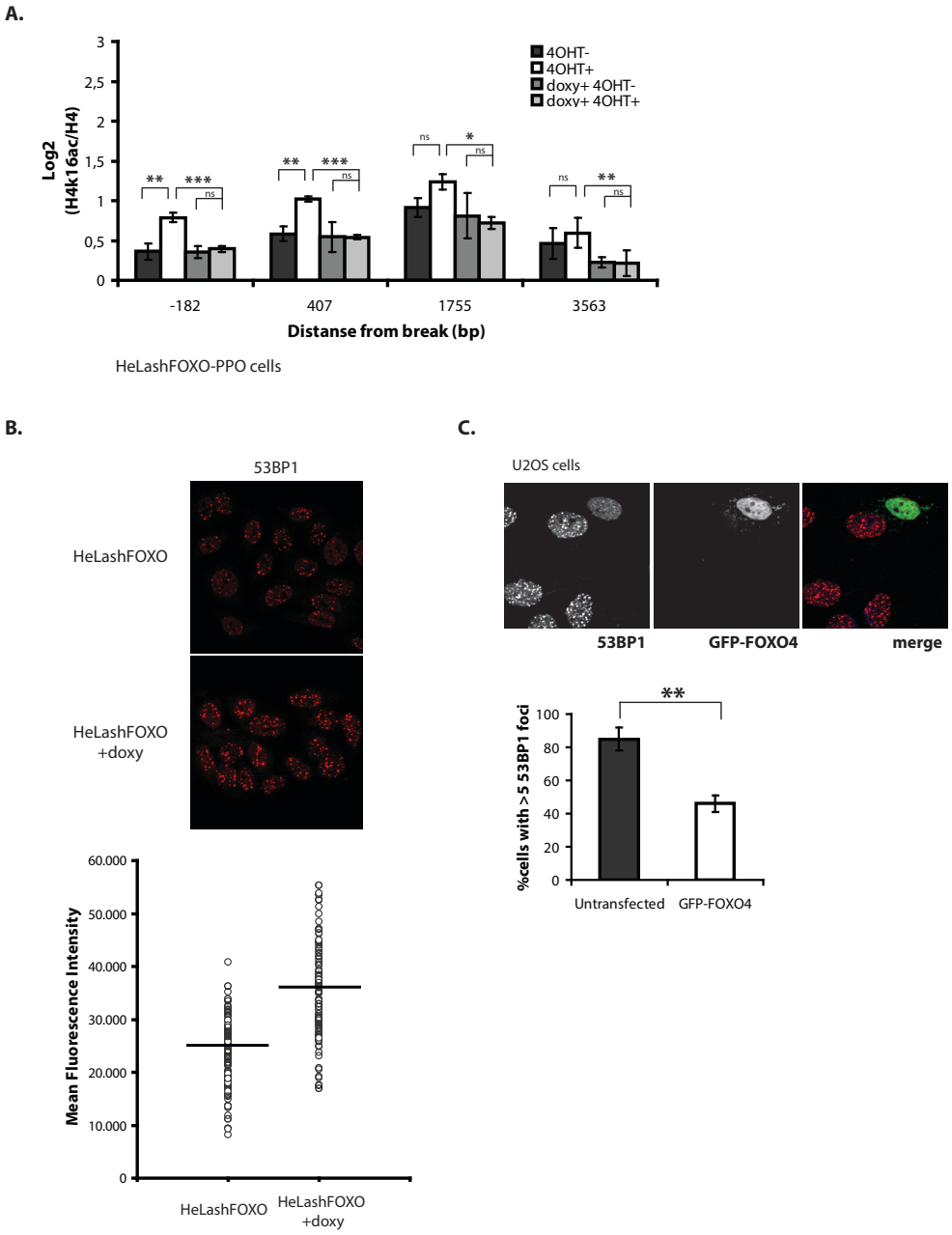

Figure 5. FOXOs regulate 53BP1 localization in a H4K16ac-dependent manner (A) HeLashFOXO-PPO cells were cultured in the presence or absence of doxycycline for 72 hrs before I-PPO was induced by 4-OHT for 16 hrs. Cells were used for ChIP against H4 and H4K16ac and Q-PCR was per-formed with oligonucleotide primer pairs surrounding the single chromosome 1 I-PpoI cleavage site. Data are represented as relative IP (H4K16ac over total H4). Results represent the mean ±SD of three independent experiments **(B)** HeLashFOXO cells treated or not with doxycycline and FOXO4 siRNA for 72 hrs were subjected to 2 Gy IR and fixed after 3 hrs. Representative immunofluorescence image of 53BP1 foci and quantification of the mean fluorescence intensity per cell. 120 cells per condition were quantified **(C)** U2OS cells were transfected with GFP-FOXO4 and 48 hrs post-transfection were treated with 2 Gy IR. 2 hrs post IR cells were fixed and immunostained with the indicated antibodies. Representative immunofluorescence image of 53BP1 and quantification of the 53BP1 foci. *P<0.05, **P<0.01, ***P<0.001, ns: not-significant

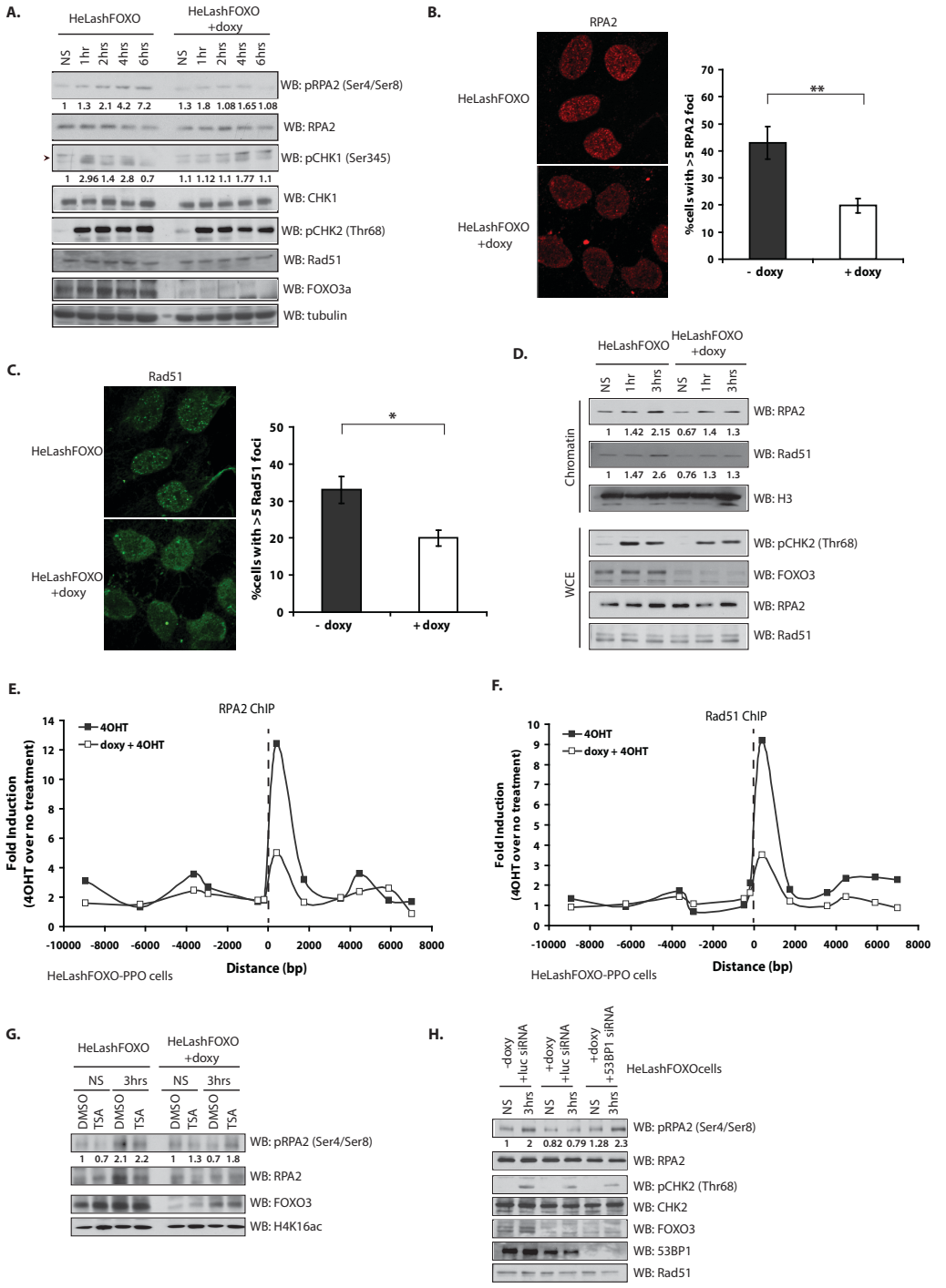

Figure 6. FOXOs regulate DNA end resection of DSBs. (A) HeLashFOXO cells were cultured in the presence or absence of doxycline to deplete FOXO1 and FOXO3a and the levels of FOXO4 were further reduced by transfection of FOXO4 specific siRNA for 72 hrs. Cells were subjected to 2 Gy IR and samples were collected at the indicated timepoints. Representative immunoblots of the indicated proteins. Lower panels show quantification of the western blots. Arrowhead indicates height

well as the formation of RPA2-containing IRIF **(Figure 6B)** was significantly impaired. DNA end resection results in the exposure of ssDNA, thereby activating ATR that subsequently phosphorylates CHK1 (46, 47); we found CHK1 phosphorylation to be diminished in the FOXO-depleted cells **(Figure 6A)**. Importantly, CHK2 phosphorylation, which occurs independently of end resection (48), was unaffected by the cellular FOXOs status. Also, BRCA1 and γH2AX foci assembly was unaffected in cells depleted of FOXOs **(Supplementary Figures 6A and 6B)**. Interestingly, previous reports indicated that FOXO3 depletion would severely inhibit ATM signaling after DNA damage (10, 24); however our data on CHK2 and γH2AX after depletion of all FOXO members fail to recapitulate these studies. FOXOs however appeared to regulate the assembly of Rad51 on sites of DNA repair, as in cells depleted of FOXOs we observed reduced formation of Rad51 foci **(Figure 6C)** and, by means of chromatin fractionation, impaired DNA damage-induced recruitment of Rad51 to the chromatin **(Figure 6D)**. In agreement, ChIP analysis showed that both RPA and Rad51 exhibit impaired distribution around the single DSB in Chromosome 1 induced with the I-PpoI system in FOXOs-depleted cells **(Figures 6E and 6F)**. Collectively, these results indicate a role of FOXOs in regulating DNA-end resection of DSBs.

The dependency on FOXOs for the regulation of H4K16ac around DSBs prompted us to further look into the effect of increasing H4K16ac levels in cells with FOXOs silencing. Treatment with thrichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, markedly increased the levels of H4K16ac and restored RPA phosphorylation after IR in FOXO depleted cells **(Figure 6G)**. Moreover, co-depletion of 53BP1 with FOXOs rescued the defect of RPA2 phosphorylation induced by the knock-down of FOXOs **(Figure 6H)**. These results indicate that FOXOs modulate DNA end resection by increasing H4K16 acetylation on the damaged chromatin and thereby antagonizing 53BP1 from the DNA ends.

Homologous recombination is impaired in cells depleted of FOXO transcription factors, in a 53BP1 manner

Regulation of DNA-end resection by FOXOs suggests a role of FOXO in HR repair. To test this possibility, we employed an HR reporter system previously described by Puget et al (U2OS#18 cells (49)). Knock-down of all FOXOs in these cells resulted in a reproducible and significant reduction in HR repair efficiency of

(Figure 6, legent continuation)

of phosphorylated CHK1 **(B)** HeLashFOXO cells treated or not with doxycycline and FOXO4 siRNA for 72 hrs were subjected to 2 Gy IR and pre-extracted and fixed after 3hrs. Representative immunofluorescence image of RPA and quantification of the RPA foci **(C)** HeLashFOXO cells treated or not with doxycycline and FOXO4 siRNA for 72 hrs were subjected to 2 Gy IR and pre-extracted and fixed after 3hrs. Representative immunofluorescence image of Rad51 and quantification of the Rad51 foci **(D)** Chromatin fractionation of HeLashFOXO cells treated or not with doxycycline and FOXO4 siRNA for 72 hrs, at the indicated times post IR. Representative immunoblot of the indicated proteins. Lower panels show quantification of the western blots **(E-F)** HeLashFOXO-PPO cells were cultured in the presence or absence of doxycycline for 72hrs before I-PPO was induced by 4-OHT for 16 hrs. Cells were used for ChIP against RPA2 and Rad51 and Q-PCR was performed with oligonucleotide primer pairs surrounding the single chromo-some 1 I-PpoI cleavage site. Data are represented as fold induction over no treatment. Results represent the mean of two independent experiments **(G)** HeLashFOXO cells cultured with or without doxycycline for 72 hrs were treated with 5 μM TSA for 3 hrs before IR (2 Gy). Samples were collected 3 hrs post irradiation. Representative immunoblots of the indicated proteins. Lower panels show quantification of the western blots **(H)** HeLashFOXO cells cultured in the presence or absence of doxycycline were transfected with luciferase or 53BP1 siRNA for 72 hrs before treatments. Cells were subjected to 2 Gy IR and samples were collected 3 hrs post IR. Representative immunoblot of the indicated proteins. The lower panel shows a quantification of the western blots. *P<0.05, **P<0.01

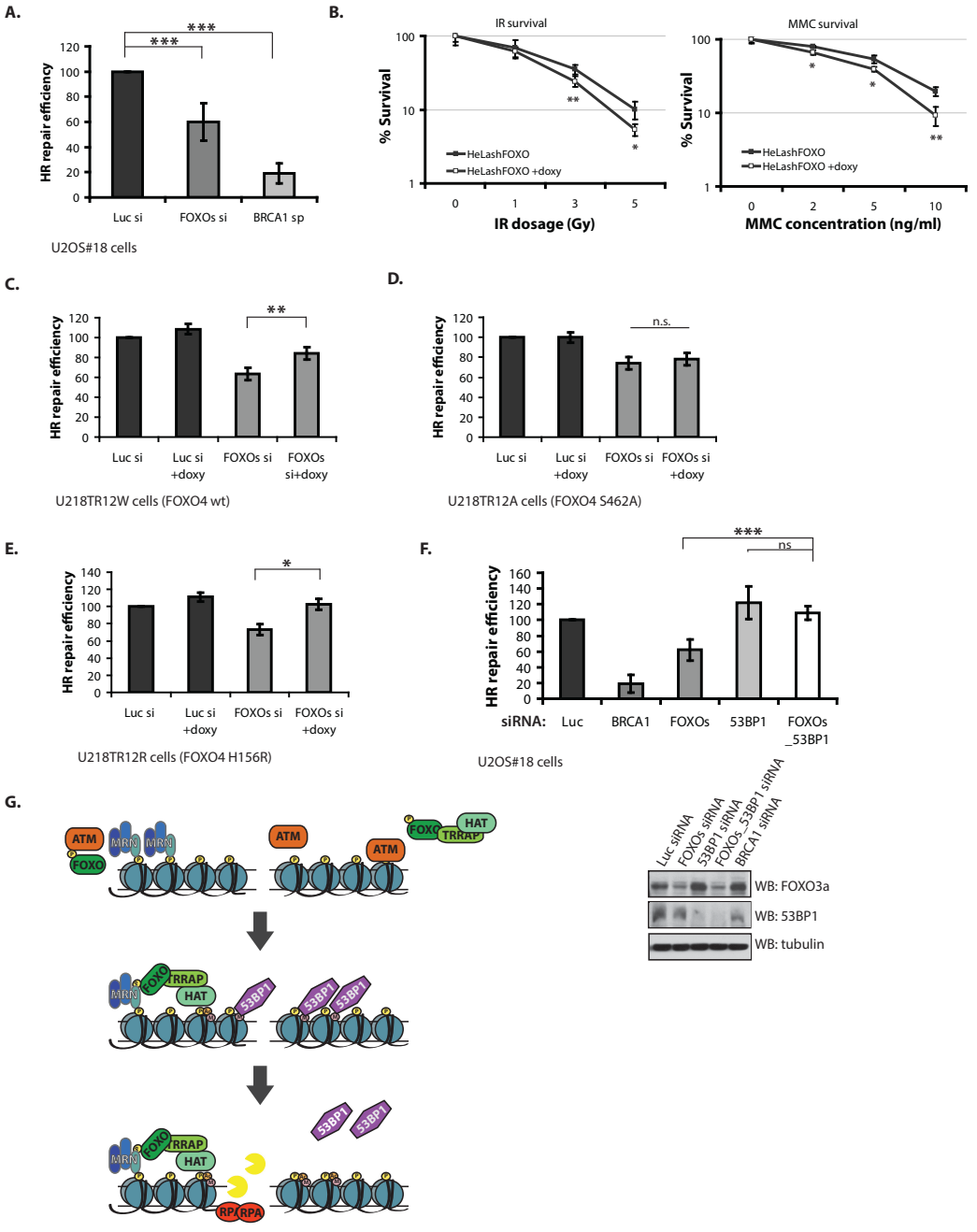

Figure 7. FOXOs protect cells from genotoxic stress by regulating homologous recombination. (A) U2OS#18 cells were transfected with the indicated siRNAs for 24 hrs before the I-SceI plasmid was transfected together with mCherry. Cells were collected 48 hrs post plasmid transfection and analysed by FACS. Results depict the means ±SD of 10 independent experiments **(B)** HeLashFOXO cells were cultured in the presence or absence of doxycline to deplete FOXO1 and FOXO3a and the levels of FOXO4 were further reduced by transfection of FOXO4 specific siRNA. Cells were subjected to clono-genic survival assay with increasing doses of IR and MMC. Results depict the means ±SD of three repli-cates from three independent experiments **(C)** U2-18TR 12W cells were treated with the indicated siRNAs and transfected with I-SceI as

approximately 40% **(Figure 7A and Supplementary Figure 7A)**. Likewise, depleting each of the FOXOs alone resulted in reduction of HR efficiency of approximately 25-30% **(Supplementary Figure 7B)**, suggesting this to be redundant function of all FOXO members. Importantly, under the conditions employed in our study, FOXOs depletion does not impact basal survival and/or cell cycle progression **(Supplementary Figures 7C and 7D)**. We further performed clonogenic survival assays to determine the contribution of FOXOs to survival following a genotoxic insult. Treatment of HeLashFOXO cells with increasing doses of IR or Mitomycin C (MMC) resulted in significantly reduced survival and/or proliferation of the FOXO depleted cells **(Figure 7B and Supplementary Figure 7E)**.

To control for possible off-target effects of FOXO siRNA in U2OS#18 cells, we assessed whether adding back siRNA resistant FOXO4 could rescue the HR deficiency. Transient FOXO4 overexpression is known to induce an arrest in the G1 phase (50, 51). To circumvent the problem of data interpretation due to cell cycle regulatory effects, we generated a cell line stably expressing doxycycline-inducible siRNA-resistant wild type FOXO4 (U2-18TR12W cells). The cells, 48hrs post siRNA transfection, were synchronized in G1/S by a thymidine block for 24hrs. Subsequently, cells were released to the cell cycle and doxycycline was added to induce the expression of FOXO4 for 16 hrs before sample collection. We found FOXO4 add-back to partially but significantly restore the HR defect **(Figure 7C and Supplementary Figure 7F)**, without appreciable effects on the cell cycle progression **(Supplementary Figure 4G)**.

To assess whether indeed FOXO recruitment to the sites of DNA damage, or their transcriptional activity are required for their function in HR, we generated stable cell lines to perform add-back with the S462A mutant (U2-18TR12A cells) or the H156R mutant (U2-18TR12R cells) after FOXOs depletion. Add-back of FOXO4 S462A, which is not recruited to the DNA lesions, did not rescue the HR defect **(Figure 7D and Supplementary Figure 7F)**, suggesting that recruitment to the damaged chromatin is crucial for FOXOs role in HR. In agreement, add-back of wild type FOXO4 in cells co-depleted of FOXOs and NBS1 did not rescue the HR deficiency **(Supplementary Figure 7H)**. The FOXO4 H156R mutant, is unable to induce FOXO4-dependent transcription **(Supplementary Figure 7I)** however is recruited to sites of DNA damage similarly to the wild type protein. This mutant rescued the HR deficiency after FOXOs deletion **(Figure 7E and Supplementary Figure 7F)**, suggesting that FOXOs regulate HR independent of their transcriptional activity.

As our data indicate that FOXOs regulate DNA end resection by the exclusion of 53BP1 from the DNA ends, we looked into the dependency of 53BP1 for the effect of FOXOs in HR. To this end, we knocked-down either FOXOs or 53BP1 alone or together in U2OS#18 cells and assessed the effects in HR efficiency. As seen in

(Figure 7, legent continuation)
in (A). 16 hrs post I-SceI transfection cells were synchronized with thymidine for extra 24hrs and subsequently released by excessive PBS washes. Doxycycline was added for 16hrs before samples were collected and analysed by FACS. Results depict the means ±SD of 8 independent experiments **(D)** U2-18TR 12A cells were treated with the indicated siRNAs and prepared and analysed as in (C). Results depict the means ±SD of 6 independent experiments **(E)** U2-18TR12R cells were treated with the indicated siRNAs and prepared and analysed as in (C). Results depict the means ±SD of 6 independent experiments **(F)** U2OS#18 cells were transfected with the indicated siRNAs for 24 hrs before the I-SceI plasmid was transfected together with mCherry. Cells were collected 48 hrs post plasmid transfection and analysed by FACS. Results depict the means ±SD of 5 independent experiments **(G)** Proposed model of FOXOs function in homologous recombination repair. DSBs induction results in ATM activation and recruitment of the MRN complex to the sites of damage. Activated ATM phosphorylates its substrates, including the histone variant H2AX and FOXOs. Phosphorylated FOXO interacts with NBS1 and is retained to the sites of DNA damage. On sites of DNA damage FOXO facilitate the recruitment of TRRAP with a HAT complex, eventually affecting the levels of H4K16 acetylation around the DSB. Increased H4K16ac results in exclusion of 53BP1 from DSBs ends and in increased DNA-end resection by CtIP and other nucleases (for details see text) *P<0.05, **P<0.01, ***P<0.001, ns: not-significant

Figure 7F, HR was restored when FOXOs were co-depleted with 53BP1, further supporting our conclusion that FOXOs regulate HR initiation by antagonizing 53BP1.

DISCUSSION

In this study we reveal a novel role of FOXO transcription factors in the maintenance of cellular genomic stability. We report that all FOXOs exhibit similar behavior in response to DSBs and this is in agreement with the notion that FOXOs act redundantly to regulate downstream responses, as was also shown by conditional deletion of all three FOXOs in mice (52). We show here that in response to DNA damage, in particular DSBs, all FOXO proteins are phosphorylated by ATM and accumulate on the sites of DNA damage in a manner that requires ATM-mediated phosphorylation of FOXO, as well as their interaction with the MRN complex. We further show that all FOXOs bind TRRAP and, by mediating the recruitment of TRRAP to the DSBs, regulate the levels of H4K16 acetylation and thereby 53BP1 DSB-localization. Herewith, FOXOs regulate DNA-end resection and HR initiation for the error-free repair of DSBs. A model for the function of FOXO proteins in DNA repair by HR is provided in **Figure 7G**.

Several HATs were proposed to mediate histone acetylation around DSBs (53) and the scaffold protein TRRAP facilitates the assembly of HAT complexes at the sites of DNA damage (32, 34). Here, we provide mechanistic insight into the process of TRRAP-HAT recruitment to the sites of damage for HR initiation. We show that FOXOs bind to TRRAP and thereby recruit the TRRAP-HAT complex to the DSBs. TRRAP was shown to require intact MRN for its association with the DSBs (33) and interestingly, we also report here that FOXOs require binding to the MRN component NBS1 for their recruitment to sites of DNA damage. Thus, we propose that FOXOs stably associate with TRRAP and regulate epigenetic changes for transcriptional programs and, once FOXOs are recruited to the damaged chromatin by their interaction with NBS1, they mediate the recruitment of TRRAP as well. In turn, histone acetylation around DSBs was suggested to facilitate chromatin de-compaction and accessibility of repair factors, to promote HR repair (32). Moreover, the recently revisited model of “access-repair-restore” (54, 55) proposes that chromatin re-organization and epigenetic changes after DNA damage are also crucial in the regulation of the DDR signaling. Indeed, histone acetylation marks, amongst which H4K16ac, also have active roles in directing DDR signaling towards a certain repair pathway. More specifically, H4K16ac was shown to inhibit the binding of 53BP1 to the damaged chromatin, resulting in alleviation of the DNA end resection inhibition imposed by 53BP1 and thereby facilitating HR repair (35).

The role of FOXOs towards HR appears mostly modulatory, rather than essential. Intriguingly, this is similar to what was reported for other previously described components of the DDR, including TRRAP (32), (also see RHINO (56), LEDGF(p75) (57), Ino80 (58), SIRT6 (59), hnRNPU-like proteins (60), SIRT1 (61)). These factors, like FOXOs and TRRAP, show a small yet significant contribution to HR and are proteins associated with chromatin remodeling and transcriptional regulation. Considering that transcription is silenced around DNA lesions (62), the assembly of these factors at the sites of DNA damage suggests that they function in a manner independent of gene transactivation. In this study we show that FOXOs, via TRRAP, mediate the deposition of acetyl-marks on the histone tails proximal to the DSB and thereby facilitate the assembly of repair complexes for the “fine-tuning” of the response towards HR repair. Therefore, we propose that, similar to FOXO, other transcription factors and chromatin remodelers involved in DDR, may support epigenetic changes that can result in a tipping of the balance in DNA repair pathway choice.

Previous studies suggested FOXOs to act upstream of ATM, by regulating ATM transcription and ATM activation upon DNA damage (10, 63). However, contrary to these studies, we did not obtain evidence

that ATM levels or activity are significantly impaired in FOXOs depleted cells, as we could not observe changes in γH2AX and CHK2 regulation following FOXOs depletion. Importantly, we show FOXOs to function downstream of ATM, rather than upstream, and to partake in a limited domain of the whole of the ATM regulated DDR. In agreement with a previous study showing S/TQ phosphorylation of FOXO1 after DNA damage (8), we show that in response to DSBs all FOXO proteins are phosphorylated by ATM and accumulate on the sites of damage, in a manner that requires their ATM-mediated phosphorylation as well as their interaction with the MRN complex. This recruitment to the damaged chromatin is a prerequisite for the role of FOXOs in DNA repair by HR, as a mutant form that can no longer be phosphorylated by ATM and bind to NBS1, cannot regulate HR repair. The reason for this discrepancy i.e. our observed lack of significant ATM regulation by FOXO is unknown, but could be trivial e.g. due to the use of different cell types. Also, the depletion of a single (FOXO3 in previous reports) versus all FOXO proteins, as in this study, may account for the differences observed, although we find all FOXO members to be recruited to laser-micro irradiation-induced DNA damage sites, suggesting that FOXO1 and/or FOXO4 could act redundant to FOXO3 in regulating ATM. Alternatively, FOXO-mediated ATM regulation could be part of a feedback loop. However, we cannot envision how this feedback loop could take place, as the complete loss of γH2AX foci reported by Tsai et al in the FOXO3 depleted cells would prohibit NBS1 chromatin retention (64), thereby excluding FOXO from the sites of damage to regulate ATM. Moreover, how FOXO recruited to DSBs would regulate ATM activity in such a feedback loop remains enigmatic. Overall, our results reveal a role for FOXO in the DDR, downstream of ATM and in the regulation of HR.

DNA damage is strongly associated with aging, as stochastic DNA damage accumulates over time and genetic syndromes with DNA repair deficiency exhibit premature aging (1). However, delayed aging due to improved DNA repair has not yet been demonstrated and therefore it remains unclear whether the accrual of DNA mutations over time is the cause or consequence of the aging process. FOXOs are considered prototype gerontogenes, as their expression in model organisms is directly correlated with longevity. Even though most of the data linking FOXOs to aging come from studies in the post-mitotic C. elegans, FOXOs have also been associated with aging in higher eukaryotes (65-67). In this respect, the data presented in this study, showing that FOXOs positively regulate HR, may be taken to suggest that increased prevalence of error-free DNA repair can positively contribute to increased lifespan. In agreement, SIRT1, which is also positively implicated in longevity, has similarly been shown to regulate HR (61). Interestingly, recent studies suggest a switch from NHEJ to HR to occur during aging and this switch is proposed to serve as a homeostatic mechanism to ensure increased genomic stability and thereby longevity of the “aged” cells (68, 69). Moreover, premature aging in DNA repair-deficient mice is associated with gene expression changes that reflect reduced signaling by the somatotrophic axis (IGF1/GH) (70). Reduced IGF1 signaling results in FOXO activation and this could be taken to suggest that FOXO-dependent protective mechanisms are activated in this manner to reduce DNA damage load. FOXOs can reduce DNA damage due to control of the cellular redox state or, as our present findings suggest, FOXOs, through error-free DNA repair, are part of a reciprocal feedback system that acts as a homeostatic mechanism to prevent excessive DNA damage.

Apart from the implications for the role of FOXOs in aging, our findings also add a new layer to the mechanism by which FOXOs act as tumor suppressors; by promoting faithful DNA repair and maintaining genome stability. Interestingly, this function of FOXOs appears to be independent of their established role as transcription factors, as HR is regulated to the same extend by the H156R mutant as by the wild type protein. Moreover, genome-wide transcriptional profiling in HeLashFOXO cells after IR treatment did not identify any FOXO-dependent transcriptional changes linked to HR repair (**Addendum**). Another transcription-

independent function of FOXOs in regulating tumorigenesis has been recently reported; Zhao et al showed that cytosolic FOXO1 induces autophagy in response to stress signals, eventually regulating tumor growth, independently of its binding to DNA elements (71). Additionally, other transcriptional regulators have been shown to regulate DNA repair, independently of gene transactivation. P53 was shown to inhibit, whereas NF-kappa B was found to activate HR, by means of direct regulation of DNA repair complexes rather than gene transcription (72, 73).

Pathways regulating FOXO subcellular localization and function are commonly de-regulated in human cancers. PTEN phosphatase and PKB kinase are often mutated in spontaneous arising tumors, resulting in overactivation of the PI3K/PKB pathway and inactivation of FOXOs (74). More specifically, upon PKB phosphorylation, FOXOs are retained in the cytoplasm (75, 76), thus making it impossible to access sites of DNA damage and mediate end resection and HR for DSBs repair. In support of this, cancer cells with hyperactive PI3K/PKB pathway are characterized by increased genomic instability and low rates of HR (77). Thus, an intriguing hypothesis is that under these conditions, the observed genomic instability can also be attributed to FOXO cytoplasmic retention. Interestingly, sporadic breast cancers with hyperactive PI3K/PKB pathway exhibit "BRCAness" phenotypes (78), with a higher prevalence of basal-like triple negative tumors (79). In line with this, we find that FOXOs regulate DNA end resection in a way similar to BRCA1, by regulating the exclusion of 53BP1 from the DSBs. Importantly, the sensitivity of cells with FOXOs depletion on agents such as IR and MMC opens new possibilities for the successful treatment of patients with mutations in components of the PI3K/PKB pathway. In fact, a recent study in prostate cancer cells defective in PTEN showed increased susceptibility of the PTEN-/- genotype to these agents (80).

Methods

DNA constructs and primers

The S462A and the H156R FOXO4 mutants were generated by site directed mutagenesis with Phusion™ High-Fidelity DNA Polymerase (Finnzymes) and the primers used were the following: S462AFW: 5'-ccctactgaagctgcagcccaagacagaatgc-3', S462ARev: 5'-gggatgacttcgacgtcgggttctgtcttacg-3', H156RFW: 5'-ggaagaactcgatccgccggaacctgtccctgcacagc-3', H156RRev: 5'-gctgtgcagggacaggttccggcggatcgagttcttcc-3'. The GLOFLAG3-Flag-FOXO4 and GFP-FOXO4 constructs were previously described (80).

For the cloning of the FOXO1 and FOXO3a inducible shRNA, the pH1tet-flex/FH1t(INSR)UTG vector system (Taconic Artemis) was utilized. The short hairpin targeting FOXO1 and FOXO3a, flanked by BbsI and XhoI recognition sequences (sequence: 5'- tcccgtgccctacttcaaggataagttcaagagacttatccttgaagtagggcacttttttctcgag-3') was first inserted in the pH1tet-flex vector digested with BbsI/XhoI. Subsequently, the H1 tetO-shRNA cassette was amplified with primers introducing PacI sites. After digestion with PacI, the H1tet-shRNA was inserted in the FH1t(INSR)UTG vector. Flag-ATM (81) and pHA-ER-PpoI (21) were kind gifts of Prof. M. Kastan (Duke University Cancer Institute) and pBMN-FKBP-L106P-YFP (82) was from Dr. Wandless (Stanford University). DNA fragments coding for the degradation domain FKBP-L106P and for HA-tagged PpoI endonuclease were subcloned in frame in BamHI/XbaI sites of pcDNA4TO (Invitrogen) resulting in pcDNA4TO-FKBP-PpoI plasmid that allows induction of PpoI expression by combination of the tetracycline and proteasomal inhibitor treatment.

Cell culture, RNA interference and treatments

The human osteosarcoma U2-OS, the human embryonic kidney HEK293T cells and the cervical HeLa cells (ATCC) were cultured in DMEM (Gibco) supplemented with 10% FBS, penicillin-streptomycin and glutamine. AT cells (GM05849, Corriel Institute) and RPE cells were grown in EMEM and DMEM/Ham F12 respectively. U2-OS#18 with the HR reporter (47) were a kind gift of Prof. Roland Kanaar (Erasmus MC, Rotterdam). To generate the U2-18TR12 cell line, U2-OS#18 cells were transfected with pcDNA6 TR (Invitrogen) and stable transformants were selected with 5 μg/ml blasticidin. The U2-18TR12 derived cell lines expressing the wild type and mutant forms of FOXO4 were generated by transfection of

pcDNA4TO-FOXO4 constructs and subsequent selection with zeocine (Invitrogen) for approximately 2 weeks. U2OS cells stably expressing the Tet repressor were transfected with pcDNA4TO-FKBP-PpoI and selected by treatment with zeocine and subsequent clonal selection. For expression of PpoI endonuclease, cells were induced with doxycycline for 16hrs and a proteasomal inhibitor MG132 was added for the last two hours before harvesting. The HeLashFOXO cells, for FOXO1 and FOXO3a inducible silencing, were generated by lentiviral delivery of the FH1t(FOXO1/3)UTG plasmid in HeLa cells and FACS sorting of the GFP positive cells. For the generation of HeLashFOXO-PPO cells, the pBABE-HA-ER-I-PpoI construct was delivered to HeLashFOXO cells by retroviral transduction and transformed cells were selected with puromycin for 2 weeks. A14-GFP-FOXO4 cells were a kind gift of M. van Triest.

The FOXO1 and FOXO3a targeting siRNA was designed based on a homologous region between the two mRNAs (5'-gugcccuacuucaaggauaag-3') and the FOXO4 siRNA was designed to target region in the 3'UTR of human FOXO4 (5'-agucaugccuggaagcuuu-3'). A siRNA targeting luciferase was used as negative control. All siRNAs were synthesized by Dharmacon research. Transfection of cells with the above siRNA oligonucleotides was performed with Hyperfect (Qiagen) according to the manufacturer's instructions.

Ionizing radiation was delivered by a Cs-137 source; ATM kinase activity was inhibited by the selective ATM inhibitor Ku-55933 (Merck chemicals) used at 10 μM and added to the cells 1 hr prior to treatments; caffeine (Sigma Aldrich) was used at 5 mM for 4 hrs prior to DNA damage induction; TSA (Sigma Aldrich) was used at 0.5 μM for 18hrs or 5 μM for 6 hrs before sample collection.

Cell lysis and Western blot

For co-immunoprecipitation studies cells were lysed in PKB lysis buffer (50 mM Tris pH 7.5, 200 mM NaCl, 1% NP40, 1 mM EDTA, 1 mM Na3VO4, 50 mM NaF, 1 μM leupeptin, 0.1 μM aprotinin) supplemented with benzonase (Merck). After lysing at 4ºC for 30 min, the lysates were pre-cleared by centrifugation at 14,000 rpm for 10 min. Subsequently, proteins were immunoprecipitated with anti-GFP-trapA beads (ChromoTek) or with anti-Flag beads (Sigma-Aldrich) or with Protein A-Sepharose beads (GE Healthcare) conjugated with the antibody of interest. For Chromatin Fractionation, soluble cytosolic proteins were extracted by incubating cells in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 0.1% Triton X-100, 1 mM Na3VO4, 50 mM NaF, 1 μM leupeptin, 0.1 μM aprotinin) at 4°C for 5 min and spinning down at 1,300xg for 4 min. The soluble nuclear fraction was obtained by extraction of pelleted nuclei with buffer B (10 mM HEPES, pH 7.9, 3 mM EDTA, 0.2mM EGTA, 1mM DTT, 1 mM Na3VO4, 50 mM NaF, 1 μM leupeptin, 0.1 μM aprotinin) and spinning down at 2,000xg for 4 min. Finally, insoluble chromatin was washed with buffer B, re-suspended in SDS sample buffer and briefly sonicated. Total cell extracts were prepared by scraping cells in Laemmli sample buffer.

Proteins were run on SDS-PAGE and transferred to PVDF membrane. Antibodies used were: rabbit anti-ATM/ATR substrate antibody (Cell Signaling), rabbit anti-FOXO4 pS462, rabbit anti-phosphorylated CHK2 (Thr68, Cell Signaling), rabbit anti-phosphorylated CHK1 (Ser345, Cell Signaling), mouse anti-Flag (Sigma-Aldrich), mouse anti-GFP (Rosche), rabbit anti-53BP1 (Santa Cruz), rabbit anti-NBS1 (Novus Biologicals), goat anti-TRRAP (Santa Cruz), rabbit anti-MRE11 (83), rabbit MDC1 (Abcam), mouse anti-RPA (Abcam), rabbit anti-phosphorylated RPA (S4/S8, Abcam) and rabbit anti-Rad51 (Santa Cruz).

Homologous recombination assay

The U2-OS#18 cell line with the "nested intron" reporter for the study of sister chromatid recombination was previously described (47). 24hrs after siRNA transfection, cells were transfected with an I-SceI expressing vector along with pcDNA3.0-mcherry. 48hrs later, cells were collected and analysed by FACS, to determine the percentage of cells positive for both GFP and mcherry.

For the FOXO4 add-back experiments, the U2-18TR12-W (with inducible expression of wild type FOXO4), U2-18TR12-A (S462A FOXO4) and the U2-18TR12-R (H156R FOXO4) cell lines were used. To correct for possible differences in the cell cycle status between different samples, samples were prepared as following: 16 hrs post I-SceI transfection, cells were synchronized in G1/S with thymidine for 24 hrs. Subsequently cells were released in cycle by extensive washing with PBS and doxycycline was added to the medium 16 hrs prior to FACS measurements.

Laser micro-irradiation, FRAP and immunofluorescence

For induction of DNA damage at defined subnuclear regions, cells were grown in glass bottomed dishes (WillCo Wells). Prior to micro-irradiation, cells were pre-sensitized with 0.5 mg/ml Hoechst 33342 (Invitrogen) for 30 min at 37ºC. Local damage was induced by a 405 nm laser diode (4 mW, 50% power, 50 iterations) of a Zeiss LSM510 laser-scanning microscope, equipped with a ×40 NA 1.3 objective. The kinetics of recruitment of the GFP-tagged FOXOs to sites of DNA damage was determined by the relative fluorescence (RF) (25), which was calculated as RF(t)=(It–IpreIR)/ Imax–IpreIR), where It is the fluorescence intensity at each time point of measurement, IpreIR the fluorescence intensity at the laser exposed areas immediately before micro-irradiation and Imax the maximum fluorescence at the end of the measurements. To compare the kinetics of different FOXO4 mutants, as well as the effect of silencing different components of the DDR on FOXO recruitment, we determined the fold increase of GFP fluorescence within the microirradiated areas (RFfold) (25), which was calculated as RFfold(t) = It/IpreIR, where IpreIR is again the fluorescence intensity measured in the laser-exposed areas immediately before irradiation. Values were corrected for non-specific fluorescence bleaching.

Fluorescence recovery after photobleaching was performed by bleaching selected areas with a 488 nm laser (100% power, 50 iterations) and imaging in 1 sec intervals. An unbleached region of the same size was used as a control.

For immunofluorescence studies, cells grown on glass coverslips were fixed in 4% formaldehyde (Merck Chemicals) for 15 min at room temperature, permeabilized in 0.2% Triton X-100, and immunostained with the combination of antibodies specified in the figure legends. Where indicated, the cells were pre-extracted before fixation for 5 min at 4ºC in the CSK buffer (10 mM PIPES pH 7.0, 50 mM NaCl, 300 mM sucrose, 2 mM MgCl2, 3 mM CaCl2, 0.5% (v/v) Triton X-100). Confocal fluorescence images were captured using a Zeiss LSM510 laser-scanning microscope.

SILAC

For SILAC labeling, A14 cells were cultured in medium consisting of SILAC DMEM without arginine, lysine and glutamine (PAA, E15-086), supplemented with, 10% dialized FBS (Gibco), penicillin-streptomycin, glutamine, 73 µg/ ml L-Lysine (light/ K0 (Sigma, A6969) or heavy/K8 (Sigma, 608041 or Silantes, 211603902)) and 29.4 µg/ ml arginine light/R0 (Sigma, A6969) or heavy/R10 (Sigma, 608033 or Silantes, 201603902)). Cells were cultured in SILAC medium until labeling efficiency exceeded 95%.

Cells were treated with 200 µM H2O2 for 30 min and subsequently nuclear extracts were prepared. Briefly, cells were trypsinized, subsequently the cells were swollen using a hypotonic buffer and lysed by dounce homogenizing in the presence of 0.15% NP40 and complete protease inhibitors. After centrifugation, the pellet consisting of nuclei was lysed by 90 min incubation in 2 volumes of nuclear lysis buffer (420mM NaCl, 20mM HEPES pH 7.9, 20% v/v glycerol, 2mM MgCl2, 0.2mM EDTA, 0.1% NP40, complete protease inhibitor w/o EDTA (Roche) and 0.5mM DTT). GFP-pulldowns were performed with GFP-Trap_A beads (Chromotek). Proteins were eluted from the GFP-beads with 0.1M Glycine pH2.5 and subsequently trypsinized on FASP columns (30 kD columns). The FASP procedure is performed as described previously (84). Peptides were separated using an EASY-nLC (Proxeon) connected online to an LTQ-Orbitrap Velos mass spectrometer (Thermo) as described (85). Raw data were analyzed using MaxQuant version 1.2.2.5 and mapped using protein database IPI mouse V3.68 fasta. Resulting protein groups were further analyzed using Perseus. Identifications were filtered for standard contaminants, reverse hits, number of peptides (>1) and unique peptides (>0). Ratios were logarithmitized (log2) and groups (consisting of forward and reverse) were defined. Scatter plots were made using a custom R script.

Chromatin immunoprecipitation (ChIP) and quantitative PCR (Q-PCR)

To perform ChIP, 40 million cells per condition were fixed with 1% formaldehyde for 10 min at room temperature and the reaction was quenced by 0.125 M Glycine for 5 min. Subsequently cells were lysed in Darnham lysis buffer (5 mM PIPES, 85 mM KCl, 0.5% NP-40, protease inhibitor cocktail Roche) and nuclear extracts were collected by low speed centrifugation and re-suspended in RIPA buffer (1x PBS, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, supplemented with Roche protease inhibitor cocktail). Chromatin was then sonicated to acquire DNA fragments of about 500-1,000 bp which were then used for immunoprecipitations. Antibodies used were: rabbit IgG (Santa Cruz), rabbit anti-RPA (Millipore), rabbit anti-Rad51 (Santa-Cruz), rabbit anti-H4 (Abcam), rabbit anti-H4K20me2 (Active Motif), rabbit anti-H4K16ac (Active Motif), rabbit anti-TRRAP (abcam) and rabbit anti-FOXO3a (Santa-Cruz).

Antibody generation

To prepare antibodies directed against phosphoSer462 of FOXO4, rabbits were immunized with the peptide PTEAAS(phospho)QDRMPQDLDC. The peptide was synthesized in house using Fmoc chemistry, analysed with LC-MS and coupled to KLH (Thermo). The conjugated peptide was diluted in either Complete or Incomplete Freund's adjuvant (Thermo) and injected every three weeks for a total of 4 times. The resulting antibody was purified from serum using a smaller epitope tagged with a biotinylated lysine (AAS(phospho)QDRK(Biotin)) coupled to streptavidine-agarose beads (Thermo).

Clonogenic survival assay

To assess genotoxic stress sensitivity, HeLashFOXO cells were either untreated or treated with doxycycline for 48 hrs before they were plated in limited amount. Sixteen hrs post-plating, cells were treated with increasing amounts of ionizing radiation or Mitomycin C. Cells were left to form colonies for 10–14 days at 37 °C and doxycycline was refreshed every 4 days. Colonies were stained with 0.5% (v/v) crystal violet and counted. Results were normalized to plating efficiencies of untreated cells.

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P.C. designed and performed experiments and co-wrote the manuscript, L.S. and M. v T. performed experiments, H.R.V. generated the pS462 antiserum and performed the FOXO SILAC, M. vdB. performed the FOXO SILAC, L.M and R.H.M provided important reagents, designed experiments and discussed results, B.M.T.B. designed experiments and co-wrote the manuscript

References

1. Hoeijmakers JHJ (2009) DNA Damage, Aging, and Cancer. New England Journal of Medicine 361(15):1475-1485.
2. Ciccia A & Elledge SJ (2010) The DNA Damage Response: Making It Safe to Play with Knives. Molecular Cell 40(2):179-204.
3. Wyman C & Kanaar R (2006) DNA Double-Strand Break Repair: All's Well that Ends Well. Annual Review of Genetics 40(1):363-383.
4. Freitas AA & de Magalhaes JP (2011) A review and appraisal of the DNA damage theory of ageing. Mutation Research/Reviews in Mutation Research 728(1â€“2):12-22.
5. Schumacher Br, Hoeijmakers JH, & Garinis GA (2009) Sealing the gap between nuclear DNA damage and longevity. Molecular and Cellular Endocrinology 299(1):112-117.
6. van der Horst A & Burgering BMT (2007) Stressing the role of FoxO proteins in lifespan and disease. Nat Rev Mol Cell Biol 8(6):440-450.
7. Charitou P & Burgering BM (2013) Forkhead Box(O) in Control of Reactive Oxygen Species and Genomic Stability to Ensure Healthy Lifespan Antioxidants & Redox Signaling 19(12):1400-1419.
8. Matsuoka S, et al. (2007) ATM and ATR Substrate Analysis Reveals Extensive Protein Networks Responsive to DNA Damage. Science 316(5828):1160-1166.
9. Tran H, et al. (2002) DNA Repair Pathway Stimulated by the Forkhead Transcription Factor FOXO3a Through the Gadd45 Protein. Science 296(5567):530-534.
10. Tsai W-B, Chung YM, Takahashi Y, Xu Z, & Hu MCT (2008) Functional interaction between FOXO3a and ATM regulates DNA damage response. Nat Cell Biol 10(4):460-467.
11. Huang H, Regan KM, Lou Z, Chen J, & Tindall DJ (2006) CDK2-Dependent Phosphorylation of FOXO1 as an Apoptotic Response to DNA Damage. Science 314(5797):294-297.

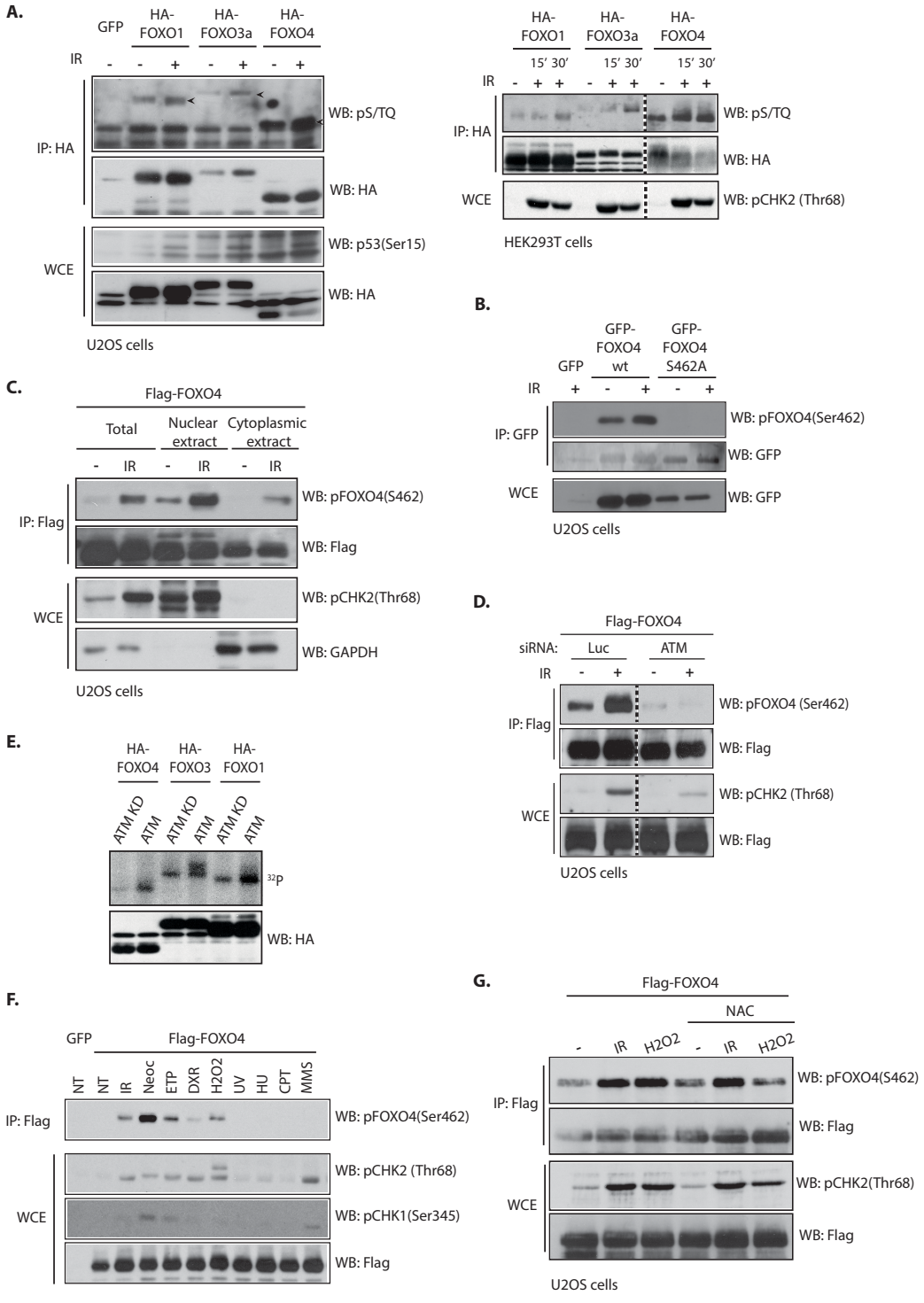
12.Lei H & Quelle FW (2009) FOXO Transcription Factors Enforce Cell Cycle Checkpoints and Promote Survival of Hematopoietic Cells after DNA Damage. Molecular Cancer Research 7(8):1294-1303.
13.Stracker TH & Petrini JHJ (2011) The MRE11 complex: starting from the ends. Nat Rev Mol Cell Biol 12(2):90-103.
14.Kim S-T, Lim D-S, Canman CE, & Kastan MB (1999) Substrate Specificities and Identification of Putative Substrates of ATM Kinase Family Members. Journal of Biological Chemistry 274(53):37538-37543.
15.O'Neill T, et al. (2000) Utilization of Oriented Peptide Libraries to Identify Substrate Motifs Selected by ATM. Journal of Biological Chemistry 275(30):22719-22727.
16.Sarkaria JN, et al. (1999) Inhibition of ATM and ATR Kinase Activities by the Radiosensitizing Agent, Caffeine. Cancer Research 59(17):4375-4382.
17.Hickson I, et al. (2004) Identification and Characterization of a Novel and Specific Inhibitor of the Ataxia-Telangiectasia Mutated Kinase ATM. Cancer Research 64(24):9152-9159.
18.Shiloh Y (2003) ATM and related protein kinases: safeguarding genome integrity. Nat Rev Cancer 3(3):155-168.
19.Bhatti S., et al. (2011) ATM protein kinase: the linchpin of cellular defenses to stress. Cellular and Molecular Life Sciences 68(18):2977-3006.
20.Guo Z, Kozlov S, Lavin MF, Person MD, & Paull TT (2010) ATM Activation by Oxidative Stress. Science 330(6003):517-521.
21.Berkovich E, Monnat RJ, & Kastan MB (2007) Roles of ATM and NBS1 in chromatin structure modulation and DNA double-strand break repair. Nat Cell Biol 9(6):683-690.
22.Lukas J, Lukas C, & Bartek J (2011) More than just a focus: The chromatin response to DNA damage and its role in genome integrity maintenance. Nat Cell Biol 13(10):1161-1169.
23.Jackson SP & Bartek J (2009) The DNA-damage response in human biology and disease. Nature 461(7267):1071-1078.
24.Chung YM, et al. (2013) FOXO3 signalling links ATM to the p53 apoptotic pathway following DNA damage. Nat Commun 3:1000.
25.Lukas C, et al. (2004) Mdc1 couples DNA double-strand break recognition by Nbs1 with its H2AX-dependent chromatin retention. EMBO J 23(13):2674-2683.
26.Tang ED, Nunez G, Barr FG, & Guan K-L (1999) Negative Regulation of the Forkhead Transcription Factor FKHR by Akt. Journal of Biological Chemistry 274(24):16741-16746.
27.Yu X, Chini CCS, He M, Mer G, & Chen J (2003) The BRCT Domain Is a Phospho-Protein Binding Domain. Science 302(5645):639-642.
28.Manke IA, Lowery DM, Nguyen A, & Yaffe MB (2003) BRCT Repeats As Phosphopeptide-Binding Modules Involved in Protein Targeting. Science 302(5645):636-639.
29.Haince J-Fo, et al. (2008) PARP1-dependent Kinetics of Recruitment of MRE11 and NBS1 Proteins to Multiple DNA Damage Sites. Journal of Biological Chemistry 283(2):1197-1208.
30.Hari FJ, Spycher C, Jungmichel S, Pavic L, & Stucki M (2010) A divalent FHA/BRCT□binding mechanism couples the MRE11/RAD50/NBS1 complex to damaged chromatin. EMBO reports 11(5):387-392.
31.Polo SE & Jackson SP (2011) Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. Genes & Development 25(5):409-433.
32.Murr R, et al. (2006) Histone acetylation by Trrap-Tip60 modulates loading of repair proteins and repair of DNA double-strand breaks. Nat Cell Biol 8(1):91-99.
33.Robert F, et al. (2006) The Transcriptional Histone Acetyltransferase Cofactor TRRAP Associates with the MRN Repair Complex and Plays a Role in DNA Double-Strand Break Repair. Molecular and Cellular Biology 26(2):402-412.
34.Murr R, Vaissiere T, Sawan C, Shukla V, & Herceg Z (2007) Orchestration of chromatin-based processes: mind the TRRAP. Oncogene 26(37):5358-5372.
35.Tang J, et al. (2013) Acetylation limits 53BP1 association with damaged chromatin to promote homologous recombination. Nature Structural & Molecular Biology 20(3):317-325.
36.Huen MSY, et al. (2007) RNF8 Transduces the DNA-Damage Signal via Histone Ubiquitylation and Checkpoint Protein Assembly. Cell 131(5):901-914.
37.Botuyan MV, et al. (2006) Structural Basis for the Methylation State-Specific Recognition of Histone H4-K20 by 53BP1 and Crb2 in DNA Repair. Cell 127(7):1361-1373.
38.Huyen Y, et al. (2004) Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks. Nature 432(7015):406-411.
39.Kolas NK, et al. (2007) Orchestration of the DNA-Damage Response by the RNF8 Ubiquitin Ligase. Science 318(5856):1637-1640.
40.Mailand N, et al. (2007) RNF8 Ubiquitylates Histones at DNA Double-Strand Breaks and Promotes Assembly of Repair Proteins. Cell 131(5):887-900.
41.Hsiao K-Y & Mizzen CA (2013) Histone H4 deacetylation facilitates 53BP1 DNA damage signaling and double-strand break repair. Journal of Molecular Cell Biology.
42.Bouwman P, et al. (2010) 53BP1 loss rescues BRCA1 deficiency and is associated with triple-negative and BRCA-mutated breast cancers. Nat Struct Mol Biol 17(6):688-695.
43.Bunting SF, et al. (2010) 53BP1 Inhibits Homologous Recombination in Brca1-Deficient Cells by Blocking Resection of DNA Breaks. Cell 141(2):243-254.
44.Heyer W-D, Ehmsen KT, & Liu J (2010) Regulation of Homologous Recombination in Eukaryotes. Annual Review of

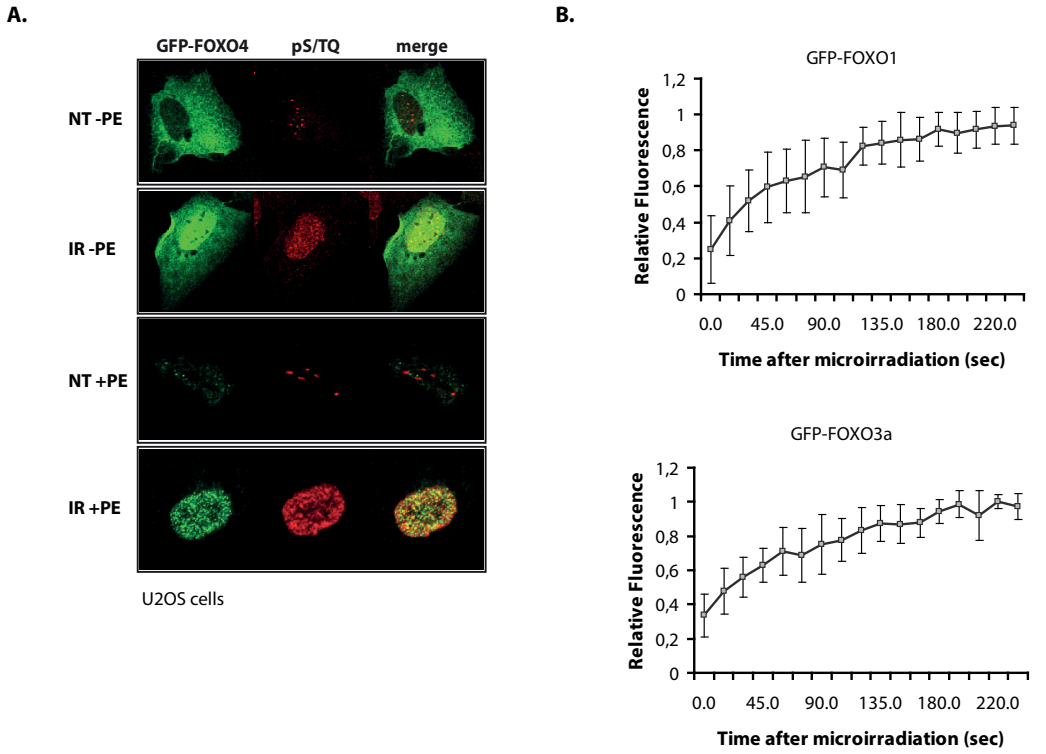
Genetics 44(1):113-139.
45.Shao R-G, et al. (1999) Replication-mediated DNA damage by camptothecin induces phosphorylation of RPA by DNA-dependent protein kinase and dissociates RPA:DNA-PK complexes. EMBO J 18(5):1397-1406.
46.Zou L & Elledge SJ (2003) Sensing DNA Damage Through ATRIP Recognition of RPA-ssDNA Complexes. Science 300(5625):1542-1548.
47.Shiotani B & Zou L (2009) Single-Stranded DNA Orchestrates an ATM-to-ATR Switch at DNA Breaks. Molecular Cell 33(5):547-558.
48.Sartori AA, et al. (2007) Human CtIP promotes DNA end resection. Nature 450(7169):509-514.
49.Puget N, Knowlton M, & Scully R (2005) Molecular analysis of sister chromatid recombination in mammalian cells. DNA Repair 4(2):149-161.
50.Medema RH, Kops GJPL, Bos JL, & Burgering BMT (2000) AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature 404(6779):782-787.
51.Kops GJPL, et al. (2002) Control of Cell Cycle Exit and Entry by Protein Kinase B-Regulated Forkhead Transcription Factors. Molecular and Cellular Biology 22(7):2025-2036.
52.Paik J-H, et al. (2007) FoxOs Are Lineage-Restricted Redundant Tumor Suppressors and Regulate Endothelial Cell Homeostasis. Cell 128(2):309-323.
53.Gong F & Miller KM (2013) Mammalian DNA repair: HATs and HDACs make their mark through histone acetylation. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis (0).
54.Smerdon MJ (1991) DNA repair and the role of chromatin structure. Current Opinion in Cell Biology 3(3):422-428.
55.Soria G, Polo Sophie E, & Almouzni G (2012) Prime, Repair, Restore: The Active Role of Chromatin in the DNA Damage Response. Molecular Cell 46(6):722-734.
56.Cotta-Ramusino C, et al. (2011) A DNA Damage Response Screen Identifies RHINO, a 9-1-1 and TopBP1 Interacting Protein Required for ATR Signaling. Science 332(6035):1313-1317.
57.Daugaard M, et al. (2012) LEDGF (p75) promotes DNA-end resection and homologous recombination. Nat Struct Mol Biol 19(8):803-810.
58.Gospodinov A, et al. (2011) Mammalian Ino80 Mediates Double-Strand Break Repair through Its Role in DNA End Strand Resection. Molecular and Cellular Biology 31(23):4735-4745.
59.Kaidi A, Weinert BT, Choudhary C, & Jackson SP (2010) Human SIRT6 Promotes DNA End Resection Through CtIP Deacetylation. Science 329(5997):1348-1353.
60.Polo Sophie E, et al. (2012) Regulation of DNA-End Resection by hnRNPU-like Proteins Promotes DNA Double-Strand Break Signaling and Repair. Molecular Cell 45(4):505-516.
61.Uhl M, et al. (2010) Role of SIRT1 in homologous recombination. DNA Repair 9(4):383-393.
62.Shanbhag NM, Rafalska-Metcalf IU, Balane-Bolivar C, Janicki SM, & Greenberg RA (2010) ATM-Dependent Chromatin Changes Silence Transcription In cis to DNA Double-Strand Breaks. Cell 141(6):970-981.
63.Yalcin S, et al. (2008) Foxo3 Is Essential for the Regulation of Ataxia Telangiectasia Mutated and Oxidative Stress-mediated Homeostasis of Hematopoietic Stem Cells. Journal of Biological Chemistry 283(37):25692-25705.
64.Celeste A, et al. (2002) Genomic Instability in Mice Lacking Histone H2AX. Science 296(5569):922-927.
65.Willcox BJ, et al. (2008) FOXO3A genotype is strongly associated with human longevity. Proceedings of the National Academy of Sciences 105(37):13987-13992.
66.Flachsbart F, et al. (2009) Association of FOXO3A variation with human longevity confirmed in German centenarians. Proceedings of the National Academy of Sciences 106(8):2700-2705.
67.Kuningas M, et al. (2007) Haplotypes in the human Foxo1a and Foxo3a genes; impact on disease and mortality at old age. Eur J Hum Genet 15(3):294-301.
68.Engels WR, Johnson-Schlitz DM, Flores C, White L, & Preston C (2007) A Third Link connecting Aging with Double Strand Break Repair. Cell Cycle 6(2):131-135.
69.Gorbunova V & Seluanov A (2005) Making ends meet in old age: DSB repair and aging. Mechanisms of Ageing and Development 126(6â€“7):621-628.
70.Niedernhofer LJ, et al. (2006) A new progeroid syndrome reveals that genotoxic stress suppresses the somatotroph axis. Nature 444(7122):1038-1043.
71.Zhao Y, et al. (2010) Cytosolic FoxO1 is essential for the induction of autophagy and tumour suppressor activity. Nat Cell Biol 12(7):665-675.
72.Volcic M, et al. (2012) NF-kB regulates DNA double-strand break repair in conjunction with BRCA1-CtIP complexes. Nucleic Acids Research 40(1):181-195.
73.Bertrand P, Saintigny Y, & Lopez BS (2004) p53's double life: transactivation-independent repression of homologous recombination. Trends in Genetics 20(6):235-243.
74.Liu P, Cheng H, Roberts TM, & Zhao JJ (2009) Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov 8(8):627-644.
75.Brunet A, et al. (1999) Akt Promotes Cell Survival by Phosphorylating and Inhibiting a Forkhead Transcription Factor. Cell 96(6):857-868.
76.Kops GJPL, et al. (1999) Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature 398(6728):630-634.
77.Guirouilh-Barbat J, Wilhelm T, & Lopez BS (2010) AKT1/BRCA1 in the control of homologous recombination and genetic

stability: the missing link between hereditary and sporadic breast cancers. Oncotarget 1(8).
78.Turner N, Tutt A, & Ashworth A (2004) Hallmarks of 'BRCAness' in sporadic cancers. Nat Rev Cancer 4(10):814-819.
79.Moulder SL (2010) Does the PI3K Pathway Play a Role in Basal Breast Cancer? Clinical breast cancer 10:S66-S71.
80.Fraser M, et al. (2012) PTEN Deletion in Prostate Cancer Cells Does Not Associate with Loss of RAD51 Function: Implications for Radiotherapy and Chemotherapy. Clinical Cancer Research 18(4):1015-1027.

Figure S1 (related to Figure 1). (A) U2OS and HEK293T cells transiently transfected with HA-FOXO1, HA-FOXO3a and HA-FOXO4 were subjected to IR (5 Gy). Immunoblots were prepared as in (Figure 1A) **(B)** Validation of the specificity of the FOXO4 pS462 antibody. U2OS cells were transiently transfected with GFP-FOXO4 and GFP-FOXO4 S462A and were subjected to IR (5 Gy, 30 min) before sample collection **(C)** U2OS cells transiently transfected with Flag-FOXO4 were treated with IR (5Gy) and 30min post-irradiation cells were collected and subjected to nuclear -cytoplasmic fractionation **(D)** U2OS cells were transfected with siRNAs against luciferase or ATM for 72 hrs before IR treatment and sample preparation. Dashed lines indicate discontinuous sections of the same gel **(E)** ATM and ATM KD expressed in HEK293T cells were isolated and utilized for in vitro kinase assay with FOXO1 and FOXO3 as substrates **(F)** U2OS cells transiently transfected with Flag-FOXO4 were treated with the indicated DNA damaging agents: IR 5 Gy for 30min, Neocarzinostatin (Neoc) 200 ng/µl for 1hr, Etoposide (ETP) 20 µM for 1hr, Doxorubicin (DXR) 1 µM for 1hr, H2O2 100µM for 30min, UV 20 J/m2 for 2hrs, Hydroxyurea (HU) 4mM for 4hrs, Camptothecin (CPT) 1 µM for 1hr and Methyl methanesulfonate (MMS) 1 mM for 1 hr **(G)** U2OS cells were transfected with Flag FOXO4 and 24hrs post transfection were treated or not for 16 hrs with NAC. Subsequently, cells were treated with 5 Gy IR or 100 µM H2O2 for 30min

SUPPLEMENTARY DATA





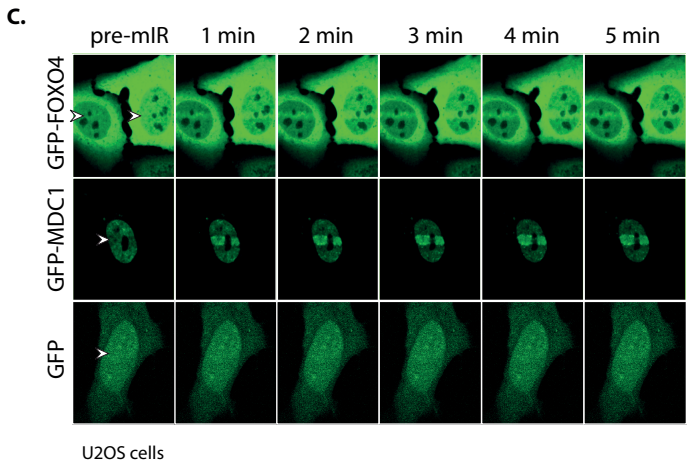


Figure S2 (related to Figure 2). (A) GFP-FOXO4 is recruited to sites of DNA damage induced by IR (5Gy, 30min). Cells were either pre-extracted (PE) or not prior to fixation and indirect immunofluorescence for GFP and ATM/ATR substrate (phosphoS/TQ) **(B)** Kinetics of GFP-FOXO1 and GFP-FOXO3a recruitment; U2OS cells transfected with the indicated plasmids were subjected to laser micro-irradiation. GFP fluorescence intensity was measured over a period of 5 min. Measurements are mean ±SD from 40 cells **(C)** U2OS cells transfected with GFP-FOXO4, GFP-MDC1 and GFP were subjected to laser micro-irradiation. Images were captured over a period of 5 min. Arrowhead indicates site of laser micro-irradiation.

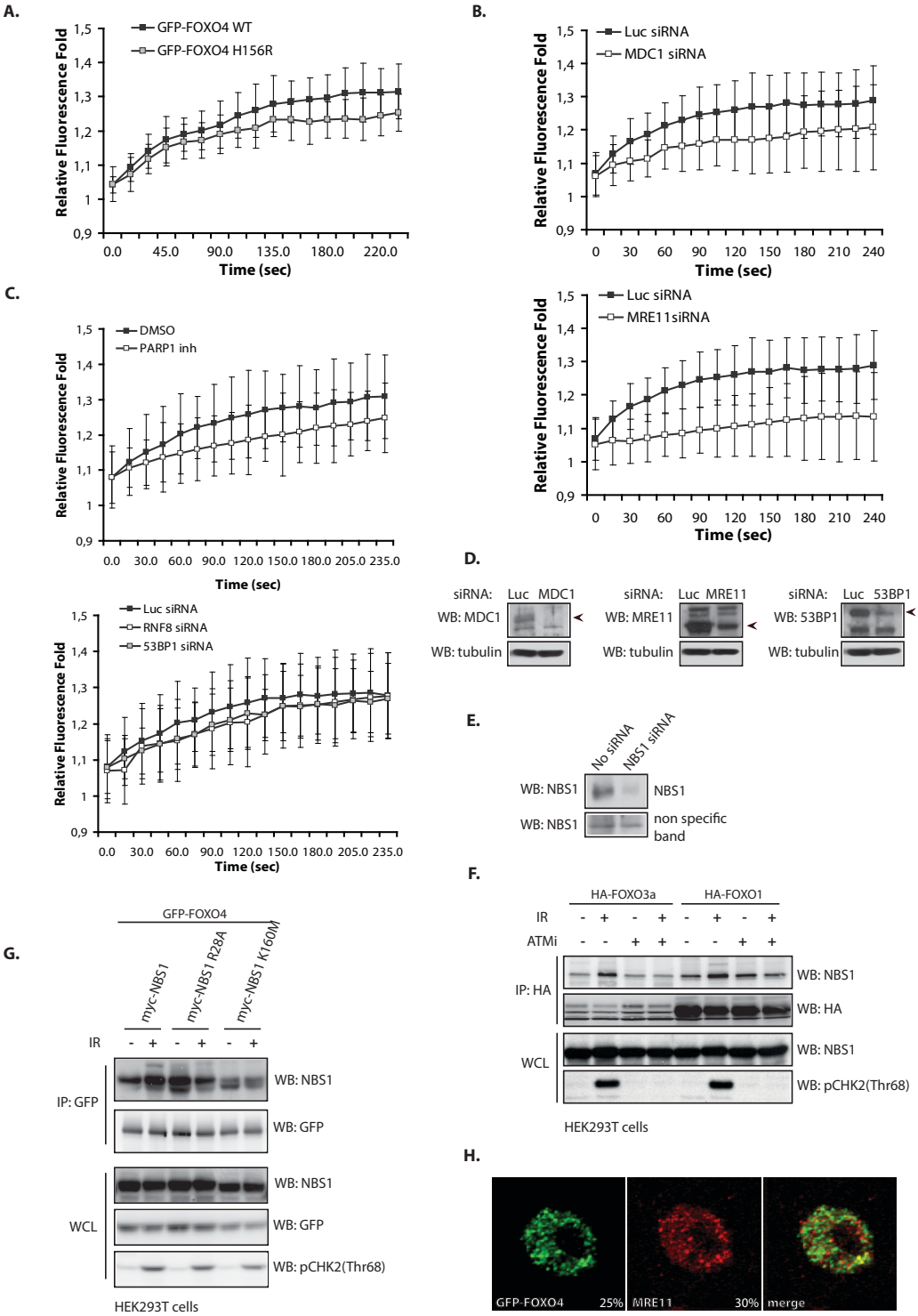

A.
GFP-FOXO4 WT
GFP-FOXO4 H156R
Relative Fluorescence Fold
Time (sec)
B.
Luc siRNA
MDC1 siRNA
Luc siRNA
MRE11siRNA
C.
DMSO
PARP1 inh
Luc siRNA
RNF8 siRNA
53BP1 siRNA
D.
siRNA: Luc MDC1
WB: MDC1
WB: tubulin
siRNA: Luc MRE11
WB: MRE11
siRNA: Luc 53BP1
WB: 53BP1
E.
No siRNA
NBS1 siRNA
WB: NBS1
NBS1
non specific band
F.
HA-FOXO3a
HA-FOXO1
IR
ATMi
IP: HA
WCL
WB: NBS1
WB: HA
WB: pCHK2(Thr68)
HEK293T cells
G.
GFP-FOXO4
myc-NBS1
myc-NBS1 R28A
myc-NBS1 K160M
IP: GFP
WB: GFP
HEK293T cells
NBS1 R28A : FHA*
NBS1 K160M: BRCT *
H.
GFP-FOXO4 25%
MRE11 30%
merge

Figure S3 (related to Figure 3). (A) Kinetics of GFP-FOXO4 H156R recruitment; U2OS cells transfected with the indicated plasmid were subjected to laser micro-irradiation. GFP fluorescence intensity was measured over a period of 5 min. Measurements are mean ±SD from 60 cells **(B)** Kinetics of GFP-FOXO4 recruitment in the absence of MDC1 and MRE11; U2OS cells transfected with siRNAs against lusiferase, MDC1 or MRE11 for 72hrs, were transfected with GFP-FOXO4 and subsequently were subjected to laser micro-irradiation. GFP fluorescence intensity was measured over a period of 5 min. Measurements are mean ±SD from 20 cells **(C)** Kinetics of GFP-FOXO4 recruitment in the absence of 53BP1 and PARP1 activity; U2OS cells transfected with siRNAs against lusiferase or 53BP1 for 72hrs, were transfected with GFP-FOXO4 and subsequently were subjected to laser micro-irradiation. DMSO or the PARP1 inhibitor Olaparib was added 1hr before micro-irradiation. GFP fluorescence intensity was measured over a period of 5 min. Measurements are mean ±SD from 20 cells **(D)** Immunoblots showing the level of knockdown of the indicated proteins (related to Figure S3B and S3C). Arrowhead indicates the height of protein of interest **(E)** Immunoblot showing the level of knockdown of NBS1 (related to Figure 3C). Non-specific band recognized by NBS1 antibody is used as a loading control **(F)** Interaction between HA-FOXO1 and HA-FOXO3 with NBS1 in response to IR (5Gy, 30 min). Where indicated, cells were pre-treated with ATM inhibitor (Ku-55933) for 1 hr prior to IR **(G)** Interaction between GFP-FOXO4 and myc-NBS1 wild type and mutants. HEK293T cells transfected with the indicated plasmids were subjected to 5 Gy IR and 30 min later cells were lysed and proteins were immunoprecipitated with ant-GFP resin **(H)** GFP-FOXO4 partially co-localizes with MRE11. Cells were treated with IR (5Gy, 30min) and subsequently pre-extracted prior to fixation and indirect immunofluorescence for GFP and MRE11. Numbers indicate the percentage of total foci that show co-localization

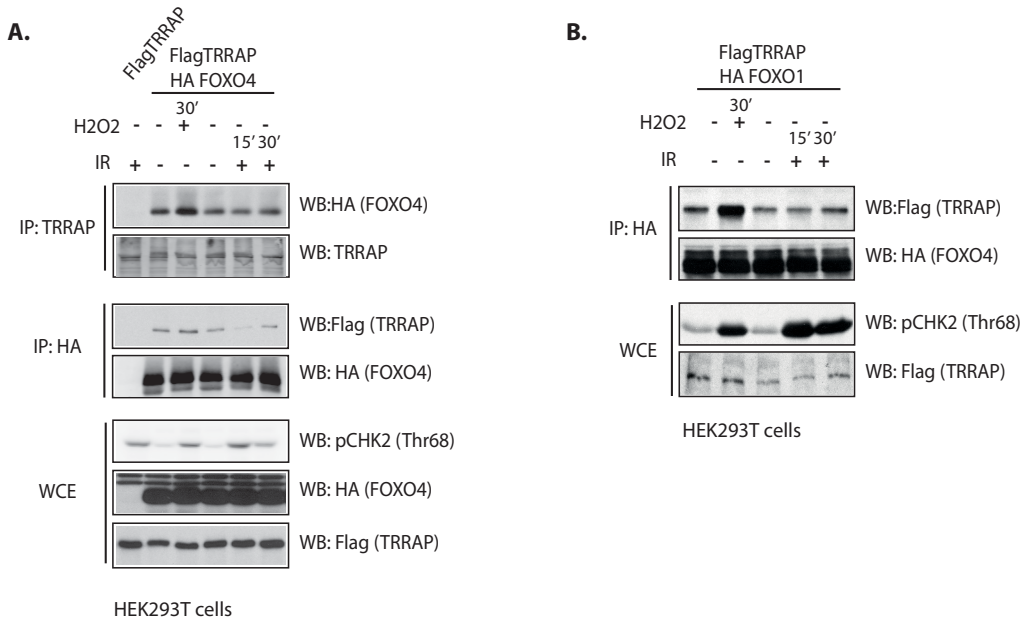


Figure S4 (related to Figure 4). (A) Interaction between FOXO4 and TRRAP. HEK293T cells were transfected with Flag-TRRAP and HA-FOXO4 and treated with 200μM H2O2 for 30 min or 5Gy IR for 15 and 30 min. Cell lysates were immunoprecipitated with either anti-TRRAP or anti-HA and the complexes were analyzed by western blot **(B)** Interaction between FOXO1 and TRRAP. HEK293T cells were transfected with Flag-TRRAP and HA-FOXO1 and treated with 200μM H2O2 for 30 min or 5Gy IR for 15 and 30 min. Cell lysates were immunoprecipitated with anti-HA and the complexes were analyzed by western blot

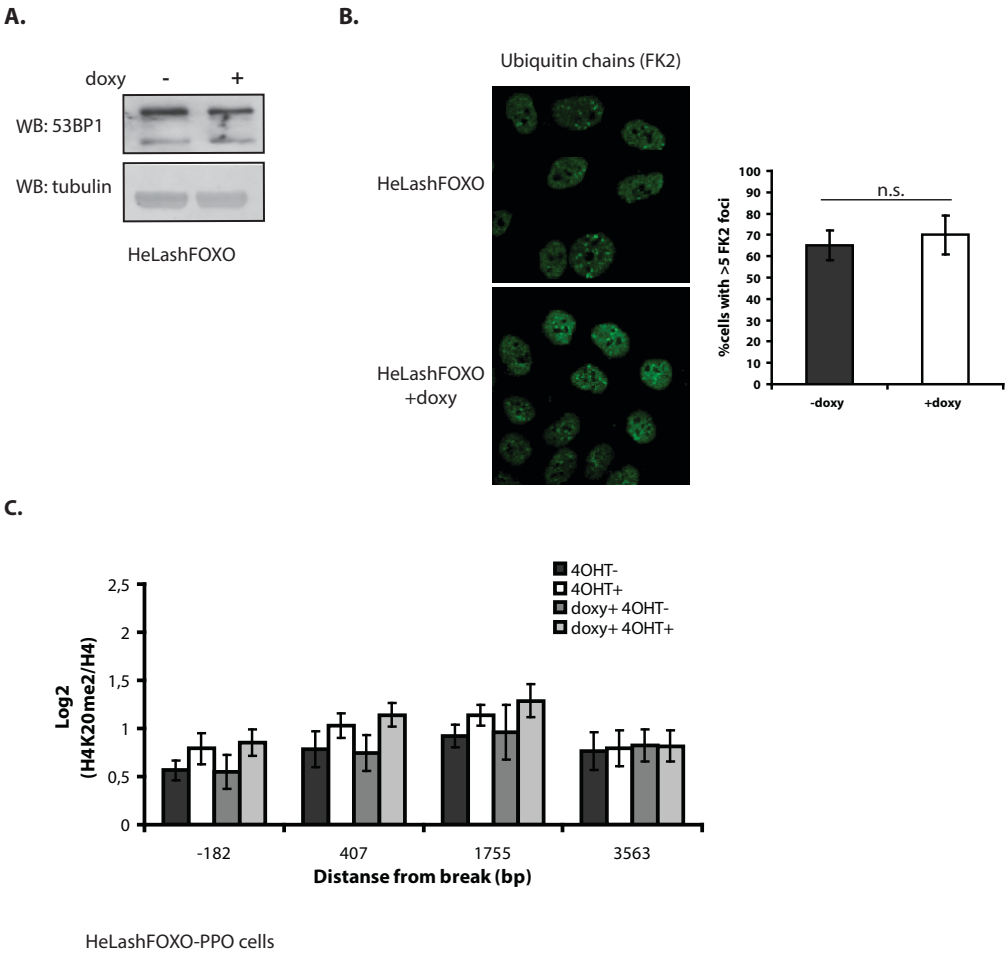


Figure S5 (related to Figure 5). (A) Immunoblots showing the level of knockdown of the indicated proteins (related to Figure 5C). Arrowhead indicates height of protein of interest **(B)** HeLashFOXO cells were cultured in the presence/absence of doxycycline and FOXO4 siRNA for 72 hrs. Cells were subjected to 2 Gy IR and were fixed after 3hrs. Representative immunofluorescence image of ubiquitin chains (FK2 antibody) **(C)** HeLashFOXO-PPO cells were cultured in the presence or absence of doxycycline for 72 hrs before I-PPO was induced by 4-OHT for 16 hrs. Cells were used for ChIP against H4 and H4K20me2 and Q-PCR was performed with oligonucleotide primer pairs surrounding the single chromosome 1 I-PpoI cleavage site. Data are represented as relative IP (H4K20me2 over total H4). Results represent the mean ±SD of three independent experiments. ns: not-significant

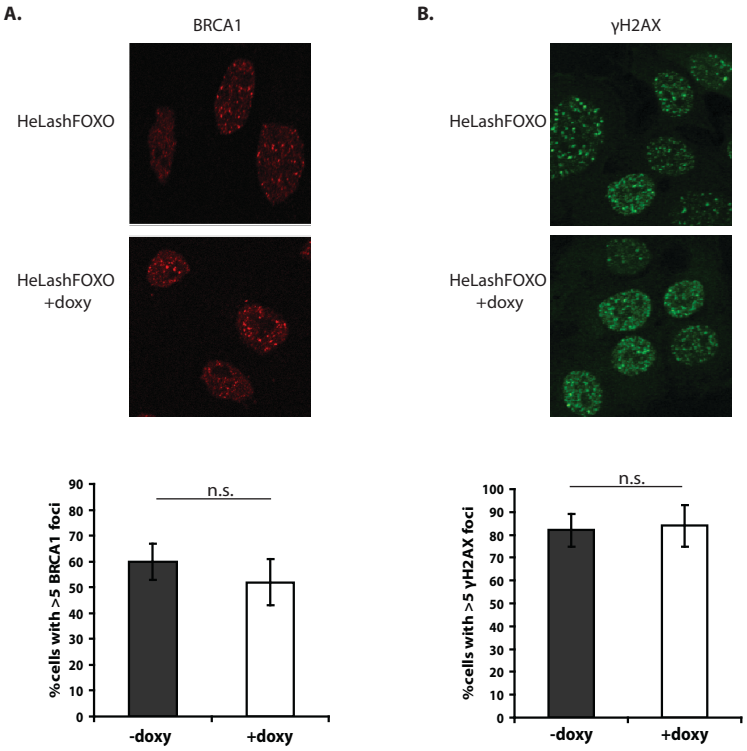
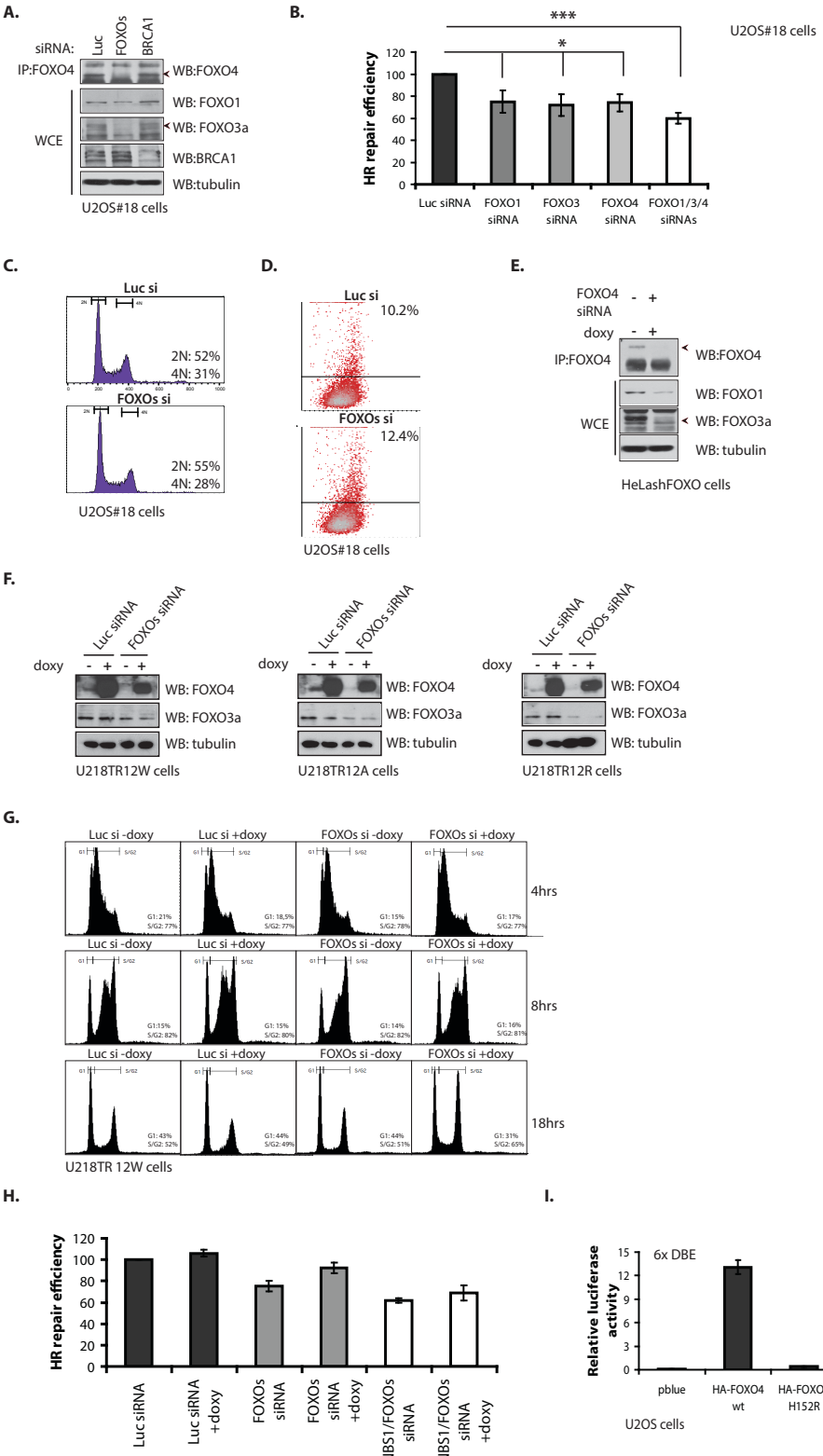


Figure S6 (related to Figure 6). HeLashFOXO cells treated or not with doxycycline and FOXO4 siRNA for 72 hrs were subjected to 2 Gy IR and fixed after 3hrs. Representative immunofluorescence image of BRCA1 and quantification of BRCA1 foci **(A)** and γH2AX **(B)** in the conditions tested. ns: not-significant

Figure S7 (related to Figure 7). (A) Immunoblots showing the level of knockdown of the indicated proteins (related to Figure 7A). Arrowhead indicates height of protein of interest **(B)** U2OS #18 cells were treated with the indicated siRNAs and prepared and analyzed as in (Figure 7A). Results depict the means ±SD of 3 independent experiments **(C)** U2OS#18 cells were transfected with the indicated siRNAs for 24 hrs before the I-SceI plasmid was transfected. Cells were collected 48 hrs post plasmid transfection, fixed and stained with PI before analysed by FACS **(D)** U2OS#18 cells were transfected with the indicated siRNAs for 24 hrs before the I-SceI plasmid was transfected. Cells were collected 48 hrs post plasmid transfection, stained with PI and analysed by FACS **(E)** Immunoblots showing the level of knockdown of the indicated proteins (related to Figure 7B). Arrowhead indicates height of protein of interest **(F)** Immunoblots showing the level of add-back for FOXO4 wild-type and the S462A and H156R mutants in the U218TR12W, U218TR12A and U218TR12R cells respectively (related to Figures 7C-E) **(G)** U2-18TR12W cells were transfected with the indicated siRNAs for 24 hrs before the I-SceI plasmid was transfected. Cells were synchronized in G1 with thymidine block and released with concomitant doxycycline addition. They were collected 4, 8 and 18 hrs post doxycycline addition, fixed and stained with PI before they were analysed by FACS **(H)** U2-18TR12W cells were treated with the indicated siRNAs and prepared and analysed as in (Figure 7C). Results depict the means ±SD of 4 independent experiments **(I)** Luciferase assay showing that the FOXO4 H156R mutant is transcriptionally inactive. U2OS cells were transfected with HA-FOXO4 wt and H156R along with the 6xDBE-luciferase plasmid (containing 6 copies of an optimal FOXO-binding site) and TK-Renilla. Samples were collected and measured 48 hrs post-transfection. *P<0.05, ***P<0.001


A.
siRNA:
Luc
FOXOs
BRCA1
IP:FOXO4
WB:FOXO4
WCE
WB: FOXO1
WB: FOXO3a
WB:BRCA1
WB:tubulin
U2OS#18 cells
B.
U2OS#18 cells
***
*
HR repair efficiency
120
100
80
60
40
20
0
Luc siRNA
FOXO1 siRNA
FOXO3 siRNA
FOXO4 siRNA
FOXO1/3/4 siRNAs
C.
Luc si
2N: 52%
4N: 31%
FOXOs si
2N: 55%
4N: 28%
U2OS#18 cells
D.
Luc si
10.2%
FOXOs si
12.4%
U2OS#18 cells
E.
FOXO4 siRNA
doxy
IP:FOXO4
WB:FOXO4
WCE
WB: FOXO1
WB: FOXO3a
WB: tubulin
HeLashFOXO cells
F.
Luc siRNA
FOXOs siRNA
doxy
WB: FOXO4
WB: FOXO3a
WB: tubulin
U218TR12W cells
U218TR12A cells
U218TR12R cells
G.
Luc si -doxy
Luc si +doxy
FOXOs si -doxy
FOXOs si +doxy
4hrs
8hrs
18hrs
U218TR 12W cells
H.
HR repair efficiency
120
100
80
60
40
20
0
Luc siRNA
Luc siRNA +doxy
FOXOs siRNA
FOXOs siRNA +doxy
NBS1/FOXOs siRNA
NBS1/FOXOs siRNA +doxy
U218TR12W cells
I.
Relative luciferase activity
15
12
9
6
3
0
6x DBE
pblue
HA-FOXO4 wt
HA-FOXO4 H152R
U2OS cells

Addendum

Transcriptional profiling in FOXO-depleted cells reveals novel FOXO-regulated processes

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2. Department of Metabolic and Endocrine Diseases, UMC Utrecht, The Netherlands

Abstract

FOXO transcription factors regulate the transcription of genes involved in cell cycle regulation, metabolism and apoptosis and are thereby key players in processes linked to cell survival and proliferation. Thus far, global gene expression analysis for FOXO-dependent gene expression has relied on either the activation of mutant FOXO that is insensitive to upstream inhibition or the analysis of cells genetically deleted for FOXOs. To complement these approaches, we describe here a gene expression analysis in HeLa cells expressing a doxycycline inducible shRNA against FOXOs, to mirror acute inhibition of FOXOs, as for example occurs after insulin stimulation. This approach identified novel target genes, whose expression is maintained by FOXO-dependent transcription. We report here on previously unidentified processes that appear to be transcriptionally regulated by FOXOs, including RNA metabolism and actin cytoskeleton turnover. Moreover, novel possible roles in metabolism and the maintenance of genomic stability are discussed.

Introduction

The Forkhead Box superfamily includes proteins that can bind DNA via their conserved wing-helix-wing DNA binding domain (1). The superfamily includes 17 gene subfamilies (FOXA-FOXR), with distinct functions (1). The FOXO family includes four members, FOXO1, FOXO3a, FOXO4 and FOXO6. Of these, FOXO6 has a more restricted expression pattern in the brain, whereas the other three members exhibit a more ubiquitous expression. FOXOs function is predominantly regulated by their subcellular localization. The PI3K-PKB (Akt) pathway negatively regulates FOXOs downstream of insulin and growth factors by inducing their cytoplasmic retention and their subsequent proteasomal degradation (2, 3). Under conditions of oxidative stress, JNK and other stress kinases induce FOXO nuclear retention and transcriptional activation ((4) and for a review (5)). FOXOs are key players in processes linked to tumor suppression and longevity. In particular, FOXOs regulate the transcription of genes involved in reactive oxygen species (ROS) scavenging, as well as cell cycle arrest and apoptosis (reviewed in (5)). Moreover, FOXO1 is activated in response to growth factor deprivation to regulate the expression of gluconeogenetic and lipid oxidation genes (6). Finally, FOXOs transcriptionally regulate the quiescent state of stem cells, thereby contributing to tissue regeneration and homeostasis maintenance (7).

Previous studies to identify FOXO regulated transcriptional changes utilized overexpression systems of constitutively active mutants (for example (8)). However, these analyses may be tainted by the fact that FOXO expression levels are beyond physiological levels and that gene induction by dominant active FOXO discovers genes that can be activated by FOXO but does not describe genes whose expression critically depends on FOXO. In a recent study gene expression profiling was performed in triple knockout (FoxO1-FoxO3-FoxO4) mice (7). While this study confirmed several of the FOXO-dependent genes discovered by the initial approach (e.g. p27kip1), it also identified several potentially novel endogenous FoxO-regulated genes in mice. The relevance of some of the identified genes was supported by the FoxO-null phenotype(s) observed, suggesting that this is a relevant approach to study transcriptional activity of endogenous proteins. Nonetheless, this analysis is also hindered by the fact that long-term deletion of critical homeostatic regulators, like FOXOs, might activate compensation mechanisms.

Based on the considerations provided above, we designed a study to identify endogenous FOXO-regulated genes in a human cellular system following acute loss of FOXO expression, by shRNA-mediated FOXO knock-

down. We further took into consideration the genetic background of the cell system to be employed. FOXOs and p53 are considered important regulators of cellular homeostasis, are regulated by similar stress signaling and share many transcriptional targets (9). Thus by and large, p53 and FOXOs act reciprocally redundant and a cell system lacking p53 would likely circumvent this redundancy. We therefore chose HeLa cells, as p53 is constitutively targeted for degradation in these cells (10, 11). In **Chapter 2** we have shown that FOXOs partake in the DNA damage response (DDR) and direct cells towards homologous recombination (HR) repair after genotoxic stress, independent of gene transactivation. However, considering the fact that FOXOs were previously shown to be transcriptionally active in response to DNA damage, (12-14), we decided to further address a possible FOXO contribution to the response to DNA double strand breaks through gene transcription. Therefore, we also performed gene expression analysis before and after gamma-irradiation (IR) of the HeLa cells stably expressing an inducible system for FOXO knockdown.

Using the approaches described, we report here the identification of several novel FOXO-target genes involved in processes including RNA stability, metabolism, transmembrane transport and cytoskeleton dynamics. Moreover, we find that FOXOs do not regulate the transcription of DNA repair genes or genes related to cell cycle arrest, under conditions of DNA damage. Collectively, our data provide new insights and avenues to follow, in order to gain a better understanding of FOXO biology.

Results and Discussion

Establishment and validation of a system for studying the effects of inducible FOXO depletion

To characterize transcriptional changes mediated by endogenous FOXO proteins, we performed global gene expression analysis in HeLa cells expressing doxycycline-inducible shRNA against FOXO1 and FOXO3 and further transfected with FOXO4 siRNA. We verified FOXO depletion in these cells, both at the RNA and protein level **(Figure 1A and 1B)**. We subsequently examined in this set-up the expression levels of selected FOXO target genes, including p27, GADD45a, RICTOR and LGR4 (8, 12, 15, 16) by PCR. We found that GADD45a and p27 do not show impaired expression in the FOXO depleted HeLa cells under basal conditions, however we observed significant reduction in the levels of RICTOR and LGR4 **(Figure 1C)**. This suggests that either in HeLashFOXO cells other (transcription) factors compensate for the FOXO-dependent regulation of these targets (p27 and GADD45a) or that endogenous FOXOs are required for their transcription specifically under certain stress conditions (for instance after oxidative stress or impaired growth factor signaling).

FOXOs transcriptionally regulate divergent cellular processes

Next, we performed global gene expression analysis in the HeLashFOXO cells, 3 days post FOXO depletion. For the identification of FOXO regulated genes, we used as criterion the change in expression by ≤0.75 fold (downregulated genes) or by ≥1.4 fold (upregulated genes) and a statistical cut-off p≤0.01. Based on these criteria we identified 33 genes that are differentially regulated in the FOXO-depleted cells compared to control HeLashFOXO cells **(Table 1)**. Using these stringent criteria, our analysis identified only caveolin from the previously established FOXO target genes (17). We further looked into the genes that exhibit significant transcriptional changes in the FOXO-depleted compared to the control HeLashFOXO cells (p≤0.01), but were below the threshold we initially set for target identification (fold induction ≤0.85 and ≥0.75) **(Supplementary Table 1)**. We consider these genes to be partially regulated by FOXOs. Amongst these genes we identified previously reported target genes, including PINK1, CDKN1C (p57kip2) and KLF7 (18-21).

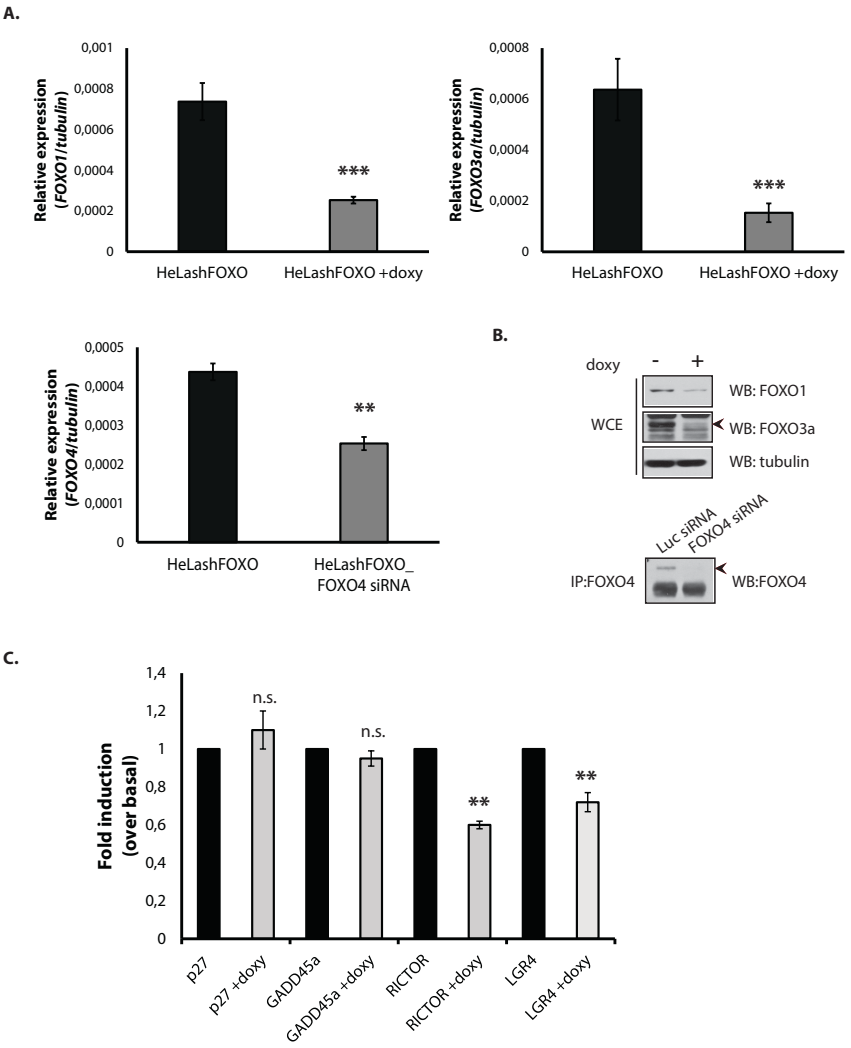

Figure 1. Efficient FOXO depletion in the HeLashFOXO cells (A) FOXO gene expression was analyzed in HeLashFOXO cells cultured in the presence or absence of doxycycline for 72 hrs **(B)** FOXO protein levels in HeLashFOXO cells cultured in the presence or absence of doxycycline for 72 hrs **(C)** Relative fold changes of previously identified FOXO target genes in the HeLashFOXO cells 72 hours post FOXO depletion

We subsequently performed a Gene Ontology (GO) terms analysis in the extended cluster of genes with impaired expression due to FOXO depletion. We identified processes previously linked to FOXO-dependent transcription, such as cell death, as well as a number of novel processes that are possibly transcriptionally regulated by FOXOs, including cytoskeletal organization and RNA metabolism **(Table 2)**. In addition, we identified novel processes that can be potentially linked to FOXO-dependent gene transactivation, such as mitochondrial assembly and function, transmembrane transport, nucleotide biosynthesis and DNA repair **(Table 2)**.

FOXO transcriptional activity is linked to gene transactivation, rather than repression (8). In agreement, we found only a few genes whose expression is upregulated upon FOXO depletion **(Table 1)**. These include genes

Table 1. Genes with significant (p≤0.01) changes in FOXO-depleted HeLa cells under basal conditions, compared to control cells (≤0.75 and ≥1.4 fold change)

Gene symbol	Gene name	Fold change
Downregulated		
GNS	glucosamine (N-acetyl)-6-sulfatase	0.615
ARPC1B	actin related protein 2/3 complex, subunit 1B, 41kDa	0.62
RAD21	RAD21 homolog (S. pombe)	0.62
ATF1	activating transcription factor 1	0.63
GNPTAB	N-acetylglucosamine-1-phosphate transferase, alpha and beta subunits	0.65
PPIC	peptidylprolyl isomerase C (cyclophilin C)	0.66
DCP2	DCP2 decapping enzyme homolog (S. cerevisiae)	0.68
IDH1	isocitrate dehydrogenase 1 (NADP+), soluble	0.68
LTA4H	leukotriene A4 hydrolase	0.69
IK	IK cytokine, down-regulator of HLA II	0.69
SCRN1	secernin 1	0.7
CAPZA1	capping protein (actin filament) muscle Z-line, alpha 1	0.7
RBM47	RNA binding motif protein 47	0.7
HSP90AA1	heat shock protein 90kDa alpha (cytosolic), class A member 2	0.72
CSRP2	cysteine and glycine-rich protein 2	0.72
UHMK1	U2AF homology motif (UHM) kinase 1	0.73
FDPS	farnesyl diphosphate synthase	0.73
TRH	thyrotropin-releasing hormone	0.73
CAV1	caveolin 1, caveolae protein, 22kDa	0.74
ALPK3	alpha-kinase 3	0.74
HMOX2	heme oxygenase (decycling) 2	0.74
NIPA1	non imprinted in Prader-Willi/Angelman syndrome 1	0.75
KRT18P27	keratin 18 pseudogene 27	0.75
RNF114	ring finger protein 114	0.75
Upregulated		
DDR1	Epithelial discoidin domain-containing receptor 1 Precursor (Epithelial discoidin domain receptor 1)	1.4
SEMA3C	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	1.4
TOM1L1	target of myb1 (chicken)-like 1	1.42
STXBP1	syntaxin binding protein 1	1.42
UBE2Q1	ubiquitin-conjugating enzyme E2Q family member 1	1.43
SMARCA1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1	1.46
PLAUR	plasminogen activator, urokinase receptor	1.5
CREM	cAMP responsive element modulator	1.68
DNER	delta/notch-like EGF repeat containing	1.7

involved in cell adhesion (DDR1), signaling components of the Notch pathway (DNER) or components of small-GTP binding proteins signaling pathways (PLAUR).

Below, we discuss some of the novel processes we now identified as potentially linked to FOXOs transcriptional activity in the HeLashFOXO cells under basal conditions.

Table 2. Gene Ontology categories enriched in the downregulated genes in FOXO depleted HeLashFOXO cells

GO term	Enrichment score	Gene names
RNA binding	3.1	DCP2, PABPC4, IMP3, RBM47, RBMS1, YBX1, MRPL3, RPL8, RPS2, RNPS1, ZMAT3, ELAVL1, NCL, EIF4A1
DNA metabolic processes (DNA repair)	1.71	RAD21, TDP1, TFAM, UNG, UBE2B
Metabolic processes/ Mitochondria	1.63	IDH1, GLO1, HMOX2, UROS, UROD, UGDH, ALDH7A1, CYP39A1, HMOX2, UROS, GSTK1, FOLR1, SRM, NQO1, NDUFB2, PTGR1, ATP6V1F, NDUFB2, PINK1, GLUL, MRPL3, MRPS16, SLC25A36, TFAM
Protein biosynthesis	1.28	EIF3B, EIF3G, EIF5A, EIF4A1
Cytoskeleton organization	0.7	ARPC1B, CAPZA1, CAV1
Apoptosis	0.66	ZMAT3, RAD21, TRAF5, GRAMD4, HMGB1, HIPK1, IL19, PHLDA1, SLC5A11, TXNDC12
Nucleotide biosynthesis	0.62	ATIC, CTPS2, ADK
Transmembrane transport	0.52	SLC16A1 , SLC16A9, SLC25A36, SLC5A11, FOLR1, ATP6V1F

FOXOs and RNA metabolism

FOXO transcription factors regulate gene expression by activating RNA polymerase II-mediated gene transcription (8). Our analysis in the HeLashFOXO cells suggests that FOXOs may also be involved in processes related to mRNA stability and processing. In particular, we identified as novel FOXO target genes the mRNA-decapping enzyme 2 (DCP2) and RNA binding motif protein 47 (RBM47) **(Table 1)** and also RNA binding motif single stranded interacting protein 1 (RBMS1), Y box binding protein 1 (YBX1), RNA binding protein S1 (RNPS1), poly(A) binding protein (PABPC4) and tRNA methyltransferase 5 (TRMT5) **(Supplementary Table 1)**. These genes are involved in various steps of RNA metabolism, such as RNA catabolism via the RISC complex and RNA splicing and polyadenylation, suggesting complex and extensive FOXO-dependent regulation of post-transcriptional gene expression. Interestingly, ribonucleoproteins including mitochondrial ribosomal protein L3 (MRPL3), mitochondrial ribosomal protein S16 (MRPS16), ribosomal protein L8 (RPL8) and ribosomal protein S2 (RPS2) were also identified as FOXO target genes, suggesting a possible involvement of FOXOs in ribosome assembly and thereby in indirect regulation of mRNA processing towards proteins.

FOXOs and metabolic processes

FOXOs are involved in the regulation of various metabolic processes in response to nutrient deprivation (22). Here, we find that FOXOs, under conditions of sufficient nutrients and active growth factor signaling, can be involved in the regulation of metabolism, albeit mediating different processes. In particular, FOXOs appear to mediate processes positively linked to the tricarboxylic acid (TCA) cycle by regulating the

expression of isocitrate dehydrogenese (IDH1) **(Table 1, Chapter 3)** and mitochondrial-associated genes **(Supplementary Table 1, Chapter 4)**. Moreover, we identify novel FOXO target-genes that are involved in nucleotide metabolism. These include CTP synthase II (CTPS2), which is required for the synthesis of cytosine (pyrimidine metabolism), as well as adenosine kinase (ADK) and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (ATIC), which are involved in purine metabolism.

FOXOs and transmembrane transport

The transfer of small molecules and ions across membranes is mediated by dedicated transmembrane proteins. The solute carrier superfamily (SLC) includes 55 families of proteins involved in the transportation of small molecules across membranes. Our analysis identified several solute carrier proteins as FOXO target genes, including SLC16A1, SLC16A9, SLC25A36 and SLC5A11 **(Supplementary Table 1)**. Interestingly, most of these proteins are located at the plasma membrane, whereas SLC5A11 is located at the mitochondrial membrane, suggesting another link between FOXOs and mitochondrial function. Of particular interest for further analysis is the SLC16A1 transporter, mediating the transportation of lactate and pyruvate and the SLC5A11 co-transporter, involved in glucose transport. Moreover, we identify folate receptor 1 (FOLR1) as partially regulated FOXO target gene **(Supplementary Table 1)**. Interestingly, folic acid is not only important for nucleotide synthesis but was also recently proposed to be required for the reversal of the mitochondrial dysfunction phenotypes with PINK1 depletion and Parkinson's disease (23). Finally, the proton (H+) transporter ATP6V1F, involved in the acidification of organelles like lysosomes, was also identified in our analysis as a FOXO-regulated gene **(Supplementary Table 1)**.

FOXOs and actin cytoskeleton dynamics

Actin remodeling is a cellular process with important implications for the communication between adjacent cells, as well as between cells and their extracellular matrix. Cells respond to signals related to cell division or cellular mobilization by rapidly remodeling their actin cytoskeleton. Moreover, actin cytoskeleton is involved in intracellular organelle trafficking, for example mitochondrial transport. Actin remodeling is a dynamic process comprising cycles of actin nucleation and polymerization and actin destabilization. Our analysis identified two genes related to actin polymerization as novel FOXO targets: Actin Related Protein 2/3 Complex, Subunit 1B (ARPC1B) and capping protein (actin filament) muscle Z-line, alpha 1 (CAPZA1) **(Supplementary Table 1)**. CAPZA1 regulates actin polymerization by capping the barbed end of the actin filament and thereby conferring stability. ARPC1B is part of the ARP2/3 complex and is involved in stimulation of actin polymerization.

FOXOs and DNA repair

The gene expression analysis we performed in the HeLashFOXO cells under basal conditions also identified genes involved in DNA repair as FOXO-regulated genes (RAD21, UNG, TDP1 and UBE2B). We decided to further look into these target genes, considering our findings, described in **Chapter 2**, that FOXOs are involved in the maintenance of genomic stability. Below we describe the processes in which these factors are involved and discuss the possible implications of FOXO-dependent regulation.

Double strand break repair

There are two major pathways for the repair of DSBs, namely the error-prone non-homologous end joining and the error-free homologous recombination (HR) repair (reviewed in (24)). RAD21 is a component of the cohesin complex, which was previously implicated in HR (25). In particular, cohesin was suggested to mediate the tethering /bridging of homologous chromosomes and thereby facilitate the exchange of genetic information of the intact sister chromatid. As described in **Chapter 2**, FOXOs mediate HR repair by

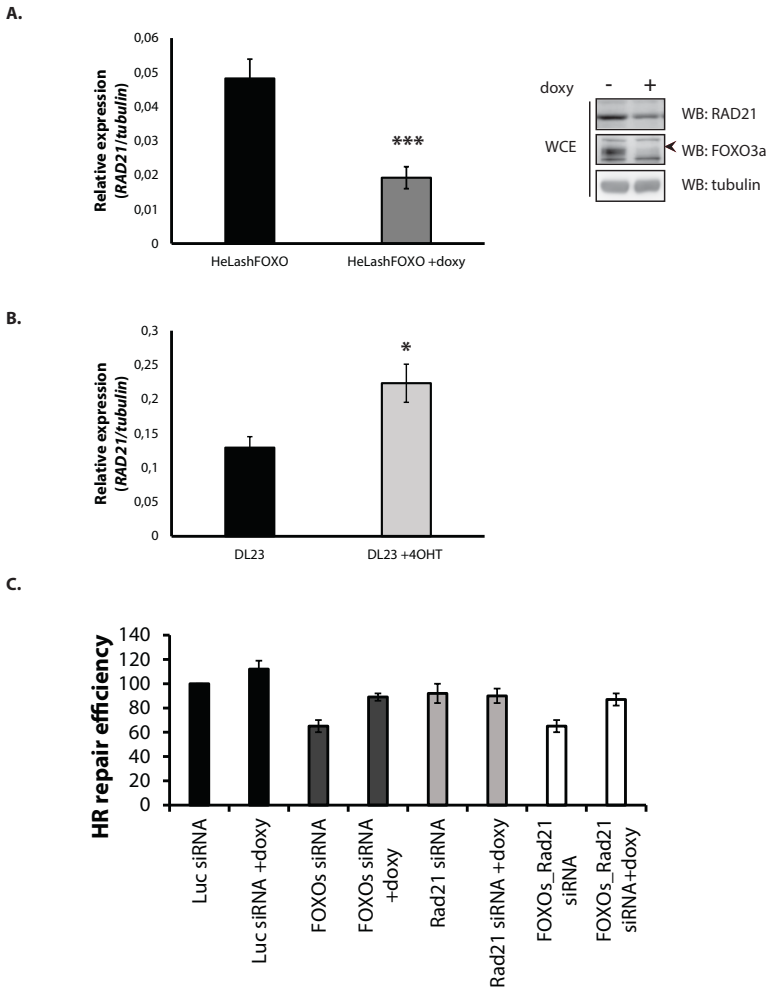

Figure 2. Rad21 is a FOXO target gene but is not involved in FOXO-dependent HR (A) Rad21 mRNA and protein levels in HeLashFOXO cells cultured in the presence or absence of doxycycline for 72 hrs **(B)** Rad21 mRNA levels in DL23 cells treated with 4-OH-T for 8 hrs **(C)** HR repair efficiency assay. U218TR12W cells were transfected with the indicated siRNAs and 24 hours later with the I-SceI enzyme together with mcherry. Cell were synchronized with thymidine block for 24 hrs before release to the cycle. FOXO expression was induced 16 hrs prior to sample collection and FACS analysis

inducing DNA end resection, yet by a transcription-independent mechanism. Nonetheless, we decided to look further into a possible contribution of *RAD21* transcription on the observed phenotype. After verifying the regulation of RAD21 by FOXOs, both under conditions of FOXOs silencing and FOXO3 overexpression **(Figure 2A)**, we assessed the functional significance of Rad21 regulation by FOXOs in HR. We employed the reporter system with inducible FOXO exogenous expression (U2TR12W cells), in which we previously observed a significant defect in HR efficiency in response to FOXO depletion and a functional add-back in the presence of wild type FOXO4 (**Chapter 2**). We found that FOXO4 add-back in the U218TR12W cells silenced for both FOXOs and Rad21, efficiently rescued HR efficiency **(Figure 2B)**, which suggests that FOXO-dependent regulation of Rad21 does not mediate HR repair but rather another function.

Oxidative damage repair

Oxidative stress induces DNA breaks as well as DNA base modifications, which pose a great mutagenic risk. The most important mechanism of base modifications repair is Base Excision Repair (BER). One of the first steps in BER is the enzymatic removal of the damaged base from the DNA helix, a task performed by dedicated enzyme termed DNA glycosylases. We identified Uracil-DNA glycosylase (UNG) as a FOXO target gene **(Supplementary Table 1)**. FOXOs are well known mediators of ROS tolerance, by promoting the transcription of genes involved in antioxidant defenses ((5) for a review and **Chapter 3**), yet, no data currently exist on the contribution of FOXOs in the repair of ROS-induced damage.

Another type of damage induced by ROS is single strand DNA breaks (SSBs); in this case the DNA ends might carry modifications that hinder further processing (26). The function of tyrosyl DNA phosphodiesterase 1 (TDP1), identified in this study as FOXO target gene **(Supplementary Table 1)**, is to facilitate the excision of such modifications. TDP1 is also involved in the processing and repair of lesions in the mtDNA (27) and in **Chapter 4** we show that FOXO depleted cells are more susceptible to mtDNA damage induced by ROS.

Post-replication repair (PRR)

Replication-blocking lesions are potentially deleterious for the cell, as they can result in replication machinery stalling, DNA breaks and eventually cell death (28). To circumvent this, cells have developed DNA damage tolerance mechanisms (DDT) that act near the blocked replication forks to "bypass" rather than repair of the lesions (29). DDT mechanisms include translesion synthesis and error-free PRR. The E2 ligase UBE2B, which we identified as a novel FOXO target **(Supplementary Table 1)**, is homologous to Rad6, a yeast DNA repair gene with important contributions to these processes. In particular, Rad6 mediates the ubiquitination of the DNA-encircling protein PCNA and the subsequent induction of translesion synthesis or error-free repair for damage bypass (30).

In summary, FOXOs appear to regulate, under standard, non-stress conditions, the expression of proteins involved in different DNA repair processes. The regulation of their transcription under basal/non-damaging conditions might reflect the elevated genomic instability in HeLa cells and thereby the activation of DNA repair processes. Alternatively, considering that some of these proteins were also identified to carry DNA repair-independent functions (for example transcriptional regulation), FOXOs might maintain their expression levels for purposes other than DNA repair. For instance, RAD21 is an important factor in regulating higher order chromatin structure (31, 32) and UBE2B was shown to mediate ubiquitination of histone 2B (33) and β-catenin (34). Moreover, the identification of genes involved in the repair of oxidative stress-induced lesions or to PRR suggests interesting potential new functions for FOXOs.

Transcriptional changes in response to IR

To study gene regulation in response to genotoxic stress, we treated cells with IR and collected samples 4 hrs afterwards. **Supplementary Table 2** summarizes the genotoxic stress-induced transcriptional changes in HeLashFOXO cells. 30 genes were found to be upregulated and 40 genes to be downregulated in response to genotoxic stress (≤0.7 fold for downregulated genes or by ≥1.4 fold for upregulated genes, statistical cut-off p≤0.01.). The transcriptional programs that we found to be altered in the HeLashFOXO cells in response to IR-induced DNA damage include cell death, cell cycle regulators and DNA repair factors. Our results are in line with previous studies performed in other cell types (35-38). Previous work has established that DNA damage results in transcriptional repression around DNA lesions (39). In our analysis we find this phenomenon mirrored in the high number of downregulated genes in HeLashFOXO cells in response to IR.

Table 3. Genes with significant changes in HeLa cells in response to IR and differential regulation in the FOXO-depleted cells (p≤0.01, ≤0.7 and ≥1.4 fold change)

Gene symbol	Gene name	Fold change (control cells)	Fold change (FOXO depleted cells)
TXNIP	thioredoxin interacting protein	1.69	1.18
SLC16A9	solute carrier family 16, member 9 (monocarboxylic acid transporter 9)	1.63	1.03
FHL2	four and a half LIM domains 2	1.58	1.34
HPS1	Hermansky-Pudlak syndrome 1	1.58	0.91
TRAF2	TNF receptor-associated factor 2	1.54	1.21
BIRC2	baculoviral IAP repeat-containing 2	1.47	1.2
PKMYT1	protein kinase, membrane associated tyrosine/threonine 1	1.4	1.18
ATF3	activating transcription factor 3	1.4	1.22
AQP3	aquaporin 3 (Gill blood group)	0.62	0.88
TRAFD1	TRAF-type zinc finger domain containing 1	0.65	0.78
RHOV	Ras homolog gene family, member V	0.68	0.87

DNA damage-associated processes transcriptionally regulated by FOXOs in HeLashFOXO cells

We further looked into the IR-modulated genes that are transcriptionally dependent on FOXOs. We found 11 IR-regulated genes (up- and down-regulated genes) to depend on FOXOs **(Table 3)**. One of these genes was thioredoxin interacting protein (TXNIP); TXNIP was previously identified as a FOXO-target gene involved in G1 arrest (40). Moreover, we identified the Hermansky-Pudlak syndrome 1 (HPS1) gene, which is involved in lysosomal organization as a novel FOXO target gene in response to IR. The induction of HPS1 by genotoxic stress could reflect the activation of autophagic processes for the clearance of cellular constituents that, apart from DNA, are also damaged by IR. Other interesting targets were four and a half LIM domains 2 (FHL2), previously reported to regulate FOXO function (41) and the TNF receptor-associated factor 2 (TRAF2), involved in JNK kinase activation (42, 43). FOXOs are known to transcriptionally mediate feedback signaling pathways (for examples see (16) and (8)) and these target genes could thus play similar roles. Interestingly, we did not find any previously suggested target genes involved in cell cycle arrest or DNA repair (for example GADD45a, MnSOD and p21) to require FOXOs for their upregulation in response to IR. This could be attributed to the functional redundancy of FOXOs with other transcriptional regulators that operate in the HelashFOXO cells during genotoxic stress.

Methods

Cell lines

For the generation of the HeLashFOXO cell line, HeLa cells were transduced by lentivirus carrying the FH1t(FOXO1/3)UTG plasmid (described in **Chapter 2**). Subsequently, the positive clones were selected by FACS sorting of the GFP positive cells. U218TR12W cell were previously described (**Chapter 2**). DL23 cells were previously described (15).

Cell culture and treatments

HeLashFOXO and U218TR12W cells were cultured in DMEM supplemented with 10% tetracycline-free serum (Lonza), penicillin-streptomycin and 2mM Glutamine. DL23 cell were cultured in RPMI supplemented with 10% tetracycline-free serum (Lonza) and penicillin-streptomycin and glutamine. To induce the silencing of FOXO1 and FOXO3, HeLashFOXO cells were grown with 1 µg/mL doxycycline for 3 days. DL23 cells were cultured in RPMI (Lonza) supplemented with 10% tetracycline-free serum (Lonza), penicillin-streptomycin and 2mM Glutamine.

To study FOXO4 add-back in the U218TR12W cells, we transfected U218TR12W with the indicated siRNAs. 24hrs post siRNA transfection, cells were transfected with an I-SceI expressing vector along with pcDNA3.0-mcherry. 16 hrs post I-SceI transfection, cells were synchronized in G1/S with thymidine for 24 hrs. Subsequently cells were released in cycle by extensive washing with PBS. Doxycycline was added to the medium to induce FOXO4 wild type expression, 16 hrs prior to sample collection and FACS analysis to determine the percentage of cells positive for both GFP and mcherry.

All siRNAs were synthesized by Dharmacon research. Transfections of siRNA were performed with Hyperfect (Qiagen), according to the manufacturer's instructions.

Ionizing radiation was delivered by a Cs-137 source (UMCU).

Total RNA Sample preparation

HeLashFOXO cells were cultured in the presence or absence of doxycycline for 72 hrs. On the day of the experiment, cells were either left untreated or treated with 5Gy IR for 4 hrs. Subsequently cells were washed twice with PBS and RNA was extracted with the RNeasy kit (Qiagen), according to the manufacturer's instructions.

Quality of the isolated RNA was tested by an Agilent 2100 Bioanalyzer digital gel run of total RNA.

Microarray and data analysis

For the microarray anlysis, cDNA made from mRNA isolated from HeLashFOXO cells as described was hybridized to a pool of cDNAs from the parental HeLa cells. All RNA amplification and labeling procedures were performed in 96-well plates (Abgene) on a customized Sciclone ALH 3000 workstation (Caliper LifeSciences), supplemented with a PCR PTC-200 (Bio-Rad Laboratories), SpectraMax 190 spectrophotometer (Molecular Devices) and a magnetic bead locator (Beckman). Labeled cRNA product was purified with RNAClean (Agencourt, GC biotech) according to the manufacturer's protocol. Hybridizations of spotted oligo-arrays (Human Operon version2 onto Codelink glass) were performed on an HS4800Pro Hybstation (Tecan). Hybridized slides were scanned on an Agilent scanner (G2565AA) at 100% laser power, 30% photo multiplier tube. After data extraction using Imagene 7.5 (BioDiscovery), print tip Loess normalization was performed44 on mean spot intensities.

Statistical analysis was performed using the Limmas files, with the following criteria: (i) p≤0.01 (ii) fold induction: ≤0.75 fold (downregulated genes) and ≥1.4 fold (upregulated genes). For analysis of downregulated genes with less stringent criteria, we looked for genes with induction ≤0.85 and ≥0.75. GO term analysis was performed by the Database for Annotation, Visualization and Integrated Discovery (DAVID).

RNA extraction and quantitative PCR (Q-PCR)

RNA was extracted with the RNeasy kit (Qiagen), with on column DNase treatment (Qiagen), according to the manufacturer's instructions. RNA was reversed transcribed with oligodT primers and the iScript cDNA synthesis kit (Biorad). Quantitative PCR was performed with FastStart SYBR Green Master mix (Roche) with the following primer sequences:

GADD45a_F: GGAGGAAGTGCTCAGCAAAG, GADD45a_R: TGGATCAGGGTGAAGTGGAT, p27_F: CCGGCTAACTCTGAGGACAC, p27_R: GGGGAACCGTCTGAAACATT, RICTOR_F: GGAAGCCTGTTGATGGTGAT, RICTOR_R: GGCAGCCTGTTTTATGGTGT, LGR4_F: CCATCAGTAGCCAAGGTGGT, LGR4_R: AAGAAACGATTCGCAGCAGT, RAD21_F: AGGAAAGGAGGAGAGGCAGA, RAD21_R: GTGGTGGAGGCATAGCTGAC.

HR reporter assay

The U218TR12W cell was used to study HR repair efficiency. U218TR12W carries the "nested intron" reporter for the study of sister chromatid recombination, as well as doxycycline inducible expression of wild type FOXO4. 24hrs after siRNA

transfection, cells were transfected with an I-SceI expressing vector along with pcDNA3.0- mcherry. 48hrs later, cells were collected and analysed by FACS, to determine the percentage of cells positive for both GFP and mcherry. To correct for possible differences in the cell cycle status between different samples, samples were prepared as following; 16 hrs post I-SceI transfection, cells were synchronized in G1/S with thymidine for 24 hrs. Subsequently cells were released in cycle by extensive washing with PBS and doxycycline was added to the medium 16 hrs prior to FACS measurements.

References

1. Lam EWF, Brosens JJ, Gomes AR, & Koo C-Y (2013) Forkhead box proteins: tuning forks for transcriptional harmony. Nat Rev Cancer 13(7):482-495.
2. Brunet A, et al. (1999) Akt Promotes Cell Survival by Phosphorylating and Inhibiting a Forkhead Transcription Factor. Cell 96(6):857-868.
3. Kops GJPL, et al. (1999) Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature 398(6728):630-634.
4. Essers MAG, et al. (2004) FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. EMBO J 23(24):4802-4812.
5. Charitou P & Burgering BM (2012) Forkhead Box(O) in Control of Reactive Oxygen Species and Genomic Stability to Ensure Healthy Lifespan Antioxidants & Redox Signaling.
6. Gross D, van den Heuvel A, & Birnbaum M (2008) The role of FoxO in the regulation of metabolism. Oncogene 27(16):2320 - 2336.
7. Paik J-H, et al. (2007) FoxOs Are Lineage-Restricted Redundant Tumor Suppressors and Regulate Endothelial Cell Homeostasis. Cell 128(2):309-323.
8. Eijkelenboom A, et al. (2013) Genome-wide analysis of FOXO3 mediated transcription regulation through RNA polymerase II profiling. Molecular Systems Biology 9(1).
9. Eijkelenboom A & Burgering BMT (2013) FOXOs: signalling integrators for homeostasis maintenance. Nat Rev Mol Cell Biol 14(2):83-97.
10. Matlashewski G, Banks L, Pim D, & Crawford L (1986) Analysis of human p53 proteins and mRNA levels in normal and transformed cells. European Journal of Biochemistry 154(3):665-672.
11. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, & Howley PM (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 63(6):1129-1136.
12. Tran H, et al. (2002) DNA Repair Pathway Stimulated by the Forkhead Transcription Factor FOXO3a Through the Gadd45 Protein. Science 296(5567):530-534.
13. Lei H & Quelle FW (2009) FOXO Transcription Factors Enforce Cell Cycle Checkpoints and Promote Survival of Hematopoietic Cells after DNA Damage. Molecular Cancer Research 7(8):1294-1303.
14. Klagge A, et al. (2011) The role of FOXO3 in DNA damage response in thyrocytes. Endocrine-Related Cancer 18(5):555-564.
15. Kops GJPL, et al. (2002) Control of Cell Cycle Exit and Entry by Protein Kinase B-Regulated Forkhead Transcription Factors. Molecular and Cellular Biology 22(7):2025-2036.
16. Chen C-C, et al. (2010) FoxOs Inhibit mTORC1 and Activate Akt by Inducing the Expression of Sestrin3 and Rictor. Developmental Cell 18(4):592-604.
17. van den Heuvel APJ, Schulze A, & Burgering BMT (2005) Direct control of caveolin-1 expression by FOXO transcription factors. Biochemical Journal 385(3):795-802.
18. Mei Y, et al. (2009) FOXO3a-dependent regulation of Pink1 (Park6) mediates survival signaling in response to cytokine deprivation. Proceedings of the National Academy of Sciences 106(13):5153-5158.
19. Evans-Anderson HJ, Alfieri CM, & Yutzey KE (2008) Regulation of Cardiomyocyte Proliferation and Myocardial Growth During Development by FOXO Transcription Factors. Circulation Research 102(6):686-694.
20. Miyamoto K, et al. (2007) Foxo3a Is Essential for Maintenance of the Hematopoietic Stem Cell Pool. Cell Stem Cell 1(1):101-112.
21. Tejera MM, Kim EH, Sullivan JA, Plisch EH, & Suresh M (2013) FoxO1 Controls Effector-to-Memory Transition and Maintenance of Functional CD8 T Cell Memory. The Journal of Immunology 191(1):187-199.
22. Gross DN, van den Heuvel APJ, & Birnbaum MJ (2008) The role of FoxO in the regulation of metabolism. Oncogene 27(16):2320-2336.
23. Tufi R, et al. (2014) Enhancing nucleotide metabolism protects against mitochondrial dysfunction and neurodegeneration in a PINK1 model of Parkinson's disease. Nat Cell Biol 16(2):157-166.
24. Ciccia A & Elledge SJ (2010) The DNA Damage Response: Making It Safe to Play with Knives. Molecular Cell 40(2):179-204.
25. Liefshitz B & Kupiec M (2011) Roles of RSC, Rad59, and Cohesin in Double-Strand Break Repair. Molecular and Cellular Biology 31(19):3921-3923.
26. Caldecott KW (2008) Single-strand break repair and genetic disease. Nat Rev Genet 9(8):619-631.
27. Das BB, Dexheimer TS, Maddali K, & Pommier Y (2010) Role of tyrosyl-DNA phosphodiesterase (TDP1) in mitochondria. Proceedings of the National Academy of Sciences 107(46):19790-19795.

28.Cox MM, et al. (2000) The importance of repairing stalled replication forks. Nature 404(6773):37-41.
29.Friedberg EC (2005) Suffering in silence: the tolerance of DNA damage. Nat Rev Mol Cell Biol 6(12):943-953.
30.Hoege C, Pfander B, Moldovan G-L, Pyrowolakis G, & Jentsch S (2002) RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature 419(6903):135-141.
31.Mishiro T, et al. (2009) Architectural roles of multiple chromatin insulators at the human apolipoprotein gene cluster. The EMBO Journal 28(9):1234-1245.
32.Wendt K & Peters J-M (2009) How cohesin and CTCF cooperate in regulating gene expression. Chromosome Res 17(2):201-214.
33.Kim J, et al. (2009) RAD6-Mediated Transcription-Coupled H2B Ubiquitylation Directly Stimulates H3K4 Methylation in Human Cells. Cell 137(3):459-471.
34.Shekhar MPV, Gerard B, Pauley RJ, Williams BO, & Tait L (2008) Rad6B Is a Positive Regulator of β-Catenin Stabilization. Cancer Research 68(6):1741-1750.
35.Tusher VG, Tibshirani R, & Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proceedings of the National Academy of Sciences 98(9):5116-5121.
36.Otomo T, Hishii M, Arai H, Sato K, & Sasai K (2004) Microarray Analysis of Temporal Gene Responses to Ionizing Radiation in Two Glioblastoma Cell Lines: Up-regulation of DNA Repair Genes. Journal of Radiation Research 45(1):53-60.
37.Kristin Rødningen O, Overgaard J, Alsner J, Hastie T, & Børresen-Dale A-L (2005) Microarray analysis of the transcriptional responseto single or multiple doses of ionizing radiation in human subcutaneous fibroblasts. Radiotherapy and Oncology 77(3):231-240.
38.Datta K, et al. (2012) Exposure to ionizing radiation induced persistent gene expression changes in mouse mammary gland. Radiation Oncology 7.
39.Shanbhag NM, Rafalska-Metcalf IU, Balane-Bolivar C, Janicki SM, & Greenberg RA (2010) ATM-Dependent Chromatin Changes Silence Transcription In cis to DNA Double-Strand Breaks. Cell 141(6):970-981.
40.Yamaguchi F, et al. (2013) FOXO/TXNIP pathway is involved in the suppression of hepatocellular carcinoma growth by glutamate antagonist MK-801. BMC Cancer 13(1):468.
41.Yang Y, Hou H, Haller EM, Nicosia SV, & Bai W (2005) Suppression of FOXO1 activity by FHL2 through SIRT1-mediated deacetylation. The EMBO Journal 24(5):1021-1032.
42.Lee SY, et al. (1997) TRAF2 Is Essential for JNK but Not NF-kB Activation and Regulates Lymphocyte Proliferation and Survival. Immunity 7(5):703-713.
43.Habelhah H, et al. (2004) Ubiquitination and translocation of TRAF2 is required for activation of JNK but not of p38 or NF-κB. The EMBO Journal 23(2):322-332.

Supplementary Data

Supplementary Table 1. Genes with significant (p≤0.01) changes in FOXO-depleted HeLa cells under basal conditions, compared to control cells (0.85>0.75 fold change). Partial regulation by FOXOs.

Gene symbol	Gene name	Fold change
SLC25A36	solute carrier family 25, member 36	0.76
ZMAT3	zinc finger, matrin type 3	0.76
NAPG	N-ethylmaleimide-sensitive factor attachment protein, gamma	0.76
C6orf62	Uncharacterized protein C6orf62 (HBV X-transactivated gene 12 protein)(HBV XAg-transactivated protein 12)	0.76
TXNDC12	thioredoxin domain containing 12 (endoplasmic reticulum)	0.76
PCOLCE2	procollagen C-endopeptidase enhancer 2	0.76
GLO1	glyoxalase I	0.76
EIF3G	eukaryotic translation initiation factor 3, subunit G	0.76
TOR1A	torsin family 1, member A (torsin A)	0.76
UNG	uracil-DNA glycosylase	0.76
COPE	coatomer protein complex, subunit epsilon	0.76
UBE2B	ubiquitin-conjugating enzyme E2B (RAD6 homolog)	0.77
YTHDF1	YTH domain family, member 1	0.77
IPO9	importin 9	0.77
TOB1	transducer of ERBB2, 1	0.77
UGDH	UDP-glucose 6-dehydrogenase	0.77
ZNF449	zinc finger protein 449	0.77
TRMT5	TRM5 tRNA methyltransferase 5 homolog (S, cerevisiae)	0.77
TRAPPC6B	trafficking protein particle complex 6B	0.78
MRPS16	mitochondrial ribosomal protein S16	0.78
NOB1	NIN1/RPN12 binding protein 1 homolog (S, cerevisiae)	0.78
FOLR1	folate receptor 1 (adult)	0.78
MARCKSL1	MARCKS-like 1	0.78
LRRC58	leucine rich repeat containing 58	0.78
IL19	interleukin 19	0.79
RYK	RYK receptor-like tyrosine kinase	0.79
FOXA1	forkhead box A1	0.79
IVNS1ABP	influenza virus NS1A binding protein	0.79
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	0.79
TBCEL	tubulin folding cofactor E-like	0.79
MED6	mediator complex subunit 6	0.79
TXNRD1	thioredoxin reductase 1	0.79
ATIC	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/ IMP cyclohydrolase	0.79
LIN7C	lin-7 homolog C (C. elegans)	0.8
NCL	nucleolin	0.8

FAM162A	family with sequence similarity 162, member A	0.8
TFAM	transcription factor A, mitochondrial	0.8
MAP1LC3B	microtubule-associated protein 1 light chain 3 beta	0.8
SLC16A1	solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	0.8
MRPL3	mitochondrial ribosomal protein L3	0.8
TRUB2	TruB pseudouridine (psi) synthase homolog 2 (E, coli)	0.8
CUTC	cutC copper transporter homolog (E, coli)	0.8
SLC5A11	solute carrier family 5 (sodium/glucose cotransporter), member 11	0.8
TDP1	tyrosyl-DNA phosphodiesterase 1	0.8
PINK1	PTEN induced putative kinase 1	0.8
FKBP3	FK506 binding protein 3, 25kDa	0.8
EIF4A1	eukaryotic translation initiation factor 4A1 pseudogene 4	0.8
NDUFB2	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2, 8kDa	0.8
CREB3L1	cAMP responsive element binding protein 3-like 1	0.8
TSN	translin	0.8
KLHL20	kelch-like 20 (Drosophila)	0.8
C1orf59	HEN1 methyltransferase homolog 1 (Arabidopsis)	0.8
NQO1	NAD(P)H dehydrogenase, quinone 1	0.81
KRT12	keratin 12	0.81
SRM	spermidine synthase	0.81
KLF7	Kruppel-like factor 7 (ubiquitous)	0.81
DPY19L1	dpy-19-like 1 (C, elegans)	0.81
CCT5	chaperonin containing TCP1, subunit 5 (epsilon)	0.81
FUZ	fuzzy homolog (Drosophila)	0.81
DEFB126	defensin, beta 126	0.81
SH2D4A	SH2 domain containing 4A	0.81
EIF3B	eukaryotic translation initiation factor 3, subunit B	0.81
GSTK1	glutathione S-transferase kappa 1	0.81
ZNF451	zinc finger protein 451	0.82
TMEM138	transmembrane protein 138	0.82
UROD	uroporphyrinogen decarboxylase	0.82
EDEM1	ER degradation enhancer, mannosidase alpha-like 1	0.82
SMEK2	SMEK homolog 2, suppressor of mek1 (Dictyostelium)	0.82
TGIF2	TGFB-induced factor homeobox 2	0.82
FYN	FYN oncogene related to SRC, FGR, YES	0.82
TRIM36	tripartite motif-containing 36	0.82
BZW2	basic leucine zipper and W2 domains 2	0.82
FAM64A	family with sequence similarity 64, member A	0.82
MCM2	minichromosome maintenance complex component 2	0.82
DUSP14	dual specificity phosphatase 14	0.82
BLMH	bleomycin hydrolase	0.82

ATP6V1F	ATPase, H+ transporting, lysosomal 14kDa, V1 subunit F	0.83
ELAVL1	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R)	0.83
TRAF5	TNF receptor-associated factor 5	0.83
LOC401387	Leucine-rich repeat and death domain-containing protein	0.83
C11orf59	RhoA activator C11orf59 (p27Kip1-releasing factor from RhoA)(p27RF-Rho)	0.83
ELAC1	elaC homolog 1 (E, coli)	0.83
GINS2	GINS complex subunit 2 (Psf2 homolog)	0.83
HIPK1	homeodomain interacting protein kinase 1	0.83
HMGB1	high-mobility group box 1	0.83
FBXO6	F-box protein 6	0.83
TRIM16L	tripartite motif-containing 16-like	0.83
ARL4A	ADP-ribosylation factor-like 4A	0.83
FSTL4	follistatin-like 4	0.83
CTPS2	CTP synthase II	0.83
UROS	uroporphyrinogen III synthase	0.83
SMYD2	SET and MYND domain containing 2	0.83
GLUL	glutamate-ammonia ligase	0.83
YBX1	Y box binding protein 1	0.83
CIAO1	cytosolic iron-sulfur protein assembly 1	0.83
PTGR1	prostaglandin reductase 1	0.83
PAPPA	PAPPA antisense RNA (non-protein coding)	0.84
IMP3	IMP3, U3 small nucleolar ribonucleoprotein, homolog (yeast)	0.84
RHOT1	ras homolog gene family, member T1	0.84
PUS3	pseudouridylate synthase 3	0.84
CD164	CD164 molecule, sialomucin	0.84
ACRV1	acrosomal vesicle protein 1	0.84
ADAM7	ADAM metallopeptidase domain 7	0.84
ALDH7A1	aldehyde dehydrogenase 7 family, member A1	0.84
PCDHA9	protocadherin alpha 9	0.84
BHLHE40	basic helix-loop-helix family, member e40	0.84
ATMIN	ATM interactor	0.84
HOXC9	homeobox C9	0.84
RALGAPA2	Ral GTPase activating protein, alpha subunit 2 (catalytic)	0.84
ZNF133	zinc finger protein 133	0.84
LUZP1	leucine zipper protein 1	0.84
G3BP1	GTPase activating protein (SH3 domain) binding protein 1	0.84
RBMS1	RNA binding motif, single stranded interacting protein 1	0.84
LSM14A	LSM14A, SCD6 homolog A (S, cerevisiae)	0.84
PABPC4	poly(A) binding protein, cytoplasmic 4 (inducible form)	0.84
ADK	adenosine kinase	0.84
RPL8	ribosomal protein L8	0.84

CYP39A1	cytochrome P450, family 39, subfamily A, polypeptide 1	0.84
SLITRK5	SLIT and NTRK-like family, member 5	0.84
NMUR1	neuromedin U receptor 1	0.84
RNPS1	RNA binding protein S1, serine-rich domain	0.84
ILF2	interleukin enhancer binding factor 2, 45kDa	0.84
IFRD2	interferon-related developmental regulator 2	0.84
FAM117B	family with sequence similarity 117, member B	0.84
PHLDA1	pleckstrin homology-like domain, family A, member 1	0.84
PPIA	peptidylprolyl isomerase A (cyclophilin A)	0.85
EIF5A	eukaryotic translation initiation factor 5A	0.85
SH3BP2	SH3-domain binding protein 2	0.85
ISX	intestine-specific homeobox	0.85
VAC14	Vac14 homolog (S, cerevisiae)	0.85
COL9A3	collagen, type IX, alpha 3	0.85
SPEG	SPEG complex locus	0.85
SLC16A9	solute carrier family 16, member 9 (monocarboxylic acid transporter 9)	0.85
GRAMD4	GRAM domain containing 4	0.85
POLE3	polymerase (DNA directed), epsilon 3 (p17 subunit)	0.85
RPS2	ribosomal protein S2	0.85

Supplementary Table 2. Genes with significant changes in HeLa cells in response to IR (p≤0.01, ≤0.7 and ≥1.4 fold change)

Gene symbol	Gene name	Fold change
Upregulated		
IL32	interleukin 32	1.4
ATF3	activating transcription factor 3	1.4
PKMYT1	protein kinase, membrane associated tyrosine/ threonine 1	1.41
UBE2Q1	ubiquitin-conjugating enzyme E2Q family member 1	1.44
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.45
ALDH1B1	aldehyde dehydrogenase 1 family, member B1	1.45
TGFB2	transforming growth factor, beta 2	1.45
TFPI2	tissue factor pathway inhibitor 2	1.45
GCLC	glutamate-cysteine ligase, catalytic subunit	1.46
ITIH1	inter-alpha (globulin) inhibitor H1	1.47
BIRC2	baculoviral IAP repeat-containing 2	1.47
TRAF2	TNF receptor-associated factor 2	1.54
CCND1	cyclin D1	1.56
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	1.57
FGF2	fibroblast growth factor 2 (basic)	1.57
FHL2	four and a half LIM domains 2	1.58
HPS1	Hermansky-Pudlak syndrome 1	1.58
RND3	Rho family GTPase 3	1.6
EXO1	exonuclease 1	1.62
SLC16A9	solute carrier family 16, member 9 (monocarboxylic acid transporter 9)	1.63
TXNIP	thioredoxin interacting protein	1.69
SOD2	superoxide dismutase 2, mitochondrial	1.73
DKK1	dickkopf homolog 1 (Xenopus laevis)	1.9
BIRC3	baculoviral IAP repeat-containing 3	1.92
IL8	interleukin 8	1.93
GADD45A	growth arrest and DNA-damage-inducible, alpha	1.95
OLR1	oxidized low density lipoprotein (lectin-like) receptor 1	1,96
C8orf4	Uncharacterized protein C8orf4 (Thyroid cancer protein 1) (TC-1)	2.29
CTGF	connective tissue growth factor	2.45
CYR61	cysteine-rich, angiogenic inducer, 61	2.88
Downregulated		
CCNB1	cyclin B1	0
INSIG1	insulin induced gene 1	0
PLK1	polo-like kinase 1	0
CKS2	CDC28 protein kinase regulatory subunit 2	0
DEPDC1	DEP domain containing 1	0

HMMR	hyaluronan-mediated motility receptor (RHAMM)	0.54
AURKA	aurora kinase A	0.54
FAM83D	family with sequence similarity 83, member D	0.54
CDCA3	cell division cycle associated 3	0.55
CENPF	centromere protein F, 350/400kDa (mitosin)	0.56
TTK	TTK protein kinase	0.56
ASPM	asp (abnormal spindle) homolog, microcephaly associated (Drosophila)	0.56 _ 0.65
TPX2	TPX2, microtubule-associated, homolog (Xenopus laevis)	0.58
UBE2C	ubiquitin-conjugating enzyme E2C	0.58
GPSM2	G-protein signaling modulator 2	0.58
CENPE	centromere protein E, 312kDa	0.59
CENPA	centromere protein A	0.59
BUB1B	budding uninhibited by benzimidazoles 1 homolog beta (yeast)	0.6
DLGAP5	discs, large (Drosophila) homolog-associated protein 5	0.61
AQP3	aquaporin 3 (Gill blood group)	0.62
ARL6IP1	ADP-ribosylation factor-like 6 interacting protein 1	0.62
CDC25C	cell division cycle 25 homolog C (S, pombe)	0.62
MTUS1	microtubule associated tumor suppressor 1	0.63
SGOL2	shugoshin-like 2 (S, pombe)	0.64
FAM64A	family with sequence similarity 64, member A	0.65
TRAFD1	TRAF-type zinc finger domain containing 1	0.65
BUB1	budding uninhibited by benzimidazoles 1 homolog (yeast)	0.67
TAC1	tachykinin, precursor 1	0.67
BRD8	bromodomain containing 8	0.67
KIF20A	kinesin family member 20A	0.67
C13orf34	Protein aurora borealis (HsBora)	0.68
RHOV	ras homolog gene family, member V	0.68
KIF20B	kinesin family member 20B	0.69
KIF2C	kinesin family member 2C	0.69
NEK2	NIMA (never in mitosis gene a)-related kinase 2	0.69
HMGB2	high-mobility group box 2	0.69
GTSE1	G-2 and S-phase expressed 1	0.69
CDCA8	cell division cycle associated 8	0.69
NUF2	NUF2, NDC80 kinetochore complex component, homolog (S, cerevisiae)	0.69
C15orf23	Putative TRAF4-associated factor 1	0.7

Chapter 3

FOXOs support the metabolic adaptation and survival of hypoxic and tumor cells by regulating the expression of IDH1

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Abstract

FOXO transcription factors are key players in cellular processes that ultimately determine cell survival, proliferation and death. FOXOs are bona fide tumor suppressors however their cell protective functions are often "hijacked" by tumors cells, eventually implicating FOXOs in tumor promotion. In this study we identified FOXOs as transcriptional regulators of the isocitrate dehydrogenase 1 (IDH1). We show that FOXOs in untransformed cells, by regulating the levels of IDH1, maintain the cytosolic levels of α-ketoglutarate, a cofactor required for the function of several histone and DNA methyltransferases, and NADPH, which is required for the cellular antioxidant defenses. At the same time, FOXOs contribute to the survival and proliferation of hypoxic cells, which are dependent on IDH1 for de-novo lipid synthesis by the reductive carboxylation pathway. In transformed cells carrying mutant IDH1, FOXOs are also required for their survival and proliferation, as they maintain the levels of mutant IDH1 and thereby of the proposed oncometabolite 2-hydroxyglutarate. Combined, our data provide a new paradigm of the dual role of FOXOs in tumor suppression-promotion.

Introduction

The Forkhead Box(O) (FOXO) family of transcription factors includes four members, namely FOXO1, FOXO3a, FOXO4 and FOXO6; of these, FOXO6 is expressed predominantly in the brain whereas the other three members exhibit a more ubiquitous expression. FOXOs are evolutionary conserved proteins and their homologues in diverse organisms ranging from the nematode C. elegans to mice are involved in lifespan extension and tumor suppression (reviewed in (1)). FOXOs integrate various environmental signals into specific transcriptional programs related to cell proliferation, metabolism and reactive oxygen species (ROS) detoxification (2, 3). The activity of FOXOs is negatively regulated by growth factors and nutrient availability through the PI3K-PKB (Akt) pathway. In particular, PKB phosphorylates FOXOs, resulting in their cytoplasmic sequestration and degradation (4, 5). Different environmental signals including ROS, hypoxia and genotoxic stress positively regulate FOXO activity by promoting their nuclear retention ((6-8) and Chapter 2).

An emerging hallmark of cancer, as this was recently introduced by Hanahan and Weinberg (9) is the reprogramming of cellular bioenergetics. Cancer cells reprogram their metabolism to sustain high growth and proliferation rates by the continuous production of energy and "building blocks" for macromolecular synthesis (10). The metabolic reprogramming induced in cancer cells was first described in the seminal work of Otto Warburg, who identified the process of aerobic glycolysis; that is the preference of cancer cells to utilize glycolysis as their main energy source, even under conditions of ambient oxygen. Furthermore, the recent identification of cancer-associated mutations in key metabolic enzymes, including the isocitrate dehydrogenases (IDH) 1 and 2 (11-13) reinforced the notion that metabolic changes in cancer do not merely reflect a 'conditio sine qua non' but that defined metabolic changes can also drive tumorigenesis (14). There are three IDH isoforms, namely IDH1 that resides in the cytoplasm and peroxisomes and the two mitochondrial isoforms IDH2 and IDH3. IDH enzymes convert isocitrate to alpha-ketoglutarate (α-KG), a cofactor required for the function of dioxygenases, a class of enzymes including DNA and histone demethylases (15, 16). The conversion of isocitrate to α-KG catalyzed by IDH1 and IDH2 also results in the generation of NADPH, which offers reductive power required in biosynthetic processes and serves as an antioxidant. Moreover, IDH1 and IDH2 have the ability to perform the reverse reaction; that is the conversion of α-KG to isocitrate with the concomitant consumption of NADPH, a process termed reductive carboxylation. Reductive carboxylation

is primarily utilized by hypoxic cells and cells with severe mitochondrial dysfunction and, to a considerably lesser extent, it may also occur under physiological conditions in fast proliferating cells (17-19). In fact, cancer cells, when growing under hypoxic conditions, which is assumed to occur within the inner mass of solid tumors, depend on reductive carboxylation executed by IDH1 and IDH2 to sustain the levels of cellular citrate that is required for *de novo* lipogenesis and growth.

Apart from the requirement of wild type IDH1 and IDH2 for the proliferation of hypoxic cancer cells, IDH proteins were initially linked to cancer by the identification of mutations in IDH1 and IDH2 in over 70% of lower-grade gliomas, as well as in glioblastomas and acute myelogenous leukemias (AML) (11-13). Mutant IDH1 and IDH2 proteins have altered enzymatic activity and further convert α-KG to 2-hydroxyglutarate (2-HG), a putative oncometabolite, as it was found to be sufficient in driving tumorigenesis under certain conditions (20, 21). The mechanism by which 2-HG drives tumorigenesis is still not fully understood however recent studies have suggested three potential mechanisms; namely interference with normal mitochondrial function, dysregulation of cellular redox and, the most prominent one, competitive inhibition of dioxygenases (for a recent review (22)). In human, over 80 different dioxygenases have been identified that can be divided into various subclasses and are involved in several important biological processes. For example, Ten-Eleven Translocation enzymes (TET1, TET2 and TET3) and the JmjC-domain histone demethylases are involved in in DNA and histone demethylation respectively. Inhibition of these enzymes by 2-HG results in epigenetic alterations similar to ones commonly found in a cancer setting (23, 24).

Previous gene expression analyses to identify FOXO-regulated genes were performed with either constitutively active FOXO mutants or in the setting of a stable full FOXO knockout. However FOXOs are known homeostasis regulators (3) and therefore it is anticipated that their overexpression or prolonged depletion can result in the activation of compensatory mechanisms and thus may introduce a bias in the analysis. We thus performed a gene expression analysis after inducing a transient FOXO depletion with shRNA. We identified several metabolic genes to be regulated by FOXO-dependent transcription, one of which was IDH1. In this study we show that FOXO transcription factors directly regulate the transcription of IDH1 and thereby regulate the levels of cytosolic α-KG and NADPH. We provide evidence that, in cells grown in hypoxic conditions, FOXOs are required for their survival and proliferation. Moreover, we show that in cells expressing mutant IDH1, FOXOs regulate the transcription of the mutant IDH1 as well, thereby controlling the production of the oncometabolite 2-HG. Consequently, loss of FOXO expression results in changes in DNA and histone demethylation and affects the proliferation of transformed cells carrying mutant IDH1. In this setting, FOXOs can be considered to act oncogenic, in contrast to their widely considered role as tumor suppressors.

Results

FOXOs regulate IDH1 levels

To identify genes regulated by the endogenous FOXOs, we performed a global gene expression analysis in HeLa cells stably expressing a doxycycline-inducible shRNA against FOXO1 and FOXO3 and further transfected with a FOXO4 specific siRNA. We decided to use HeLa cells to circumvent the contribution of p53-dependent transcription in our analysis, considering that FOXOs and p53 are regulated by similar upstream pathways and share a panel of transcriptional targets (3, 25). Comparative analysis between control and knockdown cells identified a number of genes involved in metabolic processes as FOXO-regulated genes **(Figure 1A)** and we chose to further study IDH1. To verify that endogenous FOXOs regulate the transcription

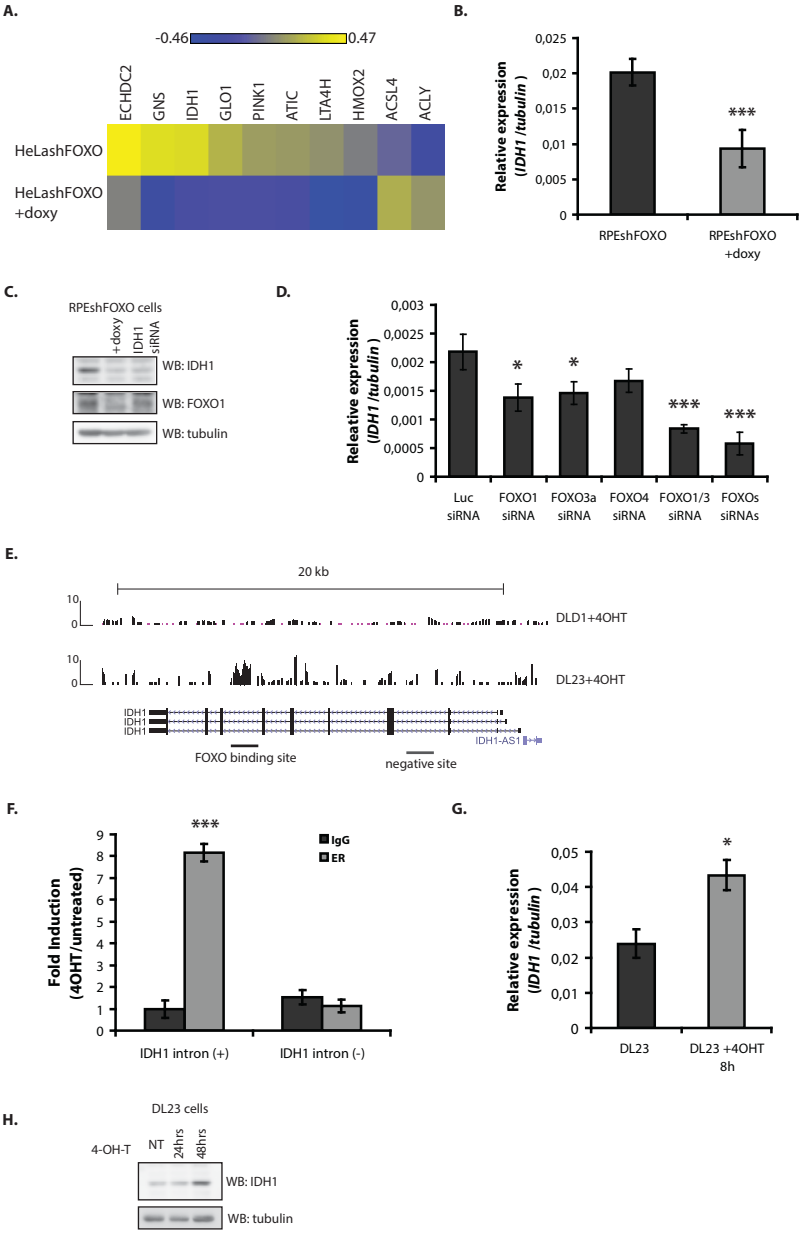

Figure 1. FOXOs regulate the expression of IDH1 (A) Heat map of selected metabolic genes showing differential expression in FOXO-depleted HeLashFOXO cells. HeLashFOXO cells were grown in the presence or absence of doxycycline for 72 hrs before RNA was collected and used for gene expression analysis **(B)** Relative mRNA levels of IDH1 in RPEshFOXO cells. RPEshFOXO cells were cultured in the presence or absence of doxycycline for 72 hrs before sample collection **(C)** Protein levels of IDH1 in RPEshFOXO cells grown in the presence or absence of doxycycline for 72 hrs before sample collection **(D)** Relative mRNA levels of IDH1 in RPE cells transfected with the indicated siRNAs. RNA samples were collected 72 hrs post siRNA transfection **(E)** Chip-sequencing analysis identified a FOXO3 binding site in the IDH1 genomic region in DL23 cells **(F)** Chip-QPCR in DL23 cells 4 hrs after FOXO3 activation. The IDH1 (+) and the IDH1 (-) introns correspond to the FOXO binding site and the negative site in (E) **(G)** Relative mRNA levels of IDH1 in DL23 cells. DL23 cells were induced with 4-OHT for 4 hrs before sample collection **(H)** Protein levels of IDH1 in DL23 cells grown in the presence of 4-OHT for different time points before sample collection.

of IDH1, we used untransformed retina epithelium cells (RPE cells), as well as the cancer cell line HeLa, both expressing doxycycline-inducible shRNA targeting FOXO1 and FOXO3 (RPEshFOXO and HeLashFOXO cells respectively). FOXO depletion resulted in a 50% reduction of IDH1 transcripts in both cell lines **(Figure 1B and Supplementary Figure 1A)**. IDH1 protein levels were also significantly reduced in the FOXO depleted cells **(Figure 1C and Supplementary Figure 1A)**. Next, we analyzed the contribution of each individual FOXO member on IDH1 transcription, to identify whether IDH1 is a common target gene for all FOXOs or a specific trait of one FOXO isoform. After siRNA-mediated silencing of each FOXO member separately or together in RPE cells, we found that knockdown of either FOXO1 or FOXO3a significantly reduced IDH1 expression **(Figure 1D)**. There was also a small contribution of FOXO4 to IDH1 expression, albeit that this appeared weaker than the FOXO1 and/or FOXO3 contribution **(Figure 1D)**. This however may merely reflect the lower expression level of FOXO4 compared to FOXO1 and FOXO3 in these cells (data not shown). Taken together, these results show that FOXOs act redundantly to regulate IDH1 regulation. Importantly, we find a significant decrease of IDH1 levels not only after shRNA-mediated FOXO knockdown but also after inhibition of endogenous FOXO protein through treatment of NIH 3T3 cells overexpressing the insulin receptor (A14 cells, (26)) with insulin for 4 hrs **(Supplementary Figure 1B)**. Evaluation of the other IDH isoforms (IDH2, IDH3A, IDH3B and IDH3G) revealed that their levels remained unchanged or even increased following prolonged FOXOs knockdown **(Supplementary Figure 1C)**, suggesting that compensatory mechanisms are activated in response to a decrease in IDH1 expression.

Analysis of data from previous chromatin immunoprecipitation (ChIP)-sequencing analysis of FOXO3(A3) in DL23 cells (27) identified a potential FOXO3 binding site in an IDH1 intron **(Figure 1E)**. We therefore performed a FOXO3 ChIP in DL23 cells after FOXO3(A3) activation. Subsequent analysis of IDH1 genomic regions verified the FOXO3 occupancy selectively on this region **(Figure 1F)**. The binding appears to be linked to IDH1 transactivation, as FOXO3(A3) activation in DL23 cells significantly increased the IDH1 mRNA **(Figure 1G)** and protein **(Figure 1H)** levels.

Reduced levels of alpha-ketoglutarate (α-KG) and NADPH in FOXO depleted cells

IDH1 proteins function in the cytoplasm to convert isocitrate to α-KG. To determine whether the reduced level of IDH1 expression in FOXO depleted cells has an effect on this conversion, we measured α-KG levels in RPEshFOXO cells after FOXO knock-down. We found that the levels of α-KG were reduced by 50-60% after FOXO depletion. The reduction in α-KG levels was similar to that following siRNA-mediated IDH1 depletion **(Figure 2A)**.

Conversion of isocitrate to α-KG catalyzed by IDH1 and IDH2, also results in the generation of NADPH and this reaction provides an additional major source of NADPH for the cell, after the pentose phosphate pathway (PPP) (28, 29). NADPH provides reductive power for several biosynthetic reactions and is then oxidized to NADP+, allowing a new round of reduction and oxidation to commence. Thus we analyzed the ratio of NADPH over NADP+ in cells depleted of FOXOs. To circumvent the contribution of the PPP to NADPH generation in our analysis, we grew cells in medium without any glucose but supplemented with glutamine and pyruvate to maintain mitochondrial metabolism. We found that under these conditions the NADPH/NADP+ ratio was significantly reduced in the FOXO depleted cells, similar to cells transfected with siRNA against IDH1 **(Figure 2B)**. Cytoplasmic NADPH is utilized to reduce GSSG (glutathione disulfide - oxidized) to GSH (glutathione - reduced), which is subsequently used for ROS detoxification (30). FOXOs are well known for their contribution in reducing intracellular ROS levels (31, 32), therefore we assessed the ratio of GSH/GSSG in cells depleted of

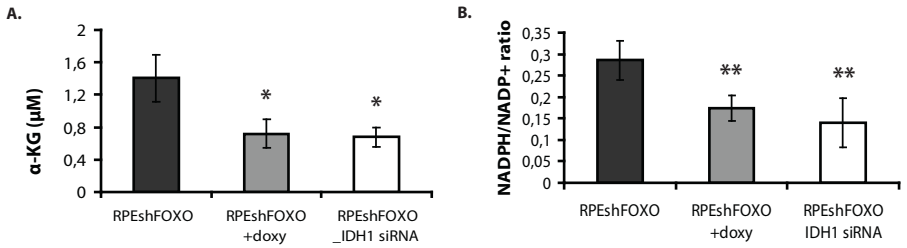

Figure 2. FOXOs maintain the levels of α-KG and NADPH (A) α-KG levels in RPEshFOXO cells and RPEshFOXO cells depleted of FOXOs (+doxy) or transfected with IDH1 siRNA. RPEshFOXO cells were cultured in the presence or absence of doxycycline for 72 hrs before sample collection and analysis by GC-MS. IDH1 silencing by siRNA served as a positive control. Results represent the mean±SD of three independent experiments **(B)** NADPH/NADP+ ratio in RPEshFOXO cells and RPEshFOXO cells depleted of FOXOs (+doxy) or transfected with IDH1 siRNA. RPEshFOXO cells were cultured in the presence or absence of doxycycline for 48 hrs and thereafter the medium was changed to medium with no glucose, supplemented with pyruvate and glutamine for another 48 hrs before sample collection. IDH1 silencing by siRNA served as a positive control. Results represent the mean±SD of three independent experiments. *P<0.05, **P<0.01

FOXOs and grown under conditions that should inhibit a PPP contribution. We found a small but significant reduction in the levels of reduced glutathione in the FOXO depleted cells **(Supplementary Figure 2)**.

In hypoxia FOXOs regulate IDH1 expression and cell proliferation

FOXOs are activated under hypoxic conditions to mediate cell survival (6). Considering that IDH1 is required for the metabolic rewiring and growth of hypoxic cells (17), we set out to investigate the contribution of FOXO-mediated IDH1 transcription to cellular survival under hypoxia. To this end, we grew RPEshFOXO cells at 1% O2 and evaluated IDH1 levels after FOXO depletion. We found that FOXO depletion also abrogates IDH1 transcription in hypoxic cells **(Figure 3A)**. We then assessed the growth of cells depleted from FOXOs under normoxic and hypoxic conditions; FOXO depletion has a small effect in normoxic growth (Supplementary

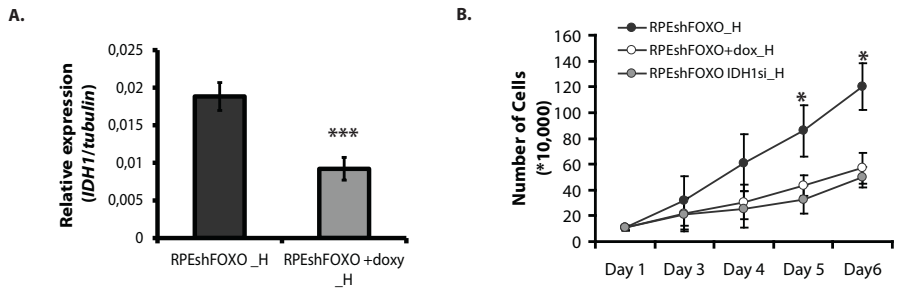

Figure 3. FOXOs regulate the metabolic adaptation to hypoxia by IDH1 transcription (A) Relative mRNA levels of IDH1 in RPEshFOXO cells grown in hypoxia. RPEshFOXO cells were cultured in the presence or absence of doxycycline for 48 hrs before transferred to a hypoxia chamber. 24 hrs afterwards, samples were collected **(B)** Growth rates of RPEshFOXO cells grown in hypoxia. 50,000 RPEshFOXO cells were plated in the presence or absence of doxycycline or transfected with IDH1 siRNA and kept in ambient O_2 for 8 hrs before they were transferred to a hypoxia chamber. Cells were allowed to proliferate in hypoxia for several days, during which cells were collected and counted every 24 hrs. Results represent the mean±SD of three independent experiments. *P<0.05, ***P<0.001

Figure 3) but the effect is significantly exacerbated in cells grown in hypoxia **(Figure 3B)**, similar to IDH1 depletion by siRNA. Combined, these results suggest that FOXOs contribute to the survival and proliferation of hypoxic cells by a mechanism involving IDH1 transcription.

FOXOs regulate the expression of the IDH1 R132C mutant in HT1080 cells and are required for their proliferation

Correlation studies in gliomas revealed a negative correlation between mutations that support FOXO inactivation (PTEN mutation, EGFR amplification) and IDH1 mutation. We therefore investigated whether FOXOs also regulate the expression of the mutant IDH1 (33, 34). We used HT1080 cells, a fibrosarcoma-derived cell line that endogenously carries the IDH1 R132C mutation. In these cells we introduced the doxycycline-inducible shRNA against FOXOs and established the HT1080shFOXO polyclonal cell line. We found that upon FOXOs depletion, IDH1 transcription is significantly reduced **(Figure 4A)** and this reduction is also reflected in the IDH1 protein levels **(Figure 4B)**. We further evaluated the levels of 2-HG, which were reduced by approximately 70% in the FOXO-depleted HT1080 cells **(Figure 4C)**.

As mentioned, a potential mechanism by which mutant IDH1 mediates its oncogenic effect is by inhibition of α-KG dependent deoxygenases, by the competitive binding of 2-HG to their active center. These enzymes include histone demethylases and TET 5-methylcytosine (5mc) hydroxylases (converting 5mc to

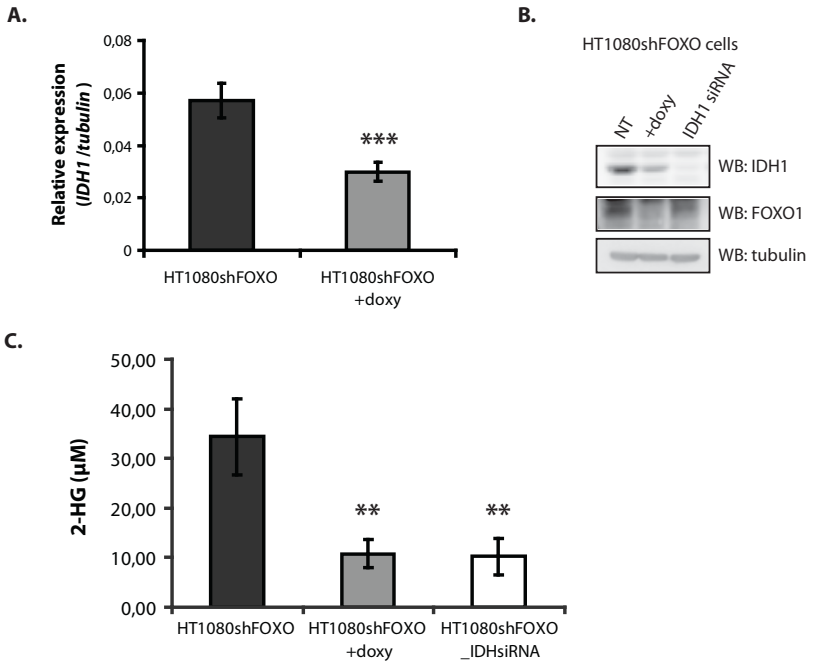

Figure 4. FOXOs regulate the expression of mutant IDH1 (A) Relative mRNA levels of IDH1 in HT1080shFOXO cells. HT1080shFOXO cells were cultured in the presence or absence of doxycycline for 72 hrs before sample collection **(B)** Protein levels of IDH1 in HT1080shFOXO cells grown in the presence or absence of doxycycline for 72 hrs before sample collection **(C)** 2-HG levels in HT1080shFOXO cells and HT1080shFOXO cells depleted of FOXOs (+doxy) or transfected with IDH1 siRNA. HT1080shFOXO cells were cultured in the presence or absence of doxycycline for 72 hrs before sample collection and analysis by GC-MS. IDH1 silencing by siRNA served as a positive control. Results represent the mean±SD of three independent experiments. **P<0.01, ***P<0.001

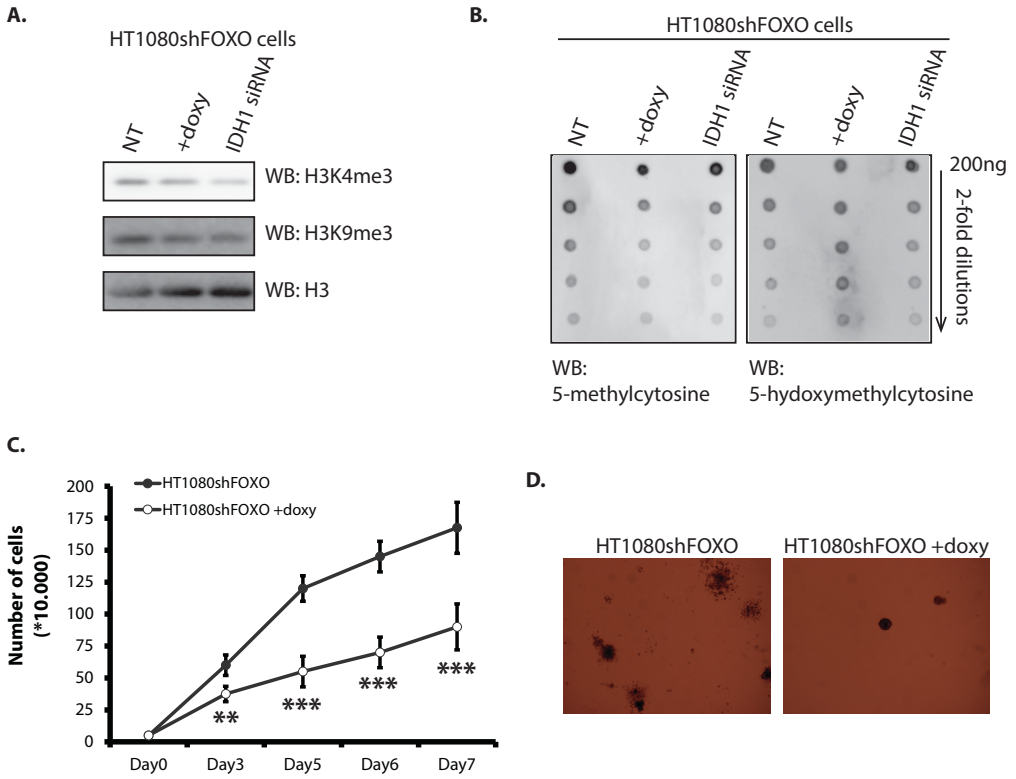

Figure 5. FOXOs are required for the proliferation of cells carrying IDH1 mutation (A) Analysis of histone methylation levels in HT1080shFOXO cells grown in the presence or absence of doxycycline for 72 hrs before sample collection. A representative western blot from three independent experiments is shown. IDH1 served as a positive control **(B)** Analysis of DNA methylation levels in HT1080shFOXO cells grown in the presence or absence of doxycycline for 72 hrs before sample collection. A representative dot-blot from three independent experiments is shown. IDH1 served as a positive control **(C)** Growth curves of HT1080shFOXO cells grown in the presence or absence of FOXOs. 20,000 cells were plated and allowed to grow for 7 days; cells were collected and counted every 24 hrs. Results represent the mean±SD of three independent experiments **(D)** Colony formation of HT1080shFOXO cells in soft agar. Soft agar assay of HT1080shFOXO cells. 20,000 cells were plated in a 0.35% top layer, on top of a 0.5% bottom layer. Doxycycline was added to the upper layer when appropriate. Colonies were allowed to grow for 21 days.***P<0.001

5-hydoxymethylcytosine (5hmc)) (22). We first assessed the effects of FOXOs depletion on the levels of histone H3 lysine 4 trimethylation (H3K4me3) and H3K9me3, which are histone marks reported to be regulated by the enzymatic activity of mutant IDH1 (35). FOXOs depletion, similar to silencing of IDH1, resulted in increased demethylation of both H3K4me3 and H3K9me3 in HT1080shFOXO cells **(Figure 5A)**. Inhibition of TET proteins by 2HG results in increased DNA methylation, which is suggested to play important roles in tumorigenesis (23). We therefore next assessed the effects of FOXO depletion on the DNA methylation status of HT1080shFOXO cells. To this end, we isolated genomic DNA following FOXO or IDH1 depletion and assessed the relative levels of 5mc and 5hmc. FOXO depletion in HT1080 cells appears to restore the function of TET proteins, as it is accompanied by a concomitant decrease in 5mc and increase in 5hmc levels **(Figure 5B)**. Collectively, these data suggest a model in which FOXO-dependent transcription of IDH1 R132C is required for the establishment of tumor promoting epigenetic changes.

FOXOs are bona fide tumor suppressors; however several studies implicated them in the survival and fitness

of cancer cells (reviewed in (36)). Considering that mutant IDH1 drives tumor progression, we assessed the contribution of FOXOs on this process. Depletion of FOXOs in HT1080 cells resulted in severe growth arrest **(Figure 5C)**, similar to the effects reported after inhibiting the enzymatic activity or reducing the levels of mutant IDH1 (37). To further explore the role of FOXOs on the tumorigenicity of HT1080 cells, we assessed the effects of their depletion in anchorage-independent growth by a soft agar assay. We observed a dramatic decrease in colony forming ability of HT1080 cells depleted of FOXOs **(Figure 5D)**, similar to what was reported for IDH1 depletion (37). Collectively, these results suggest that cells carrying mutant IDH1 require FOXO to sustain IDH1 levels and thereby their proliferative and oncogenic potential.

Discussion

In this study we provide evidence that FOXOs directly regulate the expression of IDH1 and thereby promote the metabolic adaptation and proliferation of hypoxic and cancer cells. FOXOs bind to an IDH1 intronic area and induce transactivation of the gene. By regulating the levels of IDH1, we find FOXOs to mediate different metabolic responses **(Figure 6)**. First, under physiological conditions, FOXOs contribute to the intracellular accumulation of α-KG and NADPH and thereby to the function of α-KG–dependent deoxygenases and to ROS detoxification mechanisms. Second, FOXOs via IDH1 transcription contribute to the metabolic rewiring of hypoxic cells, possibly towards reductive carboxylation for *de novo* lipid biosynthesis. Finally, we show that FOXOs also regulate the transcription of the mutant IDH1 and are therefore indispensable for the enhanced proliferative potential of cancer cells carrying IDH1 mutations.

Our data support a dual role of FOXOs in mediating both tumor suppressive and tumor promoting functions, by the regulation of IDH1 (wild type and mutant) transcription. This seemingly appears in odds with the established role of FOXOs as bona fide tumor suppressors (32). However, several lines of evidence have started to indicate that FOXOs are also implicated in cancer progression due to their role in maintaining cellular homeostasis in normal as well as in cancer cells. For example, FOXOs are involved in ROS detoxification by regulating the transcription of ROS scavengers, including MnSOD and Sestrin 3 (31, 38); cancer cells that inherently have high levels of intracellular ROS and an ensuing increased instability are known to benefit from antioxidants (39). Furthermore, FOXOs mediate error-free DNA repair and genotoxic stress resistance in rapidly proliferating cells, including cancer cells **(Chapter 2)**; this protects cancer cells by genotoxic stress-induced apoptosis and it might account for the fact that constitutively nuclear FOXO3 is associated with poor prognosis in breast tumors (40). FOXOs are also involved in the survival and maintenance of the non-proliferative state of stem cells (38, 41-43) and recently FOXO3 was shown to be required for the maintenance of leukemia initiating cells in chronic myeloid leukemia and to confer therapy resistance in leukemia cells (44). In this framework we can appreciate how the regulation of IDH1 transcription by FOXOs can serve as yet another paradigm of their dual role. In untransformed cells, FOXOs mediate the maintenance of cellular α-KG levels, thereby contributing to the function of α-KG-dependent deoxygenases in cellular differentiation and tumor suppression (23, 35, 45). Moreover, FOXOs, via the regulation of IDH1, contribute to the cytoplasmic levels of NADPH and thereby of GSH, providing yet another layer of protection against ROS and genomic instability. On the other hand, in transformed cells carrying IDH1 mutation, FOXOs are required for the maintenance of mutant IDH1 levels and thereby for the levels of the generated oncometabolite 2-HG. In the setting of cellular transformation accompanied by IDH1 mutation FOXOs function as oncogenes, as FOXO depletion in these cells results in cessation of cell growth.

FOXOs exhibit oncogenic properties also in the setting of hypoxia, as we report here FOXOs to be required

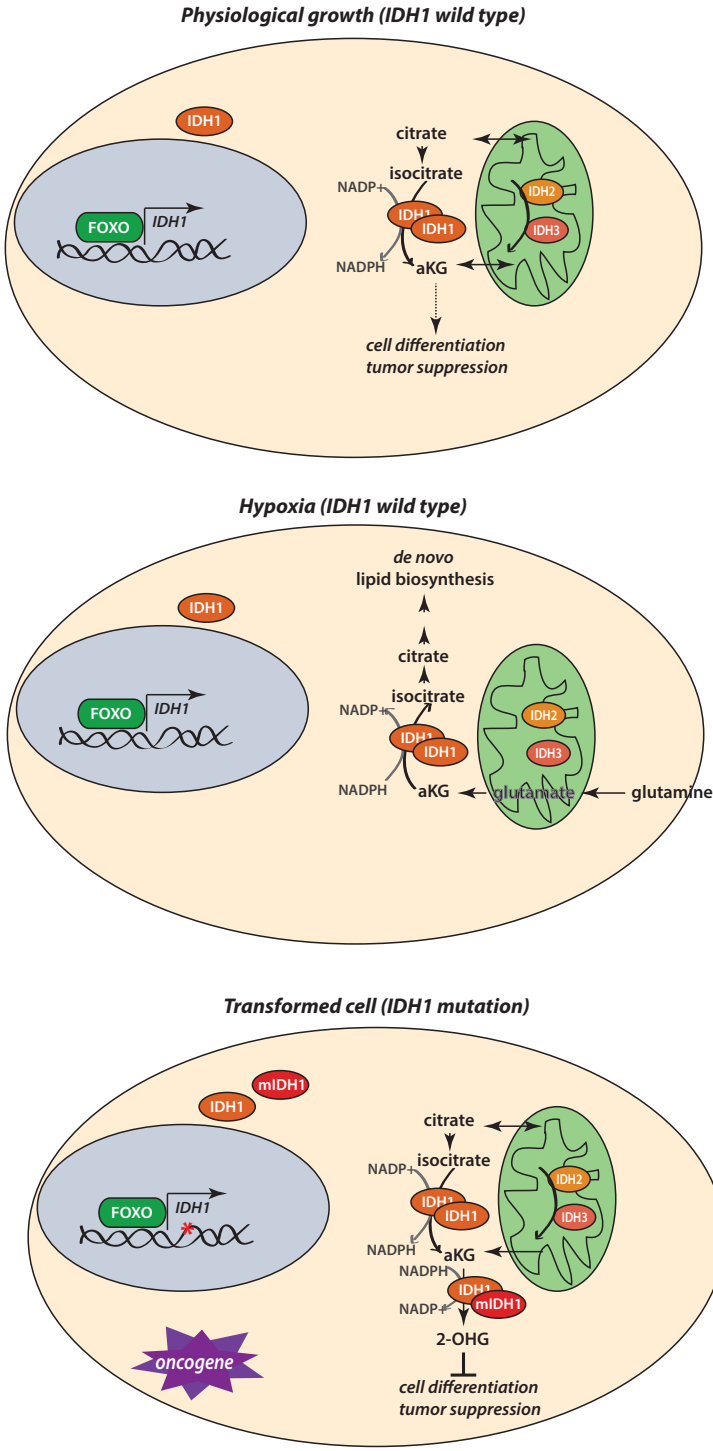

Figure 6. FOXOs mediate tumor suppressive/promoting functions by regulating IDH1 transcription. Model depicting the functions mediated by FOXOs in different genomic and physiological settings, by the regulation of IDH1 transcription (for more information refer to text).

for the proliferation of hypoxic cells, in line with a previous report (6). Hypoxic conditions are suggested to operate within many tumors, especially solid tumors. Cells grown in hypoxia cannot complete mitochondrial respiration due to lack of oxygen and sustainment of mitochondrial function may result in the accumulation of deleterious oxidative metabolism intermediates. To adapt, hypoxic cells activate Hypoxia-Inducible Factor 1 (HIF1), which in turn inhibits oxidative phosphorylation and promotes the redirection of energy metabolism towards glycolysis (46). Inhibition of mitochondrial metabolism however deprives the cells from an important metabolite; citrate. Citrate is normally transferred to the cytoplasm, where it is converted to acetyl-coA and subsequently supports *de novo* lipid synthesis. Considering that biomembrane synthesis is crucial for the proliferation and expansion of cells, it is anticipated that cells grown in hypoxia would activate adaptive mechanisms to ensure the maintenance of intracellular citrate levels. One of these mechanisms is the induction of reductive carboxylation, which relies on IDH1. IDH1 under hypoxic conditions mediates the conversion of α-KG to isocitrate, which is further converted to citrate. Cells grown in hypoxia and depleted of IDH1 show impaired lipid biosynthesis and severely impaired proliferation (17). We have observed similar growth kinetics under hypoxic conditions for both FOXO and IDH1 depleted cells, suggesting that FOXOs might also regulate reductive carboxylation for *de novo* lipid biosynthesis, via IDH1. To test this hypothesis further experiments are required, in which cells grown in hypoxic conditions in the presence of labelled glutamine are monitored for the incorporation of glutamine-derived carbon to the newly synthesized lipids (for example see (17)). Additionally, the acetyl-coA that is generated from citrate is used by HATs for protein acetylation and signal transduction (47, 48); it would therefore be interesting to study the effects of FOXO depletion on histone and protein acetylation and subsequently on cellular transcriptional programs of cells grown in hypoxia.

The metabolic rewiring of cancer cells results in the acquisition of unique characteristics compared to untrasformed cells and thereby, in principal, allows the selective targeting of transformed cells. Identification of aerobic glycolysis for example, led to the development of 18F-deoxyglucose positron emission tomography (18FDG-PET), which is an imaging technique widely used in oncology for diagnostic purposes. Recent advances point to the fact that better understanding of cancer metabolism will provide new opportunities in the potential treatment of certain cancers (49, 50). Especially in the case of mutant IDH1, specific small-molecule inhibitors have been developed that efficiently target the mutant enzymatic activity (37, 51) and hold promise for the treatment of lower-grade gliomas and AML. Identifying however also upstream components of IDH1 could potentially be equally beneficial, especially in cases that resistance mechanisms develop. In this study we identified FOXOs as regulators of IDH1 levels and thereby as important cellular components for the metabolic rewiring of cancer cells. FOXO function is in turn linked to their subcellular localization and efficient targeting of the molecular pathways that are responsible for FOXO nuclear retention in transformed cells could potentially prove beneficial for the treatment of cancer types related to IDH1.

Methods

DNA cloning and mutagenesis

For IDH1 cDNA cloning, total RNA isolated from RPE cells was reversed transcribed and full length IDH1 cDNA was amplified by PCR using the following primers: IDH1 Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGTCCAAAAAAATCAGTGGCGGTTCTGTGG and IDH1 Reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTAAAGTTTGGCCTGAGCTAGTTTGATCTTCAAG and the following PCR conditions: 98ºC for 3 min, 30 repeats of 98ºC for 30 sec, 65ºC for 30 sec and 72ºC for 2 min and a final extension step of 72ºC for 10 min. The cDNA was inserted into pDonor (Invitrogen); following the BP reaction, IDH1 coding sequence was transferred to

pcDNA3-V5 compatible with the Gateway system. For the generation of the IDH1 R132C mutant the following primers were used: R132C Forward CCTATCATCATAGGTTGTCATGCTTATGGG and R132C Reverse CCCATAAGCATGACAACCTATGATGATAGG. The pH1tet-flex/FH1t(FOXO1/3)UTG (Taconic Artemis) was previously described (Chapter 2).

Cell culture and transfections

The human retina epithelial cells RPE were maintained in DMEM/Ham F12 (Gibco) supplemented with 10% FBS (Gibco) and penicillin-streptomycin. DL23 cells were cultured in RPMI (Lonza) supplemented with 10% FBS and penicillin-streptomycin. HeLashFOXO, A14 and HT1080 cells were maintained in DMEM (Lonza) supplemented with 10% FBS, 2 mM glutamine (Lonza) and penicillin-streptomycin. DL23 and HeLashFOXO cells were previously described ((52) and Chapter 2). A14 cells were previously described (26).
To establish the RPEshFOXO and the HT1080shFOXO cells, RPE and HT1080 cells respectively were virally transduced with pH1tet-flex/FH1t(FOXO1/3)UTG and the positive clones were selected with FACS sorting.
IDH1 smartpool was purchased from Dharmacon. SiRNA transfections were performed with HiPerFect (Qiagen) and plasmid transfections were performed with FuGENE HD (Promega), according to the manufacturer's instructions.
Hypoxia was established in a hypoxic chamber (Pathology Department, UMC Utrecht).

RNA extraction and quantitative PCR (Q-PCR)

RNA was extracted with the RNeasy kit (Qiagen), with on column DNase treatment (Qiagen), according to the manufacturer's instructions. RNA was reversed transcribed with oligodT primers and the iScript cDNA synthesis kit (Biorad). Quantitative PCR was performed with FastStart SYBR Green Master mix (Roche) with the following primer sequences:
IDH1_F: TTGTCCAGATGGCAAGACAG, IDH1_R: CAGGCAAAAATGGAAGCAAT, IDH2_F: CTCATCAGGTTTGCCCAGAT, IDH2_R: GTCCGTGGTGTTCAGGAAGT, IDH3A_F: GAGATGGTATTGGCCCAGAA, IDH3A_R: TTCCTCCAGGTCCTTGAATG, IDH3B_F: TGGTGATCATTCGAGAGCAG, IDH3B_R: TGAGACTTGGCTCGTGTGAC, IDH3G_F: ATGTGTACGCGGTGTTTGAA, IDH3G_R: ATGGAGGTGGCATAGGAGTG, Tubulin_F: TACACCATTGGCAAGGAGAT, Tubulin_R: AACCAAGAAGCCCTGAAGAC, mIDH1_F: TCACCAAAGATGCTGCAGAG, mIDH1_R: TGGGGATTTCCACATTTGTT, mPBDG_F: GCCTACCATACTACCTCCTGGCT, mPBDG_R: AAGACAACAGCATCACAAGGGTT.

Protein extraction and western blot

Total proteins were collected by direct lysis in Laemli sample buffer. Proteins were run in SDS-PAGE and transferred to Polyscreen PVDF transfer membranes (PerkinElmer) or to Protran Nitrocellulose Membranes (GE Healthcare Life Sciences). Antibodies used were the following: anti-IDH1 (Cell Signaling), anti-FOXO1 and anti-FOXO3 (Santa Cruz), anti-H3K4me3 (Abcam), anti-H3K9me3 (Abcam), anti-H3 (Abcam), tubulin (Calbiochem).

Chromatin Immunoprecipitation (ChIP)

To perform ChIP, FOXO3A3 in DL23 cells was activated by addition of 4-OH-tamoxifen for 4 hrs. 40 million cells per condition were fixed with 1% formaldehyde for 10 min at room temperature and the reaction was quenced by 0.125 M Glycine for 5 min. Subsequently cells were lysed in Darnham lysis buffer (5 mM PIPES, 85 mM KCl, 0.5% NP-40, protease inhibitor cocktail Roche) and nuclear extracts were collected by low speed centrifugation and re-suspended in RIPA buffer (1x PBS, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, supplemented with Roche protease inhibitor cocktail). Chromatin was then sonicated to acquire DNA fragments of about 500 bp which were then used for immunoprecipitations. Antibodies used were: rabbit IgG (Santa Cruz) and rabbit anti-FOXO3a (Santa-Cruz).
The oligomers used for the amplification of the areas within IDH1 were:
IDH1 F: CATTCATCTGGGGAGATTCAA, IDH1 R: GTTTCATGCATGGGGTAAGG, IDH1 neg F: CTTCCATCTGGTGATGTGGTT, IDH1 neg R: TTCCCCACTATCTTTGCTCCT

α-KG and 2-HG measurements

For the determination of intracellular α-KG, RPEshFOXO cells that were cultured in DMEM containing or not doxycycline for 96 hrs, were plated in 6 wells plates (40,000 cells per well) and grown for another 16hrs. Thereafter, cells were washed with 10% NaCl, collected in ice-cold methanol and H2O and, after addition of chloroform, were allowed to extract at 4ºC for 20 min. Subsequently, samples were spinned down and the aqueous phase was used for the further analysis and

measurement by GS-MS.
Similar approach was used for the extraction and measurement of α-KG and 2-HG from HT1080shFOXO cells.

NADPH/NADP+ and GSH/GSSG measurements

For the determination of NADPH/ NADP+ and GSH/GSSG ratio, RPEshFOXO cells were grown in DMEM containing or not doxycycline for 48 hrs. Subsequently, the growth medium was switched to DMEM minus glucose (Gibco) supplemented with 10 mM pyruvate and 2 mM glutamine (Lonza) and grew for another 48 hrs. NADP+ and NADPH were measured with the NADP/NADPH Quantitation Colorimetric Kit (BioVision), according to the manufacturer's instructions. GSH and GSSG were measured with the Glutathione (total) detection kit (Enzo).

Growth rates and soft agar assays

To determine the differences in growth rates in the HT1080shFOXO cells after FOXO depletion, 20,000 cells per well were plated in 6well plates and subsequently doxycycline was added in half of the wells. After 48 hrs and every 24 hrs thereafter, cells were collected and counted using the Countess Automated Cell Counter (Life Technologies). For the study of RPEshFOXO cells growth rates in hypoxia, after plating cells were retained in normoxic conditions for 8 hrs and subsequently transferred in the hypoxic chamber for the rest of the experiment.
For the soft agar assay, 20,000 HT1080shFOXO cells were plated in a 0.35% top layer (DMEM, 10% FBS), on top of a 0.5% bottom layer (DMEM, 10% FBS). Where appropriate, the upper layer was supplemented with doxycycline. The cells were fed every 4 days with DMEM 10% FBS and doxycycline was refreshed. Cells were allowed to grow for 3 weeks and images were acquired in a Zeiss Axioskop 40 microscope.

Isolation of genomic DNA and 5-methylcytosine blot

HT1080shFOXO cells were grown in DMEM containing or not doxycycline for 96 hrs. Genomic DNA was isolated with the DNA mini kit (Qiagen), according to the manufacturer's instructions. Subsequently, 100, 50, 25, 10 and 5 ngr of genomic DNA were spotted on Hybond N membrane (GE Healthcare) and then blotted with anti-methyl cytosine antibody (Millipore) or anti hydroxyl-methyl cytosine antibody (Active motif).

Acknowledgements

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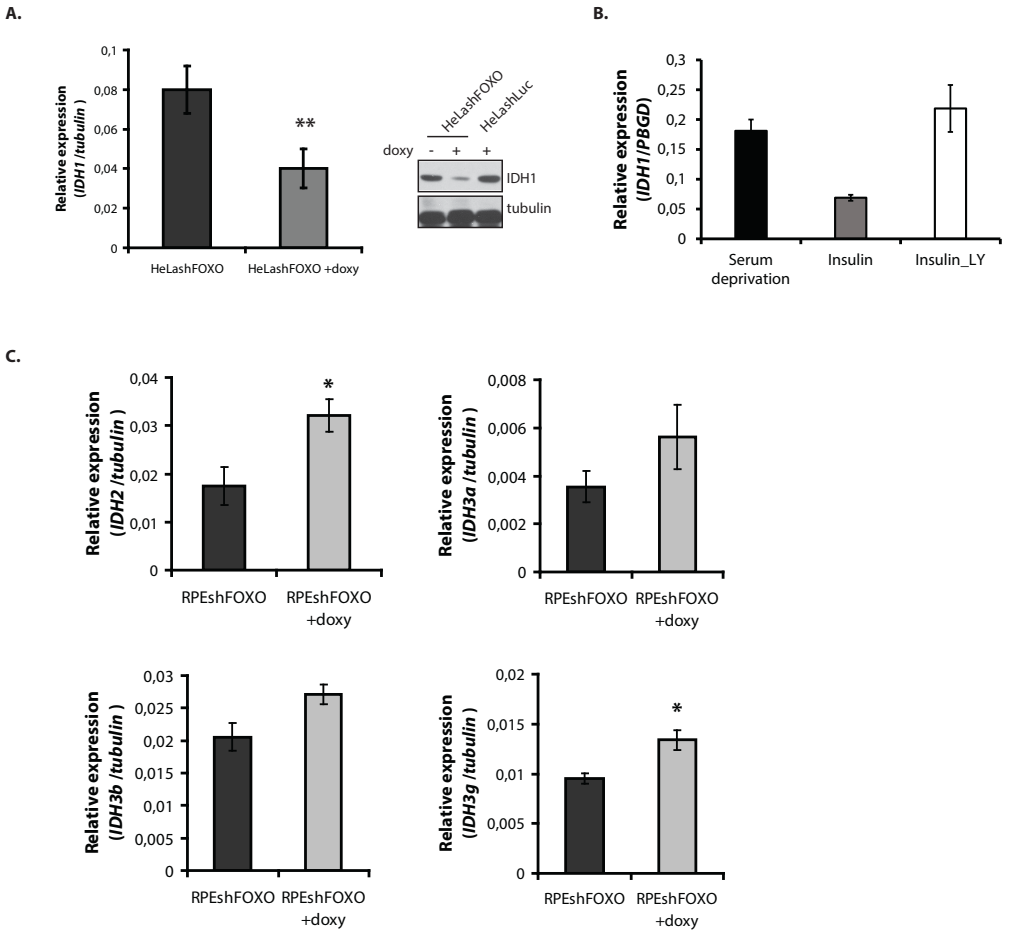
References

1. Charitou P & Burgering BM (2012) Forkhead Box(O) in Control of Reactive Oxygen Species and Genomic Stability to Ensure Healthy Lifespan Antioxidants & Redox Signaling.
2. van der Horst A & Burgering BMT (2007) Stressing the role of FoxO proteins in lifespan and disease. Nat Rev Mol Cell Biol 8(6):440-450.
3. Eijkelenboom A & Burgering BMT (2013) FOXOs: signalling integrators for homeostasis maintenance. Nat Rev Mol Cell Biol 14(2):83-97.
4. Kops GJPL, et al. (1999) Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature 398(6728):630-634.
5. Brunet A, et al. (1999) Akt Promotes Cell Survival by Phosphorylating and Inhibiting a Forkhead Transcription Factor. Cell 96(6):857-868.
6. Bakker WJ, Harris IS, & Mak TW (2007) FOXO3a Is Activated in Response to Hypoxic Stress and Inhibits HIF1-Induced Apoptosis via Regulation of CITED2. Molecular Cell 28(6):941-953.
7. Essers MAG, et al. (2004) FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. EMBO J 23(24):4802-4812.
8. Tsai W-B, Chung YM, Takahashi Y, Xu Z, & Hu MCT (2008) Functional interaction between FOXO3a and ATM regulates DNA damage response. Nat Cell Biol 10(4):460-467.
9. Hanahan D & Weinberg RA (2011) Hallmarks of Cancer: The Next Generation. Cell 144(5):646-674.
10. Ward Patrick S & Thompson Craig B (2012) Metabolic Reprogramming: A Cancer Hallmark Even Warburg Did Not Anticipate. Cancer cell 21(3):297-308.
11. Parsons DW, et al. (2008) An Integrated Genomic Analysis of Human Glioblastoma Multiforme. Science 321(5897):1807-

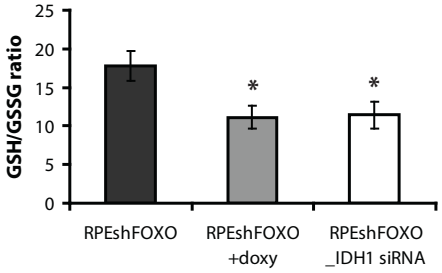
1812.
12.Yan H, et al. (2009) IDH1 and IDH2 Mutations in Gliomas. New England Journal of Medicine 360(8):765-773.
13.Yen KE, Bittinger MA, Su SM, & Fantin VR (2010) Cancer-associated IDH mutations: biomarker and therapeutic opportunities. Oncogene 29(49):6409-6417.
14.Bhagwat N & Levine RL (2010) Metabolic Syndromes and Malignant Transformation: Where the Twain Shall Meet. Science Translational Medicine 2(54):54ps50.
15.Rose NR, McDonough MA, King ONF, Kawamura A, & Schofield CJ (2011) Inhibition of 2-oxoglutarate dependent oxygenases. Chemical Society Reviews 40(8):4364-4397.
16.Hausinger RP (2004) Fe(II)/a-Ketoglutarate-Dependent Hydroxylases and Related Enzymes. Critical Reviews in Biochemistry and Molecular Biology 39(1):21-68.
17.Metallo CM, et al. (2011) Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. Nature 481(7381):380-384.
18.Mullen AR, et al. (2011) Reductive carboxylation supports growth in tumour cells with defective mitochondria. Nature 481(7381):385-388.
19.Wise DR, et al. (2011) Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of a-ketoglutarate to citrate to support cell growth and viability. Proceedings of the National Academy of Sciences 108(49):19611-19616.
20.Rakheja D, Medeiros LJ, Bevan S, & Chen W (2013) The Emerging Role of D-2-Hydroxyglutarate as an Oncometabolite in Hematolymphoid and Central Nervous System Neoplasms. Frontiers in Oncology 3.
21.Losman J-A, et al. (2013) (R)-2-Hydroxyglutarate Is Sufficient to Promote Leukemogenesis and Its Effects Are Reversible. Science 339(6127):1621-1625.
22.Losman J-A & Kaelin WG (2013) What a difference a hydroxyl makes: mutant IDH, (R)-2-hydroxyglutarate, and cancer. Genes & Development 27(8):836-852.
23.Turcan S, et al. (2012) IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. Nature 483(7390):479-483.
24.Figueroa ME, et al. (2010) Leukemic IDH1 and IDH2 Mutations Result in a Hypermethylation Phenotype, Disrupt TET2 Function, and Impair Hematopoietic Differentiation. Cancer Cell 18(6):553-567.
25.Matlashewski G, Banks L, Pim D, & Crawford L (1986) Analysis of human p53 proteins and mRNA levels in normal and transformed cells. European Journal of Biochemistry 154(3):665-672.
26.Burgering BMT & Coffer PJ (1995) Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature 376(6541):599-602.
27.Eijkelenboom A, et al. (2013) Genome-wide analysis of FOXO3 mediated transcription regulation through RNA polymerase II profiling. Molecular Systems Biology 9(1).
28.Dang CV (2012) Links between metabolism and cancer. Genes & Development 26(9):877-890.
29.Kim JY, Shin JY, Kim M, Hann S-K, & Oh SH (2012) Expression of cytosolic NADP+-dependent isocitrate dehydrogenase in melanocytes and its role as an antioxidant. Journal of dermatological science 65(2):118-125.
30.Meister A (1988) Glutathione metabolism and its selective modification. Journal of Biological Chemistry 263(33):17205-17208.
31.Kops GJPL, et al. (2002) Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature 419(6904):316-321.
32.Paik J-H, et al. (2007) FoxOs Are Lineage-Restricted Redundant Tumor Suppressors and Regulate Endothelial Cell Homeostasis. Cell 128(2):309-323.
33.Ichimura K, et al. (2009) IDH1 mutations are present in the majority of common adult gliomas but rare in primary glioblastomas. Neuro-Oncology 11(4):341-347.
34.Yan H, et al. (2009) IDH1 and IDH2 Mutations in Gliomas. New England Journal of Medicine 360(8):765-773.
35.Lu C, et al. (2012) IDH mutation impairs histone demethylation and results in a block to cell differentiation. Nature 483(7390):474-478.
36.Lam EWF, Brosens JJ, Gomes AR, & Koo C-Y (2013) Forkhead box proteins: tuning forks for transcriptional harmony. Nat Rev Cancer 13(7):482-495.
37.Rohle D, et al. (2013) An Inhibitor of Mutant IDH1 Delays Growth and Promotes Differentiation of Glioma Cells. Science 340(6132):626-630.
38.Yeo H, et al. (2013) FoxO3 coordinates metabolic pathways to maintain redox balance in neural stem cells. The EMBO Journal 32(19):2589-2602.
39.DeNicola GM, et al. (2011) Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. Nature 475(7354):106-109.
40.Chen J, et al. (2010) Constitutively Nuclear FOXO3a Localization Predicts Poor Survival and Promotes Akt Phosphorylation in Breast Cancer. PLoS ONE 5(8):e12293.
41.Boehm A-M, et al. (2012) FoxO is a critical regulator of stem cell maintenance in immortal Hydra. Proceedings of the National Academy of Sciences 109(48):19697-19702.
42.Tothova Z, et al. (2007) FoxOs Are Critical Mediators of Hematopoietic Stem Cell Resistance to Physiologic Oxidative Stress. Cell 128(2):325-339.
43.Zhang X, et al. (2011) FOXO1 is an essential regulator of pluripotency in human embryonic stem cells. Nat Cell Biol 13(9):1092-1099.
44.Sykes Stephen M, et al. (2011) AKT/FOXO Signaling Enforces Reversible Differentiation Blockade in Myeloid Leukemias.

Cell 146(5):697-708.
45.Sasaki M, et al. (2012) IDH1(R132H) mutation increases murine haematopoietic progenitors and alters epigenetics. Nature 488(7413):656-659.
46.Denko NC (2008) Hypoxia, HIF1 and glucose metabolism in the solid tumour. Nat Rev Cancer 8(9):705-713.
47.Lu C & Thompson Craig B (2012) Metabolic Regulation of Epigenetics. Cell metabolism 16(1):9-17.
48.Wellen KE & Thompson CB (2012) A two-way street: reciprocal regulation of metabolism and signalling. Nat Rev Mol Cell Biol 13(4):270-276.
49.Galluzzi L, Kepp O, Heiden MGV, & Kroemer G (2013) Metabolic targets for cancer therapy. Nat Rev Drug Discov 12(11):829-846.
50.Vander Heiden MG (2011) Targeting cancer metabolism: a therapeutic window opens. Nat Rev Drug Discov 10(9):671-684.
51.Wang F, et al. (2013) Targeted Inhibition of Mutant IDH2 in Leukemia Cells Induces Cellular Differentiation. Science 340(6132):622-626.
52.Kops GJPL, et al. (2002) Control of Cell Cycle Exit and Entry by Protein Kinase B-Regulated Forkhead Transcription Factors. Molecular and Cellular Biology 22(7):2025-2036.

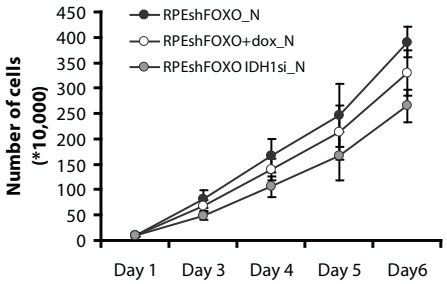
SUPPLEMENTARY DATA



Supplementary Figure S1. (A) Relative mRNA and protein levels of IDH1 in HeLashFOXO cells. HeLashFOXO cells were cultured in the presence or absence of doxycycline for 72 hrs before sample collection **(B)** Relative mRNA levels of IDH1 in A14 cells after insulin stimulation. A14 cells were cultured in serum free medium for 16 hrs before insulin stimulation for additional 4 hrs. Where indicated, cells were pre-treated with LY for 1 hr before insulin stimulation **(C)** Relative mRNA levels of IDH2, IDH3a, IDH3b and IDH3g in RPEshFOXO cells, RPEshFOXO cells were cultured in the presence or absence of doxycycline for 72 hrs before sample collection.*P<0.05, **P<0.01



Supplementary Figure S2. GSH/GSSG ratio in RPEshFOXO cells. RPEshFOXO cells were cultured in the presence or absence of doxycycline for 48 hrs and thereafter the medium was changed to medium with no glucose, supplemented with pyruvate and glutamine for another 48 hrs before sample collection. IDH1 silencing by siRNA served as a positive control.



Supplementary Figure S3. Growth rates of RPEshFOXO cells grown in normoxia. 50,000 RPEshFOXO cells were plated in in the presence or absence of doxycycline or with IDH1 siRNA transfection and kept in ambient O2. Cells were allowed to proliferate for several days, during which cells were collected and counted every 24 hrs.*P<0.05

Chapter 4

FOXOs regulate mitochondrial function and integrity at multiple levels

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Abstract

FOXO transcription factors are key players in processes related to cellular homeostasis, including reactive oxygen species detoxification, DNA repair and metabolism. Studies in model organisms have established that balanced FOXO function contributes to both tumor suppression and longevity. Mitochondrial function is also intimately linked to both cancer and aging (mitochondrial theory of aging). Here, we show that FOXOs transcriptionally regulate mitochondrial function and turnover. In the absence of FOXOs, mitochondria display impaired ATP-coupled respiration and increased ROS. Moreover, we find FOXOs to protect mtDNA from redox-induced damage. Combined, our data portray a multilayered role of FOXOs in modulating mitochondrial function and integrity.

Introduction

The Forkhead Box (O) (FOXO) family of transcription factors includes proteins that function as important homeostatic regulators, modulating processes intimately linked to cancer and aging (1). FOXO function is inhibited by insulin and growth factors, via the PI3K/PKB (Akt) pathway (2, 3), and is activated by stress signals, including oxidative stress, DNA damage and growth factor deprivation. FOXOs in turn regulate DNA repair, reactive oxygen species (ROS) detoxification and cellular growth ((4, 5) **Chapter 2**). FOXOs are also involved in the maintenance of the quiescence state of hematopoietic stem cells (HSCs) (6); in FOXO knockout mice the HSCs pool is rapidly exhausted, thereby contributing to aging phenotypes. The FOXO family in humans includes four members, namely FOXO1, FOXO3, FOXO4 and FOXO6. FOXOs are highly conserved during evolution; the FOXO homologues in lower order organisms, such as the nematode *C. elegans* (daf-16) or the fruitfly *Drosophila melanogaster* (dFOXO), are indispensable for lifespan extension (reviewed in (7)). FOXOs also appear to contribute to longevity in higher order organisms, including humans, as studies have shown a positive correlation between genetic variations in FOXO genes and long lifespan (8-10).

During the past years several theories have been proposed to shed light in the process of aging. One such theory, “the mitochondrial theory of aging” proposes that ROS, leaking from the mitochondria during the process of oxidative phosphorylation (OXPHOS), reacts with and irreversibly damages macromolecules, including proteins, lipids and nucleic acids (11, 12). ROS leakage also affects mitochondrial function, thereby contributing to a vicious cycle of mitochondrial dysfunction and increased redox stress. Accumulating macromolecular and organelle damage results in cellular dysfunction, elevated cellular clearance and tissue degeneration, eventually driving the aging process. Mitochondria contain their own genetic material, the mitochondrial DNA (mtDNA), which carries the genetic information for the synthesis of components of the respiratory chain complexes. Accrual of mtDNA mutations is observed in aging tissues and in fact mtDNA damage was also suggested as a driver of aging (13, 14). Direct evidence linking mtDNA mutations to aging comes from a study by Trifunovic et al that developed mice carrying a proof-reading-deficient version of the mtDNA polymerase (mtDNA mutator mice) and observed an increase in mtDNA deletions/point mutations and accelerated aging phenotypes in these mice (15).

FOXOs role in aging has been suggested to rely on the regulation of cellular redox and in particular the reduction of ROS levels (7). Additionally, it was noticed that in FoxO1/3/4 triple-knockout mice, cells harbor dysfunctional mitochondria (16). Therefore, it was suggested that FOXOs are required for proper mitochondrial function, yet a mechanistic explanation was lacking. In this study we provide an integrated

model of the mitochondrial processes regulated by FOXO transcriptional activity. We show that FOXOs are involved in (i) the maintenance of mtDNA integrity by regulating the levels of the mitochondrial Transcription Factor A (TFAM) and Tyrosyl DNA phosphodiesterase 1 (TDP1), (ii) the reduced leakage of mitochondrial produced ROS, by regulating the transcription of Uncoupling Protein 2 (UCP2) and (iii) the increased turnover of dysfunctional mitochondria, by regulating PTEN-Induced Kinase 1 (PINK1) levels. Also FOXOs contribute to energy production by mitochondria, likely through the modulation of the integrity of the respiratory chain complexes.

RESULTS

Bioenergetic changes induced by FOXO depletion

To understand the role of FOXOs in mitochondrial function, we first measured their contribution to mitochondrial bioenergetics. To this end we employed the Seahorse extracellular flux analyzer (XF-24) system, which allows for the interrogation of cellular bioenergetics with the sequential use of certain mitochondrial OXPHOS inhibitors (for a schematic of a typical bioenergetic profile acquired after a mitochondrial stress test using the XF-24, along with a depiction of the main bioenergetic parameters that can be determined in such a setting, refer to **Supplementary Figure 1A**). Changes in oxygen consumption rate (OCR) measured by XF-24 are an indicator of mitochondrial function and changes in medium pH due to the excretion of lactate (extracellular acidification rate, ECAR) are indicative of glycolysis (17). For our analysis we employed the untransformed retinal pigment epithelium (RPE) cells, in which we stably expressed a doxycycline-inducible shRNA targeting FOXO1 and FOXO3 (RPEshFOXO cells). As seen in **Figure 1A**, shRNA-mediated depletion of FOXOs results in alterations of the mitochondrial bioenergetic capacity. Primarily, mitochondria in FOXO depleted cells show a lower basal respiration, compared to control cells **(Figures 1A and 1B)**. This could be illustrative of either that FOXO-depleted cells have less mitochondrial mass or that their mitochondria are less functional. We therefore visualized the mitochondrial network in cells depleted of FOXOs, by immunostaining for the mitochondria-specific protein TOM20. As seen in **Figure 1C**, FOXO-depleted cells have an increased mitochondrial network compared to control cells. Similar results were obtained with the mitochondrial-specific probe Mito-tracker **(Supplementary Figure 1B)**. This suggests that the reduced basal respiration rate of the FOXO-depleted cells is probably linked to mitochondrial dysfunction.

Consequently, we further interrogated the bioenergetic profile of the FOXO-depleted cells by performing a mitochondrial stress-test. After oligomycin treatment we observed that oxygen consumption coupled to ATP synthesis (Oligomycin stimulated OCR – Basal OCR) is significantly reduced in FOXO-depleted cells **(Figure 1B, coupling efficiency and Supplementary Figure 1C)**. This indicates an involvement of FOXOs in the regulation of ATP synthesis by OXPHOS. Treatment with the proton ionophore FCCP provided variable results in the course of our experiments; however a trend towards higher spare respiratory capacity (FCCP stimulated OCR – Basal OCR) in the FOXO-depleted cells was evident **(Figure 1B, spare respiratory capacity and Supplementary Figure 1C)**. It should be noted however that the maximal respiratory capacity in the cells with acute FOXO knockdown was lower to the one of the control cells **(Figure 1A)**.

Taken together, the results from the bioenergetics analysis suggest that mitochondrial function is impaired in FOXO-depleted cells. The observed extension of the mitochondrial network could be due to changes in signaling, induced by FOXO depletion, that regulate constraints on mitochondrial biosynthesis (18), or alternatively, considering the aforementioned mitochondrial dysfunction, it could merely reflect a compensatory response. Importantly, our results cannot be attributed to the low concentration of doxycycline

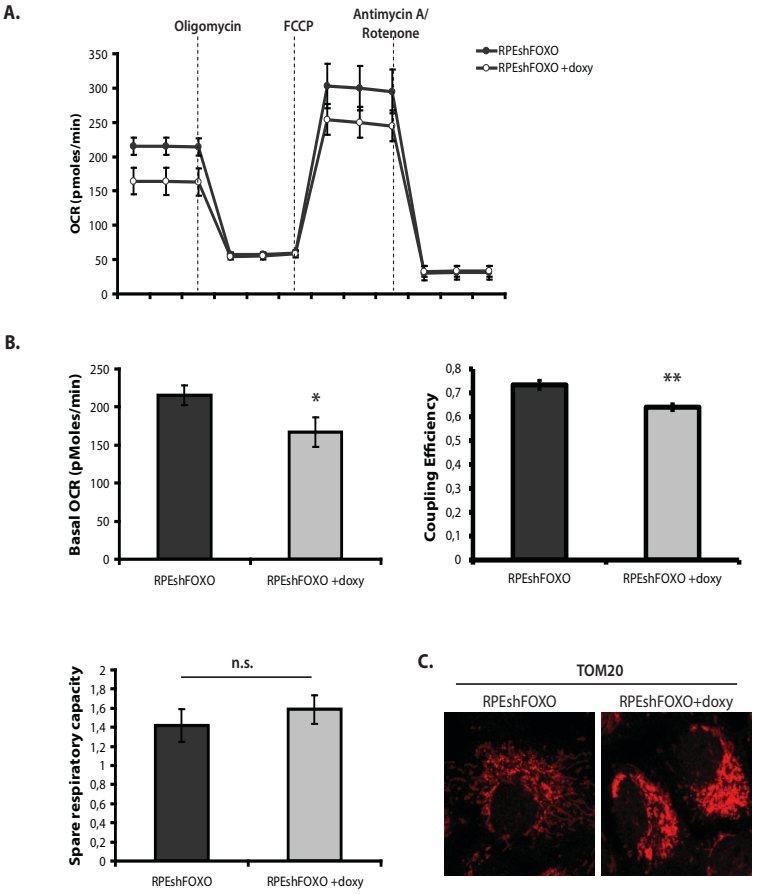

Figure 1: FOXO regulate cellular bioenergetics (A) Effects of FOXO depletion on the mitochondrial bioenergetic profile. RPEshFOXO cells were cultured in the presence or absence of doxycycline for 72 hrs. 40,000 cells were plated in XF-24 culture plates and 16 hrs later they were used for a mitochondrial stress test in the Seahorse XF-24 analyzer **(B)** Main bioenergetics parameters assessed by the XF-24 profile **(C)** Extended mitochondrial network in FOXO-depleted cells. RPEshFOXO cells were cultured in the presence or absence of doxycycline for 96 hrs. Cells were fixed and immunostained for the mitochondrial protein TOM20.*P<0.05, **P<0.01, ns: not-significant

used in the experiments, as cells expressing doxycycline-inducible shRNA targeting the luciferase gene and treated with doxycycline, exhibit a bioenergetic profile similar to control cells **(Supplementary Figure 1D)**.

FOXO depletion results in reduced ATP production and AMPK activation

To obtain a better understanding of the regulation of ATP-coupled respiration by FOXOs, we first measured ATP levels in FOXO-depleted cells. We found that depletion of endogenous FOXOs results in a significant reduction of ATP production **(Figure 2A)**, in agreement with the oligomycin-dependent response observed in the XF-24 measurements. In line with this, a careful inspection of the OCR versus ECAR profiles acquired from the XF-24 analyzer shows that FOXO depleted cells exhibit a bioenergetic profile indicative of reduced energy production **(Figure 2B)**.

Reduced intracellular ATP levels are expected to result in activation of 5' AMP-activated protein kinase (AMPK) due to an increase of the AMP/ATP ratio (19). We therefore analyzed AMPK activity, by determining

AMPK autophosphorylation, and we found increased AMPK autophosphorylation in the FOXO-depleted cells **(Figure 2C)**. AMPK activation is associated, amongst other responses, to mitochondrial biogenesis (19), which might account in part for the extensive mitochondrial network we observe in the FOXO depleted cells.

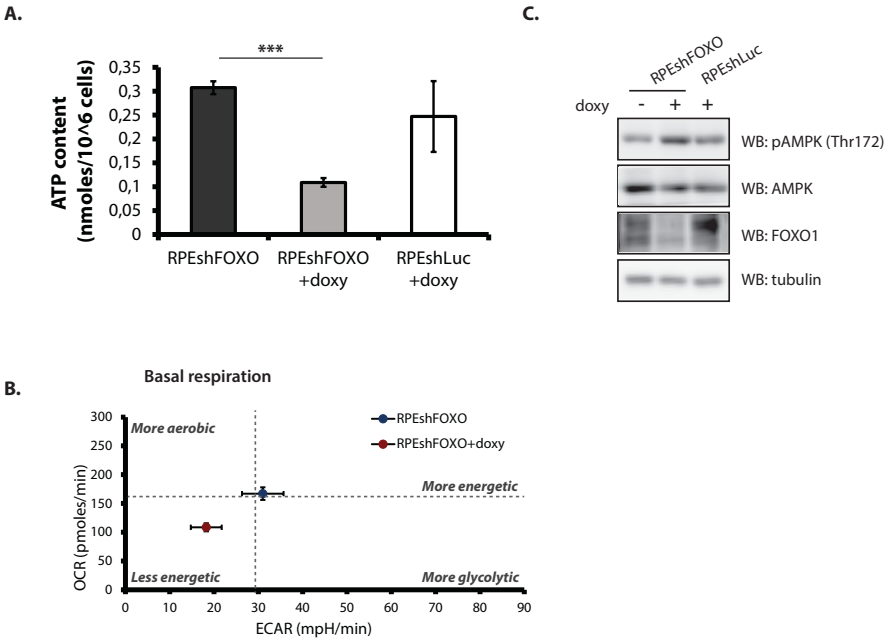

Figure 2: Energetic crisis in FOXO-depleted cells (A) ATP content in FOXO depleted cells. RPEshFOXO and RPEshLuc cells were cultured in the presence or absence of doxycycline for 96 hrs before sample collection and ATP measurement **(B)** Comparative profile of OCR versus ECAR values, as these are measured in the XF-24 (related to Figure 1A) **(C)** AMPK activity in response to FOXO depletion. RPEshFOXO and RPEshLuc cells were cultured in the presence or absence of doxycycline for 96 hrs before protein sample collection. ***P<0.001

FOXO depletion results in reduced TFAM and TDP1 expression and increased mtDNA damage

Mitochondrial dysfunction is often linked to the accrual of mtDNA mutations (20). To obtain a better mechanistic insight into the impaired mitochondrial function observed in the FOXO depleted cells, we therefore analyzed mtDNA integrity. We isolated mtDNA from RPEshFOXO cells grown under non-damaging conditions, as well as cells treated with 100 μM of hydrogen peroxide (H2O2) for 1 hr and assessed mtDNA damage by employing the long-amplicon quantitative PCR assay (21). We found that, compared to control cells, FOXO-depleted cells show increased mtDNA damage following H2O2 challenge and therefore exhibit increased susceptibility to ROS-induced genotoxic stress **(Figure 3A)**.

Mitochondrial DNA is devoid of histones and is therefore more sensitive to damage inflicted by ROS leaking into the mitochondrial matrix during OXPHOS (22, 23). To protect mtDNA from damage, certain mechanisms have been evolved. Primarily, TFAM, a nuclear-encoded protein that resides in the mitochondria, regulates the condensation of mtDNA and thereby contributes to mtDNA protection from genotoxic insults (24). Mitochondria are not equipped with the multitude of sophisticated DNA repair mechanisms that operate in the nucleus however a number of DNA repair mechanisms have been identified to operate within

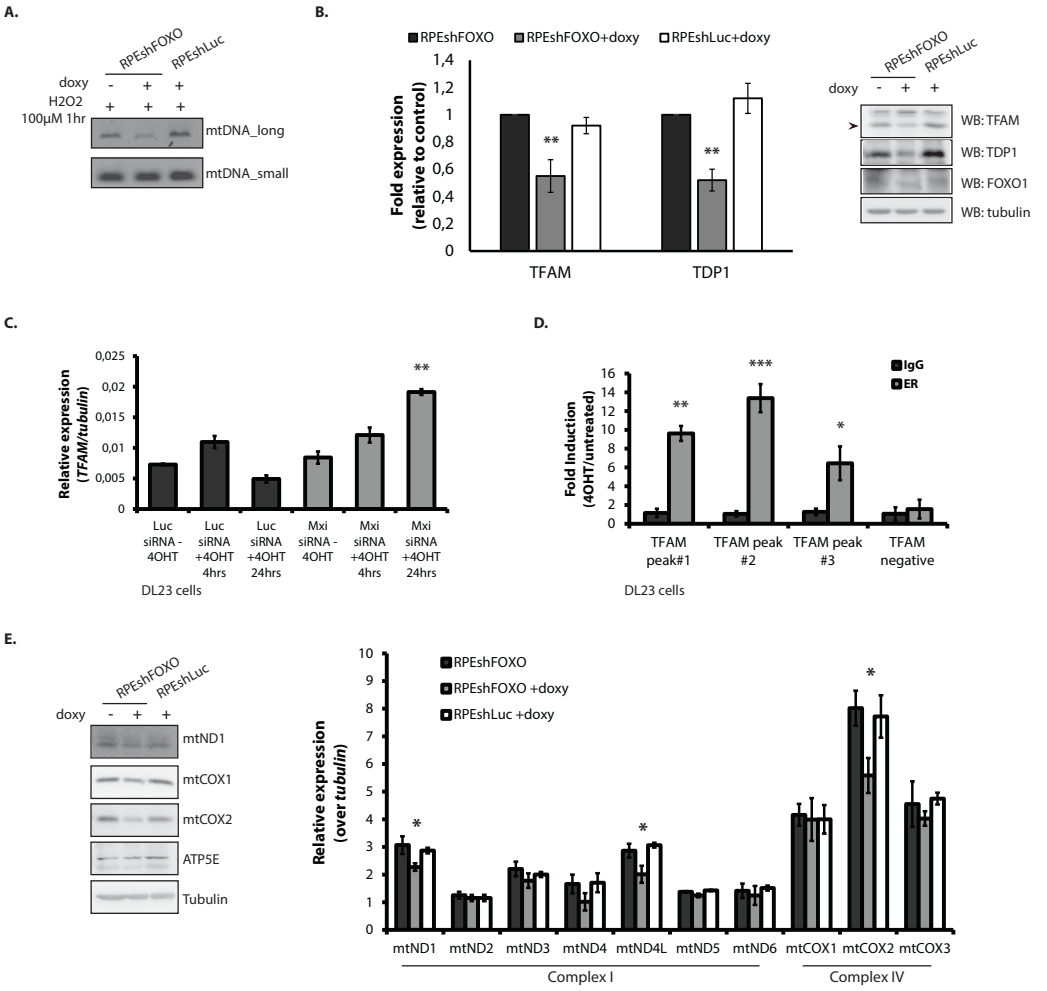

Figure 3: FOXO role in mtDNA integrity and transcription (A) MtDNA damage assessed by LA-QPCR. RPEshFOXO and RPEshLuc cells were cultured in the presence or absence of doxycycline for 96 hrs. Cells were treated with 100 μM H2O2 for 1 hr before genomic DNA isolation. 15 ng of total genomic DNA were used for the amplification of a long and a short fragment of mtDNA. Polymerization of the short amplicon (mtDNA_short) is an indication of mtDNA content, while polymerization of the long amplicon (mtDNA_long) is an indication of mtDNA damage **(B)** TFAM and TDP1 levels in FOXO-depleted cells. RPEshFOXO and RPEshLuc cells were cultured in the presence or absence of doxycycline for 96 hrs before RNA and protein sample collection. Arrowhead indicates height of TFAM protein **(C)** Bimodal regulation of TFAM in DL23 cells. DL23 cells were transfected with luciferase or MXI siRNA for 72 hrs. Cells were treated with 4-OH-tamoxifen for 4 or 24 hrs and RNA samples were collected **(D)** Direct binding of FOXO at TFAM genomic region. DL23 cells were treated with 4-OH-tamoxifen for 4 hrs, followed by ChIP-qPCR at the areas identified by ChIP-sequencing **(E)** Defects in mtDNA transcription in response to FOXO depletion. RPEshFOXO and RPEshLuc cells were cultured in the presence or absence of doxycycline for 96 hrs before RNA and protein sample collection.*P<0.05, **P<0.01, ***P<0.001

mitochondria, including base excision repair (BER), mismatch repair, single strand break repair and double strand break repair by non-homologous end-joining (25, 26). To understand the regulation of mtDNA integrity by FOXOs, we first set out to investigate the regulation of mtDNA repair components by FOXOs. A gene expression analysis performed in our laboratory, to identify transcriptional changes after FOXOs transient depletion **(Addendum)**, revealed that FOXOs regulate TFAM and TDP1 expression levels. We validated these

results in the RPEshFOXO cells, in which we found that FOXO depletion results in reduced TFAM and TDP1 mRNA and protein levels **(Figure 3B)**. TDP1 is a protein involved in the processing of DNA ends and the removal of 3'-blocking groups. Apart from the well-established role of TDP1 in the repair of topoisomerase I adducts, other roles have been proposed as well, including a function in mtDNA BER for the repair of mtDNA oxidative damage (27, 28). This suggests that FOXOs transcriptionally regulate the integrity of mtDNA both by promoting mtDNA protection from genotoxic insults but also by the orchestration of DNA repair responses.

Our data on TFAM appear to contradict a recent report that showed FOXO3 to negatively regulate TFAM expression in DLD1 cells, via the expression of MXI1 and the subsequent inhibition of c-MYC (18). To understand whether in fact this is a case of bimodal regulation, we performed a time-course of FOXO3 activation in DLD1 cells stably expressing FOXO3(A3)-ER (DL23 cells) (29). **Figure 3C** shows that early upon FOXO3 activation, TFAM expression levels show a small but significant increase, however they are suppressed at later time points. Interestingly, when we silenced the expression of MXI1, suppression of TFAM by FOXOs was alleviated, suggesting that FOXOs in DLD1 cells regulate the expression of TFAM both directly (FOXO-dependent TFAM transactivation) and indirectly (MXI1-dependent c-MYC suppression). A previous ChIP-sequencing analysis performed in our laboratory (30) identified three FOXO3 peaks around TFAM, one close to the transcription start site (TSS) and two close to the transcription termination (TES). We performed ChIP-QPCR analysis after FOXO3(A3) activation in DL23 cells and verified the specific binding of FOXO at these sites **(Figure 3D)**. The finding of FOXO3 binding to the genomic area around TFAM, in combination with the TFAM transactivation early after FOXO activation, are in line with the hypothesis that FOXOs are involved in the maintenance of a TFAM sustained expression under no-stress conditions.

Impaired expression of mtDNA encoded genes in FOXO-depleted cells

Decreased ATP production in cells depleted of FOXOs could be attributed to the accrual of mutations in the mtDNA, although it has been suggested that a certain threshold of mtDNA mutations has to be reached to have an effect on respiratory chain function (20, 22). Considering that TFAM is also involved in the induction of mtDNA transcription (24), we looked into the expression levels of mtDNA-encoded genes in the FOXO-depleted cells. Mitochondrial DNA encodes for 14 genes, along with tRNA and rRNAs (14); the mitochondrial-encoded genes are components of the electron transport chain (ETC). We next evaluated the levels of 10 mtDNA encoded genes (ND1, ND2, ND3, ND4, ND4L, ND5, ND6, COX1, COX2, COX3). FOXO-depleted cells, as seen in **Figure 3E**, exhibit reduced transcription of certain mtDNA encoded genes, namely ND1, ND4L and COX2. This suggests that the respiratory complexes I and IV may show reduced functionality in cells lacking FOXOs, which could account for the reduced ATP production.

FOXO-depleted cells have increased mitochondrial ROS and decreased ROS protection mechanisms

Mitochondrial dysfunction can result in increased cellular redox due to the elevated production and leakage of mitochondrial ROS (20, 31). To this end, we employed the mitochondria-specific probe, mito-SOX, which is an indicator of superoxide anions, and we observed higher basal mitochondrial ROS in the FOXO-depleted cells **(Figure 4A)**. FOXO activation is known to regulate the transcription of manganese-superoxide oxidase (MnSOD) and this contributes to the detoxification of mitochondrial superoxide (4). We therefore analyzed the expression level of MnSOD in cells depleted of FOXOs. Surprisingly, we found that the expression of MnSOD does not significantly change upon FOXO depletion. Thus MnSOD transcription does not critically

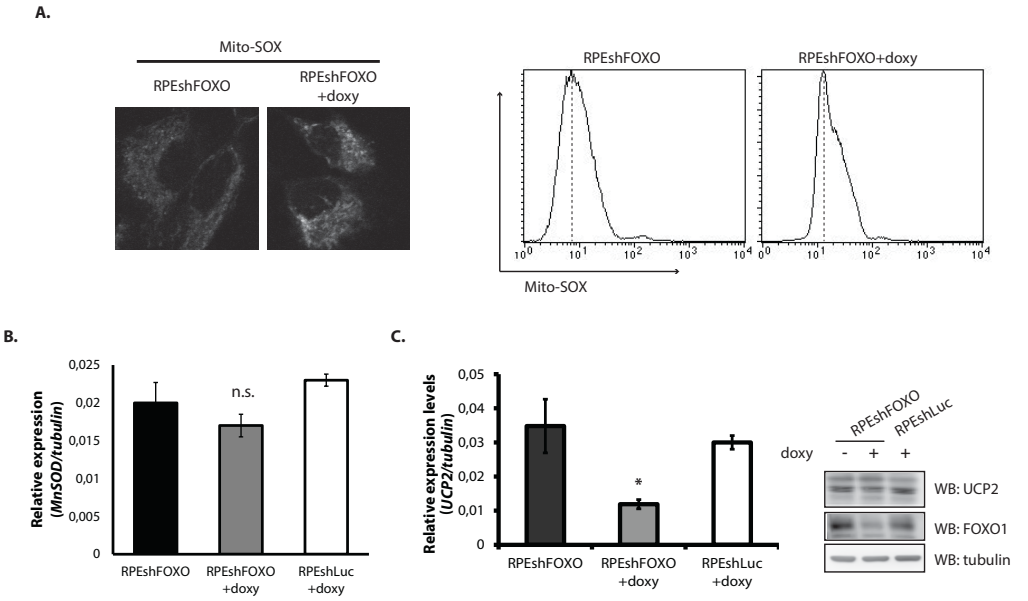

Figure 4: Increased mitochondrial ROS in FOXO-dpleted cells (A) ROS levels in FOXO-depleted cells. RPEshFOXO and RPEshLuc cells were cultured in the presence or absence of doxycycline for 96 hrs. Cells were incubated with MitoSOX for 30 min and either fixed for confocal microscopy or collected and analyzed by FACS **(B)** Relative MnSOD levels in FOXO-depleted cells. RPEshFOXO and RPEshLuc cells were cultured in the presence or absence of doxycycline for 96 hrs before sample collection **(C)** Relative UCP2 levels in FOXO-depleted cells. RPEshFOXO and RPEshLuc cells were cultured in the presence or absence of doxycycline for 96 hrs before sample collection.*P<0.05, ns:not-significant

depend on endogenous FOXOs **(Figure 4B)**, suggesting first, that other transcription factors act redundant to FOXO in the regulation of MnSOD under mild/endogenous stress conditions and second, that another FOXO target gene(s) regulate mitochondrial ROS accumulation. The family of uncoupling proteins (UCPs) are likely candidates, in this respect, as potential FOXO-target genes involved in the maintenance of mitochondrial ROS (32). We found the levels of UCP2 specifically to be significantly reduced in the FOXO-depleted cells **(Figure 4C)**, suggesting that FOXOs potentially modulate mitochondrial ROS under mild/endogenous stress conditions, by regulating UCP2 expression.

FOXOs regulate mitophagy of dysfunctional mitochondria by regulating the transcription of PINK1

Dysfunctional mitochondria are normally removed from the cell by a form of autophagy, termed mitophagy (31). Mitophagy serves as a mitochondrial quality control process activated in response to mitochondrial damage (33). To study the efficiency of mitophagy in the FOXO-depleted cells, we treated RPEshFOXO cells with the uncoupler FCCP, which induces mitochondrial membrane depolarization and activates mitochondrial clearance. We found FOXO-depleted cells to show impaired mitophagy, as this is evident by the reduced ratio of type II over type I LC3 after FCCP treatment **(Figure 5A)**.

One of the first steps of mitophagy is the accumulation on the mitochondrial membrane of the protein PINK-1, which then facilitates the accumulation of the E3 ligase Parkin (34). Parkin is then involved in the orchestration of the responses for the mitophagic clearance of dysfunctional mitochondria (34). A previous study had shown that FOXO3 can directly activate the transcription of PINK1 (35) and in the gene expression

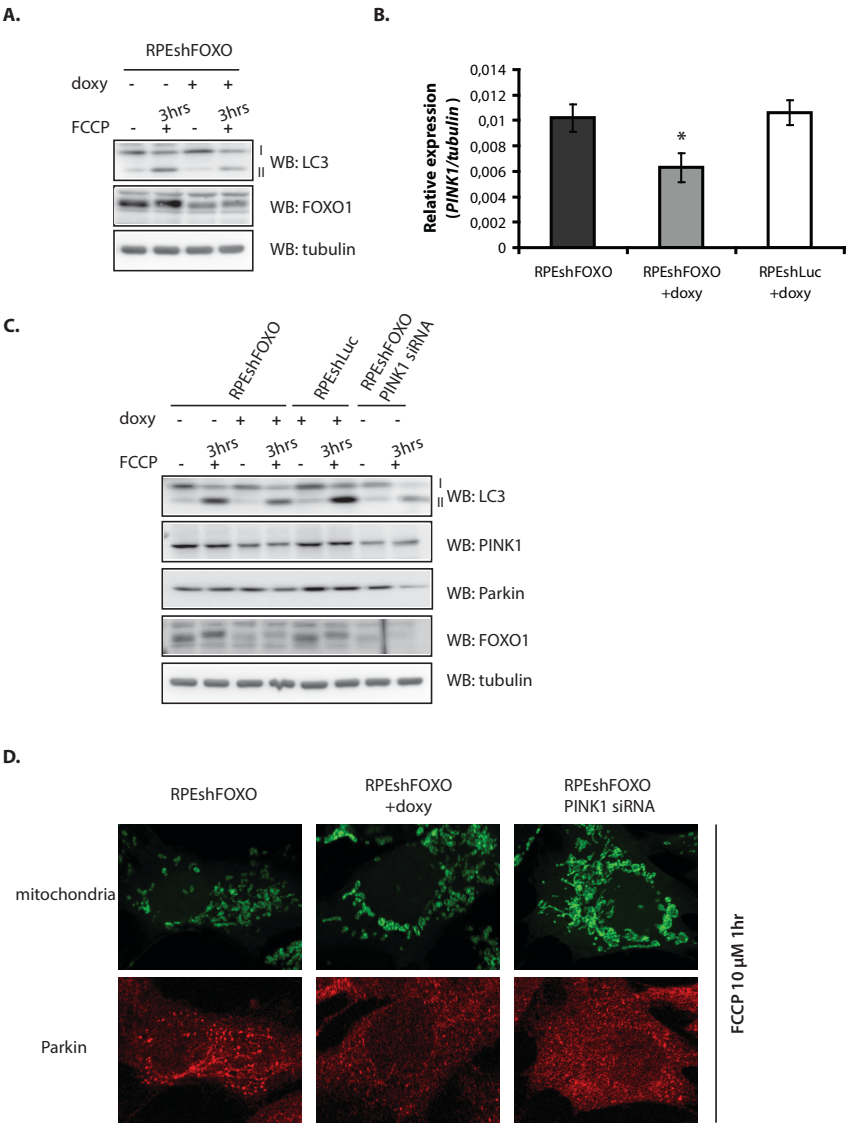

Figure 5: Impaired mitophagy in the absence of FOXO (A) Mitophagy in FOXO-depleted cells. RPEshFOXO cells were cultured in the presence or absence of doxycycline for 96 hrs. Cells were treated with 10 µM FCCP for 3 or 6 hrs before protein samples collection **(B)** PINK1 levels in FOXO-depleted cells. RPEshFOXO and RPEshLuc cells were cultured in the presence or absence of doxycycline for 96 hrs before sample collection **(C)** Mitophagy defects in response to FOXO or PINK1 depletion. RPEshFOXO and RPEshLuc cells were cultured in the presence or absence of doxycycline or RPEshFOXO cells were transfected with PINK1 siRNA for 96 hrs. Cells were treated with 10 µM FCCP for 3 hrs before protein samples collection **(D)** Mitophagy defects in response to FOXO depletion. RPEshFOXO cells were cultured in the presence or absence of doxycycline or transfected with PINK1 siRNA for 96 hrs. Cells were treated with 10 µM FCCP for 1 hr before cells were fixed and immunostained for Parkin and a mitochondrial-specific protein. *P<0.05

analysis described in **Addendum** PINK1 was also identified as a FOXO-target gene. We therefore set out to investigate PINK1 regulation by FOXOs in our system and we found that FOXO-depleted cells showed a significant reduction in PINK1 levels **(Figure 5B)**. Moreover, the defect in mitophagy observed after FOXOs

depletion was comparable to the effect of PINK1 depletion by siRNA **(Figure 5C)**. We subsequently looked into the recruitment of Parkin to mitochondria soon after FCCP treatment and we found that FOXO-depleted cells have impaired Parkin recruitment in response to FCCP-induced mitochondrial depolarization **(Figure 5D)**. All the above suggest that FOXO-depleted cells exhibit impaired mitochondrial turnover, resulting in the accumulation of dysfunctional mitochondria, as this was previously shown for PINK1 (36). Importantly, defective mitophagy in the FOXO-depleted cells, is expected to amplify a vicious circle of mitochondrial dysfunction, as accumulation of damaged organelles was shown to mitigate respiratory capacity (36).

Discussion

Here, for the first time, we provide an integrated view of the multilayered roles FOXOs play in mitochondrial function and integrity. In particular, we show FOXOs to regulate (i) the mitochondrial function for optimal coupling of oxygen consumption to ATP generation (ii) the maintenance of mtDNA stability and transcription of ETC components, (iii) the mitochondrial ROS production/leakage and (iv) the turnover of dysfunctional mitochondria (summarized in **Figure 6**).

Aging is associated with increased cellular and tissue degeneration (7). Interestingly, whereas several theories have been proposed for the aging process, including the "free radical theory" and the "DNA damage theory", a universal view on the factors that drive aging is still missing. However, it is increasingly becoming clear that the disturbance of homeostasis is a common characteristic of aging cells. Effective and balanced metabolism is in turn required for the maintenance of several homeostatic processes. Therefore, not surprisingly, different aging-associated diseases, including Parkinson's disease, Alzheimer's disease and type II diabetes are linked to disturbance of mitochondrial homeostasis, as a result of mitochondrial dysfunction (14, 37-39). In agreement, tissues with high-energy demand, such as the brain, heart and muscles, are particularly vulnerable to mitochondrial dysfunction and show accelerated degeneration with age (31, 40). We and others have shown that FOXOS are involved in redox ((4, 6)) and DNA homeostasis ((41, 42) and **Chapter 2**). Here, we report on FOXOs contribution to the regulation of mitochondrial homeostasis at multiple levels. We propose that the multilayered control of mitochondrial regulation by FOXO supports cellular health and in turn FOXO depletion or inhibition disturbs mitochondrial homeostasis and contributes to aging.

In this study, for the first time we connect FOXOs to the maintenance of mtDNA integrity. FOXOs regulate the transcription of the DNA repair protein TDP1 and the mtDNA transcription factor TFAM, which is also important for the regulation of mtDNA condensation and protection (24). This is particularly important, as accumulation of mtDNA mutations or large depletions are a common feature of mitochondria in aging tissues and are often linked to diminished OXPHOS and increased ROS leakage (43, 44). FOXOs are well established mediators of genomic stability however all data up to know were focused on their roles in nuclear DNA ((41, 42) and **Chapter 2**). Another factor which was recently assigned similarly a dual role in the maintenance of genomic integrity, by mediating mitochondrial function and mtDNA repair next to nuclear DNA repair, is the ataxia-telangiectasia kinase (ATM) (45, 46). ATM, as was also described in Chapter 2, is upstream of FOXO in the response to nuclear DNA damage. Therefore, interesting parallels are drawn between FOXOs and ATM.

Regulation of TFAM by FOXOs serves as a paradigm of how FOXO transcriptional output is intimately linked to intra- and extracellular inputs. We describe here how, under non-stressed conditions, FOXOs mediate the maintenance of TFAM levels, while two previous reports showed that under certain stress conditions TFAM expression and mitochondrial function are repressed by FOXOs (18, 47). How may these seemingly conflicting results be reconciled? FOXOs are known to function in a context-dependent fashion. For example, Ferber et

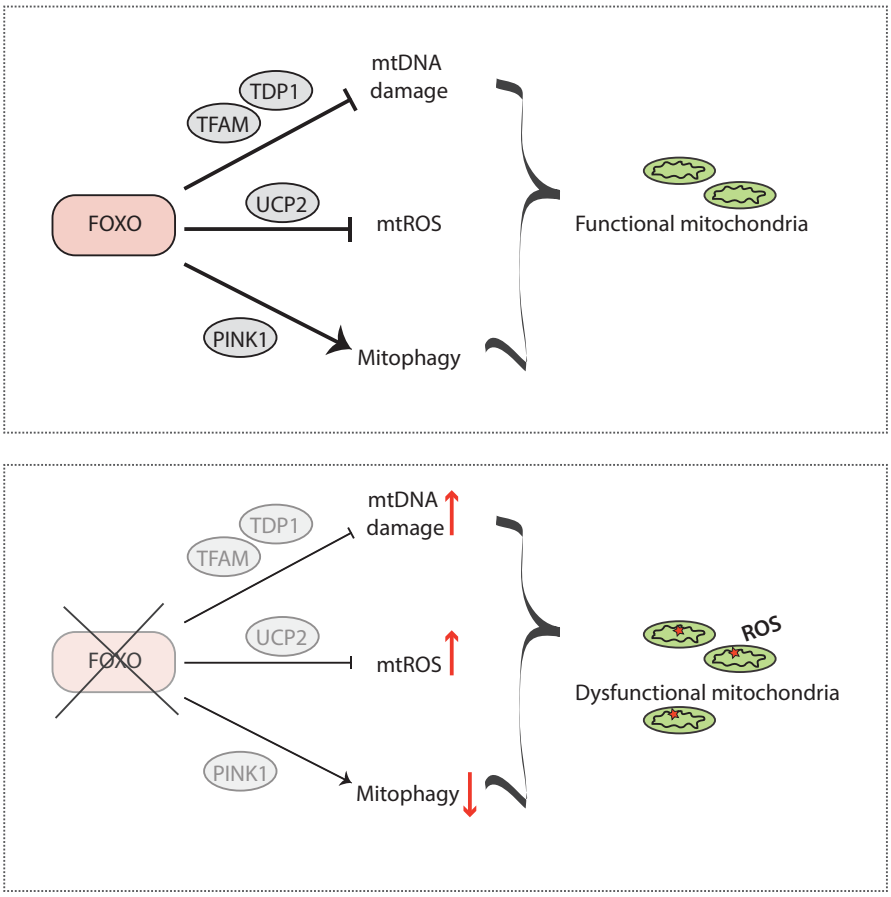

Figure 6: Integrated model depicting the multilayered role of FOXOs in mitochondrial maintenance. FOXOs regulate the transcription of genes involved in mtDNA protection (TFAM, TDP1), mitochondrial ROS production and leakage (UCP2) and mitochondrial turnover (PINK1). In FOXO-depleted cells, the relative abundance of these proteins is reduced, leading to accumulation of dysfunctional mitochondria, a phenotype associated with aging.

al, show that constitutively active FOXO3 suppresses TFAM expression in DLD1 cells (18); in DLD1 cells WNT signaling is constitutively active and c-MYC is arguably one of the most important TCF target-genes. c-MYC is a well-known mediator of mitochondrial biogenesis and function (48) and FOXOs act antagonistically with c-MYC at various levels (reviewed in (49)). In this context, repression of TFAM expression by FOXO proceeds through MXI1-mediated repression of c-MYC function and therefore the outcome of TFAM regulation by FOXO is largely dependent on the c-MYC status of the cell. Analogously, hypoxia constitutes a stress condition resulting in the activation of several stress kinases which can imprint on FOXO and regulate their function (for example c-JUN N-terminal kinase (5, 50)). FOXO function is under tight and complex control of stress kinases and other stress regulators (e.g. HDM2/USP7). Thus, the specific set of active stress regulators within a cell may also determine the outcome of TFAM regulation by FOXO.

FOXO depleted cells have increased mitochondrial mass, when compared to control cells. This may be taken to indicate activation of a compensatory mechanism(s) in response to mitochondrial dysfunction. Such compensatory mechanisms are also observed in pathologies associated with mitochondria defects (14, 51, 52). It has been proposed that cells with dysfunctional mitochondria and therefore impaired OXPHOS,

undergo a bioenergetics crisis, which subsequently activates selective proliferation of the dysfunctional mitochondria (51). Similar processes were also described in aging tissues (53). Based on the results we report here, we propose that the extended mitochondrial network in FOXO depleted cells comes from both increased mitochondrial biogenesis and decreased mitochondrial turnover (mitophagy). The bioenergetic crisis in FOXO depleted cells activated AMPK, a kinase responsive to reduced energy that is also linked to mitochondrial biogenesis. Previous work has identified AMPK as an upstream regulator of FOXO function by direct phosphorylation (54); AMPK-mediated phosphorylation directs FOXO towards transcriptional programs for oxidative stress resistance and metabolic re-wiring (54). In this study we show this to be a reciprocal regulation; FOXO function appears to be required to maintain intracellular ATP levels and thereby contributes to regulation of AMPK activity. Previous studies have in fact proposed a close collaboration between FOXO and AMPK in lifespan extension in *C. elegans* (55, 56). Interestingly, FOXOs induce a feed-forward loop for insulin signaling i.e. FOXOs induce TORC2, IRS2, and PI3K expression (reviewed in (49)), under conditions of nutrient deprivation. This sets the conditions, so that once insulin and glucose become available, cells can rapidly respond to re-enter e.g. cell cycle progression. However, such a feed-forward response should probably not be switched on when ATP levels are limiting. Thus FOXOs appear to balance energy status and AMPK activity with insulin signaling in order to optimally respond to changes in external nutrient availability. Mitophagy is a quality control mechanism operating within cells to ensure the maintenance of functional mitochondria. FOXO-depleted cells have decreased mitochondrial turnover due to defective PINK1 expression, resulting in accumulation of dysfunctional mitochondria and thereby possibly contributing to the increased mitochondrial mass of these cells. Mitophagy is particularly important for neuronal cells; several aging-associated neurodegenerative diseases are linked to mitochondrial dysfunction due to genetic perturbations of mitophagic components, including PINK1, parkin and DJ-1 (57-59). Therefore, FOXO depletion or inhibition might result in neurological defects and more work towards this direction is expected to shed new light on mechanisms of neurodegenerative diseases and potentially assist the development of novel therapies.

Methods

Cell culture

The human retina epithelial cells RPE were maintained in DMEM/Ham F12 (Gibco) supplemented with 10% FBS (Gibco) and penicillin-streptomycin. DL23 cells were cultured in RPMI (Gibco) supplemented with 10% FBS and penicillin-streptomycin. DL23 cells were cultured in RPMI (Gibco) supplemented with 10% FBS and penicillin-streptomycin.
To establish the RPEshFOXO and RPEshLuc cells, RPE cells were virally transduced with pH1tet-flex/FH1t(FOXO1/3)UTG (described in Chapter 2) and pH1tet-flex/FH1t(Luciferase)UTG respectively and the positive clones were selected with FACS sorting.

Chemicals and Oligos

Oligomycin, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), rotenone and antimycin A were purchased by Sigma and stock solutions were prepared in DMSO.
All siRNA oligos were purchased by Dharmacon.

Seahorse extracellular flux analyzer (XF-24) measurements

For measurements with the Seahorse XF-24, RPEshFOXO cells were cultured in the presence or absence of doxycycline for 72 hrs. Subsequently, 40,000 cells were plated in the Seahorse XF-24 plates and allowed to grow for another 20 hrs. Then the medium was exchanged to Seahorse XF assay medium supplemented with 2.5 mM Glucose, 5 mM Glutamine and 10 mM pyruvate for 1 hr. For the interrogation of cellular bioenergetics with pharmacological treatments, cells were

sequentially injected with 1 µM oligomycin, 1 µM FCCP and 1 µM of each Rotenone and Antimycin A.
The principle of the XF-24 function lies on the formation of a transient micro-chamber in the area between the cell monolayer and the sensor probes, allowing for the determination of changes in the extracellular oxygen levels and pH. A typical mitochondrial stress test includes the sequential use of oligomycin, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) and a combination of antimycin A and rotenone. Oligomycin is an inhibitor of the mitochondrial ATP synthase, which results in the uncoupling of OXPHOS from ATP synthesis; FCCP induces a collapse in proton gradient across the mitochondrial membrane, resulting in the uncoupling of electron flow from OXPHOS; antimycin A and rotenone are inhibitors of the Complex I and Complex III of the mitochondrial chain respectively, resulting in the total collapse of mitochondrial function (reviewed in (60)).

ATP measurement

For the measurement of intracellular ATP levels, we used the ATP bioluminescent somatic cell assay kit (Sigma Aldrich), according to the manufacturer's instructions. Briefly, RPEshFOXO cells were grown in the presence of doxycycline for 72 hrs before sample collection and ATP measurement. Cells were counted before analysis and 5 million cells per condition were used.

RNA extraction and quantitative PCR (QPCR)

RNA was extracted with the RNeasy kit (Qiagen), with on column DNase treatment (Qiagen), according to the manufacturer's instructions. RNA was reversed transcribed with oligodT primers and the iScript cDNA synthesis kit (Biorad). Quantitative PCR was performed with the FastStart SYBR Green Master mix (Roche) with the following primer sequences:
PINK1_F: CCAGGCAATTTTTACCCAGA, PINK1_R: AATGTAGGCATGGTGGCTTC, TFAM_F: CCGAGGTGGTTTTCATCTGT, TFAM_R: GCATCTGGGTTCTGAGCTTT, TDP1_F: TCAGGAAGAAGCCAATCCTG, TDP1_R: GGATGAGGTTGGAGGTGTGT, UCP2_F: GAGGTGGTCGGAGATACCAA, UCP2_R: CATAGGTCACCAGCTCAGCA, mtND1_F: CCTAAAACCCGCCACATCTA, mtND1_R: GCCTAGGTTGAGGTTGACCA, mtND2_F: ATCATCCCCACCATCATAGC, mtND2_R: TGGGGTGGGTTTTGTATGTT, mtND3_F: TTACGAGTGCGGCTTCGACC, mtND3_R: ACTCATAGGCCAGACTTAGG, mtND4_F: CTAGGCTCACTAAACATTCTA, mtND4_R: CCTAGTTTTAAGAGTACTGCG, mtND4L_F: TAGTATATCGCTCACACCTC, mtND4L_R: GTAGTCTAGGCCATATGTG, mtND5_F: TCGAATAATTCTTCTCACCC, mtND5_R: TAGTAATGAGAAATCCTGCG, mtND6_F: GGATCCTCCCGAATCAAC, mtND6_R: GTAGGATTGGTGCTGTGG, mtCOX1_F: CTTAGGGGCCATCAATTTCA, mtCOX1_R: GCAGCTAGGACTGGGAGAGA, mtCOX2_F: CCATCCCTACGCATCCTTTA, mtCOX2_R: GCCGTAGTCGGTGTACTCGT, mtCOX3_F: TCCACTCCATAACGCTCCTC, mtCOX3_R: GTGGCCTTGGTATGTGCTTT, Tubulin_F: TACACCATTGGCAAGGAGAT, Tubulin_R: AACCAAGAAGCCCTGAAGAC

Chromatin Immunoprecipitation (ChIP)

To perform ChIP, FOXO3(A3) in DL23 cells was activated by addition of 4-OH-tamoxifen for 4 hrs. 40 million cells per condition were fixed with 1% formaldehyde for 10 min at room temperature and the reaction was quenced by 0.125 M Glycine for 5 min. Subsequently cells were lysed in Darnham lysis buffer (5 mM PIPES, 85 mM KCl, 0.5% NP-40, protease inhibitor cocktail Roche) and nuclear extracts were collected by low speed centrifugation and re-suspended in RIPA buffer (1x PBS, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, supplemented with Roche protease inhibitor cocktail). Chromatin was then sonicated to acquire DNA fragments of about 500 bp which were then used for immunoprecipitations. Antibodies used were: rabbit IgG (Santa Cruz) and rabbit anti-FOXO3a (Santa-Cruz).
The oligomers used for the amplification of the TFAM predicted peaks were:
TFAM#1 F: CTTTCTCTCCTCTGCGGTTG, TFAM#1 R: GTGACCTGAAGTGGCAGCAG, TFAM#2 F: CTTCCCAGAGCTCCACTCAC, TFAM#2 R: TTGGCTTGTGACATCTCTGC, TFAM#3 F: TTCCAGGCATAAAGCTCACC, TFAM#3 R: AGCCAACTCTGAATGGCAAA, Negative F: TGAGTCTCTGCCTTCTGCAA, Negative R: AGTGAGTTGCCCAAAGTCAT

Genomic DNA isolation and long amplicon quantitative PCR (LA-QPCR)

For the isolation of genomic DNA the QIAamp DNA mini kit (Qiagen) was used, according to the manufacturer's instructions. Total genomic DNA was then used for LA-QPCR, according to previous described protocol (21, 61) with one minor modification; Phusion High-Fidelity DNA Polymerase (Thermo Scientific) was used for the amplification of both the long and short mtDNA fragment. The primers used were (21): Mito_L: TGAGGCCAAATATCATTCTGAGGGGC, Mito_S: CCCCACAAACCCCATTACTAAACCCA, Mito_SL: TTTCATCATGCGGAGATGTTGGATGG.

Protein extraction and western blot

Total proteins were collected by direct lysis in Laemli sample buffer. Proteins were run in SDS-PAGE and transferred to Polyscreen PVDF transfer membranes (PerkinElmer). Antibodies used were the following: anti-TFAM (Abcam), anti-PINK1 (Abcam), anti-FOXO1 and anti-FOXO3 (Santa Cruz), anti-pAMPK (Cell Signaling), anti-pACC (Cell Signaling), anti-tubulin (Calbiochem).

Immunofluorescence microscopy

Cells grown on coverslips were treated with 10 μM FCCP for 1 hr and subsequently fixed with 4% formaldehyde, permeabilized with 0.5% Tx100 and blocked with 2% BSA (Sigma). Antibodies used were: anti-Parkin (Abcam), anti-TOM20 (Santa Cruz) and anti-mitochondria (Millipore). Imaging was performed in a Zeiss LSM510 laser-scanning microscope.
For the imaging of mitochondrial ROS, live cells were incubated with mitoSOX red (Life Sciences) for 15 min and subsequently fixed and imaged.

Acknowledgements

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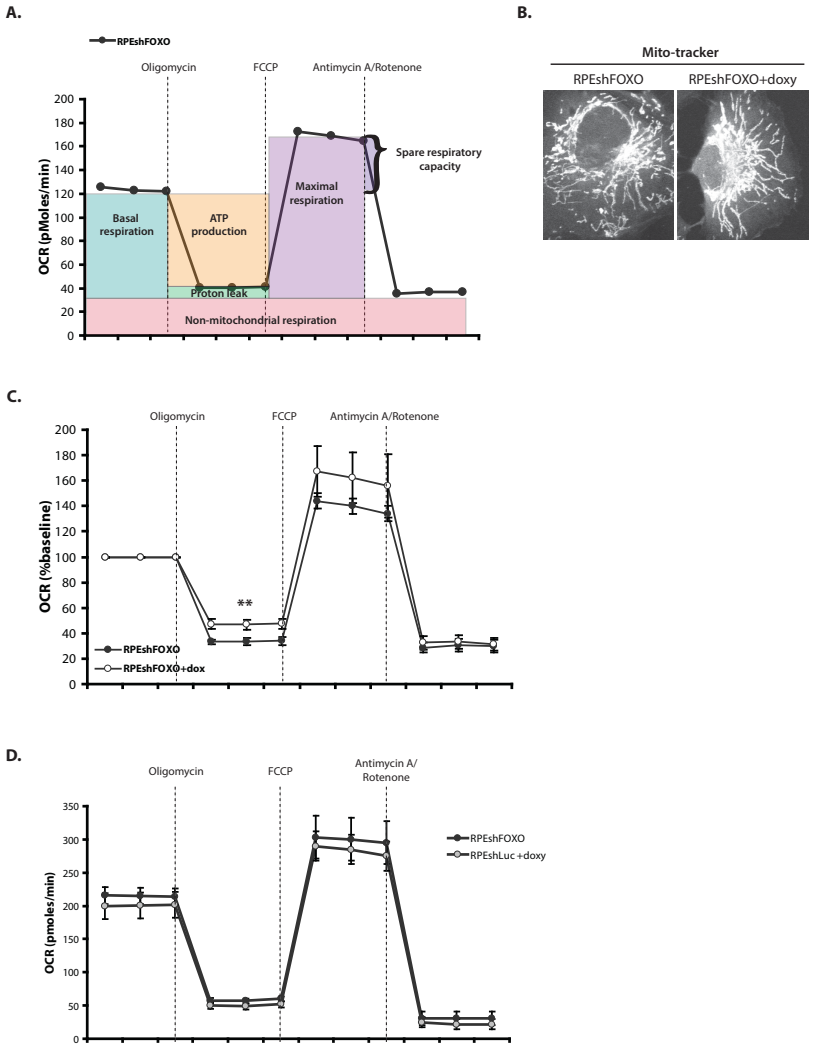
References

1. van der Horst A & Burgering BMT (2007) Stressing the role of FoxO proteins in lifespan and disease. Nat Rev Mol Cell Biol 8(6):440-450.
2. Brunet A, et al. (1999) Akt Promotes Cell Survival by Phosphorylating and Inhibiting a Forkhead Transcription Factor. Cell 96(6):857-868.
3. Kops GJPL, et al. (1999) Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature 398(6728):630-634.
4. Kops GJPL, et al. (2002) Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature 419(6904):316-321.
5. Essers MAG, et al. (2004) FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. EMBO J 23(24):4802-4812.
6. Tothova Z, et al. (2007) FoxOs Are Critical Mediators of Hematopoietic Stem Cell Resistance to Physiologic Oxidative Stress. Cell 128(2):325-339.
7. Charitou P & Burgering BM (2012) Forkhead Box(O) in Control of Reactive Oxygen Species and Genomic Stability to Ensure Healthy Lifespan Antioxidants & Redox Signaling.
8. Willcox BJ, et al. (2008) FOXO3A genotype is strongly associated with human longevity. Proceedings of the National Academy of Sciences 105(37):13987-13992.
9. Flachsbart F, et al. (2009) Association of FOXO3A variation with human longevity confirmed in German centenarians. Proceedings of the National Academy of Sciences 106(8):2700-2705.
10. Kuningas M, et al. (2007) Haplotypes in the human Foxo1a and Foxo3a genes; impact on disease and mortality at old age. Eur J Hum Genet 15(3):294-301.
11. Miquel J, Economos AC, Fleming J, & Johnson Jr JE (1980) Mitochondrial role in cell aging. Experimental Gerontology 15(6):575-591.
12. Harman D (1972) The biologic clock: the mitochondria? Journal of the American Geriatrics Society 20(4):145-147.
13. Larsson NG (2010) Somatic mitochondrial DNA mutations in mammalian aging. pp 683-706.
14. Wallace DC (2005) A MITOCHONDRIAL PARADIGM OF METABOLIC AND DEGENERATIVE DISEASES, AGING, AND CANCER: A Dawn for Evolutionary Medicine. Annual Review of Genetics 39(1):359-407.
15. Trifunovic A, et al. (2004) Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature 429(6990):417-423.
16. Sahin E & DePinho RA (2010) Linking functional decline of telomeres, mitochondria and stem cells during ageing. Nature 464(7288):520-528.
17. Wu M, et al. (2007) Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. American Journal of Physiology - Cell Physiology 292(1):C125-C136.
18. Ferber EC, et al. (2012) FOXO3a regulates reactive oxygen metabolism by inhibiting mitochondrial gene expression. Cell Death Differ 19(6):968-979.
19. Hardie DG, Ross FA, & Hawley SA (2012) AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat Rev Mol Cell Biol 13(4):251-262.
20. Bratic A & Larsson N-G (2013) The role of mitochondria in aging. The Journal of Clinical Investigation 123(3):951-957.

21.Ayala-Torres S, Chen Y, Svoboda T, Rosenblatt J, & Van Houten B (2000) Analysis of Gene-Specific DNA Damage and Repair Using Quantitative Polymerase Chain Reaction. Methods 22(2):135-147.
22.Lagouge M & Larsson NG (2013) The role of mitochondrial DNA mutations and free radicals in disease and ageing. Journal of Internal Medicine 273(6):529-543.
23.Yakes FM & Van Houten B (1997) Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. Proceedings of the National Academy of Sciences 94(2):514-519.
24.Campbell CT, Kolesar JE, & Kaufman BA (2012) Mitochondrial transcription factor A regulates mitochondrial transcription initiation, DNA packaging, and genome copy number. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms 1819(9–10):921-929.
25.Alexeyev M, Shokolenko I, Wilson G, & LeDoux S (2013) The Maintenance of Mitochondrial DNA Integrity—Critical Analysis and Update. Cold Spring Harbor Perspectives in Biology 5(5).
26.de Souza-Pinto NC, Wilson Iii DM, Stevnsner TV, & Bohr VA (2008) Mitochondrial DNA, base excision repair and neurodegeneration. DNA Repair 7(7):1098-1109.
27.Das BB, Dexheimer TS, Maddali K, & Pommier Y (2010) Role of tyrosyl-DNA phosphodiesterase (TDP1) in mitochondria. Proceedings of the National Academy of Sciences 107(46):19790-19795.
28.Dexheimer TS, Antony S, Marchand C, & Pommier Y (2008) Tyrosyl-DNA Phosphodiesterase as a Target for Anticancer Therapy. Anti-Cancer Agents in Medicinal Chemistry 8(4):381-389 (389).
29.Kops GJPL, et al. (2002) Control of Cell Cycle Exit and Entry by Protein Kinase B-Regulated Forkhead Transcription Factors. Molecular and Cellular Biology 22(7):2025-2036.
30.Eijkelenboom A, et al. (2013) Genome-wide analysis of FOXO3 mediated transcription regulation through RNA polymerase II profiling. Molecular Systems Biology 9(1).
31.Rugarli EI & Langer T (2012) Mitochondrial quality control: a matter of life and death for neurons. The EMBO Journal 31(6):1336-1349.
32.Mailloux RJ & Harper M-E (2011) Uncoupling proteins and the control of mitochondrial reactive oxygen species production. Free Radical Biology and Medicine 51(6):1106-1115.
33.Palikaras K & Tavernarakis N (2014) Mitochondrial homeostasis: The interplay between mitophagy and mitochondrial biogenesis. Experimental Gerontology (0).
34.Youle RJ & Narendra DP (2011) Mechanisms of mitophagy. Nat Rev Mol Cell Biol 12(1):9-14.
35.Mei Y, et al. (2009) FOXO3a-dependent regulation of Pink1 (Park6) mediates survival signaling in response to cytokine deprivation. Proceedings of the National Academy of Sciences.
36.Yang Y, et al. (2006) Mitochondrial pathology and muscle and dopaminergic neuron degeneration caused by inactivation of Drosophila Pink1 is rescued by Parkin. Proceedings of the National Academy of Sciences 103(28):10793-10798.
37.Santiago JA & Potashkin JA (2013) Shared dysregulated pathways lead to Parkinson's disease and diabetes. Trends in Molecular Medicine 19(3):176-186.
38.Chaturvedi RK & Flint Beal M (2013) Mitochondrial Diseases of the Brain. Free Radical Biology and Medicine 63(0):1-29.
39.Ahmad W (2013) Overlapped Metabolic and Therapeutic Links between Alzheimer and Diabetes. Mol Neurobiol 47(1):399-424.
40.Cui H, Kong Y, & Zhang H (2012) Oxidative Stress, Mitochondrial Dysfunction, and Aging. Journal of Signal Transduction 2012:13.
41.Klagge A, et al. (2011) The role of FOXO3 in DNA damage response in thyrocytes. Endocrine-Related Cancer 18(5):555-564.
42.Tran H, et al. (2002) DNA Repair Pathway Stimulated by the Forkhead Transcription Factor FOXO3a Through the Gadd45 Protein. Science 296(5567):530-534.
43.Kazachkova N, Ramos A, Santos C, & Lima M (2013) Mitochondrial DNA Damage Patterns and Aging: Revising the Evidences for Humans and Mice. Aging and Disease 4(6):337-350.
44.Zapico SC & Ubelaker DH (2013) mtDNA Mutations and Their Role in Aging, Diseases and Forensic Sciences. Aging and Disease 4(6):364-380.
45.Valentín-Vega YA, et al. (2012) Mitochondrial dysfunction in ataxia-telangiectasia. Blood 119(6):1490-1500.
46.Sharma NK, et al. (2014) Intrinsic mitochondrial DNA repair defects in Ataxia Telangiectasia. DNA Repair 13(0):22-31.
47.Jensen KS, et al. (2011) FoxO3A promotes metabolic adaptation to hypoxia by antagonizing Myc function. The EMBO Journal 30(22):4554-4570.
48.Li F, et al. (2005) Myc Stimulates Nuclearly Encoded Mitochondrial Genes and Mitochondrial Biogenesis. Molecular and Cellular Biology 25(14):6225-6234.
49.Kloet DEA & Burgering BMT (2011) The PKB/FOXO switch in aging and cancer. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1813(11):1926-1937.
50.Jin N, et al. (2000) Hypoxia Activates Jun-N-Terminal Kinase, Extracellular Signal–Regulated Protein Kinase, and p38 Kinase in Pulmonary Arteries. American Journal of Respiratory Cell and Molecular Biology 23(5):593-601.
51.Yoneda M, Chomyn A, Martinuzzi A, Hurko O, & Attardi G (1992) Marked replicative advantage of human mtDNA carrying a point mutation that causes the MELAS encephalomyopathy. Proceedings of the National Academy of Sciences 89(23):11164-11168.
52.Taylor RW & Turnbull DM (2005) Mitochondrial DNA mutations in human disease. Nat Rev Genet 6(5):389-402.
53.Herbst A, Johnson CJ, Hynes K, McKenzie D, & Aiken JM (2013) Mitochondrial Biogenesis Drives a Vicious Cycle of Metabolic Insufficiency and Mitochondrial DNA Deletion Mutation Accumulation in Aged Rat Skeletal Muscle Fibers.

PLoS ONE 8(3):e59006.
54.Greer EL, et al. (2007) The Energy Sensor AMP-activated Protein Kinase Directly Regulates the Mammalian FOXO3 Transcription Factor. Journal of Biological Chemistry 282(41):30107-30119.
55.Chen D, et al. (2013) Germline Signaling Mediates the Synergistically Prolonged Longevity Produced by Double Mutations in daf-2 and rsks-1 in C. elegans. Cell Reports 5(6):1600-1610.
56.Greer EL, et al. (2007) An AMPK-FOXO Pathway Mediates Longevity Induced by a Novel Method of Dietary Restriction in C. elegans. Current Biology 17(19):1646-1656.
57.Valente EM, et al. (2004) Hereditary Early-Onset Parkinson's Disease Caused by Mutations in PINK1. Science 304(5674):1158-1160.
58.Kitada T, et al. (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 392(6676):605-608.
59.Bonifati V, et al. (2003) Mutations in the DJ-1 Gene Associated with Autosomal Recessive Early-Onset Parkinsonism. Science 299(5604):256-259.
60.Hatefi Y (1985) The Mitochondrial Electron Transport and Oxidative Phosphorylation System. Annual Review of Biochemistry 54(1):1015-1069.
61.Furda AM, Marrangoni AM, Lokshin A, & Van Houten B (2012) Oxidants and not alkylating agents induce rapid mtDNA loss and mitochondrial dysfunction. DNA Repair 11(8):684-692.

SUPPLEMENTARY DATA



Supplementary Figure 1: (A) Representative profile with the main bioenergetic parameters after mitochondrial stress test acquired in the Seahorse XF-24 analyzer **(B)** Extended mitochondrial network in FOXO-depleted cells. RPEshFOXO cells were cultured in the presence or absence of doxycycline for 96 hrs. Cells were incubated with Mitotracker for 45 min before analysis by confocal microscopy **(C)** Relative effects of FOXO depletion on the mitochondrial bioenergetic profile. RPEshFOXO cells were cultured in the presence or absence of doxycycline for 72 hrs. 40,000 cells were plated in XF-24 culture plates and 16 hrs later they were used for a mitochondrial stress test in the Seahorse XF-24 analyzer. Results are represented as percentage of basal respiration **(D)** Mitochondrial bioenergetic profile is not affected by doxycycline treatment. RPEshFOXO and RPEshLuc cells were cultured in the presence or absence of doxycycline for 72 hrs. 40,000 cells were plated in XF-24 culture plates and 16 hrs later they were used for a mitochondrial stress test in the Seahorse XF-24 analyzer.

Chapter 5

General Discussion

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FOXO transcription factors are involved in a variety of processes including cell cycle regulation, reactive oxygen species (ROS) detoxification and cellular metabolism. FOXOs are activated, as indicated by an induction of their nuclear accumulation, under conditions of cellular stress, including growth factor deprivation, oxidative stress and DNA damage. When in the nucleus, FOXOs regulate transcriptional programs that enable an orchestrated cellular response to these environmental and intracellular signals (1, 2). The FOXO-regulated responses eventually ensure the re-establishment and/or maintenance of cellular homeostasis under stress conditions. In this thesis we have further explored the role of FOXOs in homeostatic responses, including genomic stability and metabolic homeostasis and we have identified novel FOXO-regulated processes.

Homeostasis and disease

Homeostasis is defined as the ability of a biological system, e.g. cell, organ or organism to maintain a relative constant and stable internal environment through the establishment of a dynamic equilibrium of various parameters (pH, nutrients, protein content etc). When one of the system parameters changes, the system responds by changing other parameter(s) to either return to the previously established equilibrium or to transit to a new equilibrium. The proper function of an organism is intimately linked to the maintenance of homeostasis, as the inability to maintain homeostasis directly results in a diseased state. For example, at the organismal level insulin secretion is maintained under strict control to ensure glucose availability to all cells and also to prevent hypo- or hyperglycemic conditions, even at periods of prolonged fasting; any inability to maintain insulin homeostasis results in diabetes.

At the cellular level, homeostasis is linked to the maintenance and stability of all cellular constituents including DNA, proteins and lipids, as well as ions and metabolites (3-7). Importantly, especially within the context of FOXOs, which are the subject of this thesis, homeostasis of all cellular constituents is intrinsically linked to the control of redox homeostasis; that is the maintenance of intracellular reactive oxygen species (ROS) levels to a certain threshold. ROS perform a role in cell signaling when in low levels (reviewed in (8-10)) however their increased accumulation is the prime cause of damage to cellular constituents such as DNA, proteins and lipids. Therefore ROS pose a continuous challenge to the homeostasis of these constituents.

As described in **Chapter 1**, disturbance of organismal but also cellular and molecular homeostasis is associated with aging and aging-associated diseases, such as cancer and diabetes. Cancer is often driven by increased genomic instability and is characterized by uncontrolled cellular proliferation without concomitant increase in cellular clearance. Other hallmarks of cancer are increased redox stress and metabolic changes (11). Diabetes, another aging-associated disease, is also linked to disturbance in both organismal and cellular homeostasis; decreased insulin excretion by the pancreatic β-cells or decreased sensitivity of the peripheral cells to insulin are hallmarks of diabetes (12). Other age-associated diseases are linked to disturbance of protein homeostasis, as for example Alzheimer´s and Hudington´s disease (13, 14), or, as discussed in **Chapter 4**, several neurodegenerative diseases, including Parkinson´s disease, are caused by a disturbance in the rate of mitochondrial clearance (15).

FOXOs are involved in the maintenance of genomic stability by transcription-dependent and independent mechanisms

The cellular genetic material is constantly attacked by both intra- and extra-cellular insults, for example

metabolic by-products like ROS or ultraviolet irradiation (16, 17). The induced DNA lesions range from base modifications, to single and double strand DNA breaks and are particularly deleterious for the cell, as they can result to either mutagenic events or to gross genomic region duplications, depletions or translocations (17). This ultimately results in the production of mutated peptides and proteins with altered functions or in the imbalanced cellular genomic content of the daughter cells. Disturbance of genomic homeostasis is linked to both cancer and aging (17). Cells have evolved a number of processes for the surveillance and repair of genomic DNA damage, including Base Excision Repair (BER), Nucleotide Excision Repair (NER), Mismatch Repair (MMR), Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR). The aforementioned repair pathways differ not only on the relative DNA lesions upon which they function but also on their fidelity. For example NHEJ and HR are both involved in the repair of double-strand breaks (DSBs) however NHEJ results in a rapid reconnection of the broken DNA ends that is particularly error-prone , whereas HR is essentially an error-free repair system (18, 19). Efficient DNA repair has important implications in the aging process; according to the “DNA damage theory of aging” accrual of DNA lesions is a leading cause of aging and aging-associated pathologies. Aging tissues have higher levels of DNA damage both at the nuclear and mitochondrial DNA (20, 21) and also tissue regeneration by stem cells is less efficient with aging, on the account of DNA damage accrual (22). Interestingly however this connection still remains “one-sided”, since it was never convincingly shown that more efficient DNA repair results in increased longevity. FOXOs are established longevity factors (reviewed in **Chapter 1**) and in **Chapter 2** we show that FOXOs promote error-free DNA repair, suggesting for the first time a potential positive linkage between DNA repair and prolonged longevity.

Recent advances have revealed a significant contribution of the chromatin landscape on DNA repair pathway choice and repair fidelity (reviewed in (23)). This is better portrayed by the "Access-Repair-Restore" dogma (23, 24). According to this, DNA repair involves three steps: (i) induction of epigenetics changes to allow chromatin relaxation and accessibility of repair factors, (ii) lesion repair and (iii) re-establishment of the chromatin landscape as prior to damage. Interestingly, around DNA lesions chromatin acquires epigenetic marks, including phosphorylation, methylation and ubiquitilation that are associated with transcriptional silencing (25). This transcriptional silencing is also illustrated by the results we describe in **Addendum**; the global gene expression analysis we performed after DNA damage identified a significant number of downregulated genes, again suggesting a major shutdown of gene expression after DNA damage. However, and seemingly counterintuitive, several proteins involved in the regulation or execution of transcriptional programs have been identified as important mediators of the DNA damage response and the maintenance of genomic stability (e.g. TRRAP, LEDGF, INO80, SIRT1, SIRT6 etc.) (26-30). How could this seemingly conflicting observation be reconciled? In **Chapter 2** we describe in detail the function of FOXOs in response to DSBs, which is independent of their established role in gene transactivation. FOXOs, by regulating the deposition of acetyl- marks on the histone tails proximal to the DSB, regulate the choice of HR over NHEJ for DSBs repair. Therefore, FOXOs fine-tune the response for the maintenance of genomic stability in cycling cells, by mediating epigenetic changes. Based on the above, we propose that the ability of transcription factors to recruit proteins involved in chromatin regulation for transcription, also supports most of the epigenetic changes associated with DNA repair. From the perspective of evolution, this appears a more advantageous strategy, rather than ‘reinventing the wheel’ for chromatin alterations, in response to DNA damage. Obviously, this begs the question as to how unique FOXO transcription factors are, in their role of mediating DNA repair. In fact, studies have suggested a direct involvement of NF-kappa B and p53 to the repair of DSBs (31, 32), albeit towards different directions (NF-kappa B activates and p53 inhibits HR) and by still unknown

mechanisms. Additionally, the role of FOXOs in promoting HR suggests a potential involvement in other processes as well, for example alternative lengthening of telomeres (ALT) for the maintenance of telomeric length independent of the telomerase status (33).

FOXOs appear to mediate genomic stability also by transcription-dependent mechanisms. In quiescent cells FOXOs play a protective role in genomic stability by mediating the maintenance of their quiescent state (34). Thereby, FOXOs contribute to the protection of these cells from replication-induced genotoxic stress. Additionally, FOXOs are involved in the protection/repair of mtDNA by ROS-induced damage (**Chapter 4**). This role of FOXOs appears to be linked to the transcriptional regulation of the mitochondria-resident proteins TFAM and TDP1 although other, direct, roles cannot be excluded. In fact, recent studies have suggested that FOXO members also reside in the mitochondrial matrix (35, 36). We have not acquired any data that are in direct support of this observation however it would be particularly interesting to study whether FOXOs can also regulate mtDNA stability by direct functions, even though mtDNA is naked from histones.

FOXOs mediate novel aspects of redox homeostasis

Reactive oxygen species have diverse roles in the cell, largely depending on their relative abundance. It is established that low levels of ROS are required for cellular signaling, whereas an increase in ROS levels above a threshold can result in damage in cellular constituents including proteins, lipids and DNA (8, 10). FOXOs are strongly linked to the maintenance of the cellular redox status. They rapidly respond to changes in redox levels via the formation of inter-molecular disulfide bridges (37) and regulate the transactivation of components of redox detoxification systems, including the mitochondrial resident manganese superoxide dismutase (MnSOD) and Peroxiredoxin 3, the nuclear Sestrin 3, the peroxisomal catalase and the extracellular Selenoprotein P (38-41). FOXOs maintain the redox levels in the aforementioned cellular compartments, thereby contributing to the "healthy" ROS signaling. Moreover, they protect nuclear and mitochondrial DNA, as well as other cellular constituents from ROS-induced damage. Whether FOXOs contribute to cytosolic ROS

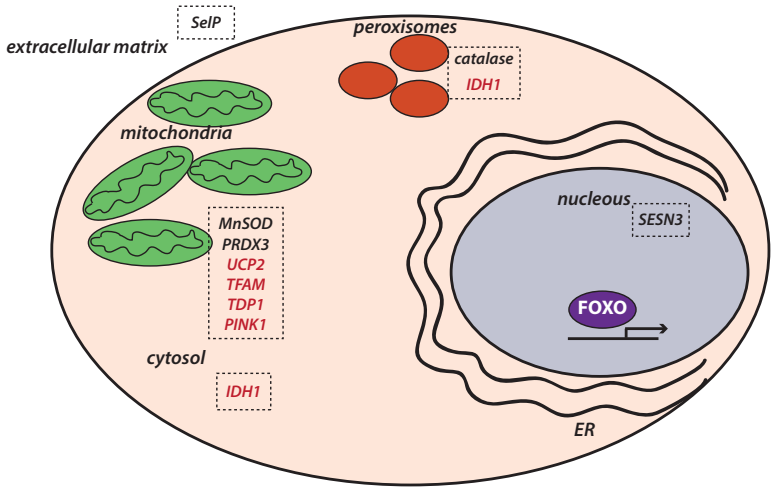

Figure 1. FOXO regulate the redox homeostasis in different sub-cellular compartments. FOXO regulate the transcription of genes involved in redox detoxification systems that function in mitochondria (MnSOD and PRDX3), peroxisomes (catalase), nucleus (SESN3) and extracellularly (SelP). FOXOs are also involved in redox homeostasis indirectly, by mediating the transcription of IDH1 and factors ensuring mitochondrial function and integrity (UCP2, TFAM, TDP1 and PINK1).

detoxification as well, was elusive until now.

In this thesis we further describe other layers of FOXO contribution to the maintenance of redox homeostasis (summarized in **Figure 1**). In **Chapter 3**, we link FOXOs to the regulation of cytosolic ROS detoxification. We describe the transcriptional regulation of isocitrate dehydrogenase 1 (IDH1) by FOXO and we further show that in the absence of FOXOs the functional output of IDH1 is severely impaired. As a result, cells in which FOXOs are absent or inhibited, are characterized by a reduced ratio of NADPH/NADP+. NADPH offers reductive power for the glutathione system (GSH-GSSG), the main ROS detoxifying system operating in the cytosol, and in FOXO depleted cells we find impaired levels of reduced glutathione. This is expected to have important implications for biosynthetic processes such as DNA and protein synthesis, as well as for intracellular trafficking (e.g. amino acid transport) and signaling. In **Chapter 4** we show that FOXOs, apart from their roles in ROS detoxification, also mediate the reduced production/leakage of ROS from mitochondria. We identify the contribution of FOXOs to the regulation of mitochondrial ROS and mtDNA stability and also in the turnover of dysfunctional mitochondria; all these processes are important for the maintenance of mitochondrial ROS and the inhibition of their aberrant generation.

Metabolic pathways under FOXO control

FOXOs function downstream of the PI3K/PKB pathway to regulate metabolic processes associated with insulin and nutrients deprivation. Under these conditions, FOXOs mediate the transcription of genes involved in the inhibition of glucose oxidation (e.g. PDK4), the activation of gluconeogenesis (e.g. G6Pase, PEPCK) and lipid oxidation (e.g. LPL) and the feedback activation of insulin sensitivity (e.g. IRS2) (42). All the above constitute a metabolic response to glucose limitation, while increasing cellular insulin sensitivity.

In **Chapter 3** we uncover novel metabolic functions of FOXO, mediated by their transcriptional target IDH1. IDH1 is involved in the conversion of isocitrate to α-ketoglutarate (α-KG) and is thereby important for the function of α-KG dependent enzymes, including epigenetic regulators such as the TET proteins (43). Therefore, by the regulation of IDH1, FOXOs maintain the cytosolic levels of α-KG and mediate processes linked to cell differentiation and tumor suppression (43). The inverse reaction, which is the conversion of α-KG to isocitrate, occurs when cells grow under hypoxic conditions or with severely compromised mitochondria. This is an anaplerotic reaction, for the replenishing of citrate intracellular levels; citrate is required for the generation of acetyl-coA that is then used for *de novo* lipid biosynthesis or in cellular signaling. Based on our findings that FOXOs regulate IDH1 levels under hypoxic conditions, we propose FOXOs as critical metabolic regulators for the maintenance of cellular signaling and the survival and proliferation of cells grown in hypoxia. Furthermore, in **Chapter 4** we describe the involvement of FOXOs in the maintenance of mitochondrial function and integrity; several metabolic processes, including the TCA cycle occur within the mitochondria and are thereby indirectly modulated by FOXO. Indeed, our findings implicate FOXOs in the regulation of mitochondrial OXPHOS and ATP production, possibly by the transcriptional regulation of TFAM. TFAM is involved not only in the maintenance of mtDNA integrity but also in the transcription of mtDNA-encoded genes (44), some of which show impaired expression in FOXO-depleted cells. Intriguingly, FOXOs have been previously suggested to negatively regulate mitochondrial biosynthesis and function (45, 46). We propose that, within the homeostasis context in which FOXOs function, their imprint on mitochondrial function may differ. For instance, in hypoxic conditions FOXOs contribute to the inhibition of the deleterious under these conditions mitochondrial function (46), yet under conditions of ambient oxygen and sufficient growth factor supply, FOXOs function to ensure the optimal mitochondrial function (**Chapter 4**).

Multifaceted roles of FOXOs in cancer and aging

Aging is associated with the disturbance of cellular and eventually organismal homeostasis. Increased cellular loss by apoptosis and senescence is not accompanied by replenishing of the lost cells, resulting in tissue degeneration and dysfunction (reviewed in **Chapter 1**). Moreover, aging-associated disorders stem from disturbed cellular homeostasis, cancer being one of them. Interestingly, whereas cancer is triggered by the loss of homeostasis, cancer cells will try to re-establish homeostasis eventually, to ensure their long-term survival. For example, in breast cancer cells with BRCA1 deletion a concomitant depletion of 53BP1 often occurs, resulting in increased error-free DNA repair and thereby functioning as a brake to prevent excessive genomic instability (47, 48).

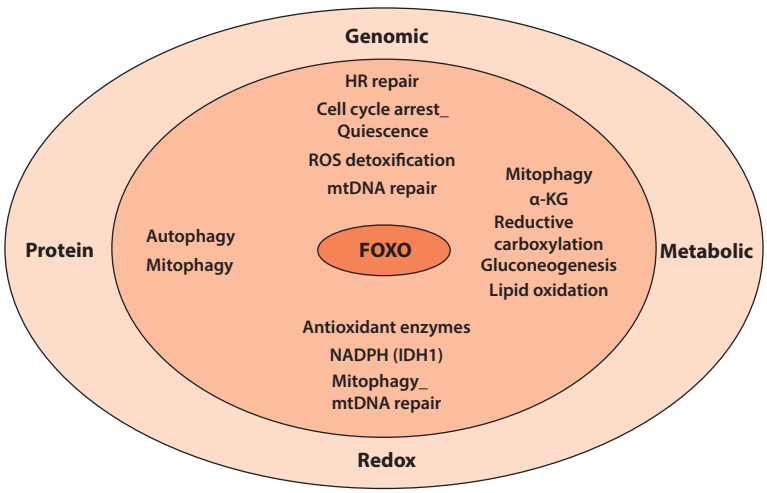

Figure 2. FOXOs mediate cellular and molecular homeostasis in multiple ways. Depicted are the main processes by which FOXOs contribute to DNA, protein, redox and metabolic homeostasis.

FOXOs are de facto homeostatic regulators mediating the responses to metabolic, redox and genotoxic stress (reviewed in (2)) **(Figure 2)**. In this thesis we further uncover novel mechanisms of FOXO-dependent homeostatic regulation, as in **Chapter 2** we show that FOXOs mediate the error-free repair of DSBs and in **Chapters 3 and 4** we uncover new roles in redox and metabolic homeostasis. All in all, FOXOs, which are established tumor suppressors (49), appear to inhibit tumor initiation by mediating genomic and redox stability. Interestingly however, in the context of homeostasis re-establishment by cancer cells, FOXOs are often activated to mediate similar homeostatic functions and thereby protect these same cancer cells (50). Also in this thesis, we find FOXOs to exhibit similar multifaceted contributions to tumor suppression/promotion (model for FOXO dual function in **Figure 3**). FOXOs mediate HR repair not only in untransformed cells but also in fast proliferating cancer cells, thereby contributing to their survival under conditions of genotoxic stress (**Chapter 2**). In **Chapter 3** we identify that regulation of IDH1 transcription by FOXOs establishes them as critical regulators for the proliferation of mutant IDH1 tumor cells and also as important mediators for the survival and proliferation of cells grown in hypoxic conditions, such as the ones suggested to operate within solid tumors. Finally, even though cancer cells are known to generate energy and "building blocks" to meet their needs mainly by the pathway of aerobic glycolysis (Warburg effect), they also have active mitochondrial metabolism and therefore mitochondria homeostasis is crucial for cancer cells as well

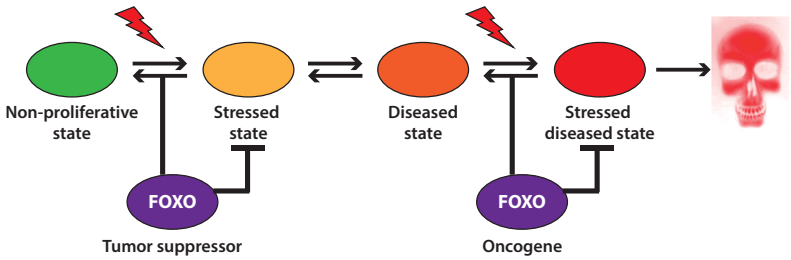

Figure 3. FOXO dual role in tumor suppression and tumor promotion. FOXOs act as homeostatic regulators in normal, as well as diseased (e.g. cancer) cells by alleviating stress and promoting restoration of the equilibrium to the original state.

(51, 52). FOXOs, by mediating the maintenance of mitochondrial integrity (**Chapter 4**), play a critical role not only to meet the energy requirements of cancer cells but also for the maintenance of the intracellular redox status in these cells. Interestingly, cancer cells can be particularly vulnerable to additional increases in cellular redox and the action of several anti-cancer agents relies on such a further increase in ROS (53, 54).

Perspectives

In conclusion, maintenance of cellular homeostasis is crucial for the survival of both untransformed, normal cells and cancer cells. However, due to the inherent genomic and metabolic differences between these two cell types, the enforced homeostatic responses are expected to have also differing underlying mechanisms between these cells. It is awaited, for example, whether the ROS detoxifying mechanisms that act within untransformed cells to efficiently lower intracellular ROS to the threshold of "healthy" signaling, will equally perform in transformed cells with an inherently higher redox status, or whether cancer cells have additional mechanisms to maintain their redox balance. The possibility of equal or distant homeostatic mechanisms between cancer cells and normal cells is important to consider, when designing novel targeted therapies. Understanding how the relative balances in cancer cells are established will provide rationale to selectively disturb them to a level that homeostasis can no longer be achieved. Moreover, targeting this way the Achilles' heel of transformed cells will ensure the survival of the "healthy" cells, thereby providing a favorable cost-effective ratio. In this context, gaining an understanding of the multifaceted role of FOXOs in cancer and aging is of particular importance.

References

1. Charitou P & Burgering BM (2012) Forkhead Box(O) in Control of Reactive Oxygen Species and Genomic Stability to Ensure Healthy Lifespan Antioxidants & Redox Signaling.
2. Eijkelenboom A & Burgering BMT (2013) FOXOs: signalling integrators for homeostasis maintenance. Nat Rev Mol Cell Biol 14(2):83-97.
3. Jarosz DF, Taipale M, & Lindquist S (2010) Protein Homeostasis and the Phenotypic Manifestation of Genetic Diversity: Principles and Mechanisms. Annual Review of Genetics 44(1):189-216.
4. Pellettieri J & Alvarado AS (2007) Cell Turnover and Adult Tissue Homeostasis: From Humans to Planarians. Annual Review of Genetics 41(1):83-105.
5. Wilson DF (2013) Regulation of cellular metabolism: Programming and maintaining metabolic homeostasis. Journal of Applied Physiology.
6. Roth DM & Balch WE (2011) Modeling general proteostasis: proteome balance in health and disease. Current Opinion in Cell Biology 23(2):126-134.
7. Papamichos-Chronakis M & Peterson CL (2013) Chromatin and the genome integrity network. Nat Rev Genet 14(1):62-75.
8. D'Autreaux B & Toledano MB (2007) ROS as signalling molecules: mechanisms that generate specificity in ROS

homeostasis. Nat Rev Mol Cell Biol 8(10):813-824.
9. de Keizer PLJ, Burgering BMT, & Dansen TB (2011) Forkhead Box O as a Sensor, Mediator, and Regulator of Redox Signaling Antioxidants & Redox Signaling 12(6):1093-1106.
10. Ray PD, Huang B-W, & Tsuji Y (2012) Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cellular Signalling 24(5):981-990.
11. Hanahan D & Weinberg RA (2011) Hallmarks of Cancer: The Next Generation. Cell 144(5):646-674.
12. Schwartz MW, et al. (2013) Cooperation between brain and islet in glucose homeostasis and diabetes. Nature 503(7474):59-66.
13. Morawe T, Hiebel C, Kern A, & Behl C (2012) Protein Homeostasis, Aging and Alzheimer's Disease. Mol Neurobiol 46(1):41-54.
14. Douglas PM & Dillin A (2010) Protein homeostasis and aging in neurodegeneration. The Journal of Cell Biology 190(5):719-729.
15. Celardo I, Martins LM, & Gandhi S (2013) Unravelling mitochondrial pathways to Parkinson's disease. British Journal of Pharmacology.
16. Alexeyev M, Shokolenko I, Wilson G, & LeDoux S (2013) The Maintenance of Mitochondrial DNA Integrity—Critical Analysis and Update. Cold Spring Harbor Perspectives in Biology 5(5).
17. Hoeijmakers JHJ (2009) DNA Damage, Aging, and Cancer. New England Journal of Medicine 361(15):1475-1485.
18. Ciccia A & Elledge SJ (2010) The DNA Damage Response: Making It Safe to Play with Knives. Molecular Cell 40(2):179-204.
19. Wyman C & Kanaar R (2006) DNA Double-Strand Break Repair: All's Well that Ends Well. Annual Review of Genetics 40(1):363-383.
20. Sedelnikova OA, et al. (2004) Senescing human cells and ageing mice accumulate DNA lesions with unrepairable double-strand breaks. Nat Cell Biol 6(2):168-170.
21. Hamilton ML, et al. (2001) Does oxidative damage to DNA increase with age? Proceedings of the National Academy of Sciences 98(18):10469-10474.
22. Mandal PK, Blanpain C, & Rossi DJ (2011) DNA damage response in adult stem cells: pathways and consequences. Nat Rev Mol Cell Biol 12(3):198-202.
23. Smerdon MJ (1991) DNA repair and the role of chromatin structure. Current Opinion in Cell Biology 3(3):422-428.
24. Soria G, Polo Sophie E, & Almouzni G (2012) Prime, Repair, Restore: The Active Role of Chromatin in the DNA Damage Response. Molecular Cell 46(6):722-734.
25. Shanbhag NM, Rafalska-Metcalf IU, Balane-Bolivar C, Janicki SM, & Greenberg RA (2010) ATM-Dependent Chromatin Changes Silence Transcription In cis to DNA Double-Strand Breaks. Cell 141(6):970-981.
26. Murr R, et al. (2006) Histone acetylation by Trrap-Tip60 modulates loading of repair proteins and repair of DNA double-strand breaks. Nat Cell Biol 8(1):91-99.
27. Daugaard M, et al. (2012) LEDGF (p75) promotes DNA-end resection and homologous recombination. Nat Struct Mol Biol 19(8):803-810.
28. Gospodinov A, et al. (2011) Mammalian Ino80 Mediates Double-Strand Break Repair through Its Role in DNA End Strand Resection. Molecular and Cellular Biology 31(23):4735-4745.
29. Kaidi A, Weinert BT, Choudhary C, & Jackson SP (2010) Human SIRT6 Promotes DNA End Resection Through CtIP Deacetylation. Science 329(5997):1348-1353.
30. Uhl M, et al. (2010) Role of SIRT1 in homologous recombination. DNA Repair 9(4):383-393.
31. Volcic M, et al. (2012) NF-kB regulates DNA double-strand break repair in conjunction with BRCA1-CtIP complexes. Nucleic Acids Research 40(1):181-195.
32. Bertrand P, Saintigny Y, & Lopez BS (2004) p53's double life: transactivation-independent repression of homologous recombination. Trends in Genetics 20(6):235-243.
33. Cesare AJ & Reddel RR (2010) Alternative lengthening of telomeres: models, mechanisms and implications. Nat Rev Genet 11(5):319-330.
34. Tothova Z & Gilliland DG (2007) FoxO Transcription Factors and Stem Cell Homeostasis: Insights from the Hematopoietic System. Cell Stem Cell 1(2):140-152.
35. Caballero-Caballero A, et al. (2013) Mitochondrial localization of the Forkhead box class O transcription factor FOXO3a in brain. Journal of Neurochemistry 124(6):749-756.
36. Jacobs K, et al. (2008) SIRT3 interacts with the daf-16 homolog FOXO3a in the Mitochondria, as well as increases FOXO3a Dependent Gene expression. Int J Biol Sci 4(5):291-299.
37. Dansen TB, et al. (2009) Redox-sensitive cysteines bridge p300/CBP-mediated acetylation and FoxO4 activity. Nat Chem Biol 5(9):664-672.
38. Kops GJPL, et al. (2002) Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature 419(6904):316-321.
39. Chen C-C, et al. (2010) FoxOs Inhibit mTORC1 and Activate Akt by Inducing the Expression of Sestrin3 and Rictor. Developmental Cell 18(4):592-604.
40. Chiribau CB, et al. (2008) FOXO3A Regulates Peroxiredoxin III Expression in Human Cardiac Fibroblasts. J. Biol. Chem. 283(13):8211-8217.
41. Walter PL, Steinbrenner H, Barthel A, & Klotz L-O (2008) Stimulation of selenoprotein P promoter activity in hepatoma cells by FoxO1a transcription factor. Biochemical and Biophysical Research Communications 365(2):316-321.

42.Gross DN, van den Heuvel APJ, & Birnbaum MJ (2008) The role of FoxO in the regulation of metabolism. Oncogene 27(16):2320-2336.
43.Hausinger RP (2004) Fe(II)/a-Ketoglutarate-Dependent Hydroxylases and Related Enzymes. Critical Reviews in Biochemistry and Molecular Biology 39(1):21-68.
44.Campbell CT, Kolesar JE, & Kaufman BA (2012) Mitochondrial transcription factor A regulates mitochondrial transcription initiation, DNA packaging, and genome copy number. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms 1819(9–10):921-929.
45.Ferber EC, et al. (2012) FOXO3a regulates reactive oxygen metabolism by inhibiting mitochondrial gene expression. Cell Death Differ 19(6):968-979.
46.Jensen KS, et al. (2011) FoxO3A promotes metabolic adaptation to hypoxia by antagonizing Myc function. The EMBO Journal 30(22):4554-4570.
47.Bouwman P, et al. (2010) 53BP1 loss rescues BRCA1 deficiency and is associated with triple-negative and BRCA-mutated breast cancers. Nat Struct Mol Biol 17(6):688-695.
48.Bunting SF, et al. (2010) 53BP1 Inhibits Homologous Recombination in Brca1-Deficient Cells by Blocking Resection of DNA Breaks. Cell 141(2):243-254.
49.Paik J-H, et al. (2007) FoxOs Are Lineage-Restricted Redundant Tumor Suppressors and Regulate Endothelial Cell Homeostasis. Cell 128(2):309-323.
50.Sykes Stephen M, et al. (2011) AKT/FOXO Signaling Enforces Reversible Differentiation Blockade in Myeloid Leukemias. Cell 146(5):697-708.
51.Solaini G, Sgarbi G, & Baracca A (2011) Oxidative phosphorylation in cancer cells. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1807(6):534-542.
52.Wallace DC (2012) Mitochondria and cancer. Nat Rev Cancer 12(10):685-698.
53.Dunyaporn T, Jerome A, & Peng H (2009) Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nature Reviews Drug Discovery 8(7):579-591.
54.Raj L, et al. (2011) Selective killing of cancer cells by a small molecule targeting the stress response to ROS. Nature 475(7355):231-234.

Appendices

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Nederlandse Samenvatting

De term homeostase beschrijft een reeks processen die als doel hebben de balans in elke cel van het lichaam te handhaven. Het behoud van homeostase is essentieel voor het overleven en de werking van het lichaam. Verstoringen van dit evenwicht zijn geassocieerd met verschillende ziektes. Veroudering en kanker zijn twee voorbeelden die onlosmakelijk verbonden zijn met de verstoring van de homeostase (samenvatting in **Hoofdstuk 1**).

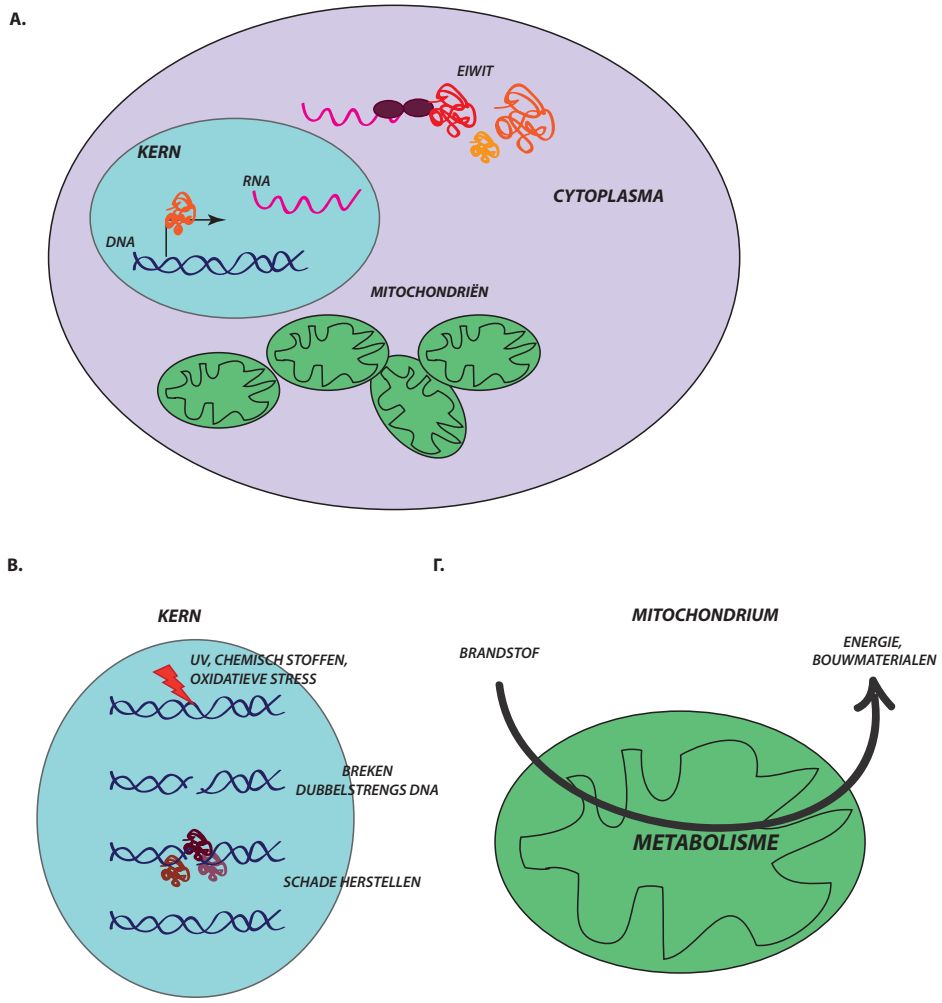
Het menselijk lichaam bestaat uit miljoenen cellen. Elke cel kan weer worden onderverdeeld in functionele compartimenten die verantwoordelijk zijn voor het uitvoeren van specifieke functies. Eén voorbeeld is de kern, het organisatorische centrum of "het brein" van de cel, die het genetische materiaal van de cel (desoxyribonucleïnezuur - DNA) bevat. De informatie die het DNA bevat wordt door de cel gebruikt voor de synthese van eiwitten. Het proces van eiwitsynthese omvat enkele stappen: (i) met behulp van speciale eiwitten, genaamd transcriptiefactoren, wordt de DNA als template gebruikt om ribonucleïnezuur (RNA) te maken (ii) vervolgens wordt in ribosomen (specifieke organellen in het cytoplasma) het RNA vertaald naar eiwitten (**Figuur 1A**). Uit studies in diermodellen is gebleken dat FOXO (FOXO1, FOXO3 en FOXO4) transcriptiefactoren een belangrijk rol spelen in de remming van kanker en het vertragen van het verouderingsproces. Deze eiwitten zijn het onderwerp van dit proefschrift.

Elke wijziging in de genetische code (mutaties in of breuken in het dubbelstrengs DNA) kan leiden tot veranderingen in de eiwitsynthese of verminderde of gewijzigde functionaliteit van eiwitten. Uiteindelijk kan dit leiden tot aandoeningen zoals kanker of veroudering. Omdat onze cellen voortdurend worden blootgesteld aan factoren (UV, oxidatieve stress, chemicaliën en andere omgevingsfactoren) die het DNA kunnen wijzigen en/of beschadigen, zijn verschillende mechanismen ontwikkeld om die schade te herstellen (**Figuur 1B**). In **Hoofdstuk 2** van dit proefschrift beschrijven wij een nieuwe functie van FOXO die te maken heeft met het repareren van schade aan het erfelijk materiaal, namelijk dubbelstrengs breuken in het DNA. Het interessante van dit mechanisme is dat het onafhankelijk werkt van de transcriptie van genen.

In het addendum analyseren we de genen die tot expressie komen in FOXO deficiënte cellen. Verder beschrijven wij een aantal genen waarvan de expressie afhankelijk is van de FOXO transcriptie factoren, waaronder vele genen betrokken bij de stofwisseling. In de Hoofdstukken 3 en 4 gaan we verder in op de genen die betrokken zijn bij de stofwisseling en beschrijven we nieuwe rollen van FOXO bij het handhaven van de metabole homeostase.

Hoofdstuk 3 gaat over het gen coderend voor het enzymeiwit IDH1 (isocitraat dehydrogenase), een nieuw transcriptioneel target van FOXO. IDH1 is in ongeveer 70 % van gliomen gemuteerd en in kleinere hoeveelheden in andere soorten kanker, waardoor dit gen een "oncogen" word genoemd. Wij hebben gevonden dat FOXO belangrijk is voor de transcriptie van IDH1, zowel van het wildtype als van het mutante gen. Verder hebben wij vastgesteld dat FOXO noodzakelijk is voor het goede verloop van stofwisselingsprocessen gecontroleerd door IDH1 en daarmee de groei van cellen onder hypoxische omstandigheden.

In **Hoofdstuk 4** bestuderen we de rol van FOXO in de mitochondriën. De mitochondriën samen vormen het "energiecentrum" of "hart" van de cel. Koolhydraten en vetten worden getransporteert naar de mitochondriën; hier worden ze gebruikt als brandstof om energie voor de cel te maken (**Figuur 1C**). Het interessante van mitochondriën is dat ze hun eigen genetisch materiaal hebben, het mitochondriaal DNA. In FOXO deficiëntie cellen observeren wij accumulatie van disfunctionele mitochondriën. Ze zijn disfunctioneel vanwege een verhoogde uitstroom van vrije zuurstof radicalen (oxidatieve stress), schade aan mitochondriaal



Figuur 1 (A) Cellen met de functionele compartimenten. De kern die het genetisch materiaal (DNA) bevat en het cytoplasma met ribosomen voor de synthese van eiwitten en mitochondriën voor energieproductie. In de kern wordt DNA als sjabloon gebruikt om RNA te maken, vervolgens gaat het RNA naar het cytoplasma voor translatie van het eiwit waar het voor codeert (B) Het genetisch materiaal is kwetsbaar voor schade veroorzaakt door factoren uit het externe en interne milieu van de cel. Specifieke eiwitten herkennen deze schade om die vervolgens te herstellen (C) De mitochondriën vormen het energiecentrum van de cel. Brandstoffen worden naar de mitochondriën vervoerd en worden in de metabole processen gebruikt om energie en bouwmaterialen voor de cel te produceren.

DNA en een verminderde productie van energie uit de beschikbare brandstof. Ook zien we een verminderd vermogen van cellen om disfunctionele mitochondriën op te ruimen.

Kortom, de in dit proefschrift beschreven bevindingen tonen de belangrijke bijdrage van FOXO aan in het handhaven van de homeostase (zie **Hoofdstuk 5** voor uitgebreide bespreking van de resultaten). Tegelijkertijd beschrijven wij voor het eerst een "dubbele" rol van FOXO. Zoals eerder beschreven, is FOXO geassocieerd met de remming van kanker, maar handhaving van de genomische en de metabole homeostase helpt juist bij het overleven van kankercellen. Verder fungeert FOXO ook als oncogen door de transcriptie van mutant

IDH1. Identificatie van de mechanismen die de activiteit van FOXO in verschillende cellulaire omgevingen (normale versus kankercellen) regelen kan bijzonder gunstig voor het ontwerpen van geneesmiddelen met selectieve werking en effectieve behandeling van kanker.

Greek summary - Συνοψη στα Ελληνικα

Ο όρος ομοιόσταση περιγράφει μια σειρά από διαδικασίες που διασφαλίζουν την διατήρηση της ισορροπίας στα κύτταρα και τον οργανισμό. Η διατήρηση της ομοιόστασης είναι απαραίτητη για την επιβίωση και τη σωστή λειτουργία του οργανισμού. Οποιαδήποτε διαταραχή της ομοιόστασης συνδέεται με ασθένεια· για παράδειγμα η γήρανση και ο καρκίνος είναι άρρηκτα συνδεδεμένα με τη διαταραχή της ομοιόστασης (ανασκόπηση βιβλιογραφίας στο **Κεφάλαιο 1**).

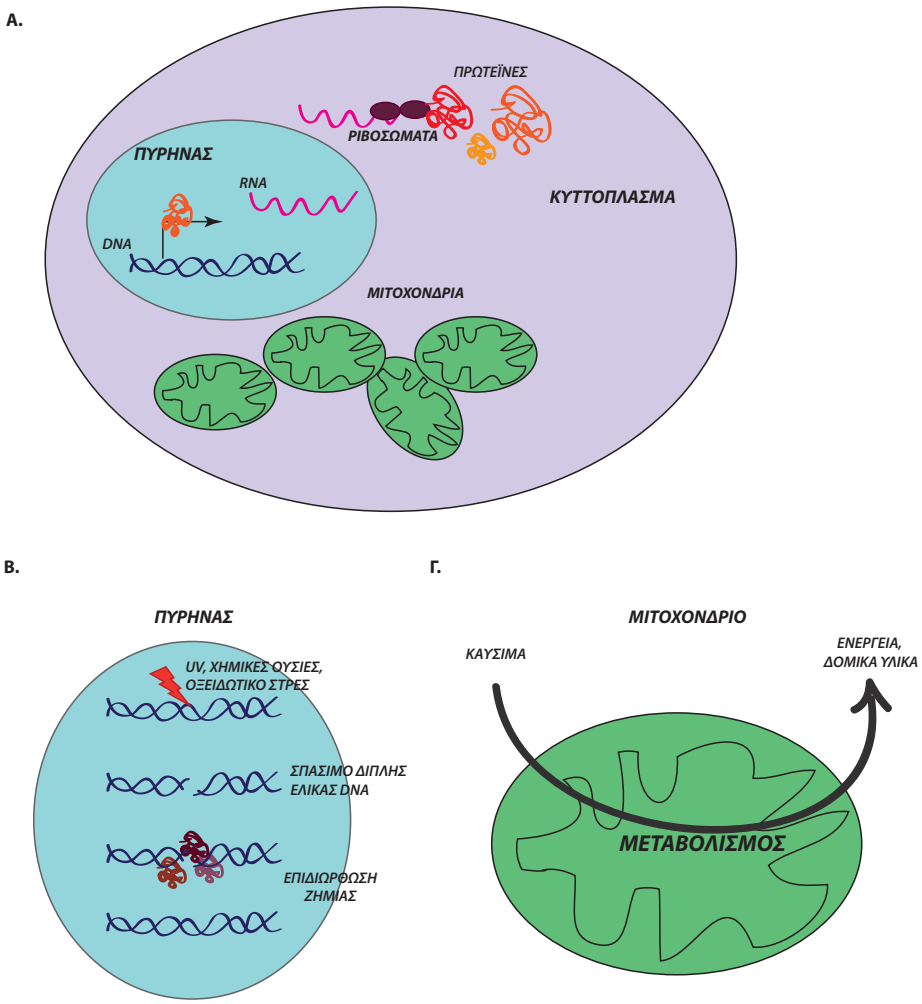
Το ανθρώπινο σώμα αποτελείται από εκατομμύρια κύτταρα. Το κάθε κύτταρο με την σειρά του είναι οργανωμένο σε λειτουργικά μέρη, υπεύθυνα για την τέλεση συγκεκριμένων ρόλων. Για παράδειγμα, το «οργανωτικό κέντρο» ή «εγκέφαλος» του κυττάρου είναι ο πυρήνας, ο οποίος περιέχει τις γενετικό υλικό του κυττάρου (δεοξυριβονουκλεϊκό οξύ-DNA). Στο DNA είναι εντυπωμένες οι γενετικές πληροφορίες για την σύνθεση των πρωτεϊνών του κυττάρου, με την μορφή κώδικα. Η διαδικασία της πρωτεϊνοσύνθεσης περιλαμβάνει ορισμένα διακριτά βήματα (i) οι γενετικές πληροφορίες μεταγράφονται από το DNA σε ριβονουκλεϊκό οξύ (RNA) με τη βοήθεια εξειδικευμένων πρωτεϊνών , των λεγόμενων «μεταγραφικών παραγόντων», (ii) το RNA μεταφράζεται σε πρωτεΐνη στο κυτταρόπλασμα σε ειδικά οργανίδια, τα ριβοσώματα (Εικόνα 1Α). Οι FOXO πρωτεΐνες (FOXO1, FOXO3 και FOXO4), οι οποίες αποτελούν το θέμα αυτής της διατριβής, είναι μεταγραφικοί παράγοντες. Η δράση των FOXO είναι συνδεδεμένη με την παρεμπόδιση της καρκινογένεσης αλλά και την καθυστέρηση της διαδικασίας γήρανσης, όπως αυτό προκύπτει από μελέτες σε ζώα-μοντέλα.

Οποιαδήποτε αλλοίωση του γενετικού κώδικα (μεταλλάξεις ή σπασίματα της διπλής έλικας του DNA) μπορεί να οδηγήσει είτε στην μη παραγωγή πρωτεΐνης είτε στην σύνθεση πρωτεϊνών με μειωμένη ή αλλοιωμένη λειτουργικότητα. Εν τέλει, μπορεί να οδηγήσει σε καταστάσεις όπως ο καρκίνος ή η γήρανση. Δεδομένου ότι τα κύτταρά μας είναι διαρκώς εκτεθειμένα σε παράγοντες που αλλοιώνουν το DNA (UV, οξειδωτικό στρες, χημικές ενώσεις και άλλοι περιβαλλοντικοί παράγοντες), διάφοροι μηχανισμοί επιδιόρθωσης της ζημιάς έχουν εξελιχθεί (Εικόνα 1Β). Στο **Κεφάλαιο 2** αυτής της διατριβής περιγράφουμε μία νέα λειτουργία των FOXO, στην επιδιόρθωση ζημιάς στο γενετικό υλικό και συγκεκριμένα σπασιμάτων στην διπλή έλικα του DNA. Ιδιαίτερο ενδιαφέρον παρουσιάζει το γεγονός ότι ο μηχανισμός που περιγράφουμε είναι ανεξάρτητος από γονιδιακή μεταγραφή.

Στο Παράρτημα περιγράφουμε μια ανάλυση της γονιδιακής έκφρασης κυττάρων που είναι ελλειμματικά στις FOXO. Περιγράφουμε έναν αριθμό γονιδίων των οποίων η έκφραση εξαρτάται από τις FOXO, ανάμεσα στα οποία πολλά γονίδια που σχετίζονται με τον μεταβολισμό. Στα Κεφάλαια 3 και 4 εντρυφούμε περαιτέρω σε αυτούς τους μεταγραφικούς στόχους και περιγράφουμε νέους ρόλους των FOXO στη διατήρηση της μεταβολικής ομοιόστασης.

Στο **Κεφάλαιο 3** περιγράφουμε έναν νέο μεταγραφικό στόχο των FOXO, το γονίδιο που κωδικοποιεί την πρωτεΐνη-ένζυμο IDH1 (ισοκιτρική δεϋδρογονάση). Μεταλλάξεις στο γονίδιο IDH1 ανευρίσκονται περίπου στο 70% των γλοιωμάτων, καθώς και, σε μικρότερα ποσοστά, σε άλλους τύπους καρκίνου, καθιστώντας το γονίδιο αυτό «ογκογονίδιο». Βρίσκουμε τις FOXO απαραίτητες για την μεταγραφή της IDH1, τόσο του άγριου τύπου, όσο και του μεταλλαγμένου γονιδίου. Επίσης δείχνουμε ότι οι FOXO είναι απαραίτητες για τις μεταβολικές διαδικασίες που ελέγχονται από την IDH1 και κατά συνέπεια για την ανάπτυξη των κυττάρων κάτω από συνθήκες υποξίας.

Στο **Κεφάλαιο 4** μελετάμε τον ρόλο των FOXO στη λειτουργία των μιτοχονδρίων. Τα μιτοχόνδρια αποτελούν το «ενεργειακό κέντρο» ή την «καρδιά» του κυττάρου. Καύσιμα με την μορφή υδρογονανθράκων ή



Εικόνα 1. (Α) Κύτταρο με τα λειτουργικά του τμήματα: ο πυρήνας που περιέχει το γενετικό υλικό (DNA) και το κυτταρόπλασμα, που περιέχει τα ριβοσώματα για την σύνθεση των πρωτεϊνών και τα μιτοχόνδρια για την παραγωγή ενέργειας. Το DNA πρώτα μεταγράφεται σε RNA μέσα στον πυρήνα και στη συνέχεια το RNA μεταφέρεται στο κυτταρόπλασμα για την μετάφραση των πρωτεϊνών **(Β)** Το γενετικό υλικό είναι ευάλωτο για ζημιά από περιβαλλοντικούς και ενδογενείς παράγοντες. Ειδικές πρωτεΐνες αναγνωρίζουν και επιδιορθώνουν τη ζημιά, ώστε το γενετικό υλικό να επανέλθει στην πρότερη κατάσταση **(Γ)** Το μιτοχόνδριο αποτελεί το ενεργειακό κέντρο του κυττάρου. Καύσιμα εισέρχονται στα μιτοχόνδρια και χρησιμοποιούνται σε μεταβολικές διαδικασίες για την παραγωγή ενέργειας και δομικών υλικών.

λιπιδίων μεταφέρονται και εισέρχονται στα μιτοχόνδρια, όπου συμμετέχουν σε μεταβολικές διαδικασίες που καταλήγουν στην παραγωγή ενέργειας και στην αποδόμηση των «καυσίμων» στα δομικά τους υλικά για την διάθεσή τους σε αναβολικές διαδικασίες του κυττάρου (Εικόνα 1Γ). Ενδιαφέρον είναι το γεγονός ότι τα μιτοχόνδρια διαθέτουν το δικό τους γενετικό υλικό, το μιτοχονδριακό DNA. Σε κύτταρα ελλειμματικά στις FOXO παρατηρούμε συσσώρευση δυσλειτουργικών μιτοχονδρίων με αυξημένη εκροή ενεργών ριζών οξυγόνου (οξειδωτικό στρες), αυξημένη ζημιά του μιτοχονδριακού DNA, καθώς και μειωμένη παραγωγή ενέργειας από τα διαθέσιμα καύσιμα. Επίσης, παρατηρούμε μειωμένη ικανότητα εκκαθάρισης των

δυσλειτουργικών μιτοχονδρίων.

Συμπερασματικά, τα ευρήματα που περιγράφονται σε αυτή τη διατριβή καταδεικνύουν την σημαντική συνεισφορά των FOXO πρωτεϊνών στην διατήρηση της ομοιόστασης (εκτεταμένη συζήτηση των αποτελεσμάτων στο **Κεφάλαιο 5**). Ταυτόχρονα, για πρώτη φορά περιγράφουμε έναν «διττό» ρόλο για τις FOXO, οι οποίες, ενώ, όπως αναφέρθηκε νωρίτερα, είναι εδραιωμένοι αντικαρκινικοί παράγοντες, κάτω από συγκεκριμένες συνθήκες μπορούν να δρουν ως ογκοπρωτεΐνες. Αναγνώριση των μηχανισμών που ρυθμίζουν τη δράση των FOXO σε διαφορετικά κυτταρικά περιβάλλοντα (φυσιολογικά έναντι καρκινικών κυττάρων) θα μπορούσε να αποβεί ιδιαίτερα πλεονεκτική για τον σχεδιασμό φαρμάκων με εκλεκτική δράση και την αποτελεσματικότερη αντιμετώπιση του καρκίνου.

Curriculum Vitae

Paraskevi (Evi) Charitou was born the 31st of May 1981 in Thessaloniki, Greece. She received her primary and secondary education in Thessaloniki, Greece. She studied Biology in the Aristotle University of Thessaloniki (AUTH) with specialization in Molecular Cell Biology. She performed her Bachelor's Internship in the Laboratory of Microbiology and Molecular Biology in a project entitled "Antiviral properties of isoborneol over Herpes Simplex virus" under the supervision of dr. Afrodite Sivropoulou and Prof. Minas Arsenakis. She obtained her Bachelor Degree in 2006 and, immediately after her graduation, she joined the Master Program "Drug Innovation" in the Department of Pharmaceutical Sciences of Utrecht University. During her Master studies she performed one internship in the Department of Cellular Protein Chemistry of Utrecht University under the supervision of Prof. Ineke Braakman on a project entitled "Studies on the primary folding defect and rescue of ΔF508 CFTR" and one internship in the Department of Pharmacology of Utrecht University under the supervision of dr. Enrico Mastrobattista on a project entitled "Development and characterization of amphiphilic, recombinantly produced peptides that self-assemble into vesicles for efficient drug delivery". In November 2008 she joined the Center for Molecular Medicine at Utrecht University and started her PhD research on the roles of FOXO transcription factors in the maintenance of genomic and metabolic stability under the supervision of Prof. Boudewijn Burgering. The results of her research are described in this thesis.

Publications

1. Hoelen H, Kleizen B, Schmidt A, Richardson J, **Charitou P**, Thomas PJ &Braakman I. (2010) The primary folding defect and rescue of ΔF508 CFTR emerge during translation of the mutant protein. *PLoS One*

2. Charitou P, Burgering BMT. (2012) FOXO transcription factors in control of reactive oxygen species and genomic stability to ensure healthy lifespan. *Antioxidants and Redox signaling*

3. Charitou P, Smits L, van Triest M, Vos HJR, van de Berg M, Macurek L, Medema R, Burgering BMT. FOXO transcription factors maintain genomic stability by regulating histone acetylation around DSBs to promote DNA-end resection and homologous recombination repair
Under review

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So, this is it! The end of a long journey! With a lot of up and downs in the way but with a strong feeling of accomplishment at the end. However, this journey would have not been the same without my "fellow-travelers", whose contribution to the completion of this thesis I would like to acknowledge.

Boudewijn, thank you for the opportunity you gave me, to perform my PhD research in your lab. Thank you for your belief in me and your support all these years. I learned a lot and I grew through this experience. I wish you the best in the future.

I would like to thank my paranymfen, **Milica and Marieke**. Milica, I always enjoyed our long walks and talks about everything; science and life! I am glad we still keep in touch and I wish to you and to Mihajlu all the best for the future! Marieke, my bench neighbor and my "lab-news newsfeed", I wish you all the best finalizing your PhD and for your next steps in your career!

Many thanks to my kamergenootjes, **David, HarmJan and Marten**! It was really unique being the girl in the "boys' room" and I enjoyed every minute of it! There was never a dull moment in that room! I also appreciate our scientific discussions and advice and also the help you gave me whenever I needed it! I wish you all the very best in everything you do! **Lydia and Miranda**, thank you sooo much for always being there, engaged and helpful in everything that I needed! I am grateful for our collaboration in the "DNA repair" project! **Tobias, Marrit, Maaike (van den Berg), Astrid and Paulien** thank you for your inputs during the work discussions and your hands-on help in the lab; I wish you all the best! I would also like to wish luck to the newbies of the lab, **Kim, Maaike (Meerlo), Sabine and Maria**!

Dear **Hans**, I always find your drive and enthusiasm, as well as your passion for new scientific concepts, very inspiring! The people of the "Bos group" **Willem-Jan, Rene, Anneke and Sarah (Consonni)**, thank you for your advice during our joined work discussions but also for the friendly and fun atmosphere in the lab! Wish you the best in everything you do! **Holger**, I would like to thank you for all the technical advice and the friendly discussions! Good luck in your next steps! **Fried**, thank you for the hearty discussions, the support and encouragement all these years! **Ingrid** thank you very much for all your help and positive attitude! **Hugo, Carla, Bas and Lukas** good luck with all scientific challenges! I am sure that all the hard work you put in the organoids will pay off!

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- Εύη -