Redefining the role of FOXO in cancer

Marten Hornsveld

ISBN: 978-90-393-6726-1

Printed by: Ipskamp Printing
Cover picture: Look and you shall see one of the ultimate products of evolution, Pygmy nightjar (*Nyctipolus hirundinaceus hirundinaceus*), Boa Nova, Brazil © Vivian Jacobs
The printing of this thesis was financially supported by: UMC Utrecht and Universiteit Utrecht

Redefining the role of FOXO in cancer

De rol van FOXO in kanker herzien
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 23 februari 2017 des middags te 4.15 uur

door

Marten Hornsveld

geboren op 14 december 1984 te Naarden

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

Copromotor: Dr. T.B. Dansen

This thesis is dedicated to Ron Hornsveld (1955-2012)

Table of contents

Chapter 1

General introduction

1

Introduction

After fertilization of a single oocyte, this one cell gives rise to the trillions of cells that constitute the body of a human adult. Every one of these divisions is carefully initiated, monitored and terminated by complex signaling networks of proteins dedicated to start the cell cycle, copy DNA without errors or damage, construct new organelles and finally divide the cellular content equally over two daughter cells. Cell proliferation needs to be tightly controlled to avoid the ectopic cell growth and subsequent disruption of organ function known as cancer.

For a healthy cell to become a cancer cell it has to acquire various traits summarized as the hallmarks of cancer (1). Two of the primary traits for cancers to initiate are "sustained proliferative signaling" and "evading growth suppression". Acquisition of the "sustained proliferative signaling" hallmark commonly arises from alteration of the receptor tyrosine kinase (RTK) pathways, driving growth factor (GFR) signaling. Mutations commonly found are in the RAS-MEK-ERK and PI3K-PKB/AKT pathways, which stimulate cell proliferation. Although mutations in GFR-signaling are well described, the mechanisms downstream of these mutations are still not fully understood. Difficulty to elude the exact effects of GFR-signaling arises from the vast complexity of how these signals are relayed in the cell. First, signals starting from RTKs undergo extensive branching as one RTK can activate both RAS-MEK-ERK and PI3K-PKB/AKT, which subsequently target diverse proteins operating in various cellular functions. Second, when GFR-signals reach the nucleus numerous transcription factors will mediate the transcription of a wide array of genes. Careful elucidation of all events downstream of GFR-signaling is required to fully understand the consequences of oncogenic mutations and a prerequisite for developing successful cancer therapies in the future.

Key transcription factors in regulating cell proliferation downstream of PI3K-PKB/AKT signaling are members of the Forkhead Box O family (FOXO). Next to GFR-signaling FOXOs operate in a plethora of cellular functions and many different aspect of life including lifespan, diabetes and cancer (2). The exact mechanism by which FOXO functions, mediates transcription and affects multifarious signaling pathways in the cell still needs to be fully understood however. This thesis aims to define the role of FOXOs in cancer and advance the understanding of the underlying mechanisms by which FOXOs contribute to tumorigenesis.

The basics of FOXOlogy

Canonical FOXO regulation

The mammalian Forkhead box O (FOXO) family of transcription factors consists of the four family members; FOXO1, FOXO3, FOXO4 and FOXO6 (FOXOs). FOXOs are conserved throughout the animal kingdom and FOXOs mediate transcription through binding to their consensus 5′-TTGTTTAC-3′ binding sites (2-4). The DNA binding domains within FOXOs are highly similar, therefore regulation of FOXO1, FOXO3, FOXO4 or FOXO6 specific target genes is expected to either arise from differential FOXO expression patterns or upstream signaling regulating the transactivation domains that vary between different FOXOs (4) . Indeed different expression patterns for FOXOs are found as differing levels of FOXO1 and FOXO3 are expressed in all tissues, FOXO6 is predominantly expressed in the brain and FOXO4 is mainly expressed in muscle cells and throughout the body at low levels (4, 5).

The Insulin-PI3K-PKB pathway is the canonical pathway in which FOXOs function. Upon activation of growth factor receptor tyrosine kinases (GFR/RTK) like the Insulin receptor (INSR), Phosphoinositide 3-kinase (PI3K) becomes activated. Activated PI3K generates Phosphatidylinositol-3 phosphate (PIP3) in the plasma membrane which functions as a docking site for phosphoinositide dependent protein kinase-1 (PDPK1) and Protein

Kinase B (PKB/AKT). PDPK1 subsequently activates PKB through phosphorylation of Ser308. In addition to Ser308 phosphorylation, PKB requires Ser473 phosphorylation by the mTORC2 complex to become fully activated (6). When fully activated, PKB can phosphorylate a wide array of target proteins amongst which are the FOXOs and members of the mTORC1 complex and stimulate glucose uptake and cell proliferation.
FOXO transcriptional activity is regulated through its shuttling between the nucleus and the cytoplasm. Phosphorylation of nuclear FOXOs by PKB at Thr24/Thr32, Ser256/Ser253/Ser197 and Ser319/Ser315/ser262 induces the binding to 14-3-3 proteins, which facilitate FOXO nuclear export and simultaneously obstruct reimporting into the nucleus (7-9). Upon loss of GFR signaling, PI3K generated PIP3 is dephosphorylated by the lipid Phosphatase and tensin homolog (PTEN), thereby antagonizing RTK signaling. Reduced PIP3 in the plasma membrane results in reduced PKB activity, loss of FOXO phosphorylation and subsequent FOXO accumulation in the nucleus. In the nucleus, FOXO mediates the transcription of a plethora of target genes involved in the cell cycle, apoptosis, redox homeostasis, metabolism, angiogenesis and aging (2, 10).
If FOXO regulates a specific set of target genes or are more general activators of gene expression remains a point of debate. It is clear however that upon FOXO accumulation in the nucleus, FOXO preferably binds DNA in promoters and enhancers covered with histone marks that are correlated to active transcription (11, 12). These observations implicate that the output of FOXO mediated transcription is heavily influenced by the epigenetic status of the DNA at the moment FOXOs reside the nucleus. Additionally, multiple proteins involved in transcription regulation have been reported to bind to FOXOs e.g. p300/CPB, β-catenin, PPARγ, Estrogen receptor, androgen receptor, SMADs, STATs, etc. (13). Combined, these observations illustrate a highly complex system in which transcriptional output of FOXOs can be different between cell lines, organs and organisms, depending on concurrent signaling and the epigenetic landscape.

FOXO activation by reactive oxygen species
Next to RTK signaling FOXO localization can be modulated through multiple other pathways even in the presence of active RTK signaling. When a cell encounters adverse conditions like elevated reactive oxygen species (ROS) levels, nutrient starvation or DNA damage, FOXO is activated in order to reestablish homeostasis (14).
When high levels of ROS are produced in the cell FOXOs move to the nucleus in two different ways. First the c-JUN terminal Kinase (JNK) becomes activated by the redox sensitive Apoptosis signal-regulating kinase 1 (ASK1) and the small GTPase Ral. Activated JNK inactivates RTK signaling by phosphorylation of Insulin Receptor Substrate (IRS1/2) adaptor proteins and FOXOs directly, hereby overriding GF-signaling dependent inactivation of FOXO (15, 16).
Second, FOXOs can be cysteine oxidized by hydrogen peroxide. Oxidized FOXOs can form disulfide bridges with nuclear importers Transportin 1 (TNPO1) and Importin 7 & 8 (IPO7/8), resulting in strong binding and nuclear translocation independently of other signals (17, 18). In order to counteract elevated ROS production in the cell, FOXO mediates the transcription of antioxidant genes like Catalase, Sestrin (SENS1/2/3), Super Oxide Dismutase 2 (SOD2), Peroxiredoxin 3 (PRDX3), Glutathion Peroxidase 1 (GPX1), GSTM1 and genes involved in the metabolic generation of the Glutathione antioxidant system and reductive entities like NADPH (19, 20) .
Especially in stem cells FOXO is essential regulators of redox homeostasis and required to prevent differentiation and mediate survival through this function. Loss of FOXO in the hematopoietic system leads to exhaustion of the hematopoietic stem cell pool, reduced repopulation capacity, enhanced proliferation and apoptosis (21-23). These defects are correlated to increased ROS levels within the stem cell compartment and could be rescued by treating mice with the antioxidant N-acetyl cysteine (NAC) (21).

Similar defects for FOXO3 loss are found in neuronal stem cells, leading to increased brain size, increased levels of ROS production, decreased self renewal and increased proliferation (24). These findings imply that FOXOs are essential for tissue homeostasis by balancing redox signaling in stem cells.

FOXO activation by nutrient depletion
Under conditions in which glucose is limited, Insulin levels will drop. Consequently INSR will become inactive, reducing PI3K-PKB signaling and thereby resulting in FOXO nuclear accumulation. In response to low glucose levels, FOXOs will mediate the transcription of genes that reduce glycolysis and stimulate ATP production by the use of glutamine through Oxidative phosphorylation in the mitochondria. Simultaneously FOXOs stimulate gluconeogenesis in the liver and autophagy in starved cells to increase the glucose supply and nutrients required for survival (25). In worms the FOXO ortholog DAF-16 functions even more drastically by mediating a low metabolic hibernation like state called Dauer as a strategy to survive adverse conditions (10). Additionally, when ATP levels in the cell drop and the ATP/AMP sensor AMP kinase (AMPK) gets activated FOXO3 get phosphorylated on Ser413, Ser588 and Ser626, stimulating the transcription of genes involved in metabolic rewiring and stress resistance (26).

Other inputs
Next to the well-studied regulation of FOXOs by PKB, JNK and AMPK, many other kinases can attenuate FOXO activity. In response to DNA damage FOXOs becomes phosphorylated by Ataxia telangiectasia mutated (ATM) kinases and might contribute to DNA damage repair and its associated cell cycle arrest. FOXOs have also been described as targets of extracellular signal-regulated kinase (ERK), Mammalian Ste20-like kinase (MST1), Cyclin dependent kinases (CDK), serum and glucocorticoid-regulated kinase (SGK), dual specificity tyrosine phosphorylation regulated kinase 1A (DYRK1A), IkB kinase (IKK) and Casein kinase 1 (CK1)(27). To which extend the phosphorylation changes FOXO activity and output is however still under investigation.
Next to phosphorylation FOXOs can also be modulated through Acetylation by actetyl transferases p300, HDACs and Situins (SIRT), as well as ubiquitylation by MDM2 and USP7 or methylation by PRMT and SET9 (28-33)

Taken together FOXOs are regulated by many different upstream signals and when activated regulate a plethora of target genes (Figure 1). Due to this great complexity it is therefore no surprise that the current understanding on how FOXOs function exactly is still limited. As FOXOs function at the crossroad of diabetes, cancer and aging it is essential to understand its functions in detail as this holds many clinical relevant implications. Strikingly, the function of FOXOs in cancer is still unclear and debated although many studies report functions for FOXOs within this disease. In fact it is still unclear if FOXOs are tumor suppressors or not after two decades of research, illustrating how complicated FOXOlogy is and stressing the need for studies dedicated to answer this question (34).

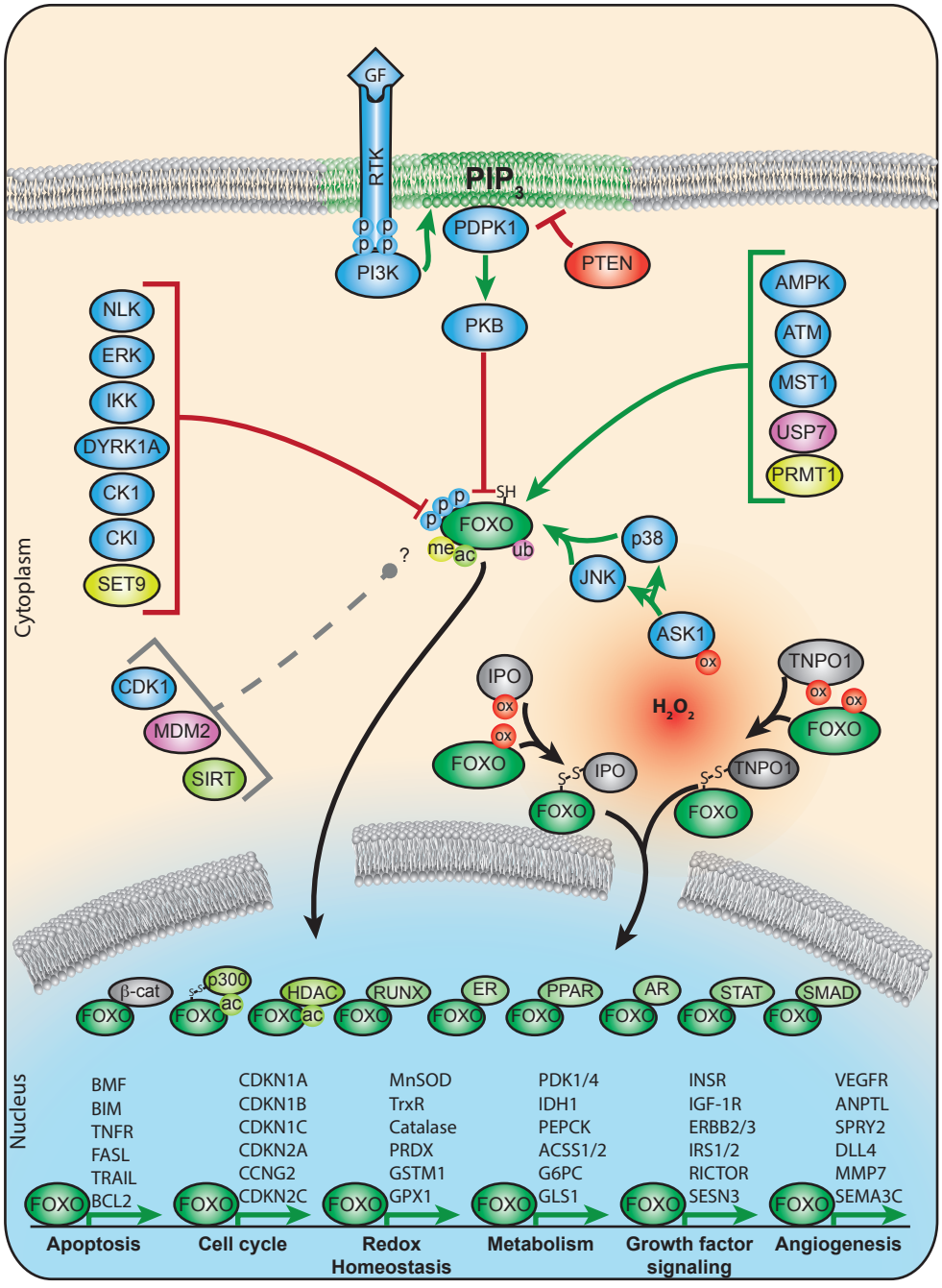


Figure 1: A schematic overview of regulators, binding partners and target genes of FOXO

1

FOXOs in cancer
The Archetype: FOXOs are tumor suppressors

Repressing the cell cycle

Already in the seminal papers that identified FOXOs are controlled by PKB signaling it was apparent that FOXOs could have tumor suppressive functions. Activation of FOXO, either by pharmacological inhibition of PI3K-PKB or the ectopic overexpression of FOXO resulted in a robust cell cycle arrest in Embryonic Kidney cells, Fibroblasts and cancer cell lines derived from colon carcinoma, glioblastoma, osteosarcoma and acute T cell leukemia (7, 8, 35, 36).

Cell proliferation starts from a quiescent state and continues by progression from G1 to S phase. In early G1, expression levels of Cyclin D (CCND1/2/3) are up regulated by GFR signaling, leading to increased levels of CyclinD-CDK4/6 complexes. Cyclin D-CDK4/6 complexes inhibit the retinoblastoma family of proteins (RB, p107 and p130), resulting in the release E2F transcription factors which induce transcription of S-phase proteins and Cyclins (37). From S-phase on Cyclin-E/A-Cdk2 complexes take over ensuring correct DNA replication and cellular growth before entering mitosis. As soon as the cell is ready to undergo mitosis Cyclin B/Cdk1 complexes become active start mitosis. At the onset of anaphase Cyclin B is quickly degraded by the APC/c ubiquitin ligase in order to ensure the end of the cell cycle and the return to quiescence.

FOXO induced cell cycle arrest is mediated through transcription of multiple cell cycle kinase inhibitors (CKI). The best described CKI downstream of FOXO is $p27^{kip1}$ (CDKN1B). Additionally to CDKN1B, FOXOs have also been described as regulators of $p21^{cip1}$ (CDKN1A), $p57^{kip2}$ (CDKN1C) and the INK4 family of CKIs, $p15^{INK4b}$ (CDKN2B), $p16^{INK4a}$ (CDKN2A), $P18^{INK4c}$ (CDKN2C) and $p19^{INK4d}$ (CDKN2D). FOXO mediated induction of CKI expression leads to inhibition of the Cyclin/CDK complexes responsible for progression through the different phases of the cell cycle and results in a robust cell cycle arrest in G0/G1, G2 or even senescence (35, 38-40).

Stimulating apoptosis

Next to functioning as repressors of the cell cycle, FOXOs are well described as inducers of apoptosis in many different cell lines (41). Apoptosis can be triggered through multiple cell intrinsic and extrinsic signals that converge on Caspase 3 and Caspase 7, which are the final and irreversible executioners. Extrinsic apoptotic stimuli comprise of Tumor necrosis factor (TNF), TNF related apoptosis inducing- (TRAIL) and FAS-ligands (FasL) that activate the TNF (TNFR) and Death (DR) receptor family of extracellular receptors respectively (42, 43).

The decision to undergo intrinsic apoptosis is determined by the status of the BAK and BAX protein. BAK/BAX are usually in an inactive conformation on the outer membrane of mitochondria. Activity is regulated through competitive binding to BAX and BAK between anti-apoptotic factors BCL2, BCL-XL or MCL1 and pro-apoptotic BH3-only proteins BIM, BAD, BID, PUMA, NOXA, BOK and BMF. Anti-apoptotic factors render BAK/BAX inactive, but upon binding to pro-apoptotic proteins BAK/BAX become activated, leading to mitochondrial outer membrane permeabilization and subsequently apoptosis (44). FOXOs can drive the expression of both extrinsic and intrinsic pro apoptotic genes including: FASL, TRAIL, TNFR, BIM and BMF, leading to cell death in both normal and cancer cells (41). Next to stimulating canonical apoptosis, FOXOs have recently been ascribed to induce anoikis in cells that detach from tissue monolayers and the extracellular matrix. Detachment from a tissue results in reduced GFR signaling and metabolic stress due to impaired glucose uptake. Under these conditions FOXOs become activated and drive the expression of BMF in order to execute anoikis and prevent metastasis (45).

FOXOs suppress tumorigenesis

The fact that FOXOs are negatively regulated down stream of the frequently mutated PI3K-PKB pathway and activation results in cell cycle arrest and apoptosis puts FOXOs forward as putative tumor suppressors. In line with the observation that FOXOs can repress cell proliferation and induce apoptosis *in vitr*o, expression of hyperactive FOXO mutants in xenograft experiments confirm primary tumor growth is impaired by ectopic FOXO activation (46, 47).

Histopathological studies focused on correlating FOXO expression and localization to disease outcome in cancer patients emphasizes a role of FOXOs as tumor suppressors. Low levels of FOXO1 expression and high levels of the inactivating FOXO1-Thr32 phosphorylation are correlated to reduced overall survival and disease free survival in soft tissue sarcoma, AML, prostate and breast cancer (48-51). Similarly high FOXO3 levels correlate to increased disease free survival in clear cell renal carcinoma, colorectal, urothelial and breast cancer (52-55). Pairing primary colorectal cancer samples with their corresponding liver metastases showed that FOXO3 levels in metastasis are significantly lower, indicating that lowering FOXO levels is required for metastasis formation (52). FOXO3 levels in ER-positive breast cancer were reported not to influence patient survival and distant metastasis, but in these cases the localization of FOXO3 in the nucleus was correlated to good prognosis and delayed metastasis formation (56). In line with the observations of FOXO1 and FOXO3, low levels of FOXO4 expression in prostate cancer cells is correlated to poor prognosis and increased metastasis formation (57).

FOXO loss enhances tumorigenesis in mice

Foxo1 knockout in mice unveiled an essential role in embryonic angiogenesis and Foxo1 therefore is essential for survival of the embryo (58, 59). *Foxo3* knockout is not embryonic lethal, but female mice become infertile due to defects in primordial follicle activation (60). Loss of *Foxo4* did not result in any notable changes in the mouse (59). Together these studies only described mild phenotypes and no relation to cancer was encountered. As noted, FOXOs are highly similar and redundant, therefore single *Foxo* knockout mice are unlikely to present with extensive defects. The seminal paper of Paik and colleagues in which conditional *Foxo1/Foxo3/Foxo4* knockout mice were generated supports this hypothesis (61). Only a mild increase in tumor incidence in aged mice was observer when two FOXO knockouts were combined e.g. *Foxo1/Foxo3* or *Foxo1/Foxo4*. But triple knockout induced at adult age in mice resulted in lymphoblastic thymic lymphomas in the spleen, liver and lymphatic system. Additionally hemangiomas were detected and widely spread in the body. This study now solidifies that FOXOs are indeed redundant and can function as tumor suppressors. Curiously, the types of tumors that are found are derived from the same lineage and solid tumors derived from epithelial tissues are hardly detected. This argues against a general role for FOXOs in prevention of tumorigenesis as it only functions in a very specific lineage.

FOXO mutations in cancer

The fact that to increase tumor incidence you need to lose six *FOXO* alleles, or 5 in males, makes it unlikely this will ever occur in tumors and is not yet reported. Mutations in single FOXOs however have been described in cancer and can contribute to tumorigenesis in different ways. First, mammalian FOXOs were found to be part of chromosomal translocations resulting in FOXO1-PAX3/7, FOXO3-MLL and FOXO4-MLL fusion proteins in alveolar rhabdomyosarcoma and acute leukemia (62-64). In all cases the DNA binding domain of PAX or MLL are fused to the transactivation domain of FOXOs and result in enhanced expression of PAX and MLL target genes and hereby contribute to tumorigenicity. On their own these fusion proteins are insufficient to drive

tumorigenesis however and additional mutations are required (63, 65).
Second, single nucleotide mutations in FOXO1 have been found enriched in the non-Hodgkin lymphomas, Follicular lymphoma and diffuse B-cell lymphoma. Interestingly, these mutations are found mostly found around in the N-terminus of FOXO1 protein and lead to a shorter form of FOXO1 (66). More detailed examination of FOXO1 mutations revealed that R19, R21 and T24 are frequently mutated (67). Phosphorylation of FOXO1 by PKB on T24 is required to induce binding to 14-3-3 proteins and keep FOXO1 cytoplasmic. The consensus binding-site on proteins for PKB is RxRxxS/T, implicating that these mutations result in either loss of PKB binding in the case of R19 and R21 mutations or disability to phosphorylate FOXO1-Thr32, all resulting in unphosphorylated active FOXO1 (67). Indeed the authors describe that these mutation increase FOXO1 nuclear localization, implicating enhanced FOXO1 activity. Contradictory to what one would expect based on FOXOs putative role as tumor suppressors, these mutations were correlated to poor prognosis and point towards a more complex role for FOXOs in cancer.

Shifting the paradigm: FOXOs as tumor supporters

Contradicting histopathological studies
In line with the reported activating mutations in FOXO1 that contribute to tumorigenesis, multiple other histopathological reports link high FOXO levels and activation to poor prognosis. In gastric cancer, phosphorylated FOXO1 is correlated to higher overall survival and lower tumor angiogenesis. These correlations suggest that active FOXO1 supports tumor growth and metastasis (68, 69).
High expression of FOXO3 is correlated to lower overall survival and recurrence free survival in Acute Myeloid Leukemia (AML) patients carrying different types of mutations (70). Studies focused on characterizing FOXO3 localization in breast and colorectal cancer described that high levels of FOXO3 nuclear localization correlate to lower overall survival (71, 72).
Combined these studies contradict the previously discussed studies that correlate high FOXO levels and low FOXO activity to good prognosis underlining the controversy on FOXOs function in cancer. Even though less numerous than studies describing FOXOs as tumor suppressors some recent studies described tumor supportive properties for FOXOs

FOXOs in cancer cell homeostasis and metastasis
As discussed above FOXOs play an important role in maintaining stem cell homeostasis and self-renewal. Especially within the hematopoietic stem cell (HSC) compartment FOXOs are essential for maintaining the stem cell populations (21).
Inhibitors targeting the common BCR-ABL mutations efficiently reduce tumor load in Chronic myeloid leukaemia (CML), but recurrence of drug resistant Leukemia Initiating cells (LICs) makes curing the disease problematic. As FOXOs are essential for maintaining HSCs, Naka and colleagues assessed if FOXO3 knockout CML cell populations contain LICs and if FOXO3 loss limits their capacity to reestablish tumors. Indeed it was found that LICs lacking FOXO3 have limited tumor reconstitution capacity (73). The mechanism by which FOXO3 maintains stem cell homeostasis and self-renewing capacity was later ascribed to the regulation of Bcl6. As a target gene of FOXO3, Bcl6 functions by repressing MYC, P53, Cyclin D2 and CDKN2A and thereby mediates LICs self-renewal capacity (74, 75).
A similar role for FOXOs was found in AML cells carrying an MLL-AF9 fusion protein mutation. LICs within the AML cell population exhibit low PKB activity and nuclear FOXO localization, implying that FOXOs are also actively maintaining the LIC population.

Hyper-activating PKB signaling within these AML cells leads to FOXO inactivation and maturation, thereby reducing the repopulating capacity. Similarly, AML cells knockout for *Foxo1/Foxo3/Foxo4* lose their repopulating capacity in bone marrow transplantation assays and is accompanied by elevated stress signaling through JNK within these cells (76).
Together these finding show that FOXO is not only required for maintaining healthy stem cell populations but also essential for cancer stem cell like populations within leukemic cancers. If FOXO fulfills a similar role in solid tumors is however still unclear and studies addressing this question are lacking to date.

FOXOs regulate metastasis
The moment tumors progress and start metastasizing, cancer becomes mortally dangerous and this is therefore a critical turning point for patient survival. Multiple studies have connected a supportive role for FOXOs in facilitating and even stimulating metastasis. The first notion of a role for FOXOs in metastasis was in aggressive breast cancer cell lines, which lose their metastatic capacity in xenograft experiments upon knockdown of FOXO3. It was shown that loss of FOXO impairs and FOXO3 activation induces invasive behavior of breast cancer cells in *in vitro* invasion and migration assays. The effects on the invasive capacity of these cells are addressed to FOXO3 mediated expression of Matrix Metalloproteinases 9 and 13 (MMP9/13), which support invasion by degrading the extra cellular matrix surrounding the tumor cells (77). In addition FOXO1 has also been linked to regulating MMP1 expression in breast cancer cells, which could stimulate metastasis (78).
FOXOs were also found to be involved in colorectal cancer metastasis, as combined nuclear localization of FOXO3 and b-catenin correlates with metastatic disease. FOXO3 hyper-activation alone leads to increased cell motility and apoptosis in DLD1 cells, but in the presence of high nuclear b-catenin levels apoptosis is repressed and transplanted cells become metastatic *in vivo* (72). The other way around, tumors generated from xenografts and patient tumors show efficient drug resistance development against PI3K/KB/TOR inhibitors in a b-catenin dependent way. Impairing b-catenin accumulation and signaling by co-treatment of these tumors with Tankyrase inhibitors, significantly reduced this adaptive resistance and increased apoptosis (79).
These observations now implicate that dependent on the context in which FOXO becomes activated, FOXO can either act as a tumor suppressor or tumor promoter. This notion is supported by the finding that in breast and ovarian cancer spheroids inhibition of the PI3K pathway results in a dichotomous response, leading to apoptosis only in cells on the inside of the spheroids. Within these spheroids a clear FOXO related gene expression profile is observed, but depending on the location at which the cell resides at the moment of activation the outcome will be different (80).
Another example of context dependent tumor supportive behavior of FOXOs is in breast cancer cells that are either positive (ER+) or negative (ER-) for the estrogen receptor. FOXO3 hyper-activation in ER+ MCF7 cells results in suppression of cell proliferation, migration, invasion and anchorage independent growth. These effects are reversed in MCF7 cell treated with RNAi targeting ER, resulting in ER- MCF7 cells (81).
Combined these studies now establish a more complex role for FOXOs tumorigenesis and metastasis. These findings reveal that studies putting FOXOs forward as tumor suppressors or tumor supporters are not necessarily contradicting, but that the context in which FOXOs are activated or lost dictate the observed phenotypes.

FOXOs mediate drug responses
Not only do FOXOs fulfill a key role in tumor biology, but they also are heavily involved in the drug response to both conventional chemotherapy as well as more modern

small molecule inhibitors. Various studies have reported FOXOs mediate apoptosis in response to chemotherapeutic drugs including 5-Fluoracil, Paclitaxel, Resveratrol, and inhibitors of BCR-ABL, PI3K or PKB (82-86).
However, FOXOs also appear to play a crucial role in drug resistance. FOXO3 was found to be a key regulator of the Multi Drug Response pump 1 (MDR1/ABCB1), thereby facilitating the acquisition of resistance against Doxorubicin in breast cancer and leukemia cells (87, 88). Additionally the role of FOXOs in maintaining cellular redox homeostasis and elevating oxidative stress resistance might also contribute to gaining resistance towards drugs that elevate ROS levels in the cells (89). For example, ovarian cancer cells generate elevated ROS levels as a side effect of Paclitaxel treatment and FOXO mediated expression of MnSOD enhances resistance to these elevated ROS levels (90).
The use of small molecule inhibitors targeting receptor tyrosine kinases and other oncogenic kinases revealed that cancer cells quickly adapt to the loss of oncogenic signaling through elaborate feedback mechanisms that re-establish growth factor signaling. Due to this plasticity, treating patients with inhibitors targeting single oncogenic kinases including PI3K or PKB is generally ineffective (91). As FOXOs are regulated downstream of PI3K-PKB it can be expected that they participate in feedback signaling within this pathway. Indeed mammalian and Drosophila FOXOs can mediate re-establishment of growth factor signaling by regulating the transcription of the Insulin receptor (92). Treatment of breast cancer or CML cells with Doxorubicin was found to induce nuclear localization of FOXO3, which in response up-regulates PIK3CA expression and thereby activates PI3K-PKB. FOXO3 induced up-regulated PI3K-PKB signaling now confers doxorubicin resistance to these cells (71, 93). In addition to PI3K, FOXOs can regulate both mTORC1 and mTORC2 complexes which inactivate and activate PKB respectively. Upon activation of FOXO1, either through over expression or PI3K inhibition, the mTORC2 component Rictor is transcribed, leading to increased mTORC2 activity and consequent PKB activity through Ser473 phosphorylation (94). In parallel FOXO1 stimulates the expression of SESN3, which in turn inactivates mTORC1 by inhibiting the GTPase Rheb, thereby repressing cell growth but enhancing PKB activity simultaneously (95).
Furthermore, FOXO3 was found to regulate multiple RTK adaptor proteins including Insulin Receptor Substrate 1/2 (IRS1/2) and Insulin Like Growth factor receptor binding proteins (IGFBP) in human colon carcinoma cells (11). The role of FOXOs in regulating GFR- feedback signaling is not only restricted to the insulin pathway as characterization of RTK expression in response to PI3K-TOR dual inhibitors revealed that FOXOs can drive the expression of different RTKs in breast cancer including c-MET, HER2, HER3 and RET (96).

This key role of FOXOs in regulating growth factor signaling feedback makes activating FOXOs as a putative strategy for cancer therapy questionable and suggests that inhibiting FOXOs might also be a suitable strategy. From this introduction it becomes clear that FOXOs are heavily involved in many aspects of cancer. Understanding the role of FOXOs in cancer is complicated. All the data presented above is scattered through many different studies in various types of cancer and most studies are limited to only the effects of FOXO loss or gain of function in a single aspect of cancer. Strikingly, up to this date no studies have been published that aim to characterize both FOXO loss and gain of function within one robust model for cancer. These kinds of studies are essential to progress our understanding on how FOXOs function in cancer and might contribute to the design of promising treatment strategies that can take advantage of FOXOs role in cell cycle regulation and the induction of apoptosis as well as impairing therapy resistance.

Thesis outline

In **chapter 2** we review how classical signal transduction cascades are intertwined with redox signaling, herewith adding another layer of complexity to signal transduction in cancer. We took the outline of the seminal review "The Hallmarks of cancer: The next generation" as a framework to discuss how signal transduction and redox signaling cooperate in all aspects of cancer (1). In **Chapter 3** we demonstrate that in order for breast cancer cells to survive ECM detachment, FOXO activation needs to be restricted to prevent accumulation of the pro-apoptotic genes BIM and the novel FOXO target gene BMF. In **chapter 4** we address the fundamental question on if FOXOs function as tumor suppressors or not. Using a robust mouse model for Invasive Lobular carcinoma we test how activating FOXO3 and loss of FOXO1 & 3 influences tumorigenesis and metastatic capacity. In this chapter we unveil that FOXOs are essential for tumorigenesis and specifically metastasis, but that tumor cells need to carefully tune FOXO activity in order to get the benefits of having FOXOs around. In **chapter 6** we aim to employ FOXO induced transcriptional programs as a prognostic marker for oncogenic PI3K pathway activity. By identifying genes that are regulated downstream of PI3K inhibition and FOXO activation in breast cancer cell lines we developed a prognostic gene set. Testing this gene set on publicly available patient data confirms the ability to predict FOXO activity. Using these gene sets, quick assessment of the tumor status can be acquired through a qPCR platform with a limited amount target genes. **Chapter 6** concerns the detailed analysis of the dynamics by which FOXO3 induces a cell cycle arrest. Using Retinal Pigment Epethelial (RPE) cells carrying a live cell cycle phase reporter system (FUCCI), we identify a novel type of cell cycle arrest in which FOXOs mediate a cell cycle exit from G2. Cells exit G2 through premature APC^{CDH1} activation by down regulating EMI1 expression and end up in a G0/G1 like state with 4n DNA. This phenomena is represents a novel way in which FOXOs regulate the cell cycle.

Chapter 2:

2

The Hallmarks of cancer from a redox perspective

M Hornsveld[1], T.B. Dansen[1]
[1]Department of Molecular Cancer Research, University Medical Center Utrecht, Utrecht

Antioxidants & Redox signaling
Volume 25, Number 6, 2016 DOI: 10.1089/ars.2015.6580

2

Abstract

Significance: For a healthy cell to turn into a cancer cell and grow out to become a tumor, it needs to undergo a series of complex changes and acquire certain traits, summarized as the "Hallmarks of Cancer". These Hallmarks can all be regarded as the result of altered signal transduction cascades and understanding these cascades is essential for cancer treatment.

Recent advances: Redox signaling is a long overlooked form of signal transduction that proceeds through the reversible oxidation of cysteines in proteins and that uses hydrogen peroxide as a second messenger.

Critical issues: In this review we provide examples that show that redox signaling is involved in the regulation of proteins and signaling cascades that play roles in every Hallmark of cancer.

Future directions: Understanding how redox signaling and 'classical' signal transduction are intertwined could hold promising strategies for cancer therapy in the future.

2

Introduction

Fundamental evolutionary processes like DNA mutation, propagation of the mutant DNA and natural selection underlie both life and the development of cancer. Elaborate signal transduction pathways ensure that cell division and cell death are tightly controlled to prevent cells with mutant DNA to disturb homeostasis of the multicellular organism. It is therefore not surprising that mutation of the DNA that encodes the players in these signal transduction pathways is what transforms normal cells into tumor cells. But evolution has yielded several safeguards to prevent uncontrolled growth in order to enable multicellular organisms to maintain homeostasis until the DNA has safely been passed on to the progeny. These safeguards can be seen as hurdles a cell has to take to change from a normal cell into a metastatic tumor that will eventually kill its host that it once was part of. Possibly the biggest challenge for the treatment of cancer is the fact that the signal transduction pathways that control whether these hurdles can be taken are also of vital importance for homeostasis of the soma; tissue maintenance and repair after all requires cell division and survival to be allowed under certain conditions.

Decades of research yielded insight into the biology and molecular mechanisms that make up the hurdles every cancer cell has to take to become a metastatic tumor. Hanahan and Weinberg originally described six mutation-driven acquired competences needed to take these hurdles in their seminal review "The Hallmarks of Cancer" (97). Ten years later, they updated this view and now Eight Hallmarks and two Enabling Characteristics have been proposed and each of these are connected to alterations in signal transduction (Table 1)(1). Indeed, a plethora of mutations in proteins fundamental to signal transduction cascades have been identified to be involved in the prevention or origin of cancer. Understanding these signaling cascades has been a main focus in cancer research, because the proteins involved in these pathways might serve as future drug targets.

An emerging mode of cellular signal transduction called redox signaling is conveyed through redox reactions at the thiol side chain of protein cysteine residues. Reactive oxygen species (ROS), for instance generated as a by-product of mitochondrial metabolism, but also produced in response to growth factor signaling by dedicated oxidases like NADPH oxidases (NOX) are a start of redox signaling cascades. Maybe it is because of the dubious connotation ROS and Free radicals have in light of pseudo-scientific health claims, or the aversion to redox reactions some biologists may have developed in high school chemistry classes, but redox signaling is more than often ignored in tumor biology. This might be a missed opportunity, because redox-signaling pathways can be a target or provide leverage in future treatment options. As a matter of fact, several therapies that are already used in the clinic have a redox-signaling component. In this review we aim to give an overview of how redox signaling and 'classical' signal transduction pathways are intertwined in every Hallmark of cancer (Table 1). In light of the Forum Issue on advances in breast cancer research, we discuss which mutations commonly found in breast cancer are involved in redox signaling and how perturbation of redox homeostasis could contribute to cancer prevention and therapy.

Redox signaling basics

Several excellent reviews have recently been published that describe the biochemistry of redox signaling in great detail (98-101). For the purpose of this review it is sufficient to understand the basics as outlined below.

Redox signal transduction

Redox signaling starts with the generation of ROS, for instance due to the incomplete reduction of molecular oxygen used to drive mitochondrial ATP generation (98, 102).

Another important source of hydrogen peroxide comes from NADPH-dependent oxidases (NOX/DUOX), which either generate extracellular hydrogen peroxide directly (by DUOX) or superoxide (by NOX) that, after dismutation to hydrogen peroxide, enters back into the cell. ROS like superoxide anions $O_2^{\bullet-}$ and hydroxyl radicals $OH^{\bullet}$ are extremely reactive and have therefore a limited diffusion range. Hydroxyl radicals are so reactive that they can arguably not be scavenged specifically because they will react with anything in their vicinity. Superoxide anions are a little less reactive, allowing diffusion over small distances. Some reports suggest that superoxide anions can even cross membranes through dedicated channels (103). Nevertheless, it is thought that it is the reaction product of the dismutation of $O_2^{\bullet-}$ (i.e. H_2O_2) that initiates the redox signaling response. The relatively stable nature of hydrogen peroxide makes it suitable to act as the second messenger in redox signaling.

Table 1: The hallmarks of cancer

Hallmarks of Cancer*	At a glace
Sustaining proliferative signaling	Oncogenic mutation or aberrant intrinsic/extrinsic growth factor signaling results in the cancer cell's capability to sustain proliferative signaling.
Evading growth suppressors	Circumventing growth suppressive mechanisms antagonizing sustained proliferative signaling is required in order for tumor cells to become hyper proliferative.
Enabling replicative immortality	Avoiding replicative senescence as a consequence of hyper proliferation and telomere erosion is required to proliferate indefinitely.
Resisting cell death	Restraining pro-apoptotic and elevating anti-apoptotic signals increases resistance to cell death and allows survival of cancer cells under adverse conditions.
Deregulating cellular energetics	Cancer cells reroute metabolic pathways to generate the building blocks and energetics required for hyperproliferation.
Genome instability and mutation**	Impaired DNA-damage repair allows mutations to build-up in the genome and fuels tumor evolution by conferring selective genotypic advantage for certain cancer cell subclones.
Inducing angiogenesis	Hypoxia induced by increased tumor mass activates the generation of tumor neo-vasculature.
Tumor promoting inflammation**	Tumor associated immune cells stimulate cancer progression by excretion of growth factors and interleukins.
Avoiding immune destruction	Immune destruction of cancer cells drives selection for low immunogenic subclones which subsequently prevail and become dominant within the tumor.
Activating invasion and metastasis	Acquiring mesenchymal properties including cell motility and anoikis resistance results in metastasic dissemination.

*Adapted from Hallmarks of Cancer: The Next Generation, Hanahan & Weinberg 2011 (1).
**Enabling characteristics which make Hallmark acquisition by cancer cells possible (1).

Redox signals are being transferred by post-translational modification of the thiolate side chain of cysteines in proteins, similar to other more well known post-translational modifications like phosphorylation, ubiquitination etc. In contrast to most other post-translational modifications, oxidative modifications have been suggested to occur largely independent of enzymatic catalysis (99). However, recent work that implicates peroxiredoxins in the oxidation of specific cysteines in for instance STAT-3 argues against random oxidation (104). Cysteine oxidation can directly influence enzyme activity or act as a linker to form complexes with other redox sensitive proteins through disulfide bridges and thus can alter the activity of signaling up- and downstream of oncogenes and tumor suppressors (105).
Oxidative post-translational modification of cysteines can be reversible (i.e. disulfide S-S, sulfenic acid S-OH) or irreversible (SO_2, SO_3), but mainly the reversible modifications occur at physiological ROS levels and that are relevant in redox signaling, because these allow the signal to be switched off again. Indeed, mass spectrometry based screens that are designed to identify reversibly oxidized proteins show that these modifications are widespread and abundant (106). An exception is the over oxidation of peroxiredoxins (PRDXs) to SO_2, which is slowly reversed by the action of the enzyme sulfiredoxin (99).

Redox signaling versus random damage: a balancing act
The damaging nature of ROS has been recognized long before it was noted that redox signaling is a physiologically relevant mode of cellular signaling. Generation of ROS is counter-balanced by multiple antioxidant systems within the cell of which PRDXs are the most abundant antioxidant proteins. Each 2-Cys class peroxiredoxin dimer has two catalytic centers that consist of a highly reactive so-called peroxidatic cysteine and a resolving cysteine. The peroxidatic cysteine reacts with H_2O_2 to form a sulfenic acid intermediate that rapidly reacts with the resolving cysteine on the other PRDX molecule in the dimer, resulting in an intermolecular disulfide and the release of H_2O. The disulfide in oxidized Peroxiredoxins as well as in other oxidized proteins and protein complexes are reduced by the action of Thioredoxin (TRX), which in turn becomes oxidized and forms an intramolecular disulfide. This leaves PRDX recycled to react with the next H_2O_2 molecule. Subsequently, oxidized TRX is reduced by Thioredoxin Reductase (TRXR) in a Nicotinamide adenine dinucleotide phosphate (NADPH) dependent reaction (100).
Besides PRDX and TRX the cell produces multiple antioxidant peptides and enzymes to counterbalance ROS-induced damage to proteins, lipids and DNA. Cells produce a large pool of the Glu-Cys-Gly tripeptide Glutathione (GSH), which can be oxidized to form a glutathione disulfide (GSSG) in a reaction that can be catalyzed by glutathione peroxidases (GPX). Reduction of GSSG to 2 GSH is mediated by glutathione reductase (GR) again in an NADPH dependent reaction (107, 108). Hence, NADPH is a key factor in redox signaling because it functions both as the supplier for reducing equivalents to recycle the PRDX/TRX and GSH systems and as the cofactor for the ROS producing NOX/DUOX enzymes. The main source of NADPH in the cell is the pentose phosphate pathway (PPP), which branches off from glycolysis at the level of Glucose-6-phosphate. The activity of the PPP can be stimulated by inhibition of glycolysis downstream of Glucose-6-phosphate, and because this pathway also generates nucleotides for DNA replication it is commonly activated in cancer. (100).
The high abundance of oxidation sensitive PRDX and GSH in the cell makes it counter intuitive that cysteines in other proteins can become oxidized with substantial stoichiometry before PRDXs are inactivated by overoxidation (109, 110). Therefore another model proposes that oxidized PRDX actually serves as an intermediate to oxidize specific target cysteines in a redox relay reaction (104, 111).

Spatiotemporal tuning of redox signaling

Redox signaling is affected by the constant changes in redox balances throughout the cell. Depending on the cells overall and localized metabolic states, changes in NAD^+/NADH and $NADP^+$/NADPH ratios allow shifting between reducing and oxidative environments. These states can vary within different organelles and compartments of the cell by localized production and reduction of ROS, which results in localized redox signals and thereby conveys specificity to these signals. As these localized redox states translate in protein oxidation or reduction, redox signaling becomes an essential part of signal transduction. Both cell intrinsic and extrinsic signals like nutrient availability, growth factor signaling, inflammation or wounding, can induce changes in the local redox state of the cell and result in for example starting the cell cycle or wound healing. The combined compartmentalized redox state including the NAD^+/NADH, $NADP^+$/NADPH and GSH/GSSG ratios as well as the local thiol proteome that together determine the outcome of redox signaling has been dubbed the Redox Code by Sies & Jones(112). Also in cancer cells the redox balance changes in response to the accumulation of mutations and tumor stage, either through local changes in production of ROS by NOX and mitochondrial respiration as well as changes in metabolism and the availability of reductive enzymes, peptides and NADPH involved in antioxidant response (113). It would be impossible to describe all redox-dependent signaling events taking place in tumor cells, but below we aim to illustrate that redox signaling plays a role in all Hallmarks of cancer and that redox signaling is hardwired in tumor biology.

Hallmarks of cancer from the redox perspective:
Sustaining proliferative signaling

Redox regulation of growth factor signaling

In healthy tissue homeostasis, stem cells give rise to cells with limited proliferative potential that will rapidly differentiate and form the tissue (Figure 1A). For tumors to arise, cells in the tissue need to overcome this limited proliferative potential. Under healthy conditions cells receive mitogenic cues from the surrounding tissue through growth factors (GF). Upon binding to extracellular growth factor receptor tyrosine kinases (RTK), cells respond by assembling the intracellular growth factor receptor complex SOS-GRB2-RAS and activation of the PI3K/PDPK1/PKB pathway (Figure 1B).
Redox signaling is essential for entering the cell cycle as making the cellular redox state more reducing impairs RTK signaling and downstream cell cycle initiation (114). Cell cycle entry and sustained proliferation is modulated by redox signaling and the latter influences the assembly of, and signaling by, these pathways at multiple levels (115). The first redox-signaling event upon GF-RTK binding is the activation of NADPH-oxidases (NOX) family members that release a burst of extracellular $O_2^{\bullet-}$. After dismutation and reentry into the cell as hydrogen peroxide, the latter reacts with the catalytic cysteines of protein-tyrosine phosphatases, including 1B (PTP1B) at Cys215, 1D (PTP1D/SHP2) at Cys459 and phosphatase and tensin homolog (PTEN) at Cys124, resulting in their inactivation (116-122). Oxidation and hence inactivation of PTPs is required to allow signaling by RTKs to proceed at full capacity. Similarly, PTEN inactivation through oxidation results in enhanced levels of PIP3 in the cell membrane to ensure a robust platform for PKB/AKT activation.

Oncogenic mutations result in elevated ROS production

Mutations in growth factor signaling complexes are among the most frequently found in cancer. Activating mutations in for instance PI3K, PKB and RAS result in a sustained mitogenic signal and primes cells to become hyper proliferative (Figure 1C). Sustained growth factor signaling results in transcription and activation of NOX downstream of

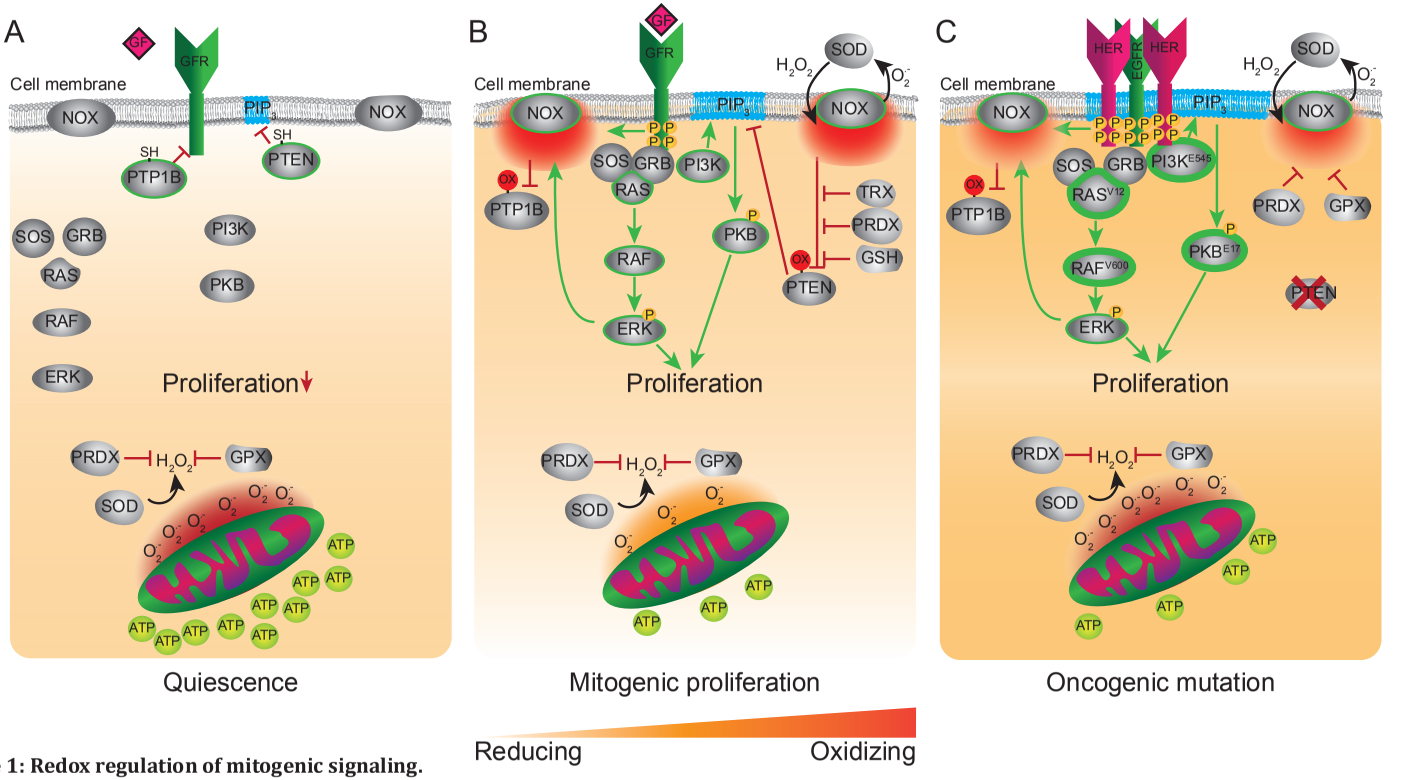


Figure 1: Redox regulation of mitogenic signaling.
(**A**) Quiescent cells have inactivated growth factor signaling, low NOX activity and elevated phosphatase activity to keep growth factor signaling low. Redox signals generated in quiescent cells are mainly derived from mitochondria as metabolism is predominantly aerobic. (**B**) Upon Growth factor receptor (GFR) binding to growth factors (GF), GFRs become active and start phosphorylating (P) their downstream substrates. This leads to the activation of NOX and production of extracellular superoxide, which after dismutation to hydrogen peroxide enters the cell and inactivates the PTP1B phosphatase,that otherwise dephosphorylates GFR substrates, ensuring full strength GFR signaling. Now SOS-GRB-RAS signaling complexes are formed, PI3K gets activated and PI3K generates PIP_3 to subsequently activate PKB/AKT mediated proliferation. PIP_3 generation is antagonized by PTEN in quiescent cells but oxidation of PTEN results in its inactivation. ERK activation downstream of RAS further stimulates NOX activation and in concert with PKB/AKT instructs the cell to start the cell cycle. (**C**) Oncogenic mutations like RAS^{V12}, $PI3K^{E545}$, RAF^{V600}, PKB^{E17} and HER amplification or loss of the PTEN tumor suppressor result in excessive mitogenic signaling. As a result of hyperactivated GF-signaling, redox signals are produced by both mitochondria and NOX and this elevated ROS production is suggested to cause the generally higher oxidative state of cancer cells.

active ERK (123). Consequently, oxidation of PTEN and PTP1B under these conditions might now be enhancing RTK signaling even further. Localized ROS production by NADPH-oxidases is clearly essential for the activation and fine-tuning of growth factor signaling (124). Excessive ROS production however, might also act as a negative feedback mechanism. It has been described that AKT/PKB can be directly oxidized on Cys297 And Cys310/311, resulting in inactivation (125-128). Also oxidation of PI3K has been detected and is proposed to be inactivating (129). Although redox signaling is essential for cell proliferation, excessive growth factor signaling can result in redox dependent negative feedback, by inhibiting mitogenic kinases and triggering stress signaling in response to damage by ROS. A recent paper suggests that the location of ROS production determines whether it acts mainly proliferative or anti-proliferative (130).

Evading growth suppression & gaining replicative immortality

Oncogenic mutations in RAS, RAF, PI3K or AKT/PKB results in sustained proliferative signaling but do not necessarily result in uncontrolled proliferation (131). In light of this review, oncogenic transformation results in a more oxidizing redox state that will trigger pathways culminating into withdrawal from the cell cycle and activation of ROS detoxification pathways. Several tumor suppressor genes are involved in repressing tumor cell proliferation or guiding the cells to a permanent cell cycle arrest known as senescence. Pathways responding to oncogene-induced stress directly regulated by redox signaling are downstream of retinoblastoma protein (RB), $p16^{INK4A}$, JNK/p38 stress kinases, p53, NRF2 and FOXO transcription factors. Of which RB, $p16^{INK4A}$ and p53 are the most prototypic tumor suppressors frequently lost or mutated in breast cancer (132, 133). Below we have briefly elaborated on the redox signaling properties of these growth-suppressive proteins and pathways.

RB is a cell cycle gatekeeper

RB functions as the main switch on the irreversible restriction point from which cells prepare to enter S-phase and hence is a gatekeeper that decides over quiescence and proliferation (133). Active RB binds and inhibits E2F transcription factors, which are responsible for cell cycle progression through S, G2 and Mitosis (Figure 2A) (134). RB is inactivated through phosphorylation by CDK4/CDK6, the activity of which depends on Cyclin D and the latter is transcribed in response to mitogenic signaling (Figure 2B) (37). Modulating RB activity in order to prevent unwanted cell cycle entry involves cell cycle dependent kinase inhibitors (CKI) including $p21^{cip1/waf1}$, $p27^{KIP1}$ and $p16^{INK4A}$, which can be induced in response to oncogenic and redox stress. Transcription of CKIs will result in CDK4/CDK6/Cyclin D and CDK2/Cyclin E complex inhibition and therefore repression of RB phosphorylation (135). Pausing the cell cycle allows the cell to recover from redox stress until conditions are considered safe again for genome duplication and cell division.

Although it is unclear if RB itself can be directly regulated through redox signaling, multiple pathways converging on RB are triggered and influenced by redox signals and will be discussed below. For example, RB can be actively dephosphorylated by the phosphatase PP2A, which is rapidly activated in response to elevated ROS levels. Whether cysteines in PP2A can be directly oxidized or whether PP2A is activated by upstream redox signals is unclear at this moment (136, 137).

$p16^{INK4A}$ puts the lock on the cell cycle

The small tumor suppressor $p16^{INK4A}$ is an essential player in the regulation of RB activity and is proposed as the main player in oncogene induced senescence. $p16^{INK4A}$

functions by binding to and inhibiting CDK4/CDK6. In response to ROS $p16^{INK4A}$ can get activated through stress kinase signaling (15, 138).
Stress kinases JNK and p38 are activated in response to redox signaling through the Apoptosis signal-regulating kinase 1 (ASK1). Under normal conditions ASK1 is kept inactive by TRX, but as the levels of ROS increase in the cell ASK1 becomes oxidized and therefore active. Active ASK1 phosphorylates p38 and JNK, which in response phosphorylate FOXO, p53 and ETS transcription factors (Figure 2C)(15, 138). Activation of FOXOs and inhibition of ETS results in upregulation of $p16^{INK4A}$ by two different mechanisms. First, ETS is a direct transcriptional repressor of $p16^{INK4A}$ and is released from this locus by p38/JNK phosphorylation (139). Second, the FOXO target gene CITED2 can modulate the activity of the $p16^{INK4A}$ transcriptional repressor BMI1 (140, 141). If the redox signal is sustained, the cell either enters $p16^{INK4A}$ mediated senescence or will undergo apoptosis (142, 143). Interestingly, cysteine oxidation of $p16^{INK4A}$ has been detected but the role hereof has not been elucidated yet (17).

P53 protects against hyper proliferation and oxidative damage
P53 acts to prevent permanent damage to the genome by responding to cellular stresses including DNA-damage, oncogene activation, oxidative stress, nutrient depletion, hypoxia, ribosome-stress and ER-stress (144). Under healthy conditions p53 levels are low due to rapid MDM2-mediated protein turnover, but when a cell encounters adverse conditions, p53 is stabilized and activated through a multitude of post-translational modification including phosphorylation, sumoylation, acetylation and methylation (Figure 2A&B)(145). Depending on the type of stress and how detrimental the stress is to the cell p53 can function in a temporary arrest and recovery from the insult, drive the cell into a permanent withdrawal from the cell cycle or activate apoptosis. P53 is redox sensitive and a number of cysteine residues, especially in the DNA binding domain, can be targeted by oxidation and glutathionylation (146, 147).

Functional effects of oxidative modifications on p53 are still poorly understood but have been found to intervene with DNA binding and differential p53 target gene regulation (148-154). P53 function in regulating the cell cycle depends largely on its target gene $p21^{cip1/waf1}$. Transcription of $p21^{cip1/waf1}$ will result in CDK4/CDK6/Cyclin D, CDK2/Cyclin E inhibition and therefore the induction of a reversible cell cycle arrest (135). In parallel with $p21^{cip1/waf1}$ function to halt the cell cycle, $p21^{cip1/waf1}$ also mediates the activation of Nuclear Factor 2 (NRF2), a major antioxidant response transcription factor (155). NRF2 is in a complex with Kelch like-ECH-associated protein 1 (KEAP1) and the Cullin 3 (CUL3) ubiquitin ligase and degraded under healthy redox conditions. As $p21^{cip1/waf1}$ levels rise in response to cellular stress, $p21^{cip1/waf1}$ competes for NRF2 binding with KEAP1 and this displacement results in stabilization and nuclear translocation of NRF2 (156). KEAP1 can also be oxidized and inactivated directly at several of its 27 cysteine residues, of which Cys151 is key to ensure NRF2 stabilization (157). When the cell has resolved the damage, p53 levels will drop and the cell will continue the cell cycle.
With respect to oxidative stress p53 is described to prevent damage caused by ROS through transcriptional regulation of several genes that contribute to maintaining redox homeostasis either directly or indirectly including: TP53-inducible glycolysis and apoptosis regulator (TIGAR), Aldehyde dehydrogenase 4 (ALDH4), Manganese Super Oxide Dismutase (MnSOD) and Glutathione peroxidase (GPX). TIGAR acts as a metabolic switch and diverts glucose from glycolysis into the Pentose Phosphate pathway (PPP) and hence elevates NAPDH production used for reducing PRDX/TRX and GSH pools. ALDH4 generates the GSH precursor glutamate as part of the proline degradation pathway. MnSOD dismutates $O_2^{\bullet-}$ into H_2O_2, which is subsequently reduced to H_2O by

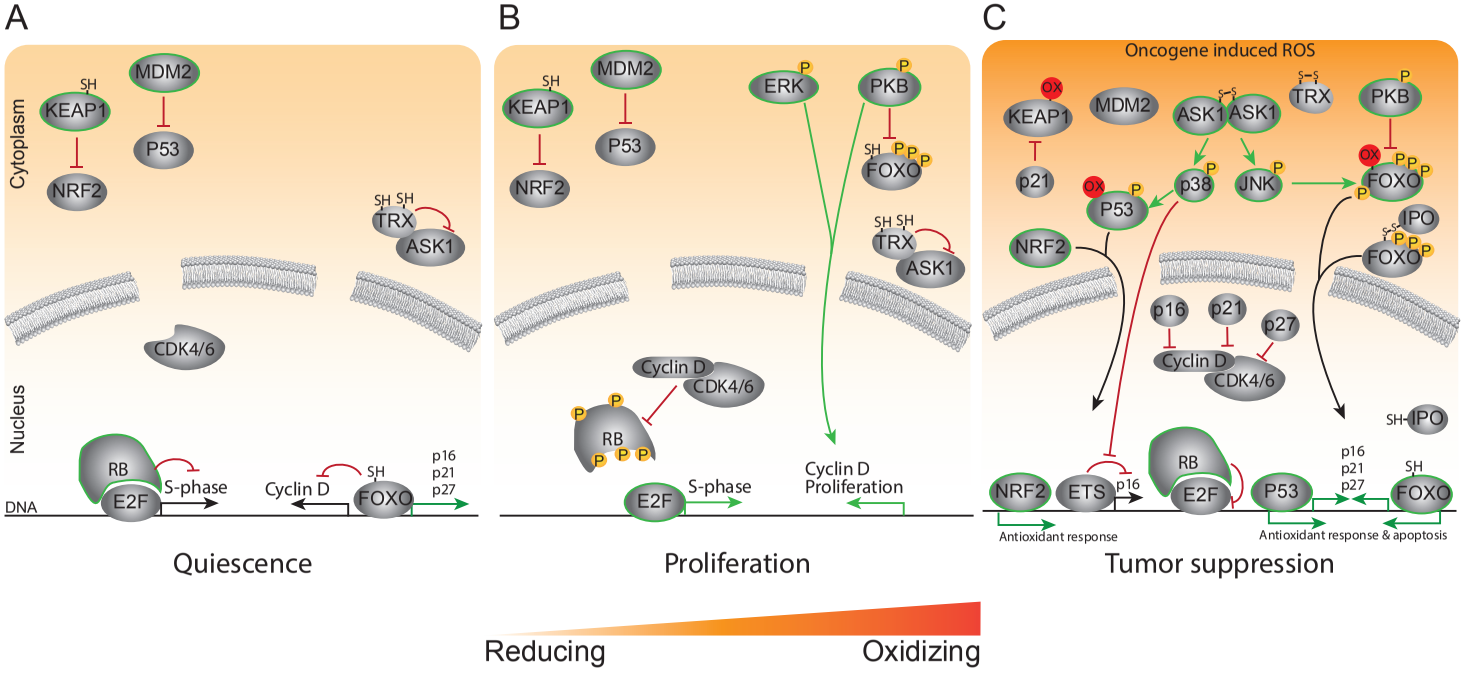


Figure 2: Redox signaling in tumor suppression
(**A**) Quiescent cells render E2F transcription factors inactive and hence prevent the cell from entering the cell cycle. RB inhibits E2F and transcription factors including FOXOs mediate the inhibition of cyclin dependent kinases by repression of Cyclin D and expression of cyclin dependent kinase inhibitors (CDKI) p16, p21 and p27. When ROS levels are low, transcription factors involved in stress response like p53 and NRF2 and stress kinase ASK1 are kept inactive by KEAP1, MDM2 and TRX respectively. (**B**) Upon mitogenic signaling FOXO is inactivated and expression of Cyclin D is stimulated. Cyclin D upregulation results in CDK4/CDK6 activation which subsequently inactivate RB and allow E2F mediated transcription of S-phase initiating genes. (**C**) Upon the acquisition of oncogenic mutations, multiple tumor suppressor pathways are triggered by the increase in ROS. ASK1 becomes activated by increased oxidation and phosphorylates (P) and activates JNK and p38. JNK and p38 subsequently in activate ETS, p53 and FOXO leading to nuclear translocation and transcription of cell cycle inhibitory and pro-apoptotic genes. Oxidation of FOXO results in binding to nuclear importers (IPO) followed by nuclear translocation. Additionally oxidation of KEAP1 relieves the degradation of NRF2.

the GPX and PRDX systems or used as the second messenger in redox signaling (144).

FOXOs maintain redox homeostasis

The family of Forkhead Box O (FOXO1, FOXO3, FOXO4, FOXO6) transcription factors plays a crucial role in regulation of the cell cycle and redox homeostasis (2, 3). FOXOs function downstream of canonical PI3K/PKB signaling, by which it is kept inactive and sequestered in the cytoplasm by 14-3-3 proteins (7, 8). Upon loss of GF-signaling and PKB activity, FOXOs are released and translocate to the nucleus where they can mediate transcription of a plethora of target genes (11, 12). FOXOs are proposed tumor suppressors as loss of FOXOs results in increased tumor formation and activation of FOXO results in cell cycle arrest and apoptosis (35, 61, 158). FOXOs mediate the transcription of $p16^{INK4A}$, $p19^{INK4D}$, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ CKIs, keeping cells in a quiescent state in the absence of GF-signaling (Figure 2A)(2). Next to their role in cell cycle regulation, FOXOs are part of the p38/JNK stress response and regulators of overall redox homeostasis (2). When cells encounter high ROS levels, FOXOs can become activated through phosphorylation by JNK. Activated JNK phosphorylates FOXOs and thereby overrules AKT/PKB mediated cytoplasmic sequestration (15). In response FOXOs will mediate the transcription of antioxidant genes like MnSOD, SOD2, Catalase, Sestrin, PRDX3, GPX1, GSTM1 and CKIs to reduce the oxidative stress and arrest the cell cycle (26, 159, 160).

FOXOs have been shown to be oxidized on several cysteines in response to low H_2O_2 levels (20). This results among others in the binding to nuclear import proteins. Oxidation of Cys150 in FOXO3 mediates the binding to Importin 7 and 8 (IPO7/8) and oxidation of Cys239 in FOXO4 was shown to result in disulfide dependent binding to Transportin 1 (TNPO1). Binding to IPO7/8 and TNPO1 subsequently results in FOXO translocation to the nucleus where it is now able to mediate transcription (17, 18). Next to redox mediated translocation, transcriptional activity of FOXOs can be directly influenced by cysteine oxidation. The acetyltransferases CBP and p300 can acetylate FOXO4 and histones in response to oxidative stress. The binding of CBP/p300 to FOXO4 is mediated by reversible covalent disulfide binding to Cys477 (31). How CBP/p300 exactly regulates FOXO transcriptional activity remains a point of debate since acetylation of histones is correlated to gene activation but acetylation of FOXOs can result in differential transcriptional activity and inactivation (32, 33, 161, 162).

Gaining replicative immortality

Additional to avoiding oncogene-induced senescence, tumor cells need to deal with telomere erosion caused by hyper proliferation. Each cell division telomere length is shortened due to the end-replication problem and in non-stem cells (that express telomerase) this will result in withdrawal from the cell cycle when telomeres become critically short (known as the Hayflick limit) (163, 164). Cancer cells deal with telomere erosion mainly through upregulation of the telomerase reverse transcriptase TERT. TERT extends telomere length by adding telomeric repeats at the end of chromosomes to maintain telomere length and thus to avoid triggering the DNA damage triggered by uncapped chromosomes (165). Although the exact mechanisms are still under investigation, redox signaling has been connected to telomere erosion by several studies and telomere erosion has been correlated to high levels of ROS (166, 167).

In parallel to its function in telomere extension, TERT functions to protect telomeres from oxidative damage both by increasing the cells antioxidant capacity through stimulating GSH generation and limiting mitochondrial ROS production (168). Also the redox-regulated endonuclease APE1, which is involved in base excision repair (as discussed in detail below), has been found to protect cells from telomere erosion in response to elevated ROS concentrations even in the presence of high TERT levels

(169). Taken together, although the underlying molecular mechanisms are far from clear, redox signaling and replicative immortality are interconnected at various levels.
To acquire the "sustained proliferation", "evasion of growth suppression" and "gaining replicative immortality" hallmarks, cancer cells must undergo oncogenic transformation. In breast cancer, mutations that give rise to these hallmarks are most commonly found in the PTEN/PI3K/PKB pathway (Figure 2C)(170). Hyperactivation of growth factor signaling overcomes the requirement for extracellular growth factor signals. Circumventing RB repressive signals generated by stress activated tumor suppressors $p16^{INK4A}$ and p53, is mainly the result of inactivating mutations in these genes. Interestingly, mutations in FOXO transcription factors are rarely reported in cancer but sustained PI3K/PKB signaling is likely sufficient to inhibit the FOXO tumor suppressor response. In addition, it is tempting to speculate that keeping FOXO transcription factors around comes with the benefit of retaining redox and metabolic adaptability, a property that might be contributing to stress resilience and tumor plasticity.

Resisting cell death

Oncogenic transformation culminates in high ROS production in the cancer cell. When a cell is exposed to levels of ROS that surpass the clearance rate and ROS become damaging, the stress response machinery is activated and this may lead to the onset of apoptosis. Apoptosis can be triggered through several cell intrinsic and extrinsic signals that converge on Caspase 3 and Caspase 7, which are the final and irreversible executioners. Extrinsic apoptotic stimuli comprise of Tumor necrosis factor (TNF), TNF related apoptosis inducing- (TRAIL) and FAS-ligands (FasL) that activate the TNF (TNFR) and Death (DR) receptor family of extracellular receptors respectively. These ligands can be secreted by different sources like immune cells, neighboring cells or autocrine signaling in response to stress or inflammatory signals. Although extrinsic and intrinsic apoptosis are mechanistically different, canonical extrinsic apoptosis signals will ultimately result in Caspase 8 mediated Caspase 3 cleavage and activation. Activation of DR and TNFR can also result in the activation of ASK1, JNK/p38 and the downstream stress response (42, 43).
From a redox perspective intrinsic apoptosis is most interesting however, as this is a redox regulated process that is executed largely by mitochondria. Only few proteins directly involved in apoptosis are described to be regulated by cysteine oxidation to date, of which BCL2 is a good example (171). The decision to undergo apoptosis is ultimately determined by the status of the BAK and BAX proteins. BAK/BAX are usually in an inactive conformation on the outer membrane of mitochondria. BAK/BAX activity is regulated through competitive binding of anti-apoptotic factors BCL2, BCL-XL or MCL1 to the pro-apoptotic BH3-only proteins BIM, BAD, BID, PUMA, NOXA, BOK or BMF. Anti-apoptotic factors render BAK/BAX inactive, but upon binding to BH3-only proteins BAK/BAX become activated, leading to oligomerization and subsequent opening of the permeability transition pore (PTP) and mitochondrial outer membrane permeabilization. Cyctochrome C (CytC) is released upon permeabilization of mitochondria. CytC is the key component of the apoptosome complex which induces the cleavage of pro-caspase 3 subsequently driving the cell into apoptosis. The onset of apoptosis can be triggered by cell autonomous and non-autonomous signals of which high ROS levels and associated oxidative stress is an example (44, 172).
BCL2 is an important anti-apoptotic protein and can be oxidized at Cys158 and Cys229, leading to interference with its anti-apoptotic functions and increased sensitivity of cells to apoptosis (171). Interestingly, hydrogen peroxide signaling has also been reported to stimulate apoptosis directly in a BAX/BAK and death receptor independent manner by oxidizing Cofillin (173). Cofillin functions in actin organization but upon oxidation of all

four cysteines (Cys39, Cys80, Cys139 & Cys147), cofillin translocates to mitochondria and induces PTP opening followed by the induction of apoptosis (173).
As discussed above, hydrogen peroxide can both directly and indirectly activate the JNK/p38, p53 and FOXO pathways, which next to ROS detoxification are also regulators of apoptotisis. Especially p53 and FOXOs are involved in the oxidative stress response, stimulating apoptosis by driving the expression of NOXA, PUMA and BIM and BMF. The apoptotic response is not solely a response to oxidative stress but also generates high levels of ROS, mostly as a result of changing mitochondrial membrane potential (MMP) (43, 102).
Not only the mitochondria are contributing to apoptosis by elevating ROS levels, but generation of ROS also appears to be part of the apoptosis inducing program of p53. Activated p53 results in the transcription of several genes involved in creating a more oxidative milieu in the cell including Quinone oxidoreductase (PIG3), proline oxidase (PIG6), PUMA and p66shc (174-177).
The expression of pro apoptotic BH3-only protein PUMA functions by directly competing for BAK/BAX binding and in this process enhances superoxide leakage from the mitochondria (152, 175). The adaptor protein p66shc is a negative regulator of RAS signaling and functions by competing with the SOS-GEF for binding tot the RAS-GRB2 complex. By doing so, p66shc expression stimulates NOX activity in a SOS-RAC1 dependent way and thereby enhances ROS production similar to the role of NOX in proliferation (178, 179). Additionally p66shc directly interacts with and oxidizes Cytochrome C, in a reaction that results in hydrogen peroxide production. Combined activation of NOX and enhanced hydrogen peroxide production in mitochondria by p66shc, PIG3 and PIG6 leads to an increased oxidative state in the cell and is proposed to function as an amplification signal stimulating the induction of apoptosis (176, 177).
Tumor cells overcome the induction of apoptosis by either losing tumor suppressor genes, most notably p53, or elevation of the apoptotic threshold. Elevated expression of BCL2, MCL1 and BCL-xl is often detected in cancer and allows tumor cells to evade apoptosis (180). Oncogenic mutant PI3K and KRAS generate upstream signals that inhibit pro apoptotic BH3-only family members. ERK activation downstream of a RAS or RAF mutation leads to the inhibitory phosphorylation of BIM and BMF (181, 182). In PI3K/PTEN mutant cells hyper-activated PKB directly inhibits BAD and BAX by phosphorylation (183, 184). Additionally, inhibition of FOXO by PKB restricts the induction of BIM and BMF transcription (185). Taken together, oncogenic mutations and concomitant loss of tumor suppressors in cancer cells are able to tilt the apoptotic/anti-apoptotic balance towards survival even in adverse situations like oxidative stress. Interestingly, high ROS levels lower the apoptotic threshold of cancer cells and even contribute to the irreversibility of apoptosis induction. This provides a rationale for targeting cancer cells by either elevating ROS production even further or repressing the antioxidant capacity, tipping the balance back towards apoptosis.

Deregulating cellular energetics

Metabolism is fundamental to redox signaling
Metabolism is central to redox homeostasis and signaling as the oxidative phosphorylation cycle in the mitochondria is one of the main sources of ROS as explained above. Furthermore, reductive entities like NADPH are generated as products of enzymatic steps in multiple metabolic routes (102, 186).
As mentioned, NADPH can be generated in the PPP but also as a product of isocitrate dehydrogenases (IDH1, IDH2, IDH3) during conversion of isocitrate to a-ketoglutarate in the cytoplasm or TCA cycle (102). The cell's reductive capacity also relies on sufficient GSH levels and as this is a glutamate-cysteine-glycine tripeptide, it relies on amino acid

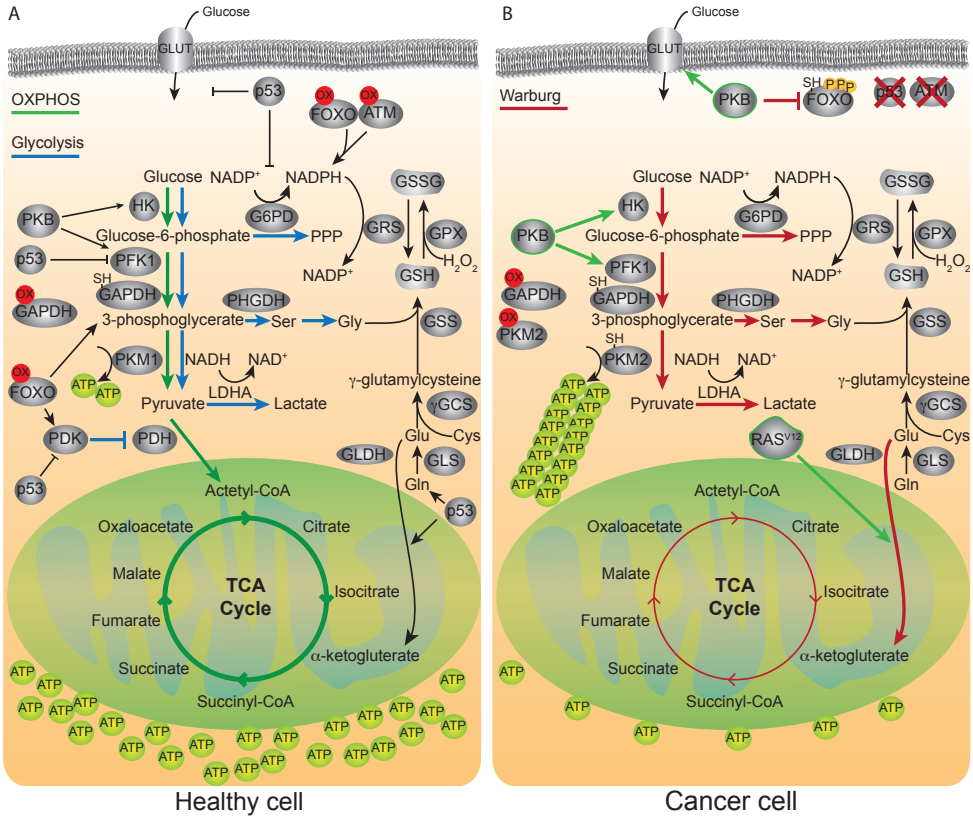


Figure 3: Redox homeostasis is mediated by cell metabolism

(**A**) In a healthy cell, metabolism is balanced between OXPHOS (green arrows) and glycolysis (blue arrows). Quiescent cells mostly utilize OXPHOS for maintaining sufficient ATP levels. Available glucose is processed to Acetyl-CoA through glycolysis and is used to fuel the TCA cycle. When cells become proliferative, mitogenic signaling stimulates glycolysis and pyruvate fermentation to lactate as well as synthesis of amino acids and nucleotides. OXPHOS generates high levels of superoxide and when the redox balance becomes more oxidizing, oxidation of FOXO, ATM, GAPDH and stress kinase activation of p53 will stimulate glycolysis and the pentose phosphate pathway (PPP) for NADPH generation by G6PD. Additionally glutamine (Gln) uptake and serine (Ser) metabolism is increased and the GSH synthesis pathway is stimulated. (**B**) Cancer cells shift to using glycolysis for ATP production although oxygen is present, known as the Warburg effect (Red arrows). Mitogenic signaling by PKB/AKT continuously stimulates glucose uptake and glycolysis by activating GLUT, Hexokinase (HK) and Phosphofructokinase 1 (PFK1). Hyperactivate RAS stimulates the synthesis of a-ketogluterate from Glutamate (Glu) to fuel the TCA cycle. Even though tumor suppressors involved in redox homeostasis are frequently lost in cancer, the build-up of ROS can be counter balanced by oxidation of GAPDH and PKM2, ensuing rerouting of glucose into the PPP to produce NADPH required for TrxR and GR.

metabolism for its synthesis (Figure 3A). Glutamate, Cysteine and Glycine are non-essential amino acids and can either be supplied by nutrient uptake from the food or synthesized de novo. Glutamate is generated from glutamine by glutaminases (GLS1 and GLS2) and can be subsequently used to generate a-ketogluterate for utilization in the TCA cycle or the generation of GSH (187). Serine metabolism is used for generation of Cysteine and Glycine (188). The first step in GSH synthesis is the generation of γ-glutamylcysteine by g-glutamylcysteine synthetase (γ-GCS) and is followed by Glycine addition through gluthation synthetase (GSS) to form GSH (Figure 3A)(189). When ROS production out-balances the reductive capacity of the cell, hydrogen peroxide itself signals to reduce mitochondrial respiration by inhibiting glycolysis and divert glucose into the PPP. Both Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the cancer

cell specific Pyruvate kinase isozyme M2 (PKM2) are essential glycolytic enzymes that can be inactivated by oxidation directly (190, 191). GAPDH converts glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate during glycolysis but upon oxidation of Cys152 GAPDH is rendered inactive and glycolysis is inhibited (192, 193). Likewise, hydrogen peroxide can directly inhibit the rate-limiting step of glycolysis by oxidizing Cys358 in PKM2 and thereby block glycolysis atop of GAPDH inhibition in cancer cells (190). Both these redox modifications lead to diversion of glucose into the PPP, driving NADPH regeneration and boosting the cellular reductive capacity. It has been shown that failure to inhibit PKM2 severely hampers tumor growth, which can be overcome by supplementation with antioxidants(190).

The Warburg effect

Highly proliferative tumor cells require great amounts of building blocks for macromolecules like amino acids, nucleotides and fatty acids. In order to maintain this hyper-proliferative state cancer cells switch their metabolism to supply the need for macromolecular biosynthesis (Figure 3B). Tumor cells enhance their glucose uptake and switch from low glycolysis rate combined with the TCA and OXPHOS, to ATP production through aerobic glycolysis followed by pyruvate fermentation to lactate. Next to having high aerobic glycolysis, cancer cells reroute glucose carbons into the PPP for enhanced production of amino acids, NAPDH, nucleotides and fatty acids. The TCA cycle is still active in tumor cells but now is often mainly dependent on glutamine instead of glucose. Rather than using the TCA cycle products for ATP generation, cancer cells utilize the TCA cycle for the biosynthesis of macromolecule precursors. This phenomenon was already observed in the early twentieth century by Otto Warburg and hence is called the Warburg effect (Figure 3B) (186, 194, 195).

Oncogenic mutations reroute metabolism

Common mutations found in cancer have a direct impact on metabolism. Oncogenic mutations in the KRAS and PI3K pathways result in stimulation of glucose uptake by enhancing membrane translocation of GLUT1 and GLUT4 glucose transporters and glycolysis (196, 197). Additionally, AKT/PKB activates Hexokinase (HK) and phosphofructokinase (PFK1/2), thereby enhancing glycolysis (198). Mutations in the KRAS pathway also stimulate glycolysis and additionally influence TCA cycle activity by feeding glutamine into the cycle as the major source of carbons (Figure 3B). Although the exact mechanism needs to be unraveled, RAS mutation increases the cells efficiency to use all available resources under low oxygen conditions by forcing glucose into glycolysis and the PPP and glutamine into the TCA cycle (196, 199, 200). Oncogenic transformation and consequential ROS production by growth factor signaling, is thus compensated for by enhancing the cell's reductive capacity by PPP dependent NADPH production and GSH production via glutamine. Interestingly, cancer cells have low TCA cycle and OXPHOS activity, but RAS enhances ROS generated by OXPHOS and this ROS production is essential for proliferation (199).

Tumor suppressors counter oncogene induced metabolic reprogramming

Several frequently mutated tumor suppressors are involved in regulating cell metabolism. Tumor suppressors respond to elevated ROS levels or nutrient starvation and stimulate ROS detoxification and catabolism, in contrast to oncogenic mutations that stimulate anabolic metabolism. When glucose becomes limited as a result of oncogene induced hyper-activation of glycolysis, ATP levels in the cell drop and p53 and FOXOs are being activated by phosphorylation downstream of the ATP/AMP ratio sensor AMPK. Activation of p53 and FOXO and their subsequent target genes instruct the cell to lower glycolysis and use fatty acids and glutamine as TCA cycle precursors

(2, 144). P53 reduces glucose uptake by suppression of *GLUT1*, *GLUT3* and *GLUT4* transcription (201, 202). Simultaneously, p53 activates the transcription of TIGAR and represses the expression of several glycolytic enzymes. TIGAR reduces the production of fructose-2,6,-bisphosphate which is the activator of phosphofructokinase 1 (PFK1) and the commitment step for glycolysis (203-205). Although glycolysis rates are low, p53 represses pyruvate dehydrogenase kinase 2 (PDK2) expression to ensure pyruvate dehydrogenase (PDH) is not inhibited by its phosphorylation and residual pyruvate can still be converted to Acetyl-CoA for use in the TCA cycle and ATP production (206). Next to lowering the glycolysis rate p53 actively stimulates the use of glutamine in the TCA cycle by inducing expression of glutaminase 2 (GLS2), which converts glutamine to glutamate (207, 208). Additionally, p53 stimulates the generation of TCA cycle and OXPHOS components Acetyl-CoA, NADH and FADH by increasing expression of carnitine palmitoyltransferase 1C (CPT1C), lipin 1 (LPIN1) and Pantothenate kinase 1 (PANK1), which mediate fatty acid transport to the mitochondria and b-oxidation (209, 210). Taken together, under nutrient deprived conditions p53 reduces glucose up-take and glycolysis rate and simultaneously ensures ATP production by the TCA cycle and OXPHOS through glutamine metabolism and b-oxidation (144).

In line with the dual role of p53 in redox homeostasis and apoptosis, p53 can also fulfill an opposite role in metabolism and is known to regulate genes that enhance glycolysis, reduce the PPP needed for NADPH and nucleotide synthesis, required in p53 DNA damage and redox response (211-214).

Next to regulating glycolysis, p53 also mediates amino acid metabolism and specifically serine metabolism, which is essential for delivering the amino acids for GSH synthesis. As serine levels drop, p53 stimulates de novo serine synthesis by inducing a p21 dependent transient cell cycle arrest (215, 216). In parallel, pyruvate kinase isoform 2 (PKM2) activity is reduced, as serine is an allosteric activator of PKM2. Combined low PKM2 activity and the p21 induced cell cycle arrest allow cancer cells to replenish the serine pool (217, 218).

Induction of FOXO activity by nutrient stress induces similar effects as seen with p53. FOXOs reduce glycolysis by regulating the expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6PD) in response to glucose deprivation (25, 219, 220). Additionally FOXOs stimulate autophagy by inducing glutamate to glutamine conversion through glutamine synthase (GS) expression, which leads to inhibition of mTOR (221). The TCA cycle can be maintained by FOXO mediated IDH1 transcription and subsequent generation of α-KG (222). In response to redox signaling, FOXOs modulate metabolism by stimulating GSH synthesis trough enhanced serine metabolism as a result of lowered PKM2 activity and upregulation of the PPP for enhanced production of NAPDH (19).

As becomes apparent, oncogenic mutations in the PI3K and KRAS pathways elevate redox signaling by increasing ROS generation through sustaining GF-signaling and antagonizing this by reprogramming metabolism and enhancing the reductive capacity of the cell. To maintain the oxidative glycolysis state, tumor cells need to either lose or inactivate tumor suppressors like p53 and FOXO to ensure these transcription factors do not deviate the cell from this state. Indeed p53 is often lost and FOXO efficiently inactivated downstream of PI3K. Interestingly, FOXOs are still functional and can be activated by AMPK, stress kinases and redox signaling. FOXOs might therefore be useful for cancer cells to adapt to adverse conditions by retaining metabolic and proliferative flexibility.

Taken together, deregulated cellular energetics is likely a necessary adaptation to the high metabolic demand and elevated ROS generation in tumor cells. Redox signaling plays a role in this adaptation at several levels and targeting cancer metabolism is currently being explored in clinical trials (223, 224).

Genome instability and mutation

Cancer is a mutation-driven disease and changes in genome integrity are widespread in cancer cells and range from single nucleotide mutations to chromosome rearrangements (genetic instability) and even the loss or gain of complete chromosomes after aberrant mitosis (Genomic instability, aneuploidy). During the course of life the genome is under constant attack as there are many circumstances that damage DNA, i.e. UV-light, radiation, toxins, metabolites and importantly ROS. The discovery that ROS are able to damage the DNA led to postulation of the free radical theory of ageing, a model where the constant need of oxygen and the consequential generation of ROS explains the degradation of an organism in time (225). This theory is found to be incorrect as the exact role and effects of endogenous ROS on genetic stability is heavily debated and ROS are equally essential for life (226, 227).

Under healthy conditions the cell encounters 10^4-10^5 insults to the DNA per day and the cell safeguards its genome by an elaborate cell homeostasis and DNA Damage Response (DDR) system (228). With respect to redox homeostasis, cells generate a reduced environment by constantly counter-balancing ROS production with the PRDX/TRX and GSH systems. In situations when protection by PRDX/TRX and GSH are insufficient and the cell suffers from damage caused by ROS, JNK/p38, p53, NRF2 and FOXO stress response is activated as delineated above. Although all these players in the oxidative stress response are connected to the DDR, repairing the actual DNA damage is highly dependent on a specific set of DDR proteins including the redox regulated kinase ATM. Most common DNA lesions are single nucleotide adducts which can impair base pairing, transcription, replication, and lead to single strand breaks (SSB). As the complementary strand is still intact these lesions are quickly resolved by nucleotide excision repair (NER) or base excision repair (BER), which remove the aberrant nucleotide or base prior to transcription coupled repair to replace lost nucleotides (Figure 4) (229, 230).

Dealing with oxidative DNA damage

The most common form of DNA damage as a result of high ROS levels is oxidation of dGTP to 8-oxodGTP (8-oxoG) and dATP to 2-hydroxy-dATP (2-OH-dATP), which can cause mismatched pairing and nucleotide substitutions in de the genome. The formation of oxidized dNTP can happen both on the DNA but also on free nucleotides prior to DNA replication. In order to prevent incorporation and accumulation of oxidized nucleotides in the DNA, cells employ multiple sanitizing mechanisms (231). Preventing incorporation of oxidized dNTP is mediated by the MTH1 proteins, which converts oxidized dNTP into monophosphates and thereby removes them from the pool of available dNTPs for replication (232). When oxidized dNTP is incorporated in the DNA or arise de novo on the DNA, oxidized nucleotides are predominantly removed by BER. Oxidized dNTPs are recognized by a set of DNA glycosylases like 8-oxoguanine DNA glycosylase (OGG1), VIII–like DNA glycosylase (NEIL1-3), endonuclease III glycosylase (NTH1) and N-methyl purine DNA glycosylase (AAG). DNA glycosylases excise the damaged base, leaving an apurinic, apyrimidinic or abasic site, which subsequently recruits and is processed by AP endonuclease APE1 followed by transcription coupled repair (233). In parallel to APE1 function in BER, APE1 functions as a major redox regulator of several transcription factors involved in stress response and DNA damage signaling. Although the exact mechanism of APE1 function as a redox regulator needs to be elucidated, the current model proposes Cys65 to be a reactive cysteine that can react with various oxidized proteins and Cys93 is expected to be the resolving cysteine (234). As a redox regulator APE1 can interact with p53, HIF1a, AP-1 and multiple tissue specific transcription factors to keep them in a reduced and active state (Figure 4) (235-237).

Next to sanitizing the oxidized nucleotide pool by MTH1 and the BER machinery, BRCA1 and 2 are essential with respect to preventing and repairing ROS induced dNTP oxidation. BRCA1 and 2 stimulate BER by enhancing transcription of NTH1, OGG1 and APE1 through direct binding of the OCT1 transcription factor (238). Not only are BRCA1 and 2 required for efficient repair of oxidized DNA lesions, they are also involved in regulating the expression of antioxidant genes by directly mediating NRF2 activity through competitive binding with KEAP1 (239-241).

ATM in redox signaling

More severe types of damage like inter-strand crosslinks and double strand DNA breaks (DSB) are more complex to repair. Repair of DSBs can be accomplished by error prone non-homologous-end-joining (NHEJ) or more secure but S/G2-phase restricted homologous recombination (Figure 4) (HR) (242, 243). Upon DSB detection cells will activate DDR Kinases ATM and ATR and attempt to perform HR mediated by MRN (MRE11-RAD51-NBS1), BRCA1 and BRCA2 complexes. Interestingly key members of the DDR, namely; p53, BRCA1, BRCA2, and ATM are frequently mutated in breast cancer. p53 and ATM are involved in regulation of the DNA damage checkpoint ensuring that cells do not progress through the cell cycle as long as DNA damage remains. As soon as the cell encounters DSBs, ATM is activated by the MRN complex and in turn activates CHK1, CHK2 and p53. Together these kinases stimulate DNA repair and keep CDK/

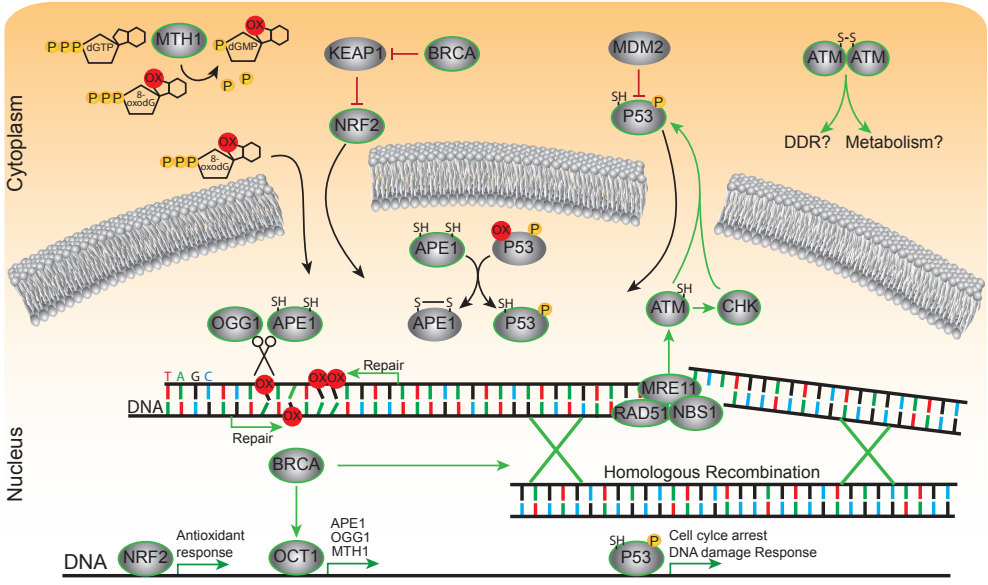


Figure 4: Preventing and repairing oxidative DNA damage

Oxidation of deoxyguanosine triphosphate (dGTP) is common and results in the formation of 8-Oxo-2'-deoxyguanosine (8-oxodG). Incorporation of 8-oxodG is prevented by MTH1. Upon incorporation of oxidized nucleotides, mismatches are induced and nucleotide excision repair is activated. OGG1 and APE1 remove the oxidized nucleotide from the DNA followed by transcription-coupled repair. APE1 additionally functions to keep p53 in a reduced and active state. BRCA1 and BRCA2 prevent oxidative DNA damage by activating NRF2 through inhibition of KEAP1 and mediate OCT1 transcriptional activity in order to enhance the expression of APE1, OGG1 and MTH1. When double strand breaks (DSB) arise in the DNA, BRCA is essential to mediate homologous recombination and ensure error free DNA repair. In parallel DSBs trigger ATM activation through recruitment of the MRE1-RAD51-NBS1 (MRN) complex, ATM subsequently activates p53 directly and by CHK phosphorylation. Redox signaling can also directly influence ATM activity independent of DNA damage, as oxidation of ATM leads to the formation of an active ATM dimer.

Cyclin complexes inactive, contributing to arresting the cell cycle and allow the cell to repair the damage (Figure 4).
Similar to BRCA1 and 2, ATM is involved in the regulation of redox homeostasis and prevention of oxidative DNA damage in parallel to its canonical role in DDR. In patients loss of ATM leads to Ataxia–telangiectasia (A-T) caused by neuronal degeneration and accompanied cancer predisposition, two phenotypes proposedly caused by defects in DDR. Additionally, cells lacking ATM exhibit increased sensitivity to oxidative stress and show impaired stress response (244). Indeed ATM can function in many other pathways next to DDR and influence redox homeostasis on multiple levels. Interestingly, ATM function outside of DDR is directed by redox signaling through oxidation of Cys2991. In DDR ATM functions as a monomeric kinase but upon Cys2991 oxidation ATM forms a homodimer, which is active independent of DNA damage (245). Although it is unclear which ATM targets are dependent on the monomeric or dimeric form, it is tempting to speculate that oxidation of ATM induces differential target regulation and makes ATM a redox sensor additional to its function in the DDR (244, 246).
Some ATM substrates that influence redox homeostasis are p53, G6PD, 4EBP1, NEMO, LKB1 and HIF1α. Activation of p53 leads to a cell cycle arrest and up regulation of anti oxidant gene expression as discussed above. Additionally, another major cue to regulate redox homeostasis is by mediating changes in cell metabolism by stimulating PPP and reducing oxidative phosphorylation. G6PD is an essential enzyme in the PPP to generate NADPH from $NADP^+$ as discussed above. ATM enhances the cells reductive capacity by elevating G6PD activity through HSP27 phosphorylation and its subsequent binding to G6PD, (247).
Taken together it is clear that redox signaling plays an important role in regulation of the activity of several DDR proteins. Altered redox homeostasis may contribute to deregulation of for instance BRCA1, BRCA2, p53 and ATM and will impair DDR and enhance the DNA mutation rate in healthy cells, predisposing them for accumulation of oncogenic mutations. Cells lacking these tumor suppressor genes are able to continue proliferation in the presence of DNA damage and thereby can more rapidly acquire mutations that provide other hallmarks of cancer.

Inducing angiogenesis

Sustained proliferation, evasion of growth suppression and escape from apoptosis will ultimately result in a neoplasm, which will increase in size over the course of time. Acquiring sufficient nutrients and oxygen becomes challenging with the gain of tumor mass and this will result in hypoxia and starvation of the core of the tumor. In response to hypoxia, cells stimulate the tumor environment to supply oxygen and nutrients. Hypoxia is the ultimate stimulus for the body to start the build-up of new vascular beds, a process called angiogenesis, and is dependent on the oxygen concentrations in the cells. The key regulator of the response to oxygen levels is hypoxia inducible factor 1 a (HIF1a) (248). Under normoxia, HIF1α is rapidly degraded by ubiquitinylation, which in turn is regulated through proline hydroxylation that is catalyzed by prolyl hydroxylases (PHDs). Hydroxy proline in HIF1α forms a docking site for the von Hippel Landau (VHL) adaptor protein that allows the Cullin E3 ligase to target HIF1α for proteosomal degradation. PHDs are the oxygen sensors in this system as it uses O_2 to catalyse proline hydroxylation on HIF1α. When oxygen levels drop, HIF1α is stabilized, binds to its cofactor HIF1b and localizes to the nucleus to mediate the transcription of angiogenesis inducing factors, most notably VEGF (248-250).

Redox regulation of HIF1α
Transcriptional activity of HIF1α can be modulated by redox signaling directly as

Cys800 is a reactive cysteine. Oxidation of Cys800 influences the binding to APE1, SIRT6 deacetylases and CBP/p300 acetyltransferases which subsequently can function as co-repressors or activators by influencing the redox and acetylation state of HIF1α as well as the chromatin state of its target genes (234, 251, 252). To what extend HIF1α mediated transcription of specific target genes is influenced by the redox state of Cys800 remains to be further characterized however.

HIF1a stimulates glycolysis through enhancing glucose import by inducing GLUT1 and GLUT3 expression (253). Glycolysis is enhanced by upregulating the expression of glycolysis enzymes: Hexokinase, PGI, PFK1, aldolase, TPI, GAPDH, PGK, PGM, enolase, PK and PFKFB1–4 (254-258). To ensure pyruvate generated by glycolysis is used for ATP production, HIF1α induces LDHA expression to convert pyruvate to lactate and expression of PDK1 to inhibit PDH mediated conversion of pyruvate to Acetyl-CoA (259-261). In parallel HIF1α induces expression of MCT4 to mediate the export of lactate out of the cell.

To stimulate angiogenesis in hypoxic tissues, HIF1α induces the expression of extracellular factors that stimulate branching and proliferation of endothelial cells (262). The induction of vascular endothelial growth factor (VEGF) is described best and is essential for angiogenesis. Additional growth factors induced by HIF1α are; placental growth factor (PGF), stromal-derived factor 1 (SDF1), angiopoietin1 and 2 (ANGPT1/2) and platelet-derived growth factor B (PDGFB) (263).

Next to stimulating angiogenesis HIF1α plays an important role in the remodeling and permeability of tissue by enhancing expression of genes that induce cell motility/EMT and extracellular matrix remodeling. In cancer cells HIF1α levels are constitutively high due to the general hypoxic nature of the tumor. However, even if tumor cells are growing under normoxic conditions HIF1α levels are high and described to be the result of either oncogenic mutations in RAS, PI3K and PTEN, leading to enhanced HIF1α translation, or the frequent loss of VHL tumor suppressors (264, 265).

Redox signaling in angiogenesis

Angiogenesis and redox signaling are highly interlinked as ROS play an essential role in stimulating blood vessel growth but are also responsible for some vascular pathologies (266). The balance in ROS levels is critical in respect to angiogenesis. Lowering ROS levels by treating cells with NAC, NOX inhibitors, ectopic SOD2 expression, mannitol and catalase inhibit angiogenesis and tumor vascularization (267-269). In line with these results, generation of mice lacking NOX1 activity show impaired angiogenesis during embryogenesis and adult neovascularization after ischemia (270). In contrast, stimulating angiogenesis through angiopoietin 1 is augmented by the loss of catalase in mice and suppressed in mice with impaired NOX signaling (271, 272).

Elevated ROS levels in tissues as well as cancer cells stimulate HIF1α stabilization independent of oxygen tension, which in turn transcribes VEGF and induces angiogenesis (273, 274). ROS signals both upstream and downstream of VEGF signaling are clearly required for stimulation of endothelial cell proliferation and angiogenesis (275, 276). Although it must be noted that the mechanism by which ROS mediate direct activation of HIF1α and VEGF induction remains to be elucidated. The oxidation of phospholipids (OxPL), 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (OxPAPC) and low-density lipoproteins (OxLDL) might explain how ROS increases HIF1α and VEGF levels. OxPL, OxPAPC and OxLDL are found to mediate VEGF expression, HIF1α stabilization and VEGF receptor activity and thereby mediate angiogenesis (277-279). Again it remains to be elucidated how these lipids transduce the information of the redox state of the cell towards HIF1a. Understanding the redox mediated induction of hypoxic signaling and associated angiogenesis is of vital importance for future cancer therapy as this hallmark is the gateway to malignant metastatic disease.

Evading immune destruction & tumor promoting inflammation

As the tumor grows and becomes vascularized it also becomes more accessible for immune cells. The immune system plays a crucial role in tumorigenesis as it can both antagonize and stimulate the growth of the tumor (280). As tumor size increases surrounding tissue becomes damaged and the tumor becomes hypoxic, both important cues for the recruitment of immune cells. The role for immune cells in the initiation and clearing of cancer is heavily debated. Cancer cells can be recognized and cleared by Th1 helper T cells, Natural killer cells (NK) and Cytotoxic T lymphocytes (CTLs) and mice lacking NK and CTLs are more susceptible to tumorigenesis (281-283). On the other hand, tumors recruit Macrophages (TAM), T regulatory cells (Treg) and Neutrophils (TAN), resulting in the suppression of tumor immune destruction and contribution to tumorigenesis (283). Especially the recruitment of TAMs and TANs is interesting from a redox perspective, since these cells utilize ROS to kill and degrade pathogens or cells in a process called respiratory burst (284). The excessive ROS production by tumor associated immune cells is proposed to be contributing to the overall oxidative state of the tumor and its surrounding tissue. Elevating ROS levels by immune cells is not only detrimental to the cancer cells but also contributes to tumorigenesis through several pathways. As discussed above, ROS plays a crucial role in HIF1α/VEGF signaling mediated angiogenesis and stimulates both tumor and immune cells to produce VEGF and other cytokines involved in this process. Indeed, lowering ROS production in tumor associated immune cells reduces angiogenesis (285, 286). The oxidative environment of the tumor as result of the immune response is also suggested to contribute to tumor evolution and plasticity as a result of a higher DNA damage rate and increased genomic instability. (280, 287).

Invasion and Metastasis

At the end stage of tumorigenesis, tumors become mortally dangerous due to cells metastasizing throughout the body. When lymphatic and blood vessels have formed and the extracellular matrix is damaged, cells can escape from the primary tumor and invade the surrounding tissue. The tumor-associated blood- and lymphoid vasculature serves as an entry point from where tumor cells are transported to distant parts in the body and colonize vital tissues. Two important changes are required for a cell to become metastatic. First, cells need to become motile in order to migrate out of the tumor by a process called epithelial to mesenchymal transition (EMT) (288). Activation of EMT in cancer cells can be induced by a plethora of signals ranging from oncogenic mutation in RAS, PI3K and b-catenin signaling pathways, to inflammation, hypoxia and cytokine signaling towards TGFβ especially. Whether redox signaling has a specific function in EMT other than its role in the previously discussed signaling pathways and processes remains a subject to be explored as this is certainly expected (289).

Second, cancer cells need to be able to survive dissemination from the tissue and its accompanying extracellular matrix, which is yet another mechanism in multicellular organisms that suppresses tumor growth. In healthy tissue, especially the epithelium of breast lumen and the intestine, cells undergo a specific type of apoptosis upon loss of cell-cell and/or ECM contact called anoikis. Redox signaling was shown to be important in the regulation of anoikis.

Redox regulation of anoikis

High levels of ROS are detected in disseminating cells and contribute to anoikis induction. Upon loss of ECM attachment, nutrient uptake, especially of glucose, is dramatically decreased. As a result of reduced glucose uptake, ATP production drops and subsequent

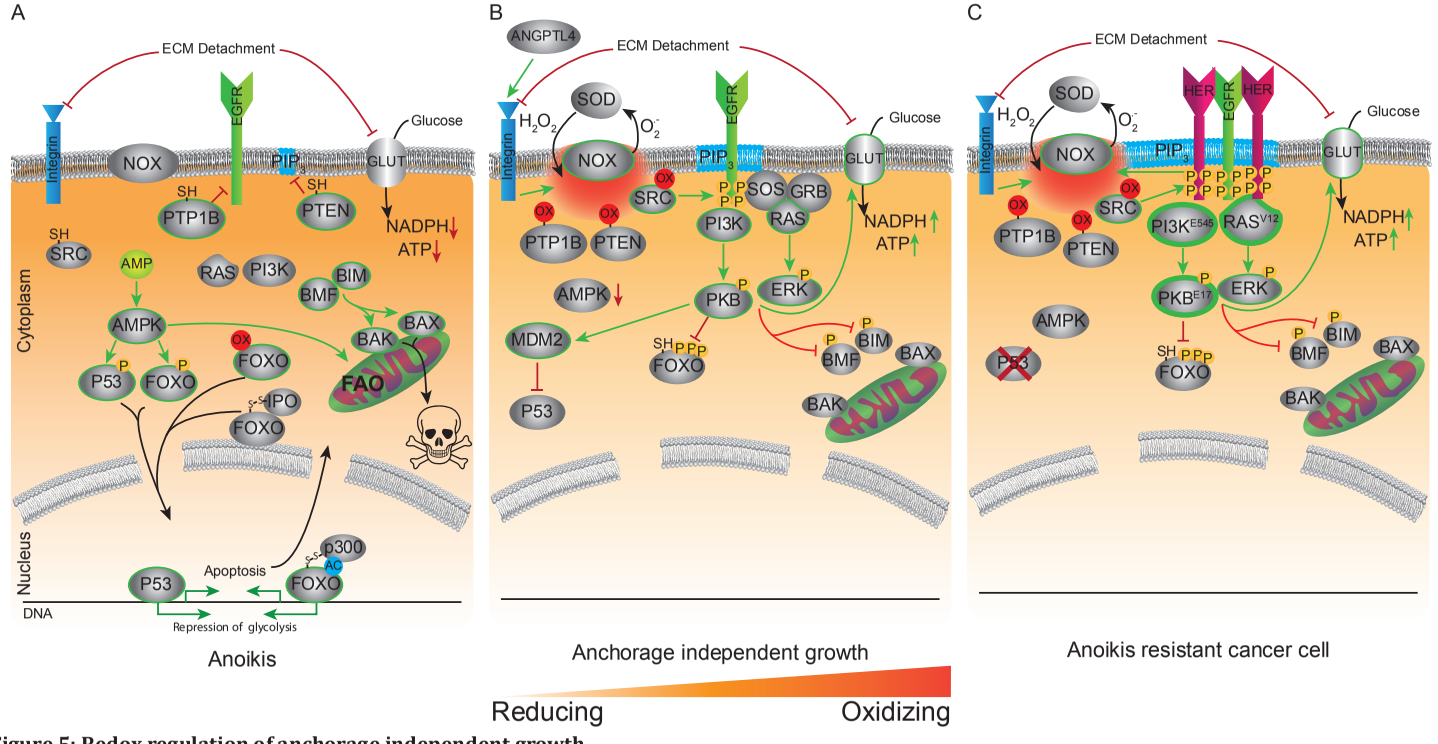


Figure 5: Redox regulation of anchorage independent growth.
(**A**) Detachment from the extra cellular matrix (ECM) leads to the inhibition of growth factor and integrin signaling and cells undergo anoikis. ECM detachment also inhibits glucose uptake, resulting in dropping ATP and NADPH levels. Loss of NADPH results §in ROS accumulation and combined with low ATP production leads to activation of AMPK and subsequent activation of p53, FOXOs and the fatty acid oxidation pathway (FAO). P53 and FOXOs translocate to the nucleus and stimulate the expression of genes involved in repressing glycolysis and activation of apoptosis. BIM and BMF are the main BH3-only proteins involved in inducing anoikis and are fully active in the absence of growth factors, which results in activation of BAK/BAX and subsequent anoikis. (**B**) Under conditions in which anchorage independent growth is required, for example during angiogenesis, cytokines like ANGPTL4 can activate integrin signaling. Activated integrin signaling triggers NOX, subsequent oxidation activates SRC kinase and inactivates PTP1B and PTEN phosphatases. SRC phosphorylates growth factor receptors (EGFR) that in turn activate the PI3K/AKT and SOS/GRB/RAS pathways. Active PKB/AKT and ERK stimulate glucose uptake by GLUT and prevent p53 and FOXO translocation. (**C**) Cancer cells overcome anoikis due to loss of tumor suppressors like p53 and hyperactivation of growth factor signaling through oncogenic mutations in PI3K, RAS, PKB/AKT or growth factor receptor amplification of HER and EGFR. Hyper activation of growth factor signaling represses FOXO activation and anoikis induction by BIM and BMF through direct phosphorylation.

NADPH production in the PPP required for GSSG and PRDX/TRX reduction is impaired, leading to detrimental ROS levels (Figure 5A). Anoikis induction and ATP production can be rescued by HER2 mediated re-establishment of glucose uptake. Additionally, treating mammary epithelial cells with antioxidants like Trolox or catalase inhibits ROS induced anoikis, allowing cells to employ AMPK induced fatty acid oxidation for ATP production (290, 291).

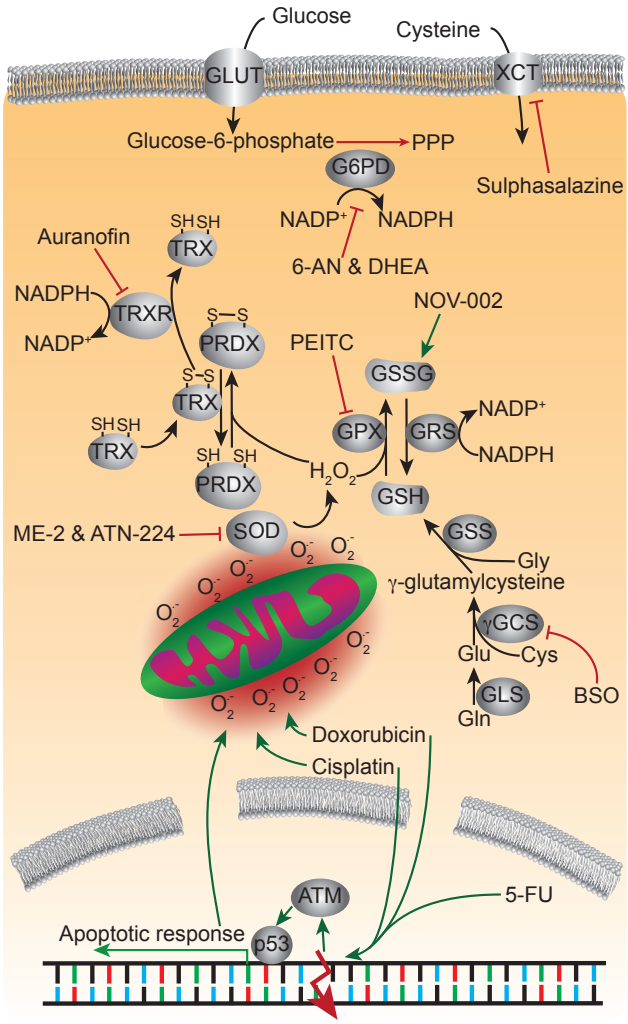
Avoiding anoikis also relies on HER2/EGFR signaling mediated inhibition of pro-apoptotic BH3-only proteins BIM and BMF (292). In healthy breast epithelium active growth factor signaling by HER2/EGFR-RAS and PI3K-PKB repress BIM and BMF activity both on the transcriptional and post-translational level. Upon aberrant localization of a tumor cell to the lumen of the mammary duct, correct ECM and cell-cell contacts are lost and growth factor signaling is decreased. Loss of growth factor signaling now results in the transcription and activation of BIM and BMF (Figure 5A)(45, 293, 294). Cells need to be able to migrate and move into tissues during development and angiogenesis and therefore need to temporarily circumvent anoikis induction. Anoikis can be repressed by the presence of HER2/EGFR activating growth factors but also other cytokines that regulate integrin activity. Upon integrin activation by cytokines like ANGLPT4, NOX1 is activated and produces ROS (Figure 5B)(295). Generation of ROS by NOX1 results in the oxidation of SRC on Cys245 and Cys487, which is essential for SRC activation (296). Active SRC subsequently phosphorylates and activates HER2/EGFR leading to downstream RAS and PI3K pathway activation and inhibition of anoikis (Figure 5B)(297, 298). In cancer cells, oncogenic mutations in and amplification of the EGF/HER2-RAS or IGFR-PI3K pathway overcome the induction of anoikis without requiring additional anoikis suppressive mechanisms and restrain BIM and BMF, allowing anchorage-independent growth and metastasis (Figure 5C)(299, 300).

Redox signaling is clearly involved in the balance between live and death upon dissemination of a cell from it's tissue. Cancer cells heavily rely on growth factor signaling to maintain the required glucose uptake for ATP production and maintaining the antioxidant capacity of the cell. Targeting redox signaling in combination with growth factor signaling might therefore hold promising therapeutically strategies especially in repressing the metastatic capacity of cancer cells.

Targeting Redox signaling in cancer

Taking all hallmarks together it becomes clear that common breast cancer mutations in RAS, PI3K, AKT/PKB and HER2 together with loss of function mutations in tumor suppressors like p53, RB and ATM results in a redox state that allows proliferation, avoids cell death via apoptosis or anoikis, stimulates angiogenesis and ultimately leads to metastatic disease. Modulating redox homeostasis as a strategy for cancer prevention and therapy is currently being explored and might hold promising opportunities. Targeting general redox homeostasis by anti- or pro-oxidant treatment can have both negative and positive effects on tumor biology and has already been tested in multiple ways (113, 224, 301, 302).

The concept of ROS induced DNA damage and consequential accumulation of mutations prompted the research into dietary antioxidant intake in cancer prevention. Several studies investigated the effects of Vitamin C, Vitamin E, selenium and b-carotene supplements on human healthspan (303-306). Although some studies report a delay in tumorigenesis and overall positive effect on healthspan of persons involved in these trials, opposite effects have also been reported and therefore the effects of dietary supplements, especially in humans, is still to be elucidated (307, 308). The effects of antioxidant supplements on cancer treatment have also yielded opposing results and can both contribute to therapy efficiency or drug resistance (224, 309). Given the



Targeting redox homeostasis

Figure 6: Targeting redox homeostasis in cancer.
Disruption of redox homeostasis can effectively kill cancer cells. Conventional chemotherapeutic agents like cisplatin, 5-FU and Doxorubicin all induce DNA damage but in parallel also elevate ROS production by disrupting mitochondrial respiration. Superoxide leaking from the mitochondria is dismutated by SOD to H_2O_2 and inhibition of SOD with ME-2 or ATN-224 therefore results in impaired superoxide detoxification, but at the same time inhibit H_2O_2-dependent redox signaling. Sulphasalazine can block the import of cysteine required for GSH synthesis and GSH synthesis can be impaired by BSO mediated inhibition of gGCS. Destabilization of the GSH/GSSG ratio by GSSG mimetic NOV-002 or inhibition of GPX mediated H_2O_2 detoxification by PEITC increases H_2O_2 levels. Interfering with the PRDX/TRX system can be accomplished by inhibition of TRXR with Auranofin impairing the recycling of the PRDX/TRX system. 6-AN and DHEA inhibit the production of NADPH by G6PD therefore less reductive entities are available, leading to elevated ROS levels.

vast amount of both positive and negative effects of redox signaling on all Hallmarks of Cancer described in this review it might not be surprising that there is no simple solution like an anti-oxidant supplement for the treatment of cancer.

Elevation of ROS in cancer cells can be detrimental for the tumor and has therefore been considered an interesting strategy for cancer therapy. Indeed several studies have sought to use strategies elevating the oxidative redox status of cells by trying to exacerbate ROS production and push cancer cells into apoptosis (310-314). Conventional cancer therapies including DNA damaging agents, irradiation and taxanes have been successful in killing cancer cells, not only by pushing cancer cells into a decrepit state due to severe genomic instability, but likely also by the accompanying ROS production (Figure 6). ROS generation as a side effect of irradiation is suggested to result from mitochondrial dysfunction and NOX activation (310, 311). DNA damaging drugs like Doxorubicin, cisplatin and 5-Fluorouracil (5-FU) all induce oxidative stress in cancer cells through different mechanisms. Doxorubicin competes with coenzyme Q_{10} in mitochondria leading to superoxide production in addition to its DNA damaging properties (312). Cisplatin leads to damaged and dysfunctional mitochondria and subsequent ROS production (313). Evoking the p53 dependent apoptotic response with its associated ROS production was shown to be the main ROS inducing mechanism for 5-FU (Figure 6) (314). As DNA damage and genetic instability strategies heavily depend on the presence and activity of tumor suppressors like p53, ATM, BRCA1, BRCA2, these drugs become less efficient upon loss of these genes, resulting in therapy resistance. Although elevated redox signaling can have opposing effects on survival of tumors, tumor cells are found to show increased sensitivity to cell death caused by elevated ROS levels. This provides a rationale for developing ROS inducing cancer therapies targeting specific antioxidants and metabolic pathways with small molecule inhibitors (113, 224, 302).

The antioxidant enzyme SOD1 is regularly found upregulated in cancer and is essential to keep cells from oxidative damage. Indeed targeting SOD1 with methoxyestradiol (2-ME) or ATN-224 inhibits superoxide conversion to peroxide, kills cancer cells selectively and are now in prostate and breast cancer clinical trails (Figure 6)(315-317). In line with the here described vital roles of redox signaling in cancer one could also speculate that the therapeutic effect of inhibition of SOD1 not only comes from elevated superoxide anion dependent damage, but also from lack of hydrogen peroxide mediated redox signaling.

GSH mediated ROS detoxification was found to be impeding therapy response and enhance resistance to DNA damaging drugs and tamoxifen treatment in breast cancer (318, 319). Targeting the synthesis of GSH or the GSH/GSSG balance in cancer and thereby abrogating ROS detoxification has yielded promising results. GCL inhibitor Buthionine Sulphoximine (BSO), inhibiting the ligation of cysteine to glutamate, directly abrogates GSH synthesis, tumor growth and resistance to cisplatin (320-323). Similar to blocking GSH synthesis, inhibition of GPX mediated GSSG reduction, blocking GSH activity or unbalancing GSH/GSSG ratios by GSSG mimetics with compounds like Phenethyl isothiocyanate (PEITC), piperlongumine and NOV-002 has been shown to be effective in multiple types of cancer (Figure 6)(324-326). Next to targeting GSH directly, targeting upstream metabolic pathways involved in GSH synthesis is proposed as a feasible strategy. Blocking cysteine import by XCT importers using Sulphasalazine, depletes GSH levels and efficiently inhibits tumor growth in xenograft models including triple negative breast cancer (327-329).

Modulation of glucose, glutamine and NADPH metabolism can also results in elevated ROS levels followed by cell death. Inhibition of TRXD by Auranofin reduces tumor cell growth and in combination with BSO results in increased drug sensitivity in otherwise drug resistant cancer cells (330, 331).

Blocking glutamine to glutamate conversion by inhibition of GLS activity using the

small molecule dibenzophenanthridine (968) effects GSH synthesis similar to blocking cysteine import by limiting the available amino acids required to generate GSH (332, 333).

Depleting the NADPH required for GSSG and TRX reduction is another promising strategy for cancer treatment. Deviation of glucose from PPP entry by inhibition of G6PD with 6-anicotinamide results in insufficient NADPH production and reductive capacity followed by elevated drug sensitivity and cell death (Figure 6) (334, 335)

Taken together redox homeostasis and signaling is clearly essential for tumorigenesis. Moreover, superoxide and hydrogen peroxide are not solely byproducts of metabolism, but function as essential signaling molecules that specifically modulate signal transduction in a plethora of pathways. Given the physiological roles of redox signaling in healthy tissue, it will be challenging to target cancer cells specifically. The cancer-associated elevated ROS production and ensuing adaptation by of the antioxidant capacity might be one way to achieve selectivity.

Another way would be by targeting acquired, redox sensitive cysteines that are unique to cancer cells due to mutation as was explored for instance for the oncogenic RasG12C mutation (336). A similar approach might be possible for other acquired cysteines in cancer (337). Tracking redox signaling experimentally is challenging and therefore it can be expected that the current knowledge on redox signal transduction is still very limited. Future research may unveil more roles for redox signaling in cancer and thereby new potential targets for treatment.

Chapter 3:

Restraining FOXO3-dependent transcriptional BMF activation underpins tumour growth and metastasis of E-cadherin-negative breast cancer

M. Hornsveld[1], M. Tenhagen[2*], R.A. van de Ven[2], A.M.M. Smits[1], M.H. van Triest[1], M. van Amersfoort[2], D.E.A. Kloet[1], T.B. Dansen[1], B.M.T. Burgering[1] and P.W.B. Derksen[2]*

[1]Department of Molecular Cancer Research, University Medical Center Utrecht, Utrecht
[2]Department of Pathology, University Medical Center Utrecht, Utrecht

[*] These authors contributed equally to this work

Cell death & Differentiation
1 April 2016; doi:10.1038/cdd.2016.33

3

Abstract
Loss of cellular adhesion leads to the progression of breast cancer through acquisition of anchorage independence, also known as resistance to anoikis. While inactivation of E-cadherin is essential for acquisition of anoikis resistance, it has remained unclear how metastatic breast cancer cells counterbalance the induction of apoptosis without E-cadherin-dependent cellular adhesion. We report here that E-cadherin inactivation in breast cancer cells induces PI3K/AKT-dependent FOXO3 inhibition, and identify FOXO3 as a novel and direct transcriptional activator of the pro-apoptotic protein BMF. As a result, E-cadherin negative breast fail to upregulate BMF upon transfer to anchorage-independence, leading to anoikis resistance. Conversely, expression of BMF in E-cadherin negative metastatic breast cancer cells is sufficient to inhibit tumour growth and dissemination in mice. In conclusion, we have identified repression of BMF as a major cue that underpins anoikis resistance and tumour dissemination in E-cadherin deficient metastatic breast cancer.

Introduction

Development and homeostasis of glandular structures like the mammary gland depend on spatiotemporal induction of apoptosis upon loss of cell-cell and cell-matrix attachment, a process known as anoikis (338, 339). Proper anoikis regulation ensures the formation of hollow lumen within a glandular epithelium structure by induction of apoptosis in selective non-polarized luminal epithelial cells that line the ductal structures (293, 340). In the mammary gland the pro-apoptotic proteins BIM and BMF induce apoptosis upon cell detachment and as such contribute to the formation of the mammary ductal lumen (294, 299).

Anoikis is regulated by an intricate regulation of the balance between pro- and anti-apoptotic proteins (172). Anti-apoptotic BCL-2 family proteins (BCL-2, BCL-xL and MCL1) compete with the pro-apoptotic molecules (BIM, PUMA, NOXA, BID, BAD or BMF) for binding to BAK and BAX to prevent mitochondrial membrane permeabilization and subsequent apoptosis. Expression of pro-apoptotic proteins can be induced by a variety of stresses including DNA damage, nutrient deprivation, heat and hypoxia (44). BMF appears to specifically function to induce anoikis in epithelial cells (299, 341). It is however still controversial whether activation of factors like BMF is induced through transcriptional activation, or by posttranslational events in the cytosol (342).

Correct execution of apoptosis in luminal mammary cells is deregulated during the early stages of breast cancer, like atypical hyperplasia and ductal carcinoma *in situ* (DCIS), resulting in filling of the mammary duct with anoikis resistant cells (343). Several studies have shown that activation of oncogenic growth factor receptor (GFR) signalling can induce aberrant filling of the luminal space (299, 340, 344). Similar effects have been observed upon inhibition of pro-apoptotic players such as BIM, BMF and p53 (294, 299, 345), indicating that either GFR activation and/or the inhibition of distal pro-apoptotic effectors underlie anchorage independence of breast cancer cells. Indeed, mutations in the PI3K and p53 pathway are among the most observed mutations in epithelial cancers including breast cancer (346). Moreover, hyperactivation of PI3K and its downstream effector AKT/PKB can lead to repression of apoptosis through phosphorylation-dependent inactivation of pro-apoptotic proteins like BAD (183, 184, 299).

Enhanced growth factor signalling can also be induced through down-regulation of the epithelial adherens junction (AJ) (347, 348). E-cadherin is the core component of the AJ and a master regulator of epithelial integrity, linking the cell membrane to the cytoskeleton (349). While loss of E-cadherin in the mammary gland is not tolerated (350-352), mammary-specific E-cadherin inactivation following loss of p53 in mice leads to the acquisition of anoikis resistance of tumour cells and subsequent dissemination, demonstrating E-cadherin loss as a prerequisite for metastatic disease progression (353).

Despite its repression by growth factor signals, the apoptotic machinery is functionally intact in cancer cells. Targeting the apoptotic machinery has become increasingly interesting in cancer therapy, as intervention strategies using novel BH-3-only protein mimetic compounds in combination with dual specificity inhibitors of PI3K and mTOR have shown promising results (80, 354). Here we have identified BMF as a direct transcriptional target of FOXO3 in breast cancer cells. Our data show that FOXO-dependent BMF expression is repressed in E-cadherin negative and metastatic breast cancer cells, and that expression of BMF is sufficient to inhibit tumour growth and dissemination in mice. We have thereby linked loss of E-cadherin to a cell intrinsic inhibition of BMF-dependent anoikis, a crucial step in malignant tumour progression.

Results

Anoikis resistant breast cancer cells restrain expression of BMF

To identify the proteins that control anchorage independence of metastatic breast cancer cells, we cultured E-cadherin expressing anchorage-dependent and anoikis resistant E-cadherin negative breast cancer cells from mouse and human origin in suspension. Under these conditions the non-metastatic mouse mammary carcinoma cell line Trp53$^{\Delta/\Delta}$-4 and human MCF7 underwent anoikis as previously demonstrated (350, 355). In contrast, mouse and human E-cadherin negative mILC1 and MDA-MB-231 cells were anoikis resistant (Fig. 1A-B).

To determine which of the key anti and pro-apoptotic molecules were induced during anoikis, we assayed the protein and mRNA expression levels of the anti-apoptotic family members *BCL2, BCL-XL* and *MCL1* and the pro-apoptotic *NOXA, PUMA, BIM, BID* and *BMF.* We observed that all cell lines up-regulated BCL2 protein levels and showed increased BID cleavage under anchorage-independent conditions regardless of E-cadherin status. For NOXA and PUMA, no changes were detected in protein levels (Fig. S1A). In line with previous findings (294, 299{Reginato, 2005 #817, 356) we detected a clear induction of BIM and BMF in the anoikis sensitive Trp53$^{\Delta/\Delta}$-4 and MCF7 (Fig. 1C&D, Fig. S1B&C). In contrast, the metastatic cell lines mILC1 and MDA-MB-231 did not show a comparable upregulation of either BIM or BMF (Fig. 1C&D, Fig. S1B&C). Subsequent qPCR experiments demonstrated that the only pro-apoptotic gene that was transcriptionally upregulated in both mouse and human E-cadherin expressing anchorage-independent cells was *BMF* (Fig. 1E&F Fig. S2A&B). We observed that although E-cadherin negative cells induced BMF under anchorage independent conditions, the expression levels were 3 to 10-fold lower relative to Trp53$^{\Delta/\Delta}$-4 and MCF7. These data demonstrate that anchorage independent E-cadherin negative cells restrain transcriptional up-regulation of *BMF*, a key inducer of anoikis.

Despite the fact that BMF is the major pro-apoptotic factor that showed uniform transcriptional upregulation in suspension settings, at the protein level both BMF and BIM are increased (Fig 1C&D and Fig S1B&C). To determine whether these two BH3-only factors are specifically required to induce anoikis, we performed loss of function studies and assessed the effect of BMF and/or BIM loss on anoikis resistance of mouse and human E-cadherin positive cells. Using two independent targeting sequences we induced knockdown and observed that loss of either BMF or BIM led to a significant increase in anoikis resistance of Trp53$^{\Delta/\Delta}$ and MCF7 cells (Fig. 2A-F). Concomitant knockdown of BMF and BIM also resulted in a significant increase in anoikis resistance when compared to controls (Fig. 2A-F). Overall our data does not indicate that dual inhibition of BMF and BIM has an additive effect when compared to the single BMF knock down experiments (Fig 2A&B). In short, our data demonstrate that BMF and BIM both contribute to the induction of anoikis in E-cadherin positive breast cancer cells. Further, our data indicate that BMF and BIM are non-redundant and have overlapping functions in the regulation of anoikis of E-cadherin expressing cells. Overall we show that BIM and BMF expression is increased upon transfer to anchorage independent conditions to induce anoikis. However, our data indicate that in contrast to BIM, the upregulation of BMF under these conditions is transcriptionally regulated.

Loss of E-cadherin results in anoikis resistance and restricts *BMF* expression

To determine if E-cadherin loss is causal to the repression of BMF expression in breast cancer, we generated E-cadherin knockout cell lines using the CRISPR/Cas9 system. Guide RNAs targeting the E-cadherin locus were expressed in Trp53$^{\Delta/\Delta}$ and MCF7 cells, which were subsequently FACS sorted based on E-cadherin expression, resulting in E-cadherin negative cell lines. In contrast to control cells, the E-cadherin knockout cell lines failed to establish E-cadherin-based cell-cell junctions and consequently

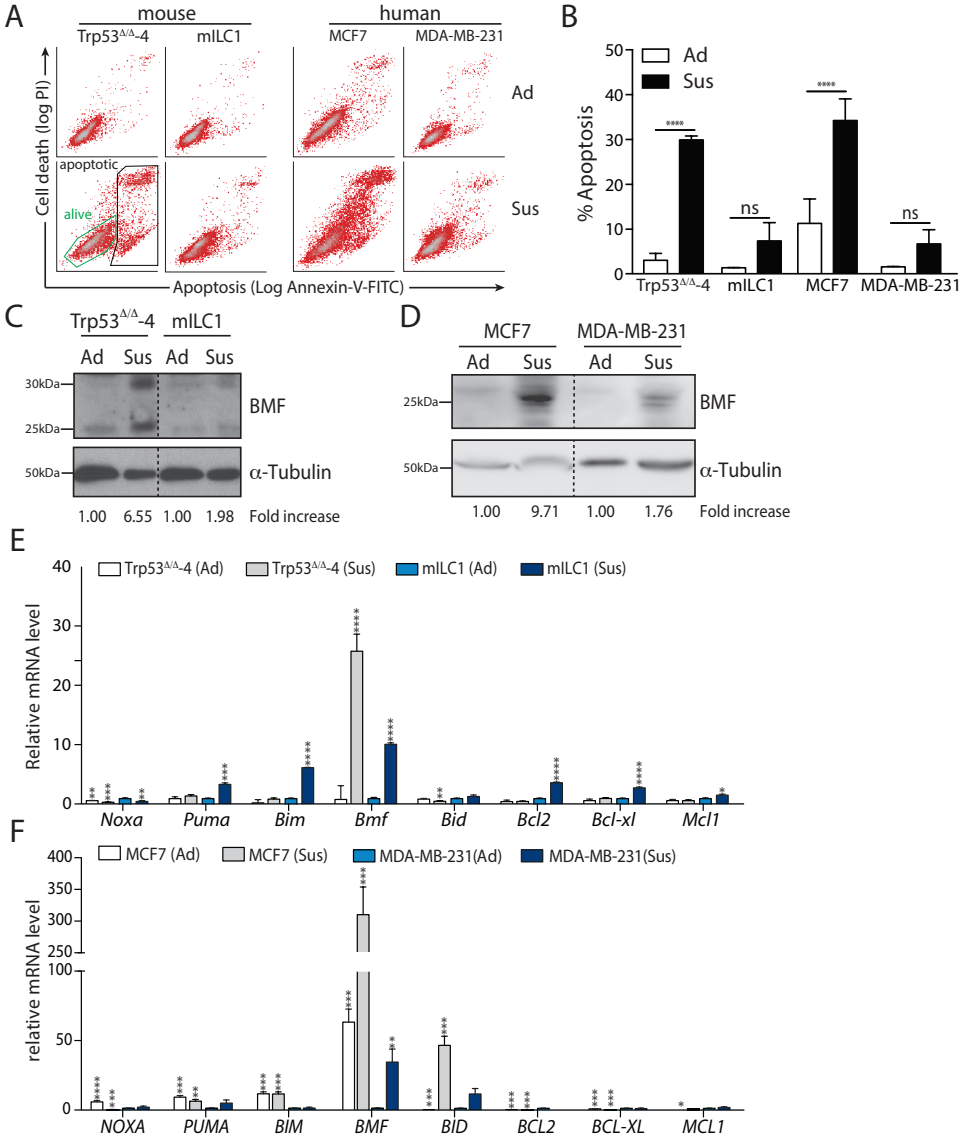


Figure1: Anchorage independent E-cadherin negative breast cancer cells restrain BMF expression. (A) Anoikis resistance in the E-cadherin negative breast cancer cell lines mILC1 and MDA-MB-231. Shown are flow cytometric analyses of apoptosis and cell death using Annexin-V-FITC and Propidiumiodide after 24 hours adherent (Ad) and suspension (Sus) conditions. Note the robust induction of anoikis in the E-cadherin expressing cells Trp53$^{\Delta/\Delta}$-4 and MCF7 (B) Quantification of anoikis induction measured by flow cytometry in (A). Data represent mean ± SD, n=3, t-test p<0.05 = *, p<0.005 = **, p<0.0005=***. (C and D) Western blot analysis of BMF and α-Tubulin protein levels in Trp53$^{\Delta/\Delta}$-4 and mILC1. Blots are cropped in correspondence to the black box (C) and MCF7 and MDA-MB-231 cells grown under adherent (Ad) and suspension (Sus) conditions. Blots are cropped corresponding to the black box (E and F) qPCR analysis of *Noxa, Puma, Bim, Bmf, Bid, Bcl2, Bcl-xl, Mcl1* mRNA expression in Trp53$^{\Delta/\Delta}$-4 and mILC1 and MCF7 and MDA-MB-231 cultured in adherent (Ad) and suspension (Sus) culture. Data are relative to mRNA levels in adherent mILC1 cells (E) or MDA-MB-231 cells (F). Data represent the mean ± SD, n=3, t-test p<0.05 = *, p<0.005 = **, p<0.0005=***.

grew dispersed as solitary cells (Fig. 3A). As expected, we observed that E-cadherin loss in mouse and human cells resulted in the acquisition of anoikis resistance (Fig. 3B). Importantly, we noted a significant reduction of *BMF* expression in E-cadherin knockout cell lines relative to control cells upon transfer to anchorage independence (Fig. 3C-D). These results confirm that loss of E-cadherin is causal to the acquisition of anoikis resistance and show that loss of E-cadherin leads to transcriptional repression of *BMF* expression upon loss of cell-matrix attachment.

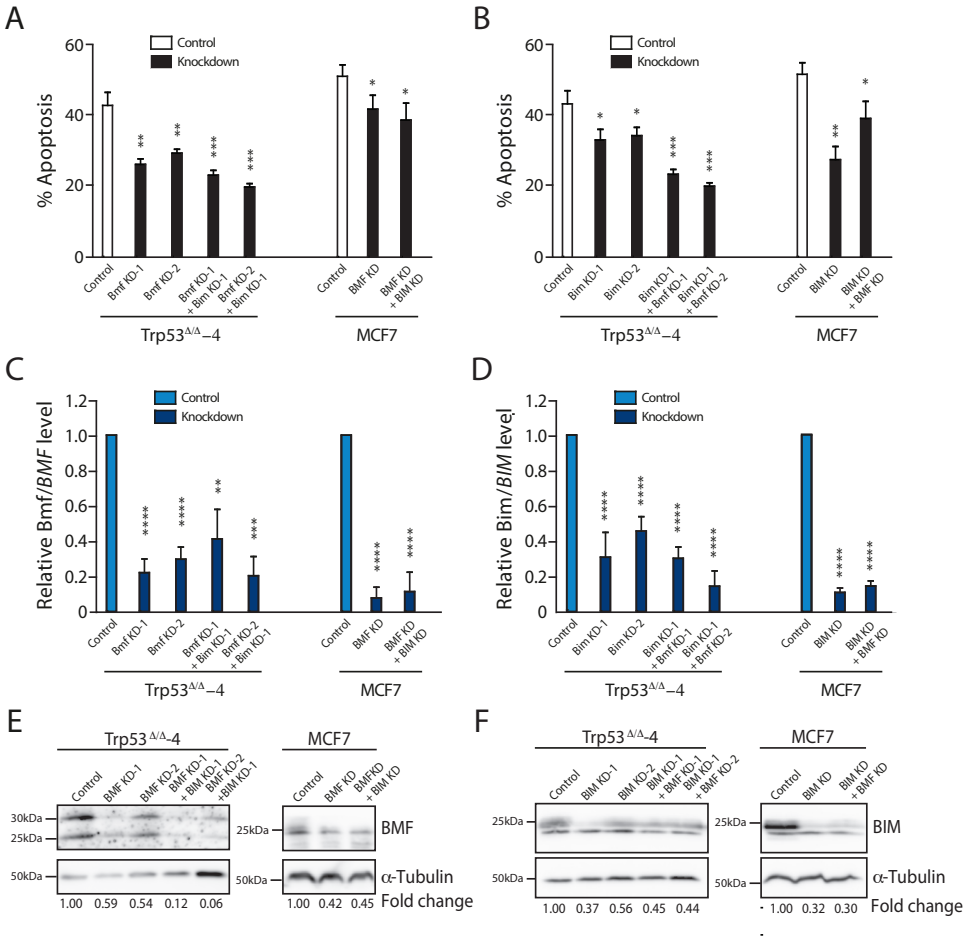


Figure 2: Loss of BMF or BIM increases anoikis resistance of E-cadherin expressing breast cancer cells

(A-B) Knockdown of BMF and BIM reduces apoptosis of Trp53$^{\Delta/\Delta}$ and MCF7 cells cultured in suspension. Flow cytometric quantifications of apoptosis using Annexin-V-FITC and propidium iodide after 24 hours in suspension culture. (C-D) qPCR analysis of BMF and BIM mRNA knockdown efficiency. Data represent the mean ± SD, n=3, t-test p<0.05 = *, p<0.005 = **, p<0.0005=***, p<0.0005=****. (E-F) Western blot analysis of BMF and BIM protein levels shows succesful BMF and BIM knockdown in Trp53$^{\Delta/\Delta}$ and MCF7 cells cultured in suspension. Blots are cropped corresponding to the black box.

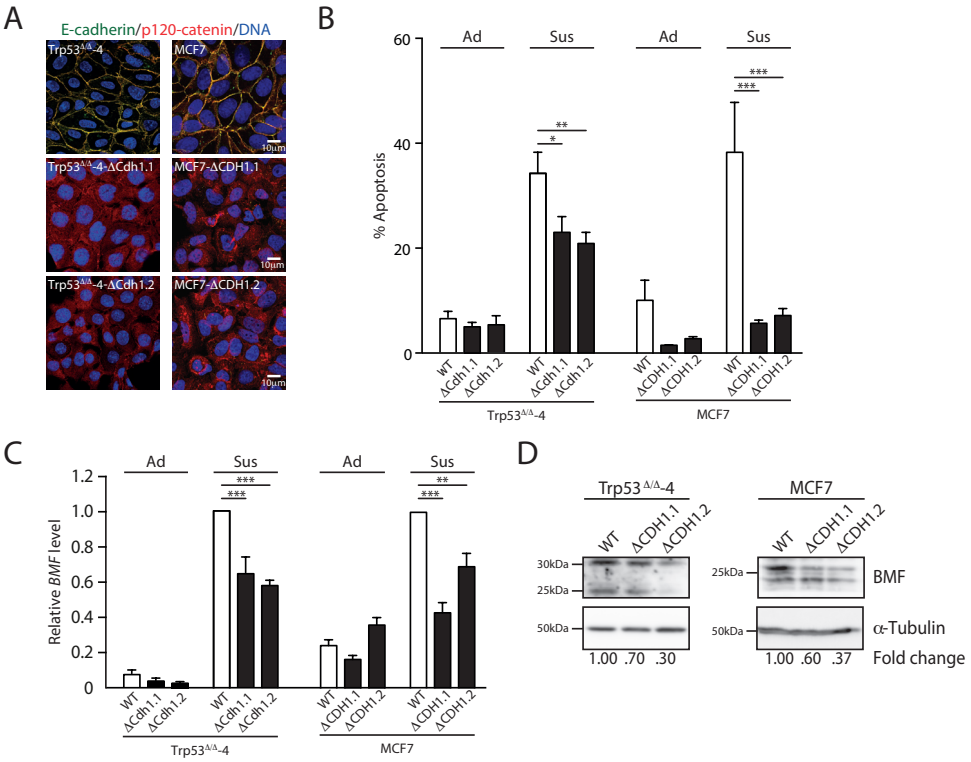


Figure 3: Loss of E-cadherin results in anoikis resistance and repression of BMF expression.
(A) Immunofluorescence analysis of Trp53$^{\Delta/\Delta}$ and MCF7 cells targeted with E-cadherin-specific gRNA. ΔCDH1.1 and ΔCDH1.2 cells grow dispersed; show loss of E-cadherin (Green), cytoplasmic translocation of p120-catenin (Red) and fail to establish cell-cell junctions. (B) Acquisition of anoikis resistance upon loss of E-cadherin in Trp53$^{\Delta/\Delta}$ and MCF7 cells. Shown are quantifications of flow cytometry analyses of apoptosis and cell death using Annexin-V-FITC and propidium iodide after 24 hours in adherent (Ad) and suspension (Sus) culture. Data represent mean ±SD, n=3, t-test p<0.05 = *, p<0.005 = **, p<0.0005=***. (C) BMF is repressed in E-cadherin knockout Trp53$^{\Delta/\Delta}$ and MCF7 cells. qPCR analysis shows *Bmf* or *BMF* expression after 24 hours in adherent (Ad) and suspension (Sus) culture conditions. Data are relative to mRNA levels in suspension cells. Data represent the mean ± SD, n=3, t-test p<0.005 = **, p<0.0005=***. (D) Western blot analysis shows increased BMF protein expression in Trp53$^{\Delta/\Delta}$ and MCF7 cells cultured in suspension. Blots are cropped corresponding to the black box

BMF expression is inhibited by growth factor signalling-dependent repression of FOXO3

Because BMF was transcriptionally regulated upon transfer to anchorage independence, we hypothesised that either direct transcription repression or inhibition of a transcription factor could underlie the differential BMF expression in anoikis resistant breast cancer cells. We therefore analysed the human genomic *BMF* promoter region and identified two FOXO consensus binding sites (*TTGTTTA*). FOXOs are directly regulated and suppressed by canonical PI3K/AKT signalling and known transcription factors for the pro-apoptotic genes *BIM, NOXA and PUMA* (2).

To assess if *BMF* is a direct FOXO target we performed a Chromatin Immunoprecipitation (ChIP) with the most ubiquitously expressed FOXO transcription factor, FOXO3. To this end we stably introduced a doxycycline (dox)-inducible FOXO3 construct in MCF7 cells and performed ChIP in the presence of the allosteric inhibitor VIII (AKTi) to prevent

upstream inhibition of FOXO3 by AKT. In line with the predicted binding sites we noticed binding of FOXO3 to both consensus sites in the *BMF* promoter (Fig. 4A). We confirmed these results by analysing induction of *BMF* upon expression of a dominant active form of FOXO3 (FOXO3.A3) (7) and showing that administration of dox indeed resulted in a robust induction of *BMF* mRNA and protein levels upon FOXO3 activation (Fig. 4C-D, Fig. S3A-C Fig. S4A&B).

To determine if FOXO3 activation was sufficient to induce anoikis in E-cadherin negative cells, we cultured mILC1-iFOXO3.A3 and MDA-MB-231-iFOXO3.A3 in suspension and assayed anchorage independent survival. Addition of dox triggered anoikis in mILC1(Fig. 4B), confirming that FOXO activation is indeed sufficient to prevent anchorage independence in E-cadherin negative breast cancer cells. Although MDA-MB-231-iFOXO3.A3 cells showed a modest increase in *BMF* expression upon expression of FOXO3 (Fig. S4B), this did not lead to anoikis, which indicates that the induced BMF levels were insufficient to cause apoptosis in these anchorage independent breast cancer cells.

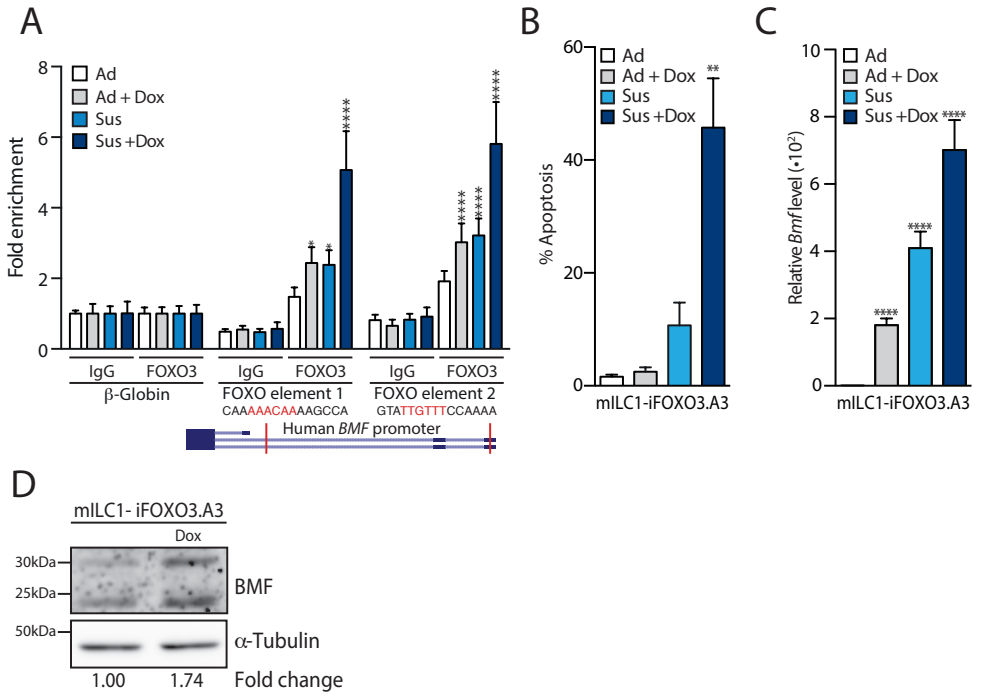


Figure 4: BMF is a direct FOXO3 target gene in breast cancer cells.
(A) BMF is a direct FOXO3 target. FOXO3 binds the *BMF* promoter after AKT inhibition and during anchorage independent growth. Average enrichment in *BMF* promoter chromatin recovery after FOXO3-ChIP in MCF7 growing under adherent (Ad), suspension (Sus) and upon FOXO3 expression in adherent and suspension conditions (Ad+Dox, Sus+Dox respectively). Data represent mean ±SD, t-test n=3, $p<0.05$ =*, $p<0.00005$ =****. (B) Activation of FOXO induces anoikis. Quantification of anoikis in mILC1-iFOXO3.A3 measured by flow cytometry, data represent mean ±SD, n=3, t-test $p<0.005$ =**. (C-D) FOXO3 activation induces *BMF* expression in mILC1. Shown are relative mRNA and protein levels in suspension upon FOXO3.A3 expression in mILC1 cells. Blots are cropped corresponding to the black box

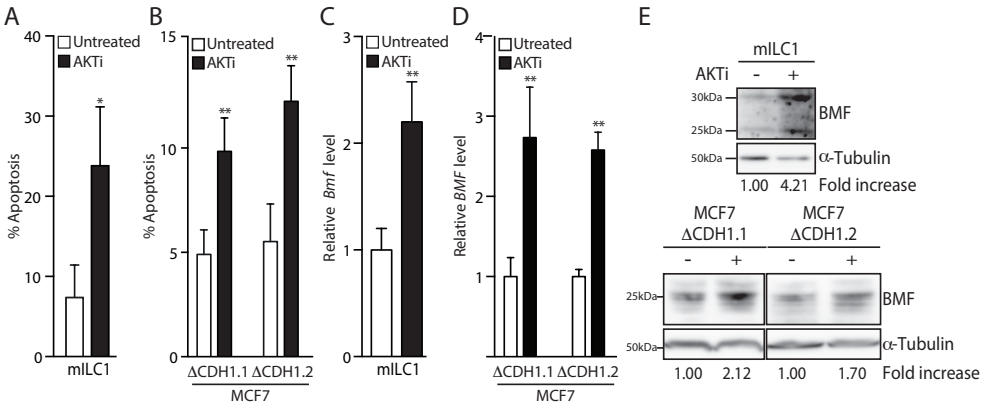


Figure 5: Growth factor receptor signals restrain BMF expression through PI3K/AKT-dependent signals

(A-B) Anoikis resistance in E-cadherin negative breast cancer cells is AKT-dependent. Shown is the quantification of anoikis induction in mILC1, MCF7-ΔCDH1.1 and MCF7-ΔCDH1.2 grown in suspension measured by flow cytometry. Data represent the mean ±SD, n=3, t-test $p<0.05$ = *, $p<0.005$ = **. (C-E) AKT inhibition results in BMF expression. Shown are qPCR analyses of *Bmf/BMF* mRNA and western blot analyses of Bmf/BMF protein levels in mILC1, MCF7-ΔCDH1.1 and MCF7-ΔCDH1.2 cultured in suspension and after treatment with AKTi. Data represent the mean ±SD, n=3, t-test $p<0.05$ = *, $p<0.005$ = **. Blots are cropped corresponding to the black box

Activation of the PI3K/AKT pathway by GFR signalling and ectopic expression of either oncogenic $PI3K^{E545K}$ or myristoylated AKT1 results in repression of anoikis in mammary epithelial cells (299). Interestingly, we previously have shown that inactivation of E-cadherin function in cancer cells results in hypersensitisation of GFR signalling without activating mutations (348). To confirm that active PI3K/AKT signalling, which is an established upstream inhibitor of FOXO, controls anoikis resistance, we cultured mILC1 cells and the MCF7-ΔCDH cells in suspension and inhibited AKT activation using AKTi and observed that AKTi resulted in a 2-fold increase in anoikis (Fig. 5A-B). More important, we could show that AKT inhibition resulted in a 2 to 3-fold upregulation of *BMF* mRNA and protein expression in anchorage-independent cells (Fig. 5C-E).
In conclusion, our data show that E-cadherin negative metastatic breast cancer cells restrain anoikis through PI3K/AKT signalling, a cue that subsequently inhibits FOXO3-dependent transcriptional activation of its pro-apoptotic target *BMF*.

Upregulation of BMF expression restrains anchorage independent tumour growth and metastasis of E-cadherin negative mammary cancer in mice

Because BMF was the major pro-apoptotic factor upregulated in anoikis sensitive breast cancer cell lines, we determined if induction of BMF expression was sufficient to induce anoikis in E-cadherin negative breast cancer cells. To this end we stably introduced a doxycycline-inducible BMF cDNA expression system (iBMF) in mILC1 and MDA-MB-231 cells (Fig. 6A) and assayed anoikis resistance. Expression of BMF was indeed sufficient to cause a marked increase in apoptosis in both cell types (Fig. 6B&C). Moreover, treating mILC1 and MDA-MB-231 cells with increasing concentrations of the BH3-mimetic drug ABT-199 induced a dose dependent execution of apoptosis (Fig. 6D), suggesting that anchorage-independent E-cadherin deficient breast cancer cells have a lower threshold for the BH3-only protein dependent execution of anoikis. Together, these results show that increased the levels of BMF, or inhibition of BCL2 using the BMF-mimetic ABT-199 is sufficient to induce apoptosis in E-cadherin negative cells, especially in an anchorage-independent setting.

Since anoikis resistance is an excellent prognosticator of E-cadherin negative breast cancer growth and metastasis (350, 353), we investigated the effect of BMF expression on tumour growth *in vivo*. To this end we orthotopically transplanted 10,000 mILC1 cells carrying either an empty vector (EV) or the iBMF expression vector in recipient mice and monitored tumour growth. Once tumours reached an average volume of 100mm^3, we induced BMF expression by feeding mice doxycycline-containing chow. Treatment of mILC1 cells carrying the empty expression vector with doxycycline either at a 100mm^3 or when metastasis was detected using bioluminescence imaging (BLI) did not result in significant changes in tumour growth (Fig. 7A). In contrast, BMF expression induced a significant reduction in tumour growth (Fig. 7B). Moreover, induction of BMF expression when metastases were detected led to a robust 19-fold decrease in primary tumour volume (Fig. 7C). Importantly, we observed a reduction in metastasis formation based upon BMF expression on thoracic BLI in dox-fed mice when compared to control mice (Fig. 7D-H).

In closing, our data establish that restriction of FOXO3-dependent BMF expression underpins anchorage-independent tumour growth and metastasis of E-cadherin negative breast cancer cells.

Discussion

Acquisition of anchorage independence through evasion of apoptosis is a hallmark of cancer (1). Metastatic cells have shifted the regulation of pro and anti-apoptotic mechanisms toward survival and have thereby gained the possibility to bypass the induction of anoikis during dissemination.

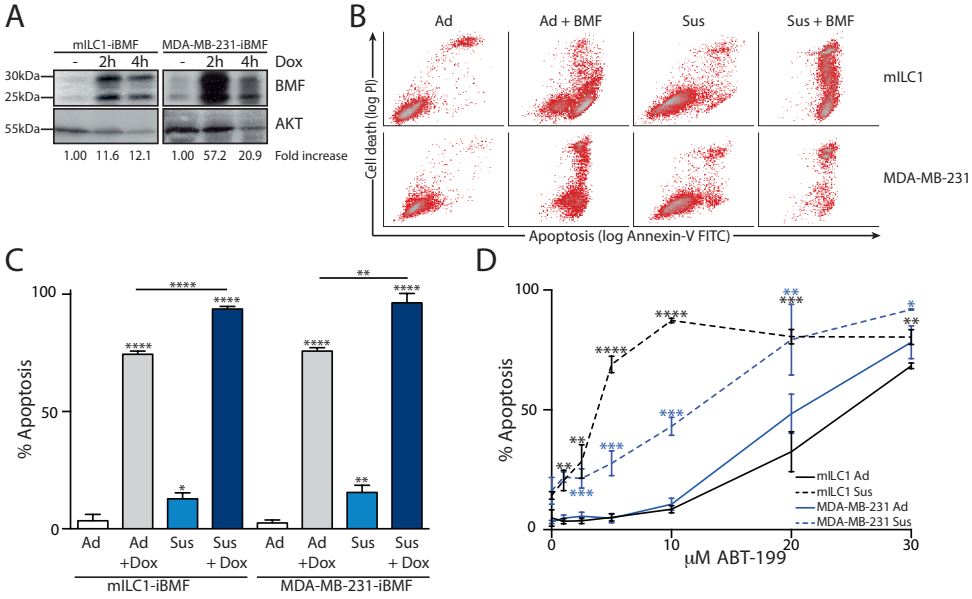
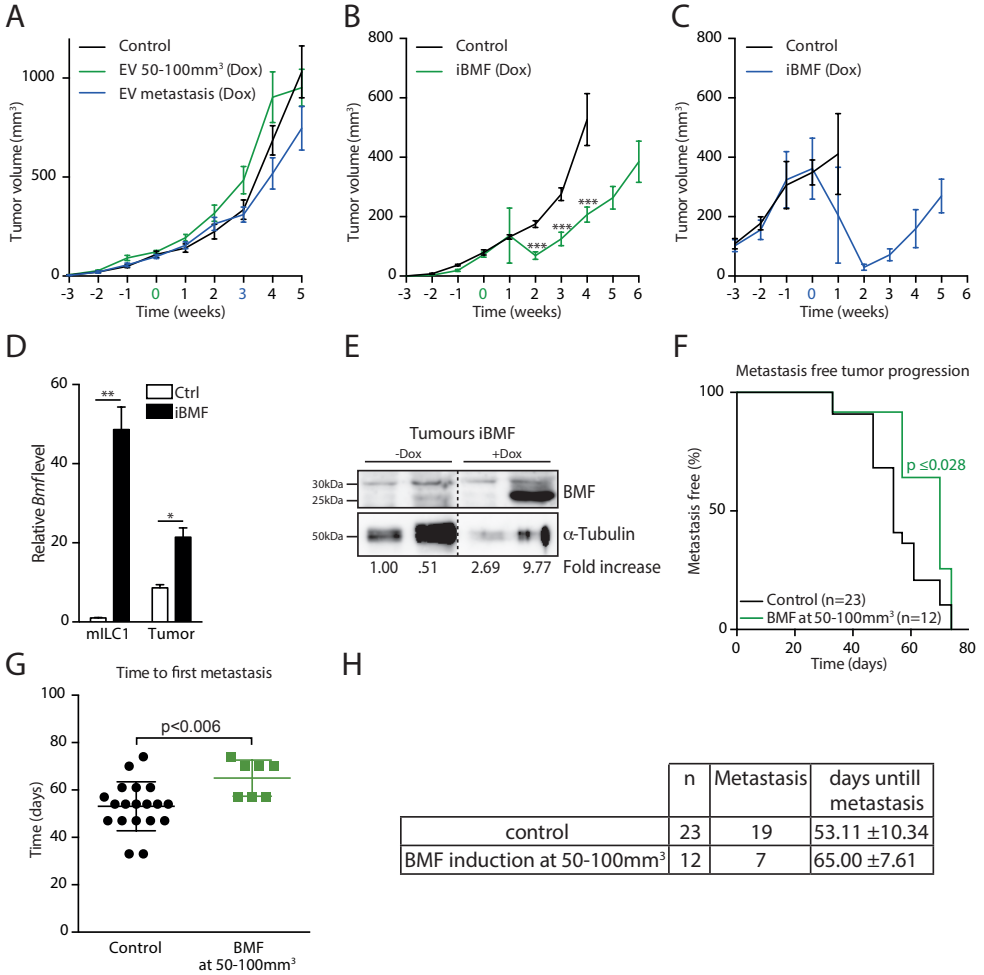


Figure 6: BMF expression is sufficient to induce apoptosis in E-cadherin negative breast cancer cells
(A) Inducible expression of BMF in mILC1-iBMF and MDA-MB-231-iBMF before and after doxycycline treatment. Blots are cropped corresponding to the black box. (B and C) BMF expression induces apoptosis in mILC1 and MDA-MB-231 cells. Flow cytometric analysis of anoikis induction by Annexin-V-FITC and Propidiumiodide staining of mILC1-iBMF and MDA-MB-231-iBMF cells (B). (C) Depicts a quantification of the anoikis induction of mILC1-iBMF and MDA-MB-231-iBMF measured in (B) data represent mean ±SD, n=3, t-test p<0.05 =*, p<0.005 =**, p<0.0005 =***, p<0.00005 =****. (D) Induction of apoptosis and anoikis by the BMF mimetic ABT-199. FACS analysis of apoptosis induction using Annexin-V-FITC and Propidiumiodide staining of mILC1 and MDA-MB-231 cells after 24 hours of treatment with ABT-199 in adherent (Ad) and suspension (Sus) conditions. Data represent mean ±SD, n=3, p<0.05 =*, p<0.005 =**, p<0.0005 =***, p<0.00005 =****.



	n	Metastasis	days untill metastasis
control	23	19	53.11 ±10.34
BMF induction at 50-100mm³	12	7	65.00 ±7.61

Figure 7: BMF expression restrains anchorage-independent tumour growth and metastasis of E-cadherin negative breast cancer cells.
(A) Doxycycline treatment does not influence tumour growth of mILC1 cells carrying the empty doxycycline-inducible expression vector (EV). (B) BMF expression inhibits mammary tumour growth in mice. Primary tumour growth of control mice (n=10) and mice treated with doxycycline (n=12), which was started when tumours reached a volume of 100mm³. Data represent the mean tumour volume ±SEM, Holm-Sidak corrected t-test p<0.0005 =***. (C) BMF expression inhibits mammary tumour growth in mice with metastatic disease. Primary tumour growth of control (n=10) and mice treated with doxycycline (n=13), which was started when lung metastasis were detected by bioluminescent imaging (>2·10³ photons/s/cm²/sr), data represent the mean tumour volume ±SEM. (D) Successful BMF expression after Dox treatment was determined by qPCR analysis of the parental mILC1-iBMF and in primary tumours derived from either untreated or Dox-treated mice. Data represent mean ±SD, n=3, t-test p<0.05 =*, p<0.005. (E) Western blot analysis of tumour lysates shows Bmf induction upon dox treatment of mice. Two untreated (-Dox) and treated (+Dox) tumours are shown respectively. (F) BMF expression inhibits metastasis formation of mILC1 cells. Kaplan-Meier curve representing metastasis free tumour progression, measured in days until metastasis were detected by bioluminescent imaging in control mice (n=23) and mice treated with doxycycline at a primary tumour volume of 50-100mm³ (n=12), Mantel-Cox test p=0.028. (G and H). Time until first metastasis are detected by bioluminescence imaging in control mice (n=19) and mice treated with doxycycline at a primary tumour volume of 50-100mm³ (n=7). Data represent individual mice with detected metastasis (>2·10³ photons/s/cm²/sr) ±SD, Welch's corrected t-test p<0.0062.

Activation of growth factor signalling in cancer cells is essential for anoikis resistance due to modulation of expression and activity of apoptotic factors (80). Others and we have demonstrated that inactivation of the E-cadherin underpins anoikis resistance in breast cancer cells, a finding that appears to depend on hyper-sensitization of GFR signalling through AKT/PKB and ERK upon dismantling of the AJ complex (347, 348, 350, 353, 357). Since these mechanisms confer constitutive activation of GFR signals in breast cancer cells, it provides a clear rationale for the maintenance of anoikis resistance in E-cadherin deficient breast cancer cells. Although AKT/PKB and ERK can control the post-translational modulation of BAD, BIM and BAX, our current data show that anoikis resistant breast cancer cells restrain *BMF* transcription, the main pro-apoptotic factor uniformly expressed in E-cadherin expressing breast cancer cells upon transfer to anchorage independence (180, 183, 184). Elegant studies by the Brugge lab have identified BIM and BMF as key anoikis regulators of luminal mammary cells, a process counterbalanced by constitutive activation of growth factor induced PI3K/AKT pathways (294, 299). However, the underlying mechanism that prevented metastatic breast cancer cells from expressing *BMF* upon loss of anchorage remained unclear. Since we detected clear changes in *BMF* mRNA levels upon transfer to anchorage independence when comparing breast cancer cells in the context of E-cadherin expression, we probed for transcriptional upstream cues, which resulted in our finding that FOXOs are key players in the regulation of *BMF* expression in breast cancer.

BMF can directly compete for BAK/BAX binding with BCL2 and thereby drive apoptosis. Our data indicate that the increase in BMF expression, rather than the levels of BLC2, is the rate-limiting factor driving breast cancer cell survival. First, the robust up-regulation in *BMF* mRNA expression in anoikis sensitive cells combined with reduced *BMF* expression in anoikis resistant cells leads us to think that a specific BMF threshold should be reached to induce anoikis. Second, although anoikis resistant cells induced *BMF* transcription in the absence of anchorage, this did not trigger anoikis, despite expressing comparable levels of BCL2 when compared to anoikis sensitive cells. In line with this we did not detect differences in expression levels of BCL2 upon transfer to anchorage independence between cell lines. Finally, we could force anoikis resistant cells to undergo apoptosis by ectopic expression of BMF or treatment with ABT-199. Together, our data show that loss of the AJ leads to growth factor receptor-induced signals that block FOXO-dependent BMF expression, a feature that is essential for survival in breast cancer cells during metastasis.

Our results indicate that FOXO-dependent transcription of *BMF* may be subject of epigenetic regulation as well. Although we have ectopically activated FOXO3 under anchorage-dependent and independent conditions, we detected further enhanced FOXO3 binding at the *BMF* promoter in suspension. The chromatin state of the BMF locus is expected to influence FOXO3 binding, as it is known that FOXO3 prefers to bind genomic regions associated with activating epigenetic marks (11, 12). Histone deacetylase (HDAC) activity has been reported as a negative regulator of *BMF* expression, because inhibition of HDACs results in BMF expression in multiple human cancer cell lines (358, 359). How BMF is regulated post transcriptionally remains to be fully characterized, but it was previously described that phosphorylation of ERK2 can inactivate BMF function (182, 360). In line with these findings, complementing HDAC inhibition with inhibitors of mitogenic signalling, *i.e.* B-RAF, augments the pro apoptotic effect of BMF (361). This might also explain why BMF induction does not directly lead to apoptosis in anchorage independent MDA-MB-231 cells that show a constitutively elevated growth factor signalling due to oncogenic KRAS and B-RAF mutations (71).

Loss of BMF has been linked to tumour suppression in several cancer types (362). Preclinical intervention strategies using BH3-only mimetics as monotherapy have been successful, but mostly in lymphoid malignancies (363, 364). Recent studies indicated

that BH3-mimetics can be used to treat non-lymphoid cancers but mostly in combination with oestrogen antagonists, proteasome inhibitors, specific PI3K-mTOR inhibitors, or chemotherapy (354, 365-370). Since previous findings from our lab demonstrated that E-cadherin negative lobular breast cancer depends on p120-catenin-mediated activation of RhoA, Rock and subsequent actomyosin contraction (355), we anticipate that dual inhibition of these pathways might be successful in E-cadherin negative cancers that are not driven by oncogenic activation of GFR pathways Although we do not yet know whether RhoA-Rock signals converge onto the GFR-AKT-FOXO axis in the regulation of anoikis resistance, the fact that FOXO expression had no effect on survival of B-RAF/KRAS mutated MDA-MB-231 cells, seems to be in line with this assumption. Moreover, given that human ILC mostly expresses ER and responds to estrogen antagonists, our findings provide an additional option to improve current treatment regimens in lobular breast cancer by using a combination BH3-mimetics and ER targeted drugs.

In conclusion, we have linked activation of GFR pathways to inhibition of FOXO3-dependent BMF expression and the regulation of anchorage-independent tumour growth and metastasis in E-cadherin negative metastatic breast cancer cells.

Materials and Methods

Cell lines

Mouse breast cancer cell lines Trp53$^{\Delta/\Delta}$-4 (KP8) and mILC1 (KEP1) were derived from tumours that developed in female *K14cre;Trp53*$^{F/F}$ and *K14cre;Cdh1*$^{F/F}$*;Trp53*$^{F/F}$ mice and cultured as described previously (353). Human breast cancer cell lines MCF7 and MB-MDA-231 were verified by STR analysis and cultured as described (348).

Constructs, lentiviral transduction and transfections

Lentiviral cDNA expression vectors expressing iBMF, iFOXO3, iFOXO3.A3 were generated using Gateway cloning in the pINDUCER20 dox-inducible expression system (371). Guide RNAs for Cdh1.1 and Cdh1.2 CRISPR were cloned into the lentiviral pSicoR CRISPR/Cas9 vector (372) using *BsmB*I restriction sites (Table. S1). After lentiviral transduction cells were selected for incorporation using puromycin and subsequently FACS-sorted based on E-cadherin expression (DECMA-1, 1:2000; Abcam #11512). BMF and BIM knockdown in Trp53$^{\Delta/\Delta}$ -4 cells was achieved by lentiviral transduction of pLKO1-shBMF (TRCN0000009716 (shBMF#1) and TRCN0000009717 (shBMF #2)) and pLKO1-shBIM (TRCN0000231244 (shBIM #1) and TRCN0000231246 (shBIM #2), Sigma), followed by puromycin selection. For MCF7, siRNA smartpools targeting BMF, BIM or control siRNA (Dharmacon M-004393-04-0005 and M-004383-02-0005) were reverse-transfected using HiPerfect (Qiagen) at a final siRNA concentration of 40 nM. 48 hours after transfection cells were harvested and seeded for anoikis assays.(371, 372)

Immunoblotting & antibodies

Proteins were detected using SDS-PAGE and subsequent western-blot analysis with primary antibodies recognizing BAD (CST-9292), BIM (CST-2819) BMF (human: CST-5889, mouse: ENZO-17A9), BID (SC-11423 Santa Cruz), AKT/PKB (CST), FOXO1 (CST-29H4), FOXO3 (H144 Santa Cruz), NOXA (SP7122p Acris), PUMA (CST-4976), BCL2 (CST-2876) used 1:2000. Primary antibodies were detected by secondary HRP conjugated antibodies targeting mouse, rabbit, and rat IgG and visualized using chemiluminescence (Biorad).

Immunofluorescence

Cells were grown on glass coverslips, fixed using ice-cold methanol and blocked with 2% BSA (Invitrogen) and 0.1% normal goat serum (Invitrogen). Cells were stained

with mouse anti-p120 (BD616134, 1:500 over night), Alexa Fluor 555-conjugated mouse anti-E-cadherin, (1:200, BD560064, 2 hours at room temp.), and DAPI (Sigma). Secondary 563-conjugated goat anti-mouse antibodies were used for visualisation on a Zeiss LSM700 confocal microscope.

Quantitative RT-PCR
mRNA was isolated from live cells using the Qiagen RNeasy kit (Qiagen). cDNA synthesis was performed using the iScript cDNA synthesis kit (BioRad). Real-time PCR was performed using SYBR green FastStart master mix (Roche) in the CFX Connect Real-time PCR detection system (BioRad). Target genes were amplified using specific primer pairs (Supplemental table 1) and specificity was confirmed by analysis of the melting curves. Target gene expression levels were normalised to *GAPDH*, *PBDG* and *TUBA1A* levels.

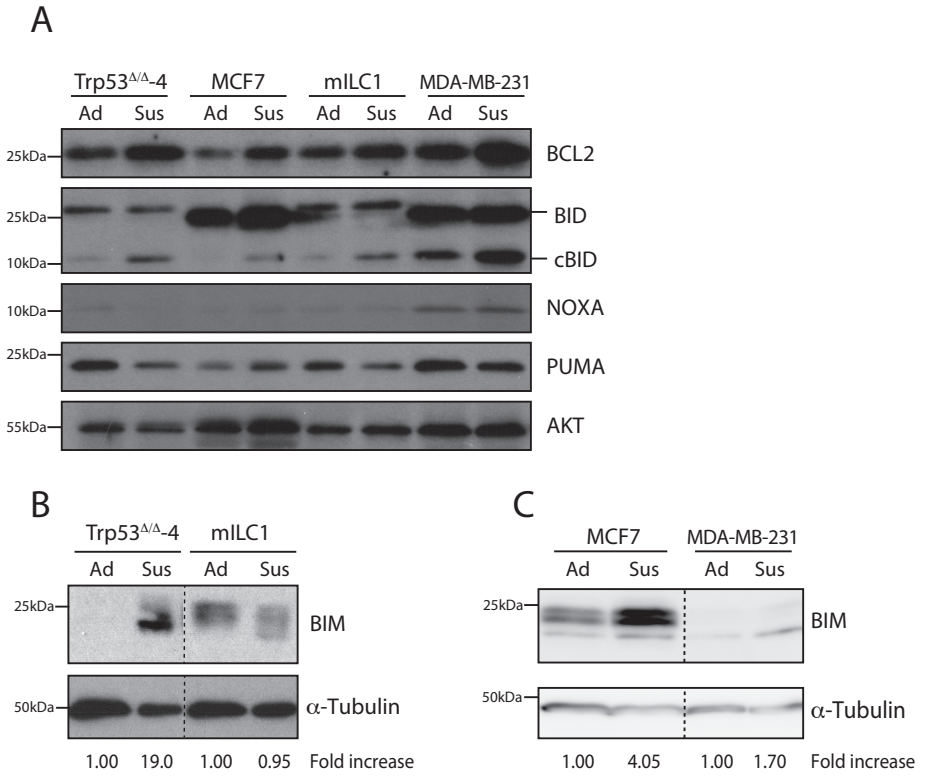
Anoikis Assays
Anoikis resistance was analysed by seeding 6-well ultra-low cluster polystyrene culture dishes (Corning) with 50,000 cells/mL. After 24 hours, cells were harvested and resuspended in 100mL of Annexin-V buffer supplemented with Annexin-V (IQ Products) and propidium iodide (Sigma-Aldrich). Anoikis was defined as the Annexin-V and propidium iodide–positive population and quantified using a FACSCalibur (BD Biosciences).

Chromatin Immunoprecipitation (ChIP)
MCF7-iFOXO3 cells were grown under adherent or suspension conditions in the absence or presence of doxycycline, and treated with the allosteric AKT inhibitor VIII 1 hour prior to harvesting to ensure full FOXO activation. Immunoprecipitations were performed on $20 \cdot 10^6$ cells with 5 µg rabbit anti-FOXO3 (Santa Cruz H144) and 5 µg of normal rabbit IgG (Santa Cruz). ChIPs were performed as previously described (373).

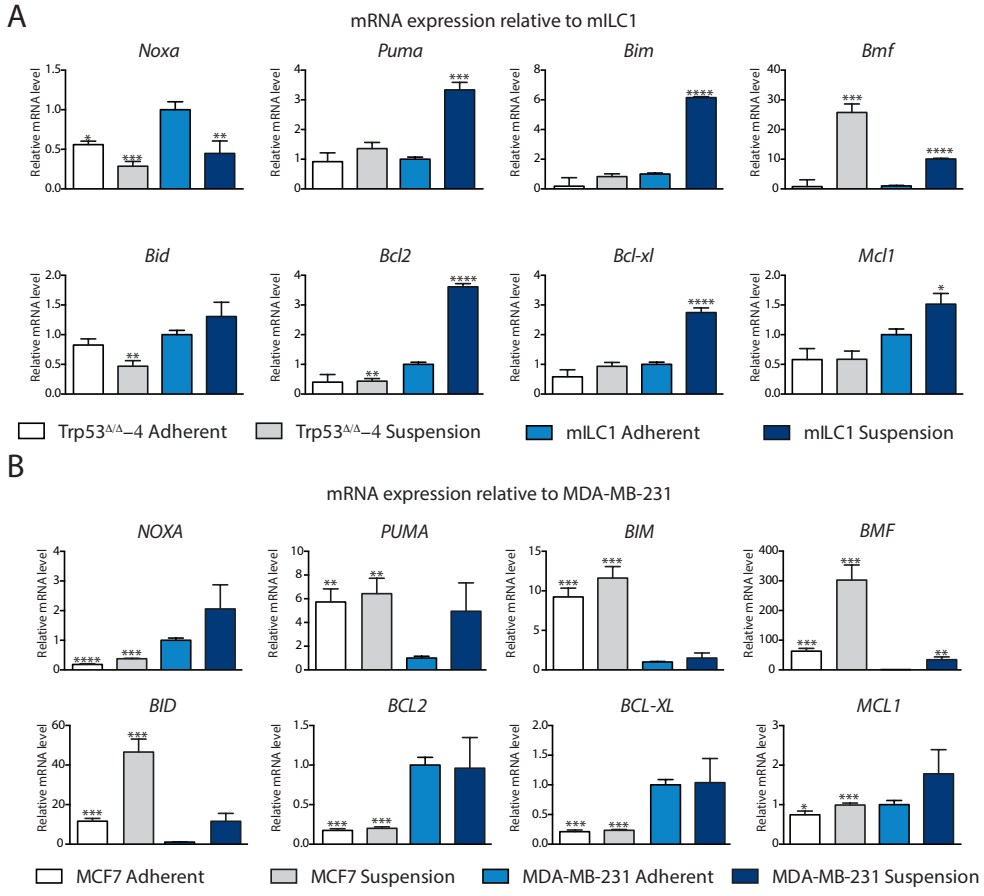
Orthotopic Transplantations and tumour watch
For longitudinal tumour growth and dissemination experiments, 10,000 mILC-1 cells were transplanted in the 4th inguinal mouse mammary gland of Nude recipient mice as described previously (355). Primary tumours were allowed to develop to a volume of $100mm^3$ at which point expression of BMF was induced by feeding doxycycline-containing chow (Ssniff). Alternatively, BMF expression was induced when lung metastasis were detected using bioluminescence imaging of the thorax ($>2 \bullet 10^3$ photons/s/cm^2/sr). Tumour volumes and lung metastases were followed in time as described using a Biospace φ imager (355). All animal experiments were approved by the Utrecht University Animal Experimental Committee (DEC-ABC no. 2012.III.05.044).

Acknowledgements
We would like to thank S. Elledge from the HHMI Boston and R. Lebbink of the molecular microbiology dept. UMC Utrecht for kindly providing the pINDUCER20 and pSicoR CRISPR/Cas9 constructs, and the UMC Utrecht Cell Biology Dept. for help with confocal imaging. The Derksen, van Diest, Dansen and Burgering labs are acknowledged for support and suggestions. Research was supported by grants from the Netherlands Organization for Scientific Research (NWO/ZonMW-VIDI 016.096.318), Foundation Vrienden UMC Utrecht (11.081) and the Dutch Cancer Society (KWF-UU-2011-5230 and KWF-UU-2009-4490)

The authors declare no conflict of interest

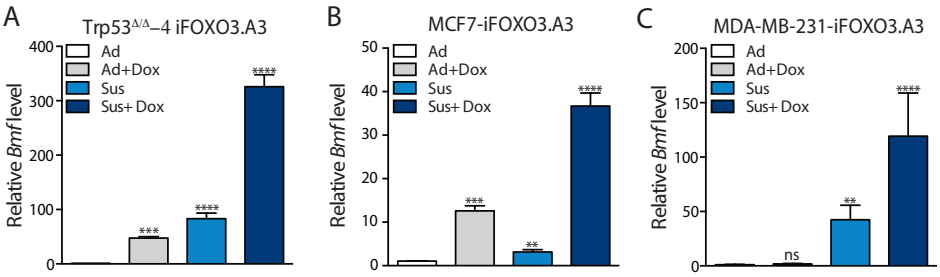


Supplemental figure 1: Induction of pro and anti apoptotic proteins in breast cancer cells.
A. Western blot analysis of showing expression of BCL2, BID, cleaved BID (cBID), NOXA, PUMA, and AKT protein levels in Trp53$^{\Delta/\Delta}$-4, MCF7, mILC1 and MDA-MB-231 cells grown under adherent (Ad) and suspension (Sus) conditions. B. BIM levels are increased in Trp53$^{\Delta/\Delta}$-4 cells upon transfer to suspension shown by Western blot analysis of BIM protein levels in Trp53$^{\Delta/\Delta}$-4 and mILC1 cells. C. BIM levels are increased in MCF7 cells upon transfer to suspension shown by Western blot analysis in MCF7 and MDA-MB-231 cells grown under adherent (Ad) and suspension (Sus) conditions.

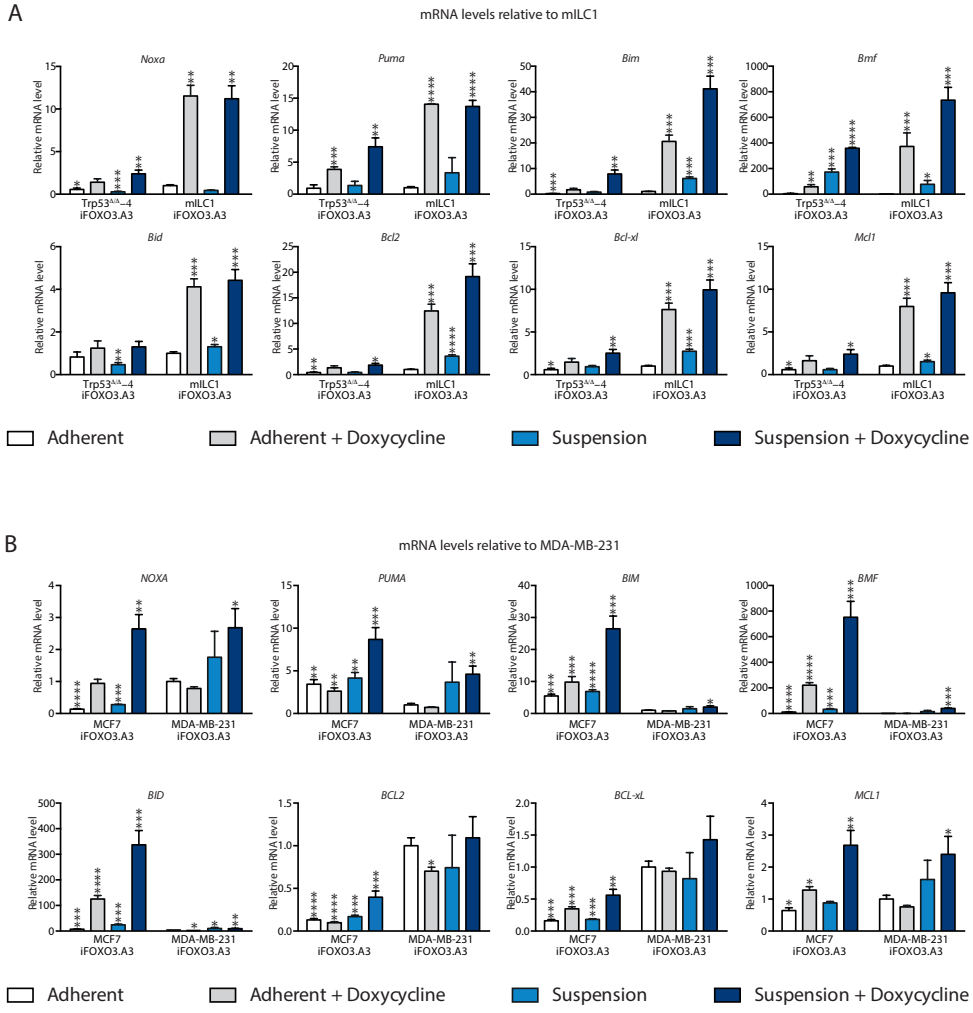


Supplemental figure 2: Transcriptional regulation of pro and anti apoptotic proteins in breast cancer cells.

A. qPCR analysis of *Noxa, Puma, Bim, Bmf, Bid, Bcl2, Bcl-xl, Mcl1* mRNA expression in Trp53$^{\Delta/\Delta}$-4 relative to mILC1 adherent cells. Data represent the mean ±SD, n=3, t-test $p<0.05$ =*, $p<0.005$ =**, $p<0.0005$=***) B. qPCR analysis of *NOXA, PUMA, BIM, BMF, BID, BCL2, BCL-XL, MCL1* mRNA expression in MCF7 relative to MDA-MB-231 adherent cells. Data represent the mean ±SD, n=3, t-test $p<0.05$ =*, $p<0.005$ =**, $p<0.0005$=***)



Supplemental figure 3: FOXO3 activation induces *Bmf/BMF* expression.
A-C. FOXO3 activation induces Bmf expression in Trp53$^{-/-}$-4, MCF7 and MDA-MB-231. Shown are qPCR analyses of Bmf/BMF mRNA before and after doxycycline treatment. Data represent the mean ±SD, n=3, t-test p<0.05=*, p<0.005 =**, p<0.0005 =***, p<0.00005 =****).



Supplemental figure 4: Transcriptional regulation of pro and anti apoptotic proteins by FOXO3.
A. qPCR analysis of Noxa, Puma, Bim, Bmf, Bid, Bcl2, Bcl-xl, Mcl1 mRNA expression in mILC1-iFOXO3.A3 and Trp53$^{-/-}$-4 cells cultured in under adherent (Ad) and suspension (Sus) conditions in the presence or absence of doxycycline (Dox). Data represent the mean mRNA expression levels ±SD relative to mILC1-iFOXO3.A3 cultured under adherent conditions. N=3, t-test $p<0.05$ =*, $p<0.005$ =**, $p<0.0005$=***. B. qPCR analysis of NOXA, PUMA,BIM, BMF, BID, BCL2, BCL-XL, MCL1 mRNA expression in MDA-MB-231-iFOXO3.A3 and MCF7-iFOXO3.A3 cells cultured in under adherent or suspension conditions in the presence or absence of Doxycycline. Data represent mean ±SD levels in mRNA expression relative to MDA-MB-231cultured under adherent conditions, n=3, t-test $p<0.05$ =*, $p<0.005$ =**,$p<0.0005$=***).

Supplemental Table 1: Oligos used in this study

qPCR Primers	Target gene	Direction	Sequence 5'--> 3'
Human	*GAPDH*	Forward	CATTTCCTGGTATGACAACG
		Reverse	CTCTTCCTCTTGTGCTCTTG
	TUBA1A	Forward	TACACCATTGGCAAGGAGAT
		Reverse	AACCAAGAAGCCCTGAAGAC
	NOXA	Forward	GGAGATGCCTGGGAAGAAGG
		Reverse	CACTCGACTTCCAGCTCTGC
	PUMA	Forward	ATTTGGCATGGGGTCTGCC
		Reverse	GAGGGCTGAGGACCACAAATC
	BIM	Forward	TCTCAGTGCAATGGCTTCCA
		Reverse	TCCAATACGCCGCAACTCTT
	BMF	Forward	CCTCCTTCCCAATCGAGTCTG
		Reverse	TCCATCTCTCCTGGGTGACT
	BID	Forward	AGCACAGTGCGGATTCTGTC
		Reverse	ACCGTTGTTGACCTCACAGT
	BCL2	Forward	GGATAACGGAGGCTGGGATGC
		Reverse	GCAGAGTCTTCAGAGACAGCC
	BCL-XL	Forward	GCCTAAGGCGGATTTGAATCTCT
		Reverse	TGGGCTCAACCAGTCCATTG
	MCL1	Forward	AAGAGGCTGGGATGGGTTTG
		Reverse	CAGCAGCACATTCCTGATGC
	ChIP-β-Globulin	Forward	ATGGGACGCTTGATGTTTTC
		Reverse	CCCTGTTACTTATCCCCTTCCT
	ChIP-FOXO binding site 1	Forward	GAGGCCACTCCGCACG
		Reverse	GGGCGGGGCCTCATCA
	ChIP-FOXO binding site 2	Forward	GGAGGCCACTCCGCAC
		Reverse	CTCATCAGCTGTTTGCGGGA
CRISPR	CDH1	gRNA_human	GCTGAGGATGGTGTAAGCGATGG
CRISPR	CDH1	gRNA_human	GAGTTTCCCTACGTATACCCTGG
CRISPR	CDH1	gRNA_mouse	ACCGCGTGTCATCAAATGGGGAAGCGG
Mouse	*Pbgd*	Forward	GCCTACCATACTACCTCCTGGCT
		Reverse	AAGACAACAGCATCACAAGGGTT
	Noxa	Forward	ATAACTGTGGTTCTGGCGCA
		Reverse	TCCTTCAAGTCTGCTGGCAC
	Puma	Forward	TACGAGCGGCGGAGACAAG
		Reverse	GTGTAGGCACCTAGTTGGGC
	Bim	Forward	CGGTCCTCCAGTGGGTATTT
		Reverse	GTCGTATGGAAGCCATTGCAC
	Bmf	Forward	GCAGCCCGCTGGAGTT
		Reverse	CAGGGTCCAGGGTGAAGAAC
	Bid	Forward	GGACTCTGAGGTCAGCAACG
		Reverse	CCTCCCAGTAAGCTTGCACA
	Bcl2	Forward	GCGTCAACAGGGAGATGTCA
		Reverse	TTCCACAAAGGCATCCCAGC
	Bcl-xl	Forward	CCGGTCTCTTCAGGGGAAAC
		Reverse	TAAGGTTCTGGCTGACTGCTC
	Mcl1	Forward	CAAAGAGGCTGGGATGGGTT
		Reverse	CAGCACATTTCTGATGCCGC
	panBMF	Forward	CAGTGCATTGCAGACCAGTT
	panBMF	Reverse	CCCCGTTCCTGTTCTCTTCT

Chapter 4:

Tuning FOXO activity is essential for tumorigenesis

M. Hornsveld[1], A.M.M. Smits[1], M. Meerlo[1], M. van Amersfoort[2], D.E.A. Kloet[1], P.W.B. Derksen[2], T.B. Dansen[1], B.M.T. Burgering[1]

[1]Department of Molecular Cancer Research, University Medical Center Utrecht, Utrecht
[2]Department of Pathology, University Medical Center Utrecht, Utrecht

Manuscript in preparation

4

Summary
FOXO transcription factors function downstream of PI3K-AKT and are often considered tumor suppressors. Conversely, FOXOs have been reported to support tumorigenesis by maintaining homeostasis, supporting metastasis and regulating drug resistance. Indeed, studies that aimed to use FOXOs expression or localization as prognostic markers for the course of cancer progression have come to diametrical conclusions. Clarifying FOXO function in cancer is therefore essential, but studies comparing the effects of both loss and hyperactivation within the same model system are lacking. To this end we characterized pathophysiological and molecular effects of FOXO expression, activation and loss in mouse invasive lobular carcinoma. Our study supports that FOXO3 activation suppresses tumor growth and metastasis, but unveils that FOXO loss is even more detrimental to tumorigenesis as FOXOs regulate cancer cell migration, invasion, anoikis resistance, PI3K activity, bioenergetics and redox homeostasis. Based on our results we conclude that FOXOs are essential for tumorigenesis but their activity needs to be carefully tuned.

Introduction

Sustained proliferative signaling is a hallmark of cancer that can for instance be provided for by many oncogenic alterations found in the highly conserved RAS-MEK-ERK and PI3K-AKT pathways {Hanahan, 2011 #789}. The PI3K-AKT pathway is commonly hyper activated in cancer and converges on the redundant Forkhead Box O family of transcription factors (FOXO1, FOXO3, FOXO4) of which FOXO1 and FOXO3 are most abundantly expressed. FOXOs are kept inactive in the cytoplasm by active AKT, but upon loss of PI3K-AKT activity FOXOs translocate to the nucleus where they control a plethora of target genes involved in redox homeostasis, cell proliferation, differentiation, metabolism and apoptosis (2, 7, 8, 11, 12). FOXOs are considered putative tumor suppressors as combined loss of *Foxo1*, *Foxo3* & *Foxo4* in adult mice results in increased tumor formation and FOXO activation arrests the cell cycle and induces apoptosis or anoikis in multiple types of tumor cells (34, 45, 61). Counter intuitively; FOXOs are rarely mutated in cancers. This could either be because mutation of 6 *FOXO* alleles is unlikely and AKT mediated FOXO inhibition is sufficient to avoid tumor suppression, or because tumor cells benefit from having FOXOs around. In line with the latter, several recent studies have suggested that FOXOs may also support tumorigenesis (72, 73, 76, 77).

Additionally, FOXOs have been reported as part of a drug resistance signaling feedback loop that re-establishes RTK-PI3K-AKT signaling after pharmacological inhibition of AKT. Inhibition of AKT results in transcription of multiple RTKs, RTK adaptor proteins like IRS2 and the mTORC2 component *RICTOR*, which concurrently reactivate RTK and AKT signaling, a response hampered in the absence of FOXOs. (94-96).

Multiple studies have aimed to use FOXO levels and localization as prognostic markers for cancer patient survival. In line with the paradoxical role of FOXOs in cancer, these studies yielded contradictory results, showing that FOXO activity (as measured by nuclear localization) and levels can both correlate with good and poor prognosis (48-57, 67-72).

It is clear that the current understanding of the role of FOXOs in cancer is insufficient. The current knowledge is scattered over many different studies in various types of cancer and most studies are limited to only the effects of FOXO loss or gain of function in single aspects of cancer. Gaining better insight into the role of FOXOs in cancer might lead to the development of promising strategies for cancer therapy (34). However, studies designed to characterize the effects of both FOXO gain and loss of function within one system are lacking.

To this end we characterized acute FOXO expression, activation and loss in a robust mouse model for invasive lobular carcinoma (mILC) that allows monitoring of both primary tumor growth and metastasis (350, 353). Our study reveals that FOXO3 hyper activation suppresses tumorigenesis and delays metastasis. Combined loss of *Foxo1* and *Foxo3* expression was found to be even more detrimental to tumorigenesis and efficiently inhibits metastasis. Further characterization of the effects of FOXO activity on mILC shows that FOXOs are essential to maintain PI3K/AKT signaling and are involved in numerous processes including cell migration, invasion, anoikis resistance, bioenergetics and redox homeostasis. Based on these observations we conclude that FOXOs are not tumor suppressors but essential for many aspects of tumorigenesis and propose that cancer cells need to carefully tune FOXO activity to maintain homeostasis.

Results

FOXO3 activation represses tumor growth and metastasis

In order to determine how FOXO levels and activity are influencing tumorigenesis we established mILC1 cell lines constitutively expressing luciferase, combined with doxycycline-inducible wild-type FOXO3 (iFOXO3) or the AKT resistant hyperactive mutant FOXO.A3 (iFOXO3.A3). We chose FOXO3 because the individual FOXOs are redundant as putative tumor suppressors and FOXO3 is the most ubiquitously expressed FOXO (Figure 1A)(7, 61, 353). It is important to use an inducible system to minimize the chances of adaptation to presence of the transgene. Cells were grown under continuous selection with neomycin that is expressed from the same construct to ensure that they do not lose the construct. Upon administration of doxycycline for 7 days, colony outgrowth was impaired in both mILC1-iFOXO3 and mILC1-iFOXO3.A3 cells (Figure 1B). To resolve whether colony formation is impaired due to apoptosis or cell cycle inhibition or both the cells were tested for apoptosis and DNA content by flow cytometry. Doxycycline treatment showed no significant increase in apoptosis for both mILC1-iFOXO3 and mILC-iFOXO3.A3 cells (Figure 1C). Induction of FOXO3.A3 did however induce a cell cycle arrest resulting in 81% of G1 arrested cells after 48 hours (Figure 1D). In mILC1-FOXO3 cells on the other hand only 61% of the cells are in G1. Induction of FOXO3 and FOXO3.A3 resulted in expression of its canonical target gene *Cdkn1b/p27*, indicating colony formation capacity is mainly restricted through inhibition of the cell cycle (Figure 1E). In case of iFOXO3 the cell cycle effect seems only temporary as p27 is induced after 16 hours, colony outgrowth is restrained but no cell cycle arrest is observed after 48 hours of FOXO3 induction. These results confirm the well-described role of FOXOs in regulation of the cell cycle (35).

Orthotopic transplantation of mILC1 cells is a robust model for invasive lobular carcinoma and the aggressive nature (tumor cells detectable in the lungs ~6 weeks after transplantation in the fat pad) of this tumor type allows studying both primary tumor growth and metastasis. In order to verify the observed tumor suppressive effects of FOXO3 and FOXO3.A3 expression *in vivo*, we orthotopically transplanted 10.000 cells into the mammary fat pad of recipient mice. Expression of FOXO3 and FOXO3.A3 was induced by feeding mice doxycycline-containing chow either 2 weeks after surgery or when tumors were 50-100mm^3 in size. Induction of FOXO3 in both early and late dox-treated mice did not influence primary tumor growth (Figure 1F). Early expression of FOXO3.A3 hampered tumor development by delaying primary tumor growth. Expression of FOXO3.A3 when primary tumors are between 50-100mm^3 resulted in inhibition of tumor growth and decreased primary tumor size, but 2 weeks after doxycycline administration primary tumor growth was re-established (Figure 1G). Detection of luciferase activity in the mouse outside the primary tumor location, primarily the lungs, was used to determine the metastatic capacity of mILC1-iFOXO3 and mILC1-iFOXO3.A3. No significant differences in metastasis free survival were detected for tumors with FOXO3 and FOXO3.A3 induction 2 weeks after surgery. In contrast, FOXO3 and FOXO3.A3 induction when tumors are 50-100mm^3 in size showed significantly delayed metastasis formation (Figure 1H&I). The average primary tumor volume was significantly larger when metastases were first detected in doxycycline treated mice, indicating that FOXO3 activation not only restrains primary tumor growth but also inhibits metastasis formation (Supplemental Figure 1A). Strong selection for cells with low amounts of viral integrations was observed by quantifying the amount of viral integrations in the genome of mILC1-iFOXO3.A3 cells isolated from untreated, early and late treated tumors by real-time PCR (Supplemental Figure 2A). Cells isolated from dox treated tumors remained resistant to neomycin selection and showed low FOXO3.A3 induction, comparable to mILC1-FOXO3.A3 cells treated for 3 weeks with

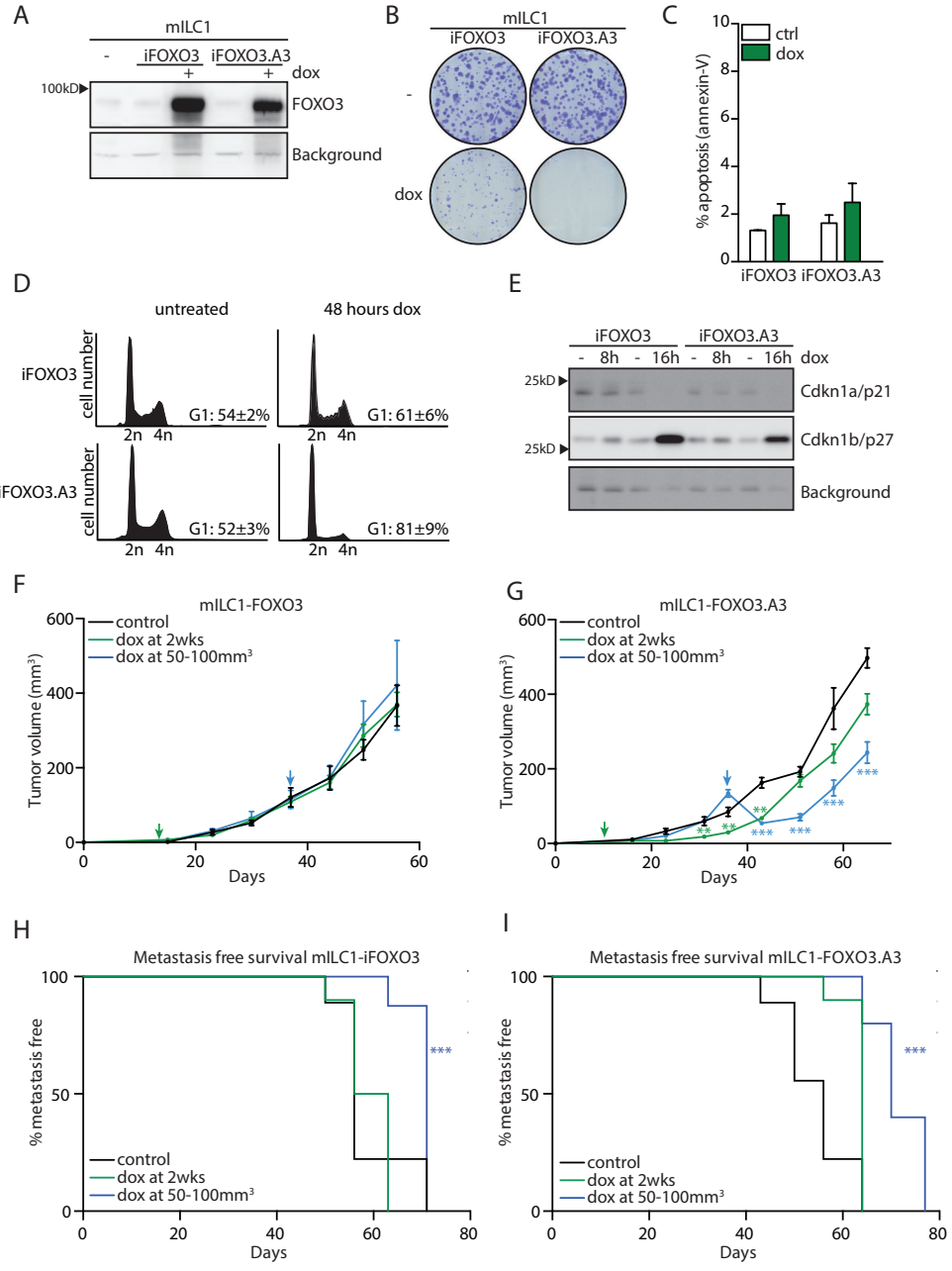

A
mILC1
iFOXO3
iFOXO3.A3
dox
100kD
FOXO3
Background
B
mILC1
iFOXO3
iFOXO3.A3
dox
C
ctrl
dox
% apoptosis (annexin-V)
iFOXO3
iFOXO3.A3
D
untreated
48 hours dox
iFOXO3
iFOXO3.A3
cell number
G1: 54±2%
G1: 61±6%
G1: 52±3%
G1: 81±9%
2n
4n
E
iFOXO3
iFOXO3.A3
8h
16h
dox
25kD
Cdkn1a/p21
Cdkn1b/p27
Background
F
mILC1-FOXO3
control
dox at 2wks
dox at 50-100mm³
Tumor volume (mm³)
Days
G
mILC1-FOXO3.A3
control
dox at 2wks
dox at 50-100mm³
Tumor volume (mm³)
Days
H
Metastasis free survival mILC1-iFOXO3
% metastasis free
control
dox at 2wks
dox at 50-100mm³
Days
I
Metastasis free survival mILC1-FOXO3.A3
% metastasis free
control
dox at 2wks
dox at 50-100mm³
Days

Figure 1: FOXO3 activation inhibits tumor growth and metastasis
A Western blot analysis of FOXO3 expression in mILC1-iFOXO3 and mILC1-iFOXO3.A3 cells after 16 hours of doxycycline. **B** Colony formation capacity of mILC1-iFOXO3 and mILC1-iFOXO3.A3 cells after 7 days of doxycycline. Shown here is a representative figure. **C** Flow cytorometric analysis of mILC1-iFOXO3 and mILC1-iFOXO3.A3 cells stained with Annexin-V and Propidium Iodide (PI) after 24 hours of doxycycline. Data represent the average of 3 experiments ±SD. Aspecific background signal was used to control for loading. **D** Flow cytometric analysis of PI staining for DNA content in mILC1-iFOXO3 and mILC1-iFOXO3.A3 cells before and after 48 hours of doxycycline. Shown is a representative histogram and the average percentage of cells with 2n DNA (G1) (n=3) ±SD **E** Western blot analysis of Cdkn1a and Cdkn1b protein levels in mILC1-iFOXO3 and mILC1-iFOXO3.A3 cells after 8 or 16 hours of doxycycline. Aspecific background signal was used to control for loading. **F & G** Primary tumor growth curves of orthotopically transplanted mILC1-iFOXO3 and mILC1-iFOXO3.A3 cells. Black lines indicate untreated tumors (n=10), green lines indicate tumors treated with doxycycline starting from 2 weeks after transplantation (n=10), blue lines indicate tumors treated with doxycycline starting from tumor size 50-100mm^3 (n=10). Data represent the mean tumor volume ±SEM, Holm-Sidak corrected t-test p<0.005 =**, p<0.0005 =***. **H & I** Kaplan-Meier curve representing metastasis free survival, measured in days until metastasis were detected by bioluminescent imaging in control mice (n=10), mice treated starting from 2 weeks after transplantation (n=10) and mice treated with doxycycline at a primary tumor volume of 50-100mm^3 (n=10), Mantel-Cox test mILC1-iFOXO3 p=0.0015 (**), mILC1-iFOXO3.A3 p<0.0001=***

doxycycline in culture, which means that they did retain and express the construct (Supplementary Figure 2B&C). The strong selection for cells expressing low levels of FOXO3.A3 explains why the suppressive effect of FOXO3.A3 on primary tumor growth is limited and temporary.
Together these results show that FOXO3 activation suppresses tumor growth and delays the establishment of metastatic disease. Previously we showed FOXO3 activation might be involved in repression of metastasis by regulating anoikis in breast cancer cells including the cells used in our mILC1 model (45). Our results now for the first time implicate that FOXO3 activation can repress metastasis *in vivo* and complement our previous findings.

FOXOs are essential for tumor growth and metastasis
Our data confirms activation of FOXOs suppresses tumorigenesis *in vivo*, in agreement with Paik et al. showing that *Foxo1, Foxo3* & *Foxo4* knockout mice display high tumor incidence and (61). But since epithelial tumors were rarely found in knockout mice and recent studies revealed also a putative tumor supportive role for FOXOs, we set out to evaluate the effects of FOXO loss on tumorigenesis in our mILC1 model (72, 73, 77).
As FOXO transcription factors are highly redundant in their function we first determined the endogenous expression levels of *Foxo1*, *Foxo3* and *Foxo4* in mILC1. Both *Foxo1* and *Foxo3* were expressed and no *Foxo4* mRNA was detected in mILC1 (supplemental figure 3A). Conveniently, *Foxo1* and *Foxo3* share sufficient sequence overlap that they can be targeted by a single shRNA, that we expressed dox-inducible in mILC1 cell lines (shFOXOs)(38, 222). Upon shFOXOs expression efficient knockdown of both *Foxo1* and *Foxo3* is observed over the course of 72 hours (Figure 2A & Supplemental figure 3B). *Foxo* knockdown over the course of 7 days reduced mILC1 colony outgrowth (Figure 2B). To resolve if colony outgrowth is impaired due to apoptosis or cell cycle inhibition, apoptosis and DNA content were assessed by flow cytometry. FOXO knockdown for 72 hours showed no significant increase in apoptosis (Figure 2C). FOXO knockdown did also not induce a cell cycle arrest but resulted in an overall increase in cell cycle time as measured by video time-lapse microscopy (Figure 2D&E). Whereas *Cdkn1b/p27* was induced upon FOXO3 activation, inactivation of FOXOs in mILC1 cells up regulates the expression of *Cdkn1a/p21* indicating that FOXO activation and FOXO loss affects the cell cycle through different mechanisms (Figure 2F).
We orthotopically transplanted 10.000 mILC1-shFOXOs cells into the mammary

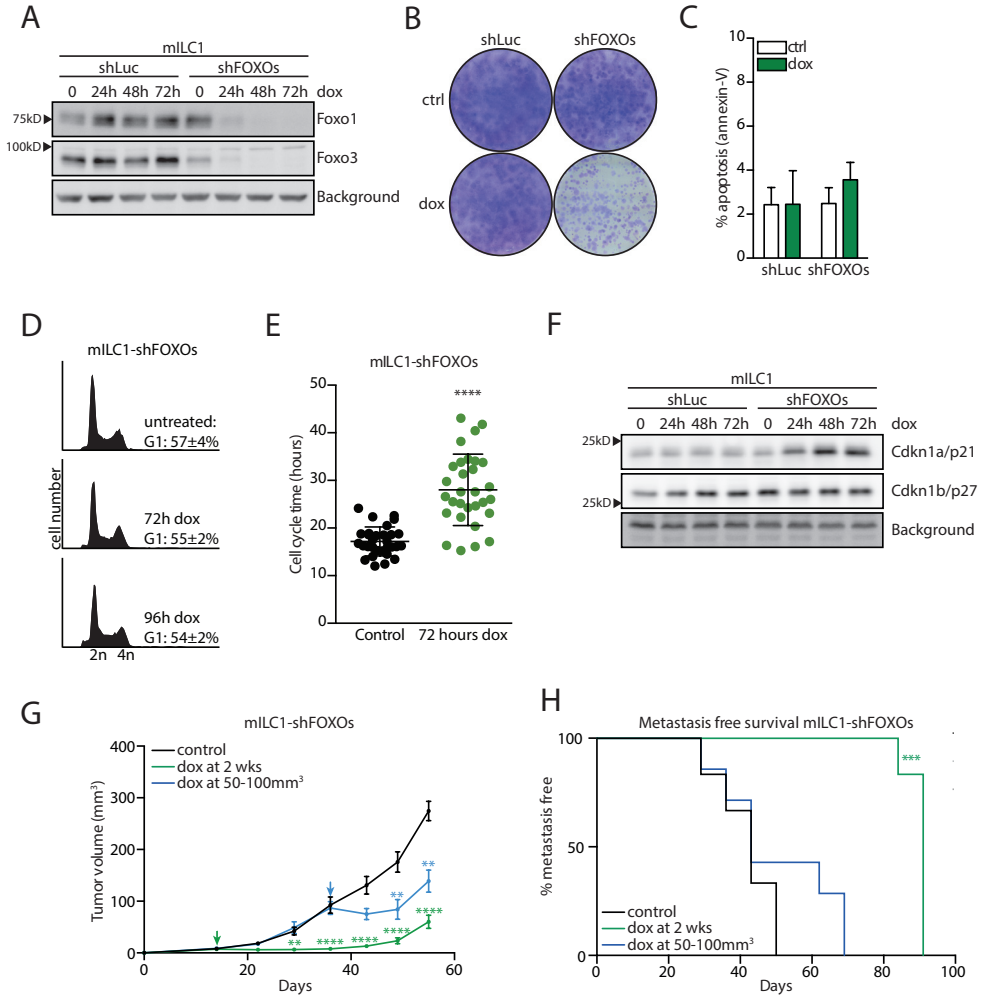


Figure 2: FOXOs are essential for tumor growth and metastasis

A Western blot analysis of Foxo1 and Foxo3 expression in mILC1-shLuc and mILC1-shFOXOs cells after 24, 48 and 72 hours of doxycycline. **B** Colony formation capacity of mILC1-shLuc and mILC1-shFOXOs cells after 7 days of doxycycline. Shown here is a representative experiment. Aspecific background signal was used to control for loading. **C** Flowcytorometric analysis of mILC1-shLuc and mILC1-shFOXOs cells stained with Annexin-V and Propidium Iodide (PI) after 72 hours of doxycycline. Data represent the average of 3 experiments ±SD. **D** Flow cytometric analysis of PI staining for DNA content in mILC1-shFOXOs cells treated with doxycycline for 72 and 96 hours. Shown is a representative histogram and the average percentage of cells with 2n DNA (G1) (n=3) ±SD **E** Quantification of time-lapse microscopy imaging of the time it takes to undergo the complete cell cycle in untreated or 72 hours doxycycline treated mILC1-shFOXOs cells. **F** Western blot analysis of Cdkn1a and Cdkn1b protein levels in mILC1-shLuc and mILC1-shFOXOs cells after 24, 48 or 72 hours of doxycycline. Aspecific background signal was used to control for loading. **G** Primary tumor growth curves of orthotopically transplanted mILC1-shFOXOs cells. Black lines indicate untreated tumors (n=10), green lines indicate tumors treated with doxycycline starting from 2 weeks after transplantation (n=10), blue lines indicate tumors treated with doxycycline starting from tumor size 50-100mm^3 (n=10). Data represent the mean tumor volume ±SEM, Holm-Sidak corrected t-test p<0.005 =**, p<0.0005 =***. **H** Kaplan-Meier curve representing metastasis free survival, measured in days until metastasis were detected by bioluminescent imaging in control mice (n=10), mice treated starting from 2 weeks after transplantation (n=10) and mice treated with doxycycline at a primary tumor volume of 50-100mm^3 (n=10), Mantel-Cox p<0.0003 (***).

fat pad of recipient mice to determine loss of FOXO expression effects *in vivo*. FOXO loss hampered tumor development significantly, delaying primary tumor growth by approximately 4 weeks. Expression of shFOXOs in primary tumors between 50-100mm^3 resulted in efficient but temporary growth inhibition (Figure 2G). Strikingly, the onset of metastasis was significantly delayed in tumors treated with FOXO knockdown from 2 weeks after surgery compared to untreated control tumors. Treating tumors at 50-100mm^3 also reduced the establishment of metastasis (Figure 2H). Average primary tumor volume was significantly larger when metastases were first detected in doxycycline treated mice, indicating that loss of *Foxo1* and *Foxo3* expression not only restrains primary tumor growth but also delays metastasis formation (Supplemental Figure 4A).

The effect of Foxo loss on tumor growth is initially quite dramatic but a relapse was observed in time. Quantifying the amount of viral integrations in the genome of mILC1-shFOXOs cells isolated from control or dox treated tumors by real-time PCR showed mild selection for cells with low amounts of viral integrations in contrast to mILC1-FOXO3.A3 tumors (Supplemental Figure 5A). Cells isolated from dox treated tumors retained GFP expression, meaning that the construct is still expressed. Additionally, efficient *Foxo1* and *Foxo3* knockdown was still observed in cells derived from the doxycycline treated tumor groups (Supplementary Figure 5B). The fact that there is little negative selection against cells carrying shFOXO construct combined with the maintenance of efficient *Foxo* knockdown suggests that tumor cells are able to adapt to *Foxo* loss by a yet unknown mechanism. Together these results show mILC1 tumors depend on the presence *Foxos* for both growth and metastasis.

FOXOs attenuate cell motility and anoikis resistance

For cells to become metastatic they need to be motile and able to survive dissemination. FOXOs have been reported to facilitate metastasis trough regulating cell motility and migration in breast and colon cancer cells (72, 77). But others and we have previously shown that FOXO activation is correlated to anoikis induction in anchorage independent cells including mILC1 (45, 80). In order to determine whether decreased metastatic potential by FOXOs correlates to changes in cell migration or prevention of anchorage independent survival we assessed how different levels of FOXO activity impact on cell motility and anoikis resistance.

Tracking cell migration by live microscopy of mILC-shFOXOs and mILC1-iFOXO3.A3 in 2D culture showed a significant inhibition of cell motility upon loss but not upon activation of FOXO (Figure 3A). mILC1 cells cultured as spheroids in a mixture of matrigel and collagen grow as large multicellular structures that invade into the matrix. Loss of FOXO results in a marked reduction of invasive growth whereas expression of FOXO3.A3 impairs spheroid formation or leads to the collapse of the 3D structure when induced after the formation of invasive protrusions (Figure 3B).

Next we determined the effect of FOXO activity on anoikis resistance. Previously we reported that activation of FOXOs induces anoikis, however the effect of FOXO loss on anoikis has not been studied (45). Significant increase in anoikis was observed in mILC1-shFOXOs cells transferred to suspension after 72 hours of doxycycline (Figure 3C). Reduced anoikis resistance by FOXO loss indicates that FOXOs are required for anchorage independent survival. Induction of anoikis by FOXO activation is mediated by transcription of the pro-apoptotic BH3-only genes BIM and BMF (45). Probing for genes involved in anoikis in mILC1-shFOXOs cells grown in suspension confirms that Bim and Bmf expression are up-regulated in a FOXO dependent manner and shows reduced Bcl2 and Bcl-xl expression levels (Figure 3D&E). Together these results show that loss of FOXOs correlates with reduced cell migration, invasion and anoikis resistance of mILC1 cells. The mechanism by which anoikis is triggered remains to be clarified however.

FOXOs are required for cancer cell homeostasis and bioenergetics

The increased sensitivity to apoptosis in cells lacking FOXO expression is not expected to be sufficient to attenuate cancer cell proliferation or trigger cell death without additional stimuli. Anchorage independent survival is reported to be dependent on active growth factor signaling and the cancer cell's ability to maintain redox and metabolic homeostasis (290, 292). FOXOs have been reported to influence cell proliferation and survival by prevention of metabolic and oxidative stress (19, 21, 76, 374). To determine if mILC1-shFOXO cells activate stress signaling we determined the phosphorylation status of JNK, p38 and c-JUN. FOXO knockdown lead to induction of stress kinase pathway activity over the course of three days, suggesting that FOXO loss leads to oxidative or metabolic stress (Figure 4A). Next we determined whether stress kinase activation correlates with increased levels of ROS and an overall more oxidative state. CellRox fluorescence intensity was increased after FOXO knockdown in cells treated with this reactive species probe, indicating that FOXO loss increases ROS levels (Figure 4B). The ratio of $NADP^+$ over NADPH, the main reductive entity in cells, increased after FOXO knockdown supporting the notion that an overall more oxidative environment reigns in cells after loss of FOXO (Figure 4C). NADPH is mainly recycled

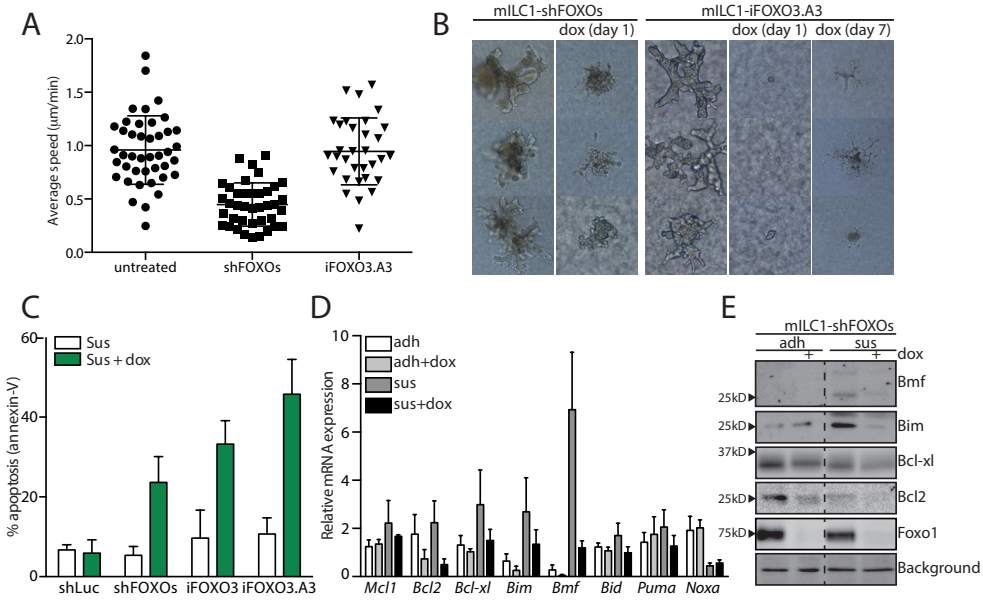


Figure 3: Loss of FOXOs reduces cell motility, invasion and anchorage independent survival.
A Cell migration speed in mm/frame (5 min) determined by tracking cell movement using live cell microscopy of mILC1-shFOXOs treated with doxycycline for 72 hours and mILC1-FOXO3.A3 cells treated with doxycycline for 8 hours. **B** Spheroid cultures of mILC1-shFOXOs and mILC1-FOXO3.A3 in Matrigel containing Colagen I cultured in the presence of doxycycline either from day 1 or after 7 days. **C** Flow cytometric analysis of anoikis induction in mILC1-shLuc, mILC1-shFOXOs, mILC1-iFOXO3 and mILC1-iFOXO3.A3 cells cultured in suspension for 24 hours and stained for Annexin-V and PI. mILC1-shLuc and mILC1-shFOXOs were pretreated with doxycycline for 72 hours. mILC1-iFOXO3 and mILC1-FOXO3.A3 were treated with doxycycline upon transfer to suspension. Data represent the average of 3 experimens ±SD. **D** RT-qPCR analysis of *Mcl1*, *Bcl2, Bcl-xl, Bim, Bmf, Bid, Puma* and *Noxa* mRNA expression levels in mILC1-shFOXOs cells. mILC1-shFOXOs cells (adh) were cultured in the presence of doxycycline for 96 hours (adh+dox) or transfered to suspension for 24 hours (sus) after 72 hours of doxycycline (sus+dox). Data represent the average of 3 experiments ±SD, data is normalized to *Gapdh* mRNA expression. **E** Western blot analysis of Bmf, Bim, Bcl-xl, Bcl2 and Foxo1 protein levels. mILC1-shFOXOs cells (adh) were cultured in the presence of doxycycline for 96 hours (adh+dox) or transfered to suspension for 24 hours (sus) after 72 hours of doxycycline (sus+dox). Picture of the same blot is cut at indicated dashed line. Aspecific background signal was used to control for loading.

from NADP+ when glucose is shunted into the pentose phosphate pathway. FOXOs are well known regulators of glucose uptake and metabolism and therefore we tested whether glucose metabolism was impaired upon FOXO knockdown. Tracking glucose uptake using 2-NBDG showed loss of FOXOs led to reduced glucose uptake (Figure 4D). To determine the effect of FOXO loss on overall bioenergetics we measured glycolytic activity and oxygen consumption rate using Seahorse technology. Basal and maximal glycolysis was lower after FOXO loss, showing cells are using their full glycolytic capacity (Figure 4E). Similarly, oxygen consumption is lower in cells lacking FOXOs and cells use their full capacity (Figure 4F). Although FOXOs clearly affect cellular bioenergetics and redox homeostasis, antioxidants e.g. Trolox, N-acetyl cysteine or EUK134, do not rescue cell proliferation or anoikis induction (Supplemental figure 6). Taken together, these data indicate that although FOXOs are essential for cellular redox homeostasis in mILC1, the role of FOXOs in cancer is more complex than just providing tumor cells with sufficient reductive capacity.

FOXOs tune growth factor signaling activity

Growth factor signaling is fundamental to tumorigenesis and anchorage independent survival is strongly dependent on RAS and PI3K pathway activity (45, 80, 292, 300). Interestingly, FOXOs have been proposed as regulators of a signaling feedback mechanism that maintains PI3K-pathway activity in response to pharmacological inhibition of this pathway (71, 94-96). How FOXOs precisely regulate PI3K feedback signaling remains to be characterized. Expression of FOXO3.A3 increases RAS and PI3K pathway activity, shown by ERK phosphorylation, AKT-Ser308 and Ser473 phosphorylation. Loss of FOXO correlated with a marked reduction in Akt-S308 and S473 phosphorylation (Figure 5A). Unchanged p70s6k phosphorylation implicated no changes in mTORC1 activity. To validate these observations *in vivo* we stained AKT-Ser473 in mILC1-iFOXO3. A3 and mILC1-shFOXOs tumors that were either untreated or doxycycline treated for a week at primary a tumor volume of 50-100mm^3. Induction of FOXO3.A3 or shFOXOs correlated to increased and reduced levels of AKT-S473 respectively (Figure 5B). FOXO activation induced mRNA expression of a panel of previously described growth factor signaling feedback components including *c-Met, Erbb2, Erbb3, Insr, Igf-1r, Pdgfra, Fgfr1, Fgfr3, Irs1, Irs2, Rictor, Sesn1 and Sesn3* (Figure 5C). FOXO knockdown resulted in decreased mRNA expression of *c-Met, Igf-1r, Insr, Erbb2, Erbb3, Rictor, Irs1, Irs2, Sesn1, Sesn3* (Figure 5D). Protein levels indeed confirm FOXOs control the expression of c-Met, Igf-1r, Insr, Erbb2, Erbb3, Irs1, Irs2, Rictor and Pdgfra (Figure 5E). Together these results show that FOXOs are important mediators of PI3K feedback signaling and do so by regulating multiple components of this pathway. Co-expression of an shRNA-insensitive mutant of FOXO3 in mILC1-shFOXOs is sufficient to maintain AKT activation and prevent stress kinase activation (Supplemental figure 7A-F). Re-establishing AKT activity by ectopic expression of PI3K^{H1047R}, myr-AKT or Rictor is however insufficient to rescue cell proliferation and anoikis induction upon loss of FOXO, even in the presence of antioxidants (Supplemental figure 7G&H). These findings stress that carefully tuning FOXO activity is essential for its complex function within the cell, as ectopic hyper activation, complete loss of FOXO3 or hyper activation of GFR signaling all overdo the endogenous function of FOXOs.

Collectively, our results show that FOXOs are involved in diverse processes that are essential for tumor cell proliferation, survival, migration, redox & metabolic homeostasis. Moreover, our findings demonstrate that tumor cells need to carefully tune FOXO activity for outgrowth and survival (Figure 5F).

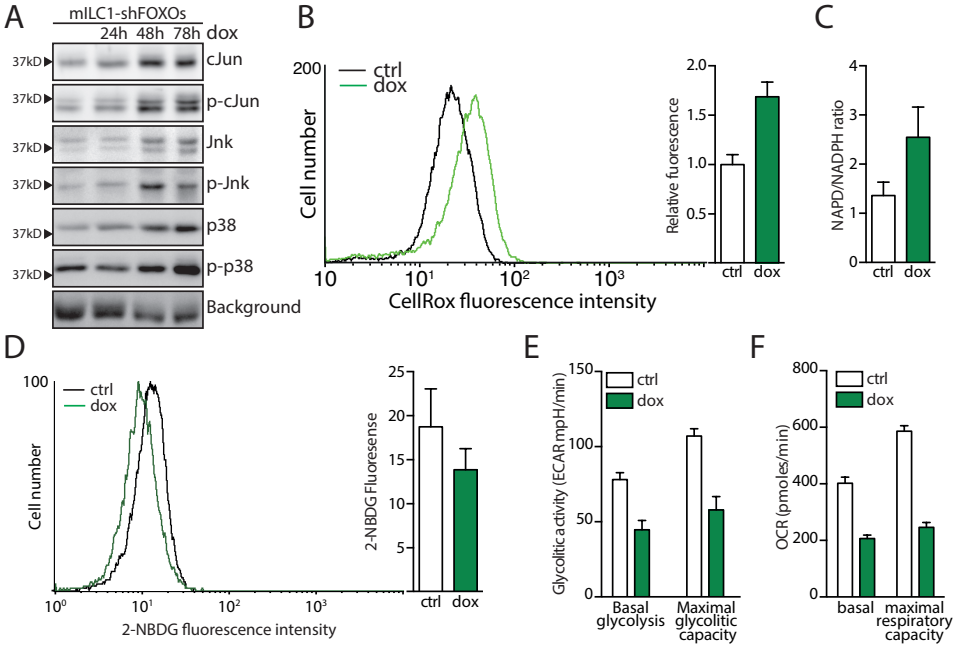


Figure 4: FOXOs are required for maintaining cellular redox homeostasis and bioenergetics
A Western blot analysis of cJun, phospo-cJun, Jnk, phospho-Jnk, p38 and phospho-p38 protein levels in mILC1-shFOXOs cells treated with doxycycline for 24, 48 or 72 hours. Aspecific background signal was used to control for loading. **B** Flow cytometric analysis of CellRox oxidation in mILC1-shFOXOs cells treated with doxycycline for 72 hours. Shown is a representative cytometric histogram and the average of three independent experiments ±SD. **C** Quantification of the ratio between NADP+ and NADPH. Data represents the average of 3 experiments ±SD, t-test $p<0.05$ = *. **D** Flow cytometric analysis of 2-NBDG uptake in mILC1-shFOXOs cells treated with doxycycline for 72 hours. Shown is a representative cytometric histogram and the average of three independent experiments ±SD. **E** Seahorse measurement of glycolytic activity and maximal glycolytic capacity based on the extra cellular acidification rate (ECAR) of mILC1-shFOXOs cells treated with doxycycline for 72 hours. Shown is the average of 5 technical replicates within a representative experiment ±SD. **F** Seahorse measurement of mitochondrial respiration (OXPHOS) and maximal respiratory capacity based on the oxygen consumption rate (OCR) of mILC1-shFOXOs cells treated with doxycycline for 72 hours. Shown is the average of 5 technical replicates within a representative experiment ±SD.

Discussion

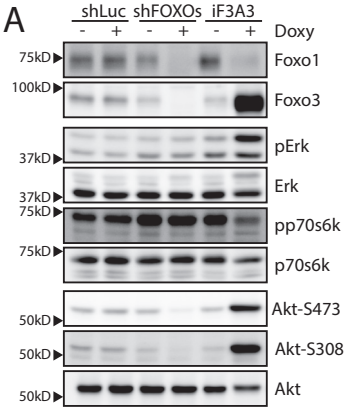
Tuning FOXO activity is essential for tumorigenesis

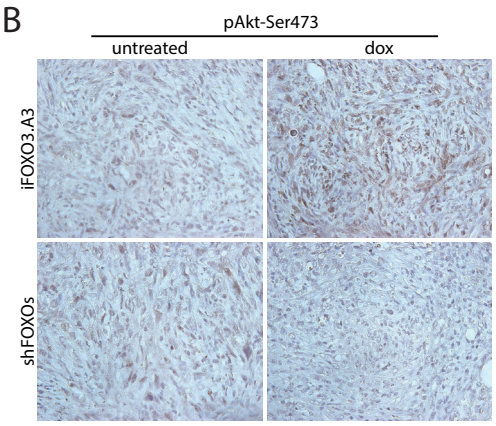
Our approach for the first time allowed determination of the effects of differential FOXO levels and activity in an orthotopic model of tumor growth and metastasis. Elevated FOXO3 levels had no effect on primary tumor growth but delayed metastasis formation. The expression of constitutively nuclear and hyperactive FOXO3.A3 strongly affected both primary tumor growth and metastasis, underlining that suppression of FOXO activity by AKT is required for tumor cells to proliferate and survive anchorage independent growth. These findings are well in line with previous studies that propose a tumor suppressive role for FOXOs based on its roles in cell cycle arrest, apoptosis and anoikis (2, 7, 34, 35, 45, 61, 80).

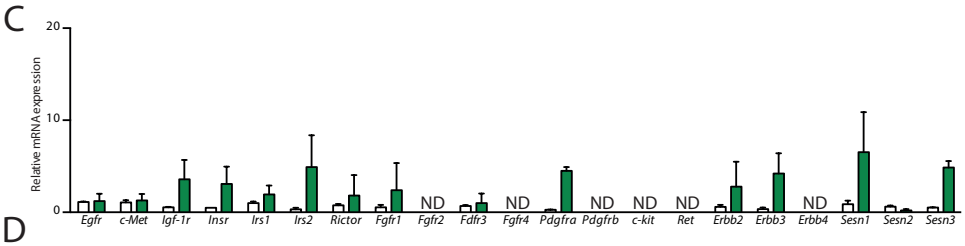
Induced loss of FOXO expression revealed that mILC1 cells are highly dependent on FOXOs for tumor growth and metastasis. These observations argue against a general role for FOXOs as tumor suppressors and show that endogenous FOXOs support cell proliferation and cellular homeostasis not only in normal cells but also in cancer cells. Previous studies already proposed that FOXOs support tumorigenesis by either facilitating cell invasion in breast and colon cancer cells or blocking differentiation in

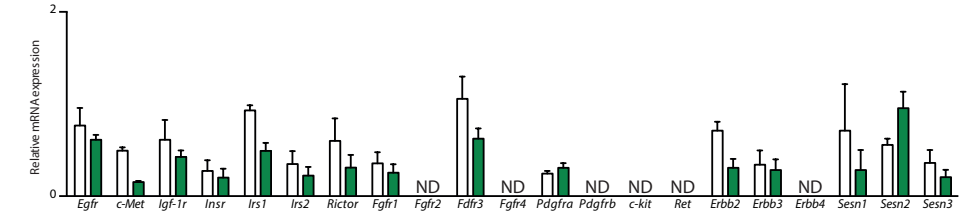
CML or AML (72, 73, 76, 77). We now show that FOXOs are involved in diverse aspects of tumor development simultaneously, including proliferation, invasion, anchorage independent growth and cellular energy & redox homeostasis.
In the paper by Paik and colleagues FOXOs are described as putative tumor suppressors due to the higher tumor incidence associated with the combined loss of Foxo1, Foxo3 & Foxo4 in mice (61). An interesting note to that study is that epithelial tumor incidence is not increased in Foxo triple knockout mice. This could be because early onset of different tumor types obscures epithelial tumorigenesis or because epithelial tumors rely more on the presence of FOXOs. The observation of a 23,1% decrease in spontaneous lung cancer incidence in Foxo3$^{-/-}$ mice treated with DMBA carcinogen insinuates a more tumor supportive role for FOXOs in epithelial cancers.
Except from the study by Paik and colleagues the paradigm of FOXOs acting as tumor suppressors is mainly build on ectopic over-expression of FOXO3.A3, activating FOXO in the context of PI3K-AKT inhibition or exposing cells to adverse conditions like excessive amounts of hydrogen peroxide. The subsequent cell cycle arrest and induction of apoptosis observed in cells involves FOXOs but whether endogenous FOXOs can do this in an endogenous context remains unanswered. Cells isolated from mILC1-FOXO3.A3 tumors showed strong selection against high expression of FOXO3.A3. However, the remaining cells retained the capability of expressing FOXO3.A3 at super-endogenous levels without suffering from reduced proliferation. This observation implicates that cells can deal with unnaturally high levels of FOXO activity to a certain extent, while still being able to proliferate and raises the question if fully active endogenous FOXO3 could inhibit proliferation without concurrent AKT inhibition. It is therefore temping to speculate that the technical aspects of how experiments on FOXOs are designed have obscured the essential role of endogenous FOXOs in cancer and led to the paradigm that FOXOs need to be repressed for cell proliferation.
Similar to FOXO3.A3 expression, tumors adapt to FOXO loss and regain their capacity to grow and metastasize. In contrast to FOXO3.A3, mILC1-shFOXO cells retain the efficient knockdown of both *Foxo1* and *Foxo3*. It might therefore be expected that cells adapt to FOXO loss by rewiring signaling in order to become FOXO independent through a yet unknown mechanism. This observation might also explain why FOXO knockout cells can be cultured and show mild phenotypes in the absence of adverse conditions, stressing the importance of conditional loss of function strategies to further elucidate FOXO function (19, 21, 24, 61, 76).

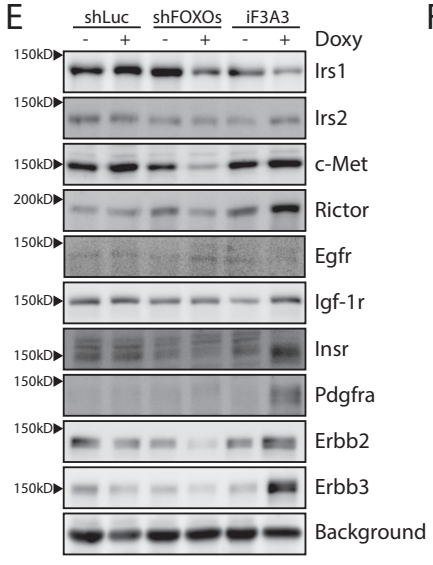
FOXOs facilitate anchorage independent survival
FOXOs have been reported to be involved in a plethora of processes essential for optimal cell function including proliferation, death, migration, redox homeostasis, bioenergetics, differentiation and lifespan (2, 375).
Others and we proposed a role for FOXOs in preventing anchorage independent survival (45, 80). The current study shows that hyper activation of FOXO3 inhibits metastasis *in vivo* and is in line with these previous observations. The reported metastasis stimulating effect of FOXO3.A3 in colon carcinoma cells was not observed in our system (72). These contradicting results illustrate that the output of FOXO activation differs between cell types and the concurrent signaling context in which FOXOs are activated. The differences between our study and that of Tenbaum and colleagues might be explained by the presence of high b-catenin levels in colon carcinoma cells compared to mILC, which represses FOXO3.A3 induced apoptosis (72, 79, 353).
We confirmed that loss of FOXOs correlates with reduced cell motility and invasion *in vitro* (77). Additionally, we show for the first time that conditional loss of FOXOs impairs the formation of metastasis *in vivo*. The exact reason by which FOXO loss impairs metastasis formation is hard to pinpoint due to its complex function in the cell. Attempts to rescue


A
shLuc shFOXOs iF3A3
- + - + - +
Doxy
75kD
Foxo1
100kD
Foxo3
37kD
pErk
37kD
Erk
75kD
pp70s6k
75kD
p70s6k
50kD
Akt-S473
50kD
Akt-S308
50kD
Akt


B
pAkt-Ser473
untreated
dox
iFOXO3.A3
shFOXOs


C
Relative mRNA expression
20
10
0
Egfr c-Met Igf-1r Insr Irs1 Irs2 Rictor Fgfr1 Fgfr2 Fdfr3 Fgfr4 Pdgfra Pdgfrb c-kit Ret Erbb2 Erbb3 Erbb4 Sesn1 Sesn2 Sesn3
ND


D
Relative mRNA expression
2
0
Egfr c-Met Igf-1r Insr Irs1 Irs2 Rictor Fgfr1 Fgfr2 Fdfr3 Fgfr4 Pdgfra Pdgfrb c-kit Ret Erbb2 Erbb3 Erbb4 Sesn1 Sesn2 Sesn3
ND


E
shLuc shFOXOs iF3A3
- + - + - +
Doxy
150kD
Irs1
150kD
Irs2
150kD
c-Met
200kD
Rictor
150kD
Egfr
150kD
Igf-1r
150kD
Insr
150kD
Pdgfra
150kD
Erbb2
150kD
Erbb3
Background

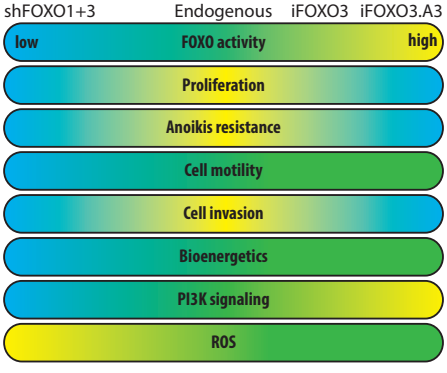

F
shFOXO1+3
Endogenous
iFOXO3
iFOXO3.A3
low
FOXO activity
high
Proliferation
Anoikis resistance
Cell motility
Cell invasion
Bioenergetics
PI3K signaling
ROS

Figure 5: FOXOs maintain PI3K pathway activity
A Western blot analysis of Foxo1, Foxo3, Erk, phosphor-Erk, Akt, Phospho-Akt-S308, phospo-Akt-Ser473, p70s6k and phosphor-p70s6k protein levels in mILC1-shLuc, mILC1-shFOXOs and mILC1-iFOXO3.A3 cells. mILC1-shluc and mILC1-shFOXOs cells were treated with doxycycline for 72 hours, mILC1-iFOXO3.A3 were treated with doxycycline for 16 hours. Total ERK, AKT and p70S6K levels were used to control for loading. **B** Immunohistochemical analysis of phospo-Akt S473 protein levels in mILC1-iFOXO3.A3 and mILC1-shFOXOs cells after treatment with doxycycline for 7 days. Shown are representative pictures of one tumor. **C** RT-qPCR of *Egfr, cMet, Igf1r, Insr, Irs1, Irs2, Rictor, Fgfr1, Fgfr2, Fgfr2, Fgfr3, Fgfr4, Pdgfra, Pdgfrb, c-Kit, Ret, Erbb2, Erbb3, Erbb4, Sesn1, Sesn2* and *Sesn3* mRNA expression levels in mILC1-shLuc, mILC1-shFOXOs and mILC1-iFOXO3. A3 cells. mILC1-shluc and mILC1-shFOXOs cells were treated with doxycycline for 72 hours, mILC1-iFOXO3. A3 were treated with doxycycline for 16 hours. Graph represents the average of 3 independent experiments ±SD, ND = not detected. **D** RT-qPCR of *Egfr, cMet, Igf1r, Insr, Irs1, Irs2, Rictor, Fgfr1, Fgfr2, Fgfr2, Fgfr3, Fgfr4, Pdgfra, Pdgfrb, c-Kit, Ret, Erbb2, Erbb3, Erbb4, Sesn1, Sesn2* and *Sesn3* mRNA expression levels in mILC1-iFOXO3.A3 after treatment with doxycycline for 16 hours. Graph represents the average of 3 independent experiments ±SD, ND = not detected. **E** Western blot analysis of Irs1, Irs2, cMet, Rictor, Egfr, Igf-1r, Insr, Pdgfra, Erbb2 and Erbb3 protein levels in mILC1-shLuc, mILC1-shFOXOs and mILC1-iFOXO3.A3 cells. mILC1-shluc and mILC1-shFOXOs cells were treated with doxycycline for 72 hours, mILC1-iFOXO3.A3 were treated with doxycycline for 16 hours. Aspecific background signal was used to control for loading. **F** A graphic model illustrating the consequences of differential FOXO activity in cancer cells.

this decrepit cell state with only antioxidants or growth factor signaling components is therefore insufficient as this likely requires more signaling and metabolic components and subtle tuning of FOXO activity is experimentally unattainable. Even though cancer cells primarily rely on the presence of FOXOs, cells can adapt to the absence of FOXOs through a yet unknown mechanism.

Targeting FOXOs in cancer
Inhibition of PI3K or AKT and the subsequent cell cycle arrest and apoptosis in cancer cells was the rationale for development of many different small molecule inhibitors targeting PI3K and AKT for cancer therapy. The effectiveness of these inhibitors in treating cancer is however limited due to the strong feedback response of cells, re-establishing PI3K/AKT activity (376). Induction of GFR-signaling components in response to PI3K/AKT inhibition is obstructed in the absence of FOXOs and FOXO3.A3 expression correlates with increased RICTOR and SESN3 expression and AKT activity (71, 94-96). We combined these observations and establish that FOXOs can indeed drive the expression of multiple components of GFR-signaling and regulate PI3K/AKT activity in mILC1. Complementary, we show that FOXOs are required for diverse aspects of cellular metabolism and that loss of FOXOs results in an overall more oxidative state. Our study emphasizes that FOXOs are essential for tumorigenesis and not operating in a binary fashion in which they are “ON” or “OFF” but rather in a gradual mode of operation in which activity is balanced to be “just right” in both healthy and cancer cells. The joined observation that loss of FOXOs is disadvantageous for tumor development, correlates with increased metabolic defects and reduces GFR-signaling, puts inhibiting FOXOs forward as an appealing strategy for cancer therapy, especially combined with blocking adaptation to FOXO loss.

Experimental Procedures

Cell culture
Mouse invasive lobular carcinoma cells mILC1 were derived from tumors that developed in female *K14cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mice and cultured as described previously (353). For doxycycline treatment 100 nM Doxycycline was used for the duration of indicated times. Antioxidant treatment was done by daily 200mM Trolox, 20mM EUK-134 or 1mM n-acetyl cysteine (NAC) treatment. 3D spheroid cultures are established by culturing

mILC1 cells in 20 ml drops containing 40% matrigel, 40% rat tail collagen I (1,5mg/ml final concentration)(Ibidi) and 20% culture medium containing 1000 cells/ml. For colony formation assays 2000 cells/well were plated and grown for 7 days in normal medium containing the indicated chemicals.

Constructs, lentiviral transduction and transfections
Third generation packaging vectors and HEK293T cells were used to generate lentiviral particles (377). Lentiviral cDNA expression vectors expressing FOXO3 and FOXO3.A3 were generated using Gateway cloning in the pINDUCER20 (Addgene #44012) dox-inducible expression system (371). Foxo1 and Foxo3 knockdown in mILC1 cells was achieved by lentiviral transduction of doxycycline inducible FH1tUTG carrying shRNA simultaneously targeting Foxo1 and Foxo3 (TRCN0000020707) (38, 378). The Lentiviral constructs pLV-CMV-PIK3CA-IRES-Puromycin, pLV-CMV-PIK3CA^{H1047R}-IRES-Puromycin, pCDH-myr-HA-Akt-puromycin (Addgene #46969) pLV-CMV-RICTOR-IRES-Hygromicin and pINDUCER20-GFP-FOXO3^{mt} were used to express PI3K, PI3K^{H1047R}, ^{myr}AKT, RICTOR and FOXO3^{mt} respectively.

Immunoblotting & antibodies
For western blot cells are lysed in sample buffer containing 0.2%SDS, 10% glycerol, 0.2% b-mercapto ethanol, 60mM Tris pH6.8. Protein concentrations were determined using Bradfort (Biorad) and equal concentrations of protein were used for Western blot. Proteins were detected using 6-15% SDS-PAGE gels and subsequent western-blot analysis with primary antibodies recognizing: BIM (CST-2819) BMF (ENZO-17A9), AKT/PKB (5178 (379)), FOXO1 (CST- 29H4), FOXO3 (H144 Santa Cruz), BCL2 (CST-2876), BCL-XL (CST-2764), PKB-S308 (CST-2965), PKB-S473 (CST-4060), ERK (SC-94), pERK (CST-4370), p70S6K (CST-2708), phospho-p70S6K (CST-9234), JNK (SC-1648), pJNK (CST-4668), cJUN (SC-1694), cJUN-S73 (CST-9164), p38 (CST-2387), p38-T180/183 (CST-9211), IRS1 (CST-2390), IRS2 (CST-4502), cMET (CST-3127), EGFR (CST-4267), ERBB2 (CST-2165), ERBB3 (CST-12708), INSR (CST-3025), IGF-1R (CST-9750), PDGFRA (CST-3174) and RICTOR (CST-2140), p27 (BD-610241), p21 (SC-471) used 1:2000. Primary antibodies were detected by secondary HRP conjugated antibodies targeting mouse, rabbit, and rat IgG and visualized using chemiluminescence (Biorad).

Orthotopic Transplantation assays
For tumorigenesis experiments, 10,000 mILC1-iFOXO3, mILC1-iFOXO3.A3 and mILC1-shFOXO cells were orthotopically transplanted in the 4th mouse mammary gland of Nude recipient mice as described previously (355). Primary tumours were allowed to develop to a volume of 50-100mm^3 at which point expression or knockdown of FOXOs was induced by feeding doxycycline-containing chow (230mg/kg) (Ssniff). Alternatively, FOXO expression or loss was induced 2 weeks after transplantation. Tumour volumes and lung metastases were followed in time as described using a Biospace φ imager and caliper (355). All animal experiments are approved by the Utrecht University Animal Experimental Committee (DEC-ABC no. 2012.III.12.135).

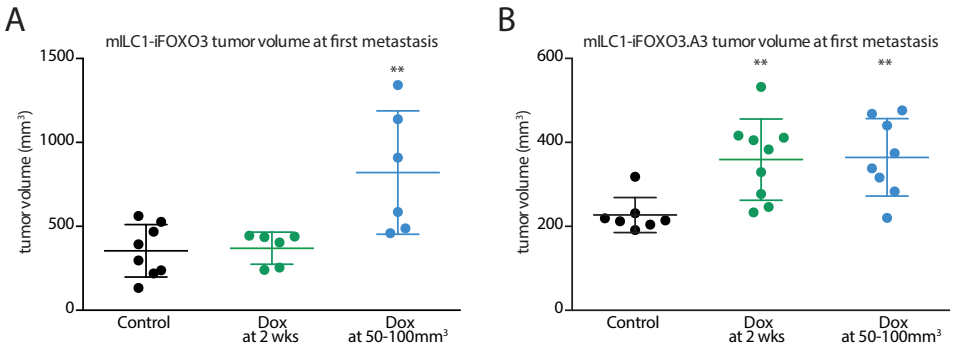
Immunohistochemistry
Mouse tissues were fixed in 4 % formaldehyde for 24 hours followed by dehydration in 70% ethanol and embedding in paraffin. Rehydrated slides were blocked for endogenous peroxidase activity in phosphate buffer (pH5.8) containing 1.5% hydrogen peroxide. Cooking samples for 20 minutes in 10mM citrate buffer (pH6) in a microwave was used for antigen retrieval. Primary and secondary HRP-conjugated antibodies were incubated overnight or 1 hour at 4°C respectively. Staining of slides was performed using diaminobenzidine (DAB) and hematoxylin.

RT-qPCR
mRNA was isolated from live cells using the Qiagen RNeasy kit (Qiagen) and cDNA synthesis was performed using the iScript cDNA synthesis kit (BioRad). Real-time PCR was performed using SYBR green FastStart master mix (Roche) in the CFX Connect Real-time PCR detection system (BioRad). Target genes were amplified using specific primer pairs (Supplemental table 1) and specificity was confirmed by analysis of the melting curves. Target gene expression levels were normalized to *GAPDH*, *PBDG* and *HRNPN1A* levels. Genomic DNA from tumors and cell lines was isolated using the DNeasy kit (Qiagen).

Flow cytometry
Anoikis was assessed by seeding 6-well ultra-low cluster polystyrene culture dishes (Corning) with 50,000 cells/ml. After 24 hours, cells were harvested and resuspended in 100mL of Annexin-V buffer supplemented with Annexin-V (IQ Products) and propidium iodide (Sigma-Aldrich). Anoikis was defined as the Annexin-V and propidium iodide–positive population and quantified using a FACSCalibur (BD Biosciences). For assessing glucose uptake cells were treated win 10mM of 2-NBDG for 1 hour prior to trypsinization and cytometric analysis. For assessing oxidative stress, cells were treated for 30 minutes with 5mM CellRox deep red prior to trypsinization and cytometric analysis.

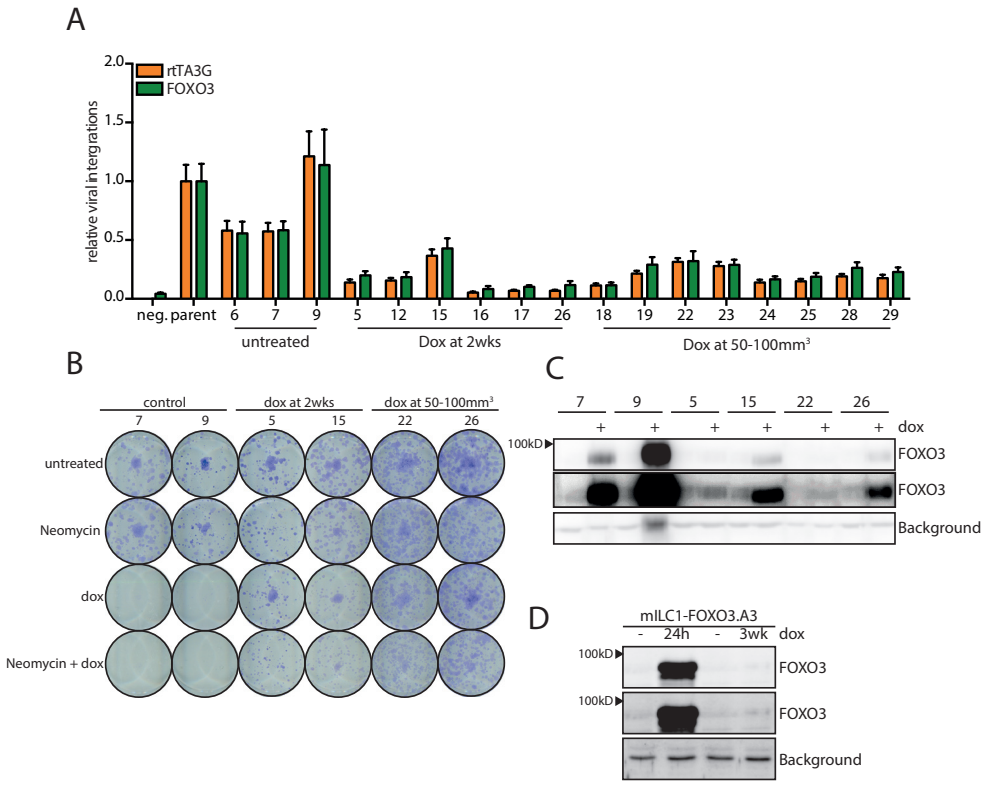
Live cell imaging and tracking
10.000 mILC1-shFOXOs cells were cultured in Lab-Tek II 8-well imaging chambers and treated with doxycycline 72 hours prior to imaging. mILC1-iFOXO3.A3 cells were treated with doxycycline 8 hours prior to imaging. DIC imaging was performed on a Olympus Real-Time imager microscope for 48 hours at 37°C and 5% CO_2. Cell tracking and quantification of cell migration was performed using ImageJ.

Seahorse & $NADP^+$/NADPH measurements
Seahorse XF-24 technology was used to determine glycolytic activity (ECAR) and mitochondrial respiration (OCR) in mILC1-shFOXO cells. 10.000 mILC1-shFOXO cells were seeded in poly-lysine coated Seahorse XF-24 plates (Seahorse Bioscience) and cultured in the presence or absence of doxycycline. For ECAR the culture medium was replaced with Seahorse XF base medium (Seahorse Bioscience) supplemented with 2mM L-glutamin and, 0.56mM NAOH 1 hour prior to measurements after which the cells were sequentially treated with 17.5mM Glucose, 8mM Oligomycin and 100mM 2-deoxy-glucose. For OCR culture medium was replaced with Seahorse XF base medium supplemented with 17.5mM Glucose, 5 mM Pyruvate, 2mM L-glutamin and, 0.56mM NAOH 1 hour prior to measurements after which the cells were sequentially treated with 8 mM oligomycine, 18mM FCCP and 2mM Rotenone & Antimycine A. OCR and ECAR were normalized based on protein levels.
NADP+/NADPH ratio was determined using Amplite™ Fluorimetric NADP/NADPH Ratio Assay Kit (AAT Bioquest).

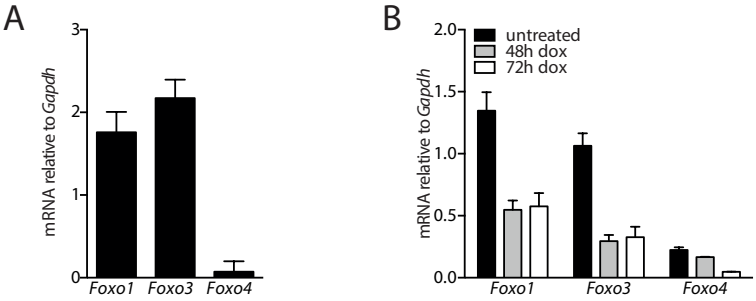


Supplemental figure 1: Average tumor size upon detection of metastasis in mILC1-iFOXO3 and mILC1-iFOXO3.A3

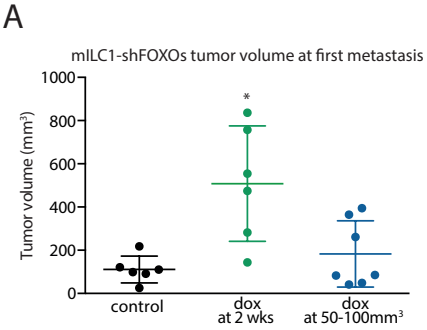
A Primary tumor volume (mm^3) of mILC1-iFOXO3 cells treated with when metastases were first detected by bioluminescent imaging. Black dots represent control tumors (n=8), green dots represent tumors treated with doxycycline 2 weeks after transplantation (n=6) and blue dots represent tumors treated with doxycycline at tumor sizes 50-100mm^3 (n=6). Graph represents the average ±SD, t-test $p<0.05$=* **B** The average primary tumor volume (mm^3) of mILC1-iFOXO3.A3 cells treated with when metastases were first detected by bioluminescent imaging. Black dots represent control tumors (n=8), green dots represent tumors treated with doxycycline 2 weeks after transplantation (n=6), blue dots represent tumors treated with doxycycline at tumor size 50-100mm^3. Graph represents the average ±SD, t-test $p<0.005$=**



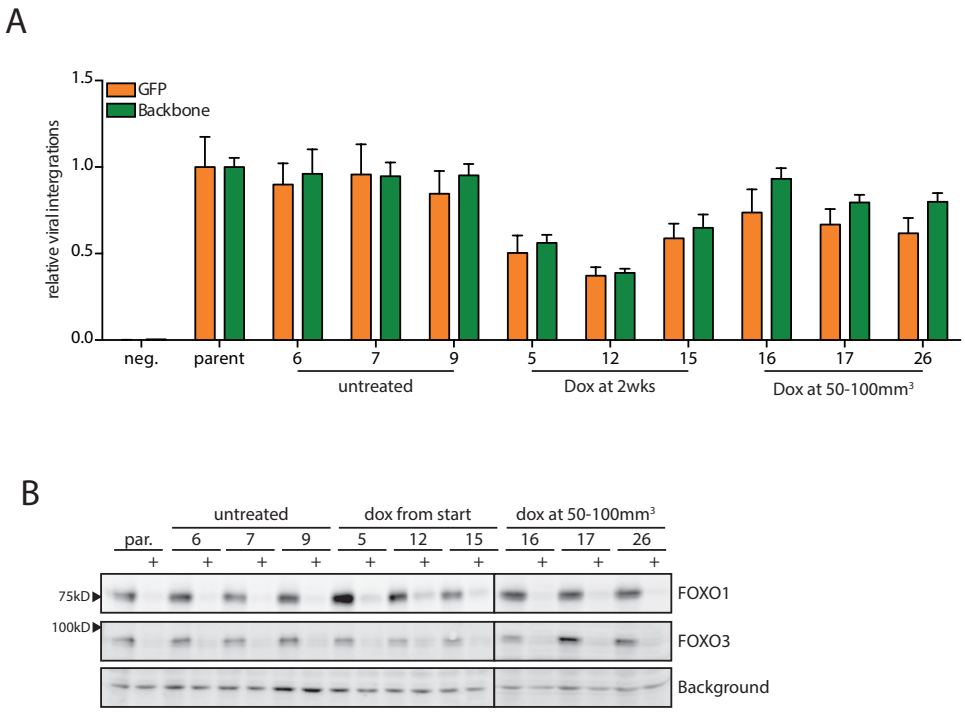
Supplemental figure 2: negative selection of FOXO3.A3 cells
A Quantitative PCR analysis of genomic DNA for pINDUCER20-FOXO3.A3 viral integrations in tumor cells isolated from tumors of mILC1-iFOXO3.A3 control, treated with doxycycline 2 weeks after transplantation and treated with doxycycline at tumor volumes of 50-100mm^3 groups. Orange bars represent relative integration levels of the rtTA3G gene contained in the pINDUCER20 construct backbone. Green bars represent relative integration levels of the FOXO3.A3 cDNA contained in the pINDUCER20-iFOXO3.A3 construct. **B** Colony formation capacity of tumor cells isolated from control, doxycycline 2 weeks after transplantation and doxycycline at tumor volume 50-100mm^3 groups, cultured in the presence of neomycin, doxycycline or neomycin and doxycycline for 7 days. **C** Western blot analysis of FOXO3 protein levels in corresponding tumor cells after treatment with doxycycline for 24 hours. Two different exposures of the FOXO3 blot are shown. Aspecific background signal was used to control for loading. **D** Western blot analysis of FOXO3 protein levels in mILC1-iFOXO3.A3 cells cultured in the presence of doxycycline for 24 hours or 3 weeks. Two different exposures of the FOXO3 blot are shown. Aspecific background signal was used to control for loading.



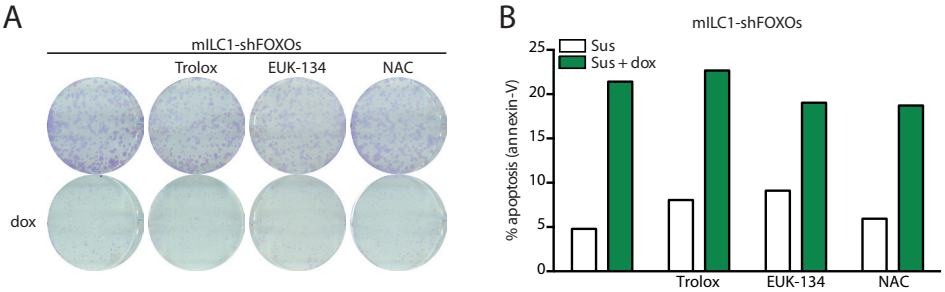
Supplemental figure 3: *Foxo1*, *Foxo3* and *Foxo4* expression levels in mILC1
A RT-qPCR analysis of *Foxo1, Foxo3* and *Foxo4* mRNA expression in mILC1 cells. Graph represents the average of 3 experiments ±SD, mRNA levels are relative to *Gapdh* mRNA levels. **B** RT-qPCR analysis of *Foxo1, Foxo3* and *Foxo4* mRNA expression after shFOXOs induction in mILC1-shFOXOs cells. Graph is a representative experiment, average of 3 replicates ±SD, mRNA levels are relative to *Gapdh* mRNA levels.



Supplemental figure 4: Average tumor size upon detection of metastasis in mILC1-shFOXOs
A Primary tumor volume (mm^3) of mILC1-shFOXOs cells treated with when metastases were first detected by bioluminescent imaging. Black dots represent control tumors (n=6), green dots represent tumors treated with doxycycline 2 weeks after transplantation (n=6) and blue dots represent tumors treated with doxycycline at tumor sizes 50-100mm^3 (n=7). Graph represents the average ±SD, t-test $p<0.05$=*



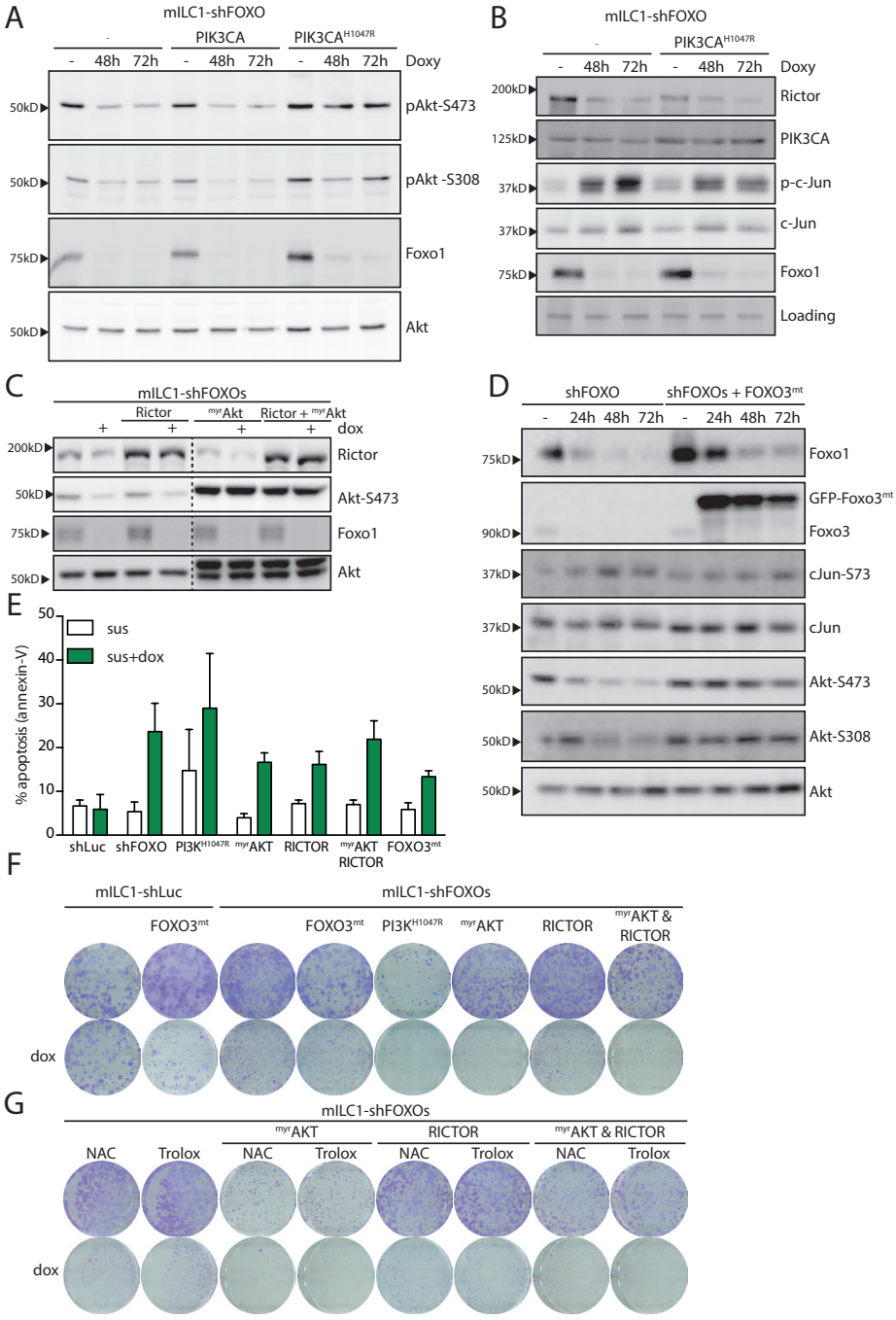
Supplemental figure 5: Adaptation of mILC1-shFOXOs cells to loss of Foxo1 and Foxo3 expression
A Quantitative PCR analysis of genomic DNA for viral integrations of FH1tUTG-shFOXOs in tumor cells isolated from tumors of mILC1-shFOXOs control, treated with doxycycline 2 weeks after transplantation and treated with doxycycline at tumor volumes of 50-100mm^3 groups. Orange bars represent relative integration levels of the GFP gene contained in the FH1tUTG construct backbone. Green bars represent relative integration levels of the shFOXO location contained in the FH1tUTG-shFOXOs construct. **B** Western blot analysis of Foxo1 and Foxo3 protein levels in corresponding tumor cells after treatment with doxycycline for 72 hours. Aspecific background signal was used to control for loading.



Supplemental figure 6: Antioxidant treatment of mILC1-shFOXOs does not rescue cell proliferation or anoikis resistance
A Colony forming capacity of mILC1-shFOXOs cells treated with doxycycline for 7 days and in combination with 200mM Trolox, 20mM Euk-134 or 1mM n-acetyl cysteine (NAC). **B** Flow cytometric analysis of mILC1-shFOXOs cells cultured in suspension for 16 hours, after 72 hour pretreatment with doxycycline or in combination with 200mM Trolox, 20mM Euk-134 or 1mM NAC, stained for stained for Annexin-V and PI. Shown is a representative experiment.

4

Supplemental figure 7: Restoring GFR-signaling in mILC1-shFOXOs cells is insufficient to rescue cell proliferation and stress kinase induction.
A Western blot analysis of Akt, Akt-S308, Akt-S473 and Foxo1 protein levels in mILC1-shFOXOs cell constitutively expressing PIK3CA or oncogenic $PIK3CA^{H1047R}$. Total Akt levels were used to control for loading. **B** Western blot analysis of Rictor, PIK3CA, cJun, phospho-cJun and Foxo1 protein levels in mILC1-shFOXOs cell constitutively expressing oncogenic $PIK3CA^{H1047R}$. Aspecific background signal was used to control for loading. **C** Western blot analysis of Rictor, Akt, Akt-S473 and Foxo1 protein levels in cells constitutively expressing RICTOR, myristoylated AKT (^{myr}AKT) or both RICTOR and ^{myr}AKT. Total Akt levels were used to control for loading. **D** Western blot analysis of Foxo1, Foxo3, cJun, phospo-cJun, Akt, Akt-S308 and Akt-S473 protein levels in mILC1-shFOXOs co-expressing a hairpin insensitive GFP-FOXO3 ($FOXO3^{mt}$). Total Akt levels were used to control for loading. **E** Flow cytometric analysis of Annexin-V and PI stained mILC1-shFOXOs constitutively expressing $PIK3CA^{H1047R}$, ^{myr}AKT, RICTOR, ^{myr}AKT & RICTOR or $FOXO3^{mt}$ cultured in suspension in the presence of doxycycline for 16 hours. **F** Colony forming capacity of mILC1-shFOXOs constitutively expressing $PIK3CA^{H1047R}$, ^{myr}AKT, RICTOR, ^{myr}AKT & RICTOR or $FOXO3^{mt}$ cultured in suspension in the presence of doxycycline for 7 days. **G** Colony forming capacity of mILC1-shFOXOs constitutively expressing ^{myr}AKT, RICTOR or ^{myr}AKT & RICTOR cultured in the presence of doxycycline for 7 days in combination with 1mM NAC or 200mM Trolox.


A
mILC1-shFOXO
PIK3CA
PIK3CA H1047R
- 48h 72h - 48h 72h - 48h 72h
Doxy
50kD
pAkt-S473
50kD
pAkt -S308
75kD
Foxo1
50kD
Akt
B
mILC1-shFOXO
PIK3CA H1047R
- 48h 72h - 48h 72h
Doxy
200kD
Rictor
125kD
PIK3CA
37kD
p-c-Jun
37kD
c-Jun
75kD
Foxo1
Loading
C
mILC1-shFOXOs
Rictor
myrAkt
Rictor + myrAkt
+ + + +
dox
200kD
Rictor
50kD
Akt-S473
75kD
Foxo1
50kD
Akt
D
shFOXO
shFOXOs + FOXO3mt
- 24h 48h 72h - 24h 48h 72h
75kD
Foxo1
GFP-Foxo3mt
90kD
Foxo3
37kD
cJun-S73
37kD
cJun
50kD
Akt-S473
50kD
Akt-S308
50kD
Akt
E
% apoptosis (annexin-V)
sus
sus+dox
0
10
20
30
40
50
shLuc
shFOXO
PI3KH1047R
myrAKT
RICTOR
myrAKT RICTOR
FOXO3mt
F
mILC1-shLuc
mILC1-shFOXOs
FOXO3mt
FOXO3mt
PI3KH1047R
myrAKT
RICTOR
myrAKT & RICTOR
dox
G
mILC1-shFOXOs
myrAKT
RICTOR
myrAKT & RICTOR
NAC
Trolox
NAC
Trolox
NAC
Trolox
NAC
Trolox
dox

Chapter 5:

A cancer tissue mRNA-based diagnostic test for PI3K activity designed from a computational pathway model predicting FOXO3 activity

H. van Ooijen[1#], M. Hornsveld[2#], C. Dam[1], A. Eijkelenboom[2], R. Velter[1], W. Verhaegh[1], B.M.T. Burgering[2#] & A. van de Stolpe[1#]

[1]Molecular diagnostics, Philips Research, Eindhoven

[2]Department of Molecular Cancer Research, University Medical Center Utrecht, Utrecht

[#]These authors contributed equally to this work

5

* This chapter only presents a limited part of the data due to an ongoing patent filing procedure*

Manuscript in preparation

5

Abstract
The PI3K signaling pathway is commonly hyper activated in a wide variety of cancers. Tumors with active PI3K signaling are potentially sensitive to PI3K pathway inhibitors but reliable diagnostic tests quickly assessing PI3K activity in patients lack. We developed a knowledge-based Bayesian computational model interpreting tissue-derived FOXO3 target gene mRNA levels. As FOXO activity is inversely correlated with PI3K activity we apply this model to determine PI3K activity in cancer samples. FOXO and PI3K activity was correctly predicted in MCF7 and MDA-MB-231 cells expressing hyperactive FOXO3. The model was used to deduct FOXO and PI3K activity in colorectal cancer patient tissue samples and predicted low PI3K activity in healthy tissue and high PI3K in cancer tissues, correlating to FOXO3 localization in healthy and colon cancer tissues. Strikingly, FOXO was predicted to be active in a subset of colorectal cancer and various normal-like, Luminal A, Luminal B, HER2 positive and triple negative breast cancer samples. This suggests that alternative pathways even in the presence of growth factor signaling activate FOXOs. The test will be further refined using this knowledge in the future and clinically validated for multiple cancer types. The final model can than be used in efforts to improve diagnosis of the mutational status of patient tumors, prediction of therapy response to PI3K pathway targeted drugs and prediction of prognosis on an individual patient basis.

Introduction

The past decade treatment of cancer has moved from conventional chemotherapy towards administration of targeted drugs directed at patient-specific tumor traits. This "precision medicine" approach requires biomarker tests that reliably predict the response to a specific targeted therapy (380). Such tests need to assess relevant molecular aspects of the tumor, specifically the mechanism driving cancer progression. Cancer progression can be driven by roughly ten to twelve cellular signal transduction pathways, and this can be relatively independent of the tissue (1, 381, 382). The PI3K pathway, one of the main cellular growth factor signaling pathways, is commonly hyper activated in cancer. The PI3K pathway can be activated through multiple aberrations in the cancer genome e.g. amplification of receptor tyrosine kinases like *HER2*, loss of lipid phosphatase *PTEN* and activating mutations in the *PIK3CA* gene that encodes the catalytic subunit of PI3K (381).

Small molecule inhibitors targeting PI3K signaling are applied in cancer treatment, for example for the treatment of HER2-driven breast cancer and hematological malignancies like CML (383). Additionally, PI3K pathway inhibitors are being used in combination with other targeted strategies like endocrine therapy in breast cancer to prevent emergence of therapy resistance (376, 384). Despite selection of potentially responsive patients based on identification of PI3K pathway mutations in their tumors, in general only a subpopulation of patients responds adequately to the drugs (385, 386). This shows that changes at the cancer genome level do not reliably predict functional PI3K pathway activity and tests assessing PI3K activity more directly are needed to improve patient treatment strategies. Such tests would also be useful to monitor therapy response and emerging drug resistance during the course of treatment.

We have previously described a novel computational approach to assess signal transduction pathway activity in cancer tissue samples. Pathway activity can be inferred by modeling of mRNA expression profile data sets in which mRNA expression changes are mediated by specific signal transduction cascades, for example by WNT or estrogen receptor (ER) signaling (387, 388) (van Ooijen 2016 submitted).

Here we report the development of an mRNA-based diagnostic test for the quantitative assessment of PI3K pathway activity. This test is based on the computational modeling principle mentioned above using Forkhead Box O (FOXO) transcription factor mediated transcription as readout. The FOXO family includes the highly redundant FOXO1, FOXO3, FOXO4 and FOXO6 (FOXO) transcription factors in humans, which operate down stream of PI3K-PKB signaling (2). In the presence of growth factor signaling FOXO is phosphorylated by PKB and reside inactively in the cytoplasm. Upon loss of growth factor signaling FOXO translocates to and accumulate in the nucleus where they mediate transcription of a plethora of target genes (7, 8, 11, 389). Oncogenic activation of the PI3K pathway in cancer cells results in FOXO inactivation and hence blocking of the FOXO transcriptional program. In principle a simple inverse relationship exists between FOXO activity and PI3K pathway activity and therefore we reasoned that FOXO target gene expression levels could be used as readout for PI3K pathway activity. Here we describe how knowledge on direct FOXO3 target genes can be used to create a Bayesian network model assessing the probability that FOXOs are in a transcriptionally active state based on measured target gene expression levels. We determined a core set of FOXO target genes that robustly predict PI3K activity using publicly available data sets combined with mRNA profiling of MCF7 and MDA-MB-231 breast cancer cell lines treated with PI3K inhibitor or carrying doxycycline inducible hyper active FOXO3 mutant (FOXO3.A3) constructs. We then validated the predictive value of this core set of FOXO target genes on patient material.

Methods

Cell culture-based model system for FOXO3 activity

MCF7 and MDA-MB-231 breast cancer cell lines were cultured in DMEM-F12 containing 10% FBS (Lonza) 100U/ml penicillin and 100mg/ml streptomycin (Lonza). Third generation packaging vectors were used to generate lentiviral particles in HEK293T cells (377). MCF7 and MDA-MB-231 cells were stably transduced with lentivirus containing pINDUCER20-FOXO3.A3, allowing for doxycycline induced expression of hyper active FOXO3 (FOXO3.A3) (7, 45, 371). MCF7 and MDA-MB-231 cells were treated with 20% FBS (Lonza) or 10mM PI3K inhibitor LY294002 (Selleckchem) for 16 hours to activate and inactivate the endogenous PI3K pathway, respectively. FOXO3.A3 expression was induced by 16 hours treatment with 100ng/ml doxycycline.

RNA isolation and Affymetrix microarray analysis

Control cells or cells treated with doxycycline, 20%FBS, LY294002 and LY294002 plus doxycycline were harvested after 16 hours of the respective incubations, and processed for RNA isolation using the RNeasy kit (Qiagen). Affymetrix HG-U133Plus2.0 microarrays were used for mRNA hybridization and processed by ServiceXS (Leiden, The Netherlands).

Western Blotting

Western blot analysis was performed using standard 6-15% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked using TBS containing 0.01% tween20 and 2% BSA for 30 minutes, followed by incubation with primary rabbit antibodies (1:2000) raised against FOXO3 (H144 Santa Cruz) at 4°C for 16 hours. Next, blots were incubated with HRP-conjugated secondary antibodies at 4°C for 30 minutes and proteins were visualized with enhanced chemiluminescence (Biorad) using a ImageQuant LAS 4000 scanner (GE healthcare).

Immunofluorescence and immunohistochemistry

For immunofluorescence, cells were grown on glass coverslips, fixed using 4% paraformaldehyde and blocked with PBS containing 2% bovine serum albumin (BSA) (Invitrogen) and 0.1% normal goat serum (Invitrogen). Cells were incubated with FOXO3 antibody (1:500 CST-75D8), secondary Alexa563 conjugated antibodies and DAPI (Sigma). Slides were imaged on a Zeiss LSM710 confocal microscope. For immunohistochemistry rehydrated paraffin sections were blocked for endogenous peroxidase activity in phosphate buffer (pH5.8) containing 1.5% hydrogen peroxide. For antigen retrieval samples were boiled ,using a microwave, for 20 minutes in 10mM citrate buffer (pH6) in. Primary FOXO3 (CST-75D8) and secondary HRP-conjugated antibodies were incubated overnight or 1 hour at 4°C respectively. HRP-conjugated antibodies were visualized using diaminobenzidine (DAB) and sections were counterstained with hematoxylin.

Development of the computational model for predicting FOXO3 activity

Our signal transduction pathway modeling approach is based on inferring pathway activity from the expression profiles using probabilistic Bayesian network inference, as previously described (388). Bayesian networks were built using the Bayes Net Toolbox for MATLAB. The model describes how expression of target genes depends on transcription complex activation and how probeset intensities in turn depend on expression of the respective target genes. Probeset intensities were taken from fRMA preprocessed Affymetrix HG-U133Plus2.0 microarrays available from Gene Expression Omnibus (GEO, www.ncbi.nlm.nih.gov/geo) and ArrayExpress (http://www.ebi.ac.uk/arrayexpress). The probabilistic relations in the Bayesian network model were

made quantitative using experimental data, enabling probabilistic reasoning on new experimental samples. Parameters describing relationships between target genes and their respective probe sets were trained on a publicly available data set of Human Umbilical Vein Endothelial Cells (HUVEC) carrying inducible hyperactive FOXO3.A3-ER (GSE16573) (390). Parameters enforcing the relationships between the transcription complex and target genes were manually set as described to improve generalization behavior of the model across different tissue types (388). PI3K pathway analysis for colon samples was performed on publicly available datasets GSE20916 and GSE8671.
For GSE17907 and GSE12276 breast cancer patients sets, the intrinsic breast cancer subtypes of included samples were determined from the microarray data according to the method described by Parker and co-workers (391) based on earlier work of Perou and co-workers (392). In short, this method calculates the distance between a sample and the centroids of the intrinsic subtypes using 50 genes in a representative training set using correlation, also known as the PAM50 algorithm. The intrinsic subtype characterisation of the GSE21653 dataset, as given by Sabatier et al., was used to determine the centroids of the intrinsic subtypes (393). PI3K pathway activity was subsequently inferred for the specific breast cancer subtypes.

Results

Selection of direct target genes for FOXO

Activity of the PI3K pathway can be inferred from FOXO3 activity as PI3K activity is inversely correlated with FOXO transcriptional activity. Development of a Bayesian network-based computational model for the PI3K-FOXO pathway is knowledge-based, therefore knowledge on FOXO target genes and availability of cell-culture-based model systems in which the pathway can be turned "ON" and "OFF" in a controlled manner are required. For optimal performance across multiple different tissue types, the mathematical model should contain direct target genes of the FOXO transcription factor. Unfortunately, pathway databases such as KEGG (www.genome.jp/kegg) and Biocarta (www.biocarta.com) are incomplete and inconsistent on this aspect (394). Hence, we manually selected target genes based on scientific evidence for each individual gene being a direct target gene of FOXO. We extensively evaluated available literature retrieved from the MEDLINE database using PubMed for FOXO target genes. In addition, target genes were extracted from Thomson-Reuters' Metacore by selecting only genes that had multiple sources of reliable evidence for being transcriptionally regulated by one or more of the FOXO family members. Ultimately, the target genes were ranked according to literature evidence using a similar methodology as described earlier (388). Only the highest ranked target genes, also included in the list published by van der Vos & Coffer or Webb & Brunet were selected as direct target genes (389, 395)(Table 1).

Computational model for FOXO activity in untransformed cells

The computational FOXO pathway model was built using the selected FOXO target genes and initially calibrated on a public GEO dataset (GSE16573), consisting of mRNA expression data from human umbilical vein endothelial cells (HUVEC) stably transfected with tamoxifen (4OHT) inducible FOXO3.A3-ER (390). HUVECs were used to provide the "ground truth" evidence for FOXO activity status in an untransformed setting. HUVEC and HUVEC-FOXO3.A3-ER in which FOXO3.A3 activity was induced with 4OHT were used for training purposes, including refining the list of target genes for the model. A second group, HUVEC treated with 4OHT, was used to calibrate our model. The probability of the PI3K pathway being active is inversely correlated with the predicted probability of the FOXO transcription factor being in the active transcribing state. This probability can be translated into odds of the transcription factor being active by taking the ratio of the probability of being active versus inactive (i.e. odds are given by p/(1-p)

Gene	Affymatrix probeset	References
AGRP	207193_at	(405, 406)
BCL2L11	1553096_s_at/1555372_at/ 1558143_a_at/ 208536_s_at/ 222343_at/ 225606_at	(407, 408)
BCL6	203140_at/ 215990_s_at	(75, 409)
BNIP3	201848_s_at/ 201849_at	(410, 411)
BTG1	1559975_at/ 200920_s_at/ 200921_s_at	(412)
CAT	201432_at/ 211922_s_at/ 215573_at	(413)
CAV1	203065_s_at/ 212097_at	(414, 415)
CCND1	208711_s_at/ 208712_at/ 214019_at	(416)
CCND2	200951_s_at/ 200952_s_at/ 200953_s_at/ 231259_s_at	(416)
CCNG2	1555056_at/ 202769_at/ 202770_s_at/ 211559_s_at/ 228081_at	(417, 418)
CDKN1A	1555186_at/ 202284_s_at	(419, 420)
CDKN1B	209112_at	(35, 185, 421)
ESR1	205225_at/ 211233_x_at/ 211234_x_at/ 211235_s_at/ 211627_x_at/ 215551_at/ 215552_s_at/ 217190_x_at/ 207672_at	(422)
FASLG	210865_at/ 211333_s_at	(7, 423)
FBXO32	225801_at/ 225803_at/ 225345_s_at/ 225328_at	(424)
GADD45A	203725_at	(425, 426)
INSR	207851_s_at/ 213792_s_at/ 226212_s_at/ 226216_at/ 226450_at	(92)
MXI1	202364_at	(427)
NOS3	205581_s_at	(428)
PCK1	208383_s_at	(429)
POMC	205720_at	(405, 430)
PPARGC1A	1569141_a_at/ 219195_at	(431)
PRDX3	201619_at/ 209766_at	(432)
RBL2	212331_at/ 212332_at	(36, 417)
SOD2	215078_at/ 215223_s_at/ 216841_s_at/ 221477_s_at	(89)
TNFSF10	202687_s_at/ 202688_at/ 214329_x_at	(433)

Table 1: Selected FOXO target genes

if p is the predicted probability of being active). Samples are classified as FOXO active in case the transcription factor activity exceeds the odds of 1 to 1 that the pathway is active and inactive in case the odds are 1 to 1 or less (Figure 1A).
The FOXO activity status predicted by the Baysian-model were consistent with the experimental FOXO activity status (Figure 1B). Vehicle treated HUVEC, HUVEC treated with 4OHT and vehicle treated HUVEC-FOXO3.A3-ER were predicted to have low FOXO activity and therefore active PI3K signaling, as expected in proliferating cells. HUVEC-FOXO3.A3-ER treated with 4OHT are predicted to have highly active FOXO, in line with the induction of FOXO3.A3. HUVEC expressing FOXO3.A3-ER-H212R, a mutant version of FOXO3 with impaired DNA binding capacity, showed no predicted FOXO activity in untreated cells and low FOXO3 activity in cells treated with 4OHT. This observation confirms the model specifically detects transcriptional changes induced by FOXOs and is sensitive as already very low levels of FOXO activity are identified. The residual transcription inducing capacity of the FOXO3-H212R mutant is expected to be the consequence of the remaining low affinity for FOXO target genes as reported previously (Figure 1B) (390).

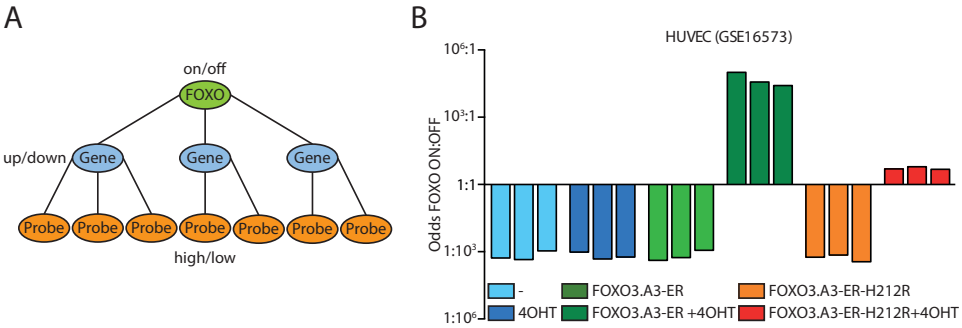


Figure 1: A Bayesian computational model predicting FOXO activity
A. A simplified model of the Bayesian network structure used as a basis for our modelling approach. The network consists of three types of nodes: the transcription factor which can be on or off, target genes which can be up or down and corresponding microarray probe sets of which the intensity can be high or low. **B.** Training of the computational FOXO3 model on a public GEO dataset GSE16573, consisting of Affymetrix microarray 2.0 Plus expression data from HUVEC containing a 4OHT-inducible FOXO3.A3-ER expression construct. Each bar represents a sample analysis result. The vertical axis indicates the probability that FOXO is "active" (values above the horizontal axis) versus "inactive" (values below the horizontal axis).

Accurate prediction of FOXO activity in breast cancer cell lines

To follow-up on the establishment and calibration of the computational model we set out to validate the model in independent breast cancer cell lines. ER positive, $PI3K^{E545K}$ mutant MCF7 and triple negative MDA-MB-231 cells were stably transduced with a doxycycline inducible FOXO3.A3 expression vector, allowing rapid and controlled induction of FOXO3.A3. Strong induction of FOXO3.A3 was observed upon treatment with doxycycline for 16 hours (Figure 2A). Endogenous FOXO3 was found predominantly in the cytoplasm in untreated and 20%FBS stimulated MCF7 cells and nuclear localization was observed in doxycycline, PI3K inhibitor LY294002, and doxycycline in combination with LY294002 treated cells (Figure2B). Endogenous FOXO3 in MDA-MB-231 cells was mainly localized in the nucleus even in untreated conditions, but a more pronounced nuclear FOXO3 localization was observed in cells treated with doxycycline, LY294002 and doxycycline combined with LY294002. Combined these results show that we can activate FOXO3 in a controlled manner, making these cell lines a suitable test case for our computational model.

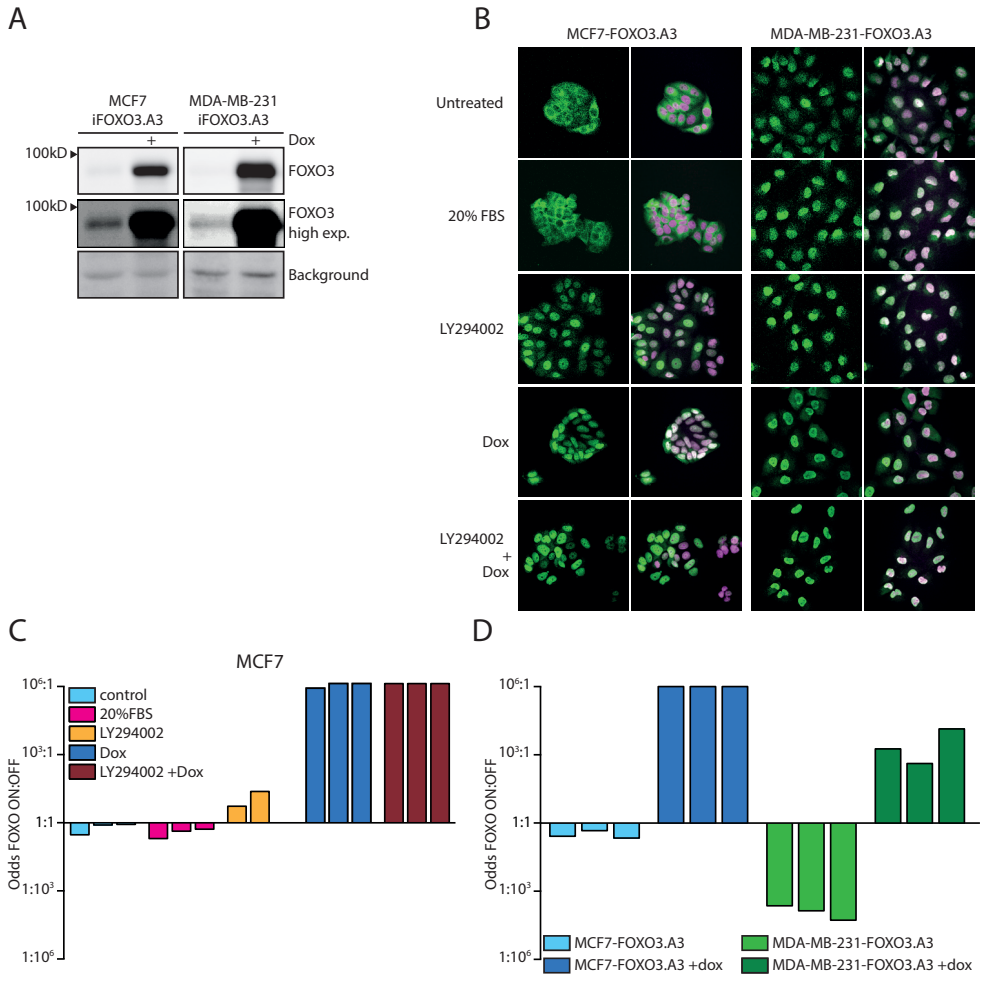


Figure 2: FOXO and PI3K activity are correctly predicted in breast cancer cell lines
A. Western blot analysis of FOXO3 expression levels in MCF7-FOXO3.A3 and MDA-MB-231 cells cultured in the absence or presence of doxycycline (dox) for 16 hours. The lower FOXO3 blot represents a longer exposure of the same blot. Aspecific background signal ws used to control for loading. **B.** Immunofluorescence staining of FOXO3 (green) and DNA (pink) in MCF7-FOXO3.A3 and MDA-MB-231-FOXO3.A3 cells treated with 20% FBS, PI3K inhibitor LY294002, doxycycline or a combination of doxycycline and LY294002 for 16 hours. **C.** Validation of the computational FOXO3 model using MCF7-FOXO3.A3 cells treated with 20% FBS, PI3K inhibitor LY294002, doxycycline or a combination of doxycycline and LY294002 for 16 hours. Each bar represents the analysis results of one sample. The vertical axis indicates the probability that the FOXO3 is "active" (values above the horizontal axis) versus "inactive" (values below the horizontal axis. **D.** Validation of the computational FOXO3 model using MCF7-FOXO3.A3 and MDA-MB-231 cells treated with doxycycline for 16 hours. Each bar represents the analysis results of one sample. The vertical axis indicates the probability that the FOXO3 is "active" (values above the horizontal axis) versus "inactive" (values below the horizontal axis).

Subsequently, we generated microarray (Affymetrix HG-U133Plus2.0) mRNA expression profile data sets from the MCF7-FOXO3.A3 cells treated with 20%FBS, LY294002, doxycycline and a combination of LY294002 and doxycycline to biologically validate the PI3K pathway model. The odds FOXOs are active were predicted to be low in untreated cells or MCF7 treated with 20%FBS. Conversely, the probability that FOXOs are active was predicted to be high in cells treated with doxycycline, LY294002 and combined treatment with doxycycline and LY294002 (Figure 2C). These results are in line with the expected localization and corresponding activity of FOXO. Newly generated microarray mRNA expression profiles for both MCF-FOXO3.A3 and MDA-MB-231-FOXO3.A3 that were either untreated or treated with doxycycline for 16 hours were used to reproduce the prediction and validate the model in MDA-MB-231-FOXO3.A3 cells. In line with the first experiment the probability that FOXOs are active in untreated MCF7 and MDA-MB-231 cells was predicted to be low and predicted to be high in doxycycline treated cells (Figure 2D). Together these results confirm that the generated Bayesian-network model based on preselected FOXO target genes and trained in an untransformed cell culture-based system can accurately predict FOXO activity in independent cancer cell line samples.

Identification of FOXO3 activity in clinical colon tissue samples

A number of selected public datasets (GSE8671 & GSE20916) with Affymetrix 2.0 Plus data were used to validate the computational model predicting FOXO activity (396, 397). First we applied the FOXO activity model on tissue samples derived from 32 patient biopsies of both normal colon tissue and adenoma tissue (GSE8671)(397). A clear difference in the probability of FOXO activation was observed between the different types of samples, predicting FOXO activation in healthy colon tissue and FOXO inactivation in adenomas (Figure 3A). Second, we applied the FOXO activity model on a broad set of patient tissues containing normal colon, colon adenoma and colon carcinoma tissues (GSE20916)(396)(Figure 3B). In normal colon mucosa FOXO was consistently predicted to be active and in adenomas FOXOs are predicted to be inactive, in line with our findings in the first data set. Additionally FOXO is predicted to be mainly inactive in adenocarcinoma and were predicted to be inactive in 28 out of 46 colon carcinoma samples. Interestingly, in data sets from colon tissue that were micro dissected into mucosa and crypt tissue, FOXO pathway activity was predicted active in the mucosa, while inactive in crypts (Figure 3B). In colon adenoma samples, whether obtained from crypt or mucosal tissue, FOXO3 activity was predicted to be inactive.

Overall the expected FOXO inactivation in highly proliferative tissue is predicted as expected but FOXOs are predicted active in a subset of colon carcinoma samples. Also FOXO can be active in a variety of cell types other than epithelial and cancer cells, among which immune cells, implying that mRNA extracted from a cancer tissue sample is in most instances derived from the mixture of cell types present in the tissue. In case the presented computational model FOXO3 is scored as transcriptionally active in such a heterogeneous sample, to take the right therapy decision for the respective patient, it may be necessary to know in which cell type FOXO is active and if the predicted activity correlates with FOXO localization. For this reason, we aimed to develop a FOXO3 immunohistochemistry (IHC) staining for identification of cytoplasmic versus nuclear localized FOXO3, which can be applied in a routine diagnostic setting and be used to validate our observed predictions. FOXO3 was mainly found cytoplasmic in normal crypt cells and present in the nucleus of normal mucosa cells in IHC staining for FOXO3 on normal colon tissue samples (Figure 3C). Colon adenoma cells were found to have FOXO3 localized mainly in the cytoplasm and colon carcinoma cells displayed heterogeneous FOXO3 localization, showing some cells have nuclear FOXO3 and other cytoplasmic FOXO3.

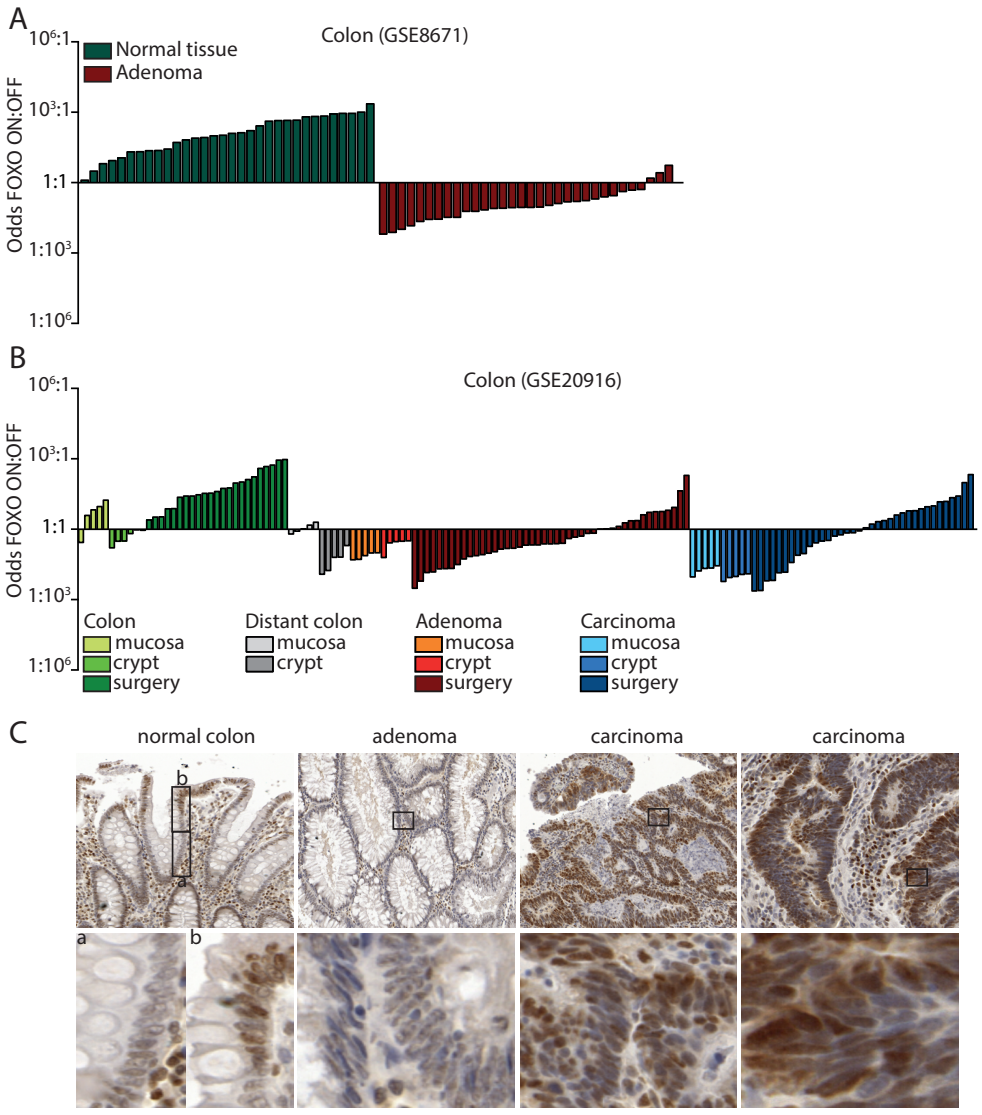


Figure 3: The predicted FOXO3 /PI3K activity in colorectal cancer correlates with FOXO3 localization
A. Prediction of FOXO activity in corresponding normal colon and colon adenoma patient samples within the GSE8671 dataset. Each bar represents the analysis results of one sample. The vertical axis indicates the probability that the FOXO3 is "active" (values above the horizontal axis) versus "inactive" (values below the horizontal axis). The green bars represent normal tissue, the red bars represent adenoma samples. **B.** Prediction of FOXO activity model in Normal colon tissue (separated in micro-dissected mucosa, micro-dissected crypt and complete surgical samples), distant colon (separated in micro-dissected mucosa and micro-dissected crypt), colon adenoma (separated in micro-dissected mucosa, micro-dissected crypt and complete surgical samples) and carcinoma (separated in micro-dissected mucosa, micro-dissected crypt and complete surgical samples) patient samples within the GSE20916 dataset. Each bar represents the analysis results of one sample. The vertical axis indicates the probability that the FOXO3 is "active" (values above the horizontal axis) versus "inactive" (values below the horizontal axis). The green bars represent normal tissue, the red bars represent adenoma samples, and the blue bars represent colon carcinoma samples. **C.** Immunohistochemical staining of FOXO3 and haematoxylin in normal colon, colon adenoma and two colon carcinoma patient samples. The lower panel is a magnification of the area indicated by the black box.

Although PI3K is expected to be active in the majority of the used colon cancer samples, causal relations are hard to determine due to a lack of genetic background information on the patients included in these datasets. We established that we can predict FOXO activity in colon cancer patient material and that these predictions are reflected by the physical localization of FOXO3 within the tissue.

Prediction of FOXO3 activity in clinical breast tissue samples

To further test the computational model we applied the model to three independent publicly available data sets on breast cancer (GSE17907, GSE12276 & GSE21653) (393, 398-400). Prior to modeling, breast cancer tumor subtyping in all data sets was performed using the PAM50 algorithm to ensure the subtypes in all data sets are similar (391). In line with normal colon tissue FOXO activity was predicted to be active in the normal breast tissue subset (Figure 4A). The relatively low grade tumors from the normal-like and Luminal A breast cancer subtype were predicted to have high FOXO activity in all data sets (Figure 4 A-C). In high-grade tumors from the Luminal B, HER2 positive and generally triple negative Basal breast cancer subsets FOXO was predicted to be active in the majority of tumors. Although our observation that FOXOs are predicted to be active in normal-like benign breast tissue is in line with the expected levels of FOXO activity in non-proliferative cells, FOXOs are also predicted to be active in cancers that are more aggressive. Interestingly, these observations indicate that even though FOXO is expected to be inactive in highly proliferative tissue commonly carrying PI3K activating alterations, FOXO can still be activated and regulate transcription. It is therefore not possible yet to infer PI3K activity based on our current computational model for FOXO activity. The computational model does however uncover that FOXOs are expected to be active in during breast tumorigenesis. If the predictions are in line with the actual PI3K and FOXO activity status within the breast cancer data sets needs to yet be controlled however to be ensure that the model is accurate.

Discussion

A computational model predicting FOXO and PI3K activity

We describe the development of a novel computational model predicting FOXO transcriptional activity in cancer patient tissue mRNA samples. The computational model was developed using established FOXO target genes and using FOXO3 as the paradigm FOXO isoform as FOXOs are redundant (61, 389, 395). Based on this subset of FOXO target genes that make up the computational FOXO3 model, we aim to asses PI3K pathway activity in a tissue sample. The Bayesian-model calibrated in FOXO-inducible HUVEC resulted in a computational model that accurately predicts FOXO activity in the breast cancer cell lines MCF7 and MDA-MB231. Prediction of PI3K/FOXO activity upon treatment of MCF7 cells with the PI3K inhibitor LY294002 showed the model is indeed capable to infer the PI3K activity status correctly. The observation that the odds PI3K is active are lower in LY294002 treated cells compared to ectopic FOXO3 activation in MCF7 might be a result of growth factor signaling feedback. Although we observed FOXO nuclear accumulation in LY294002 treated cells, pharmacological inhibition of PI3K also initiates a growth factor feedback response that re-establishes growth factor signaling and inactivates FOXO, a major component of drug resistance development in cancer cells (96, 376, 384).

The generated Bayesian-model robustly predicts FOXO and PI3K activity in controlled cell culture based models, but applying the model to patient material to infer PI3K activity proved to be more challenging. The predictions in colon tissue fall largely in line with the expected proliferative status of the different tissues in our model. Tissues with low levels of proliferation like the mucosa were predicted to have active FOXO and therefore inactive PI3K signaling and highly proliferative cancerous tissue was predicted

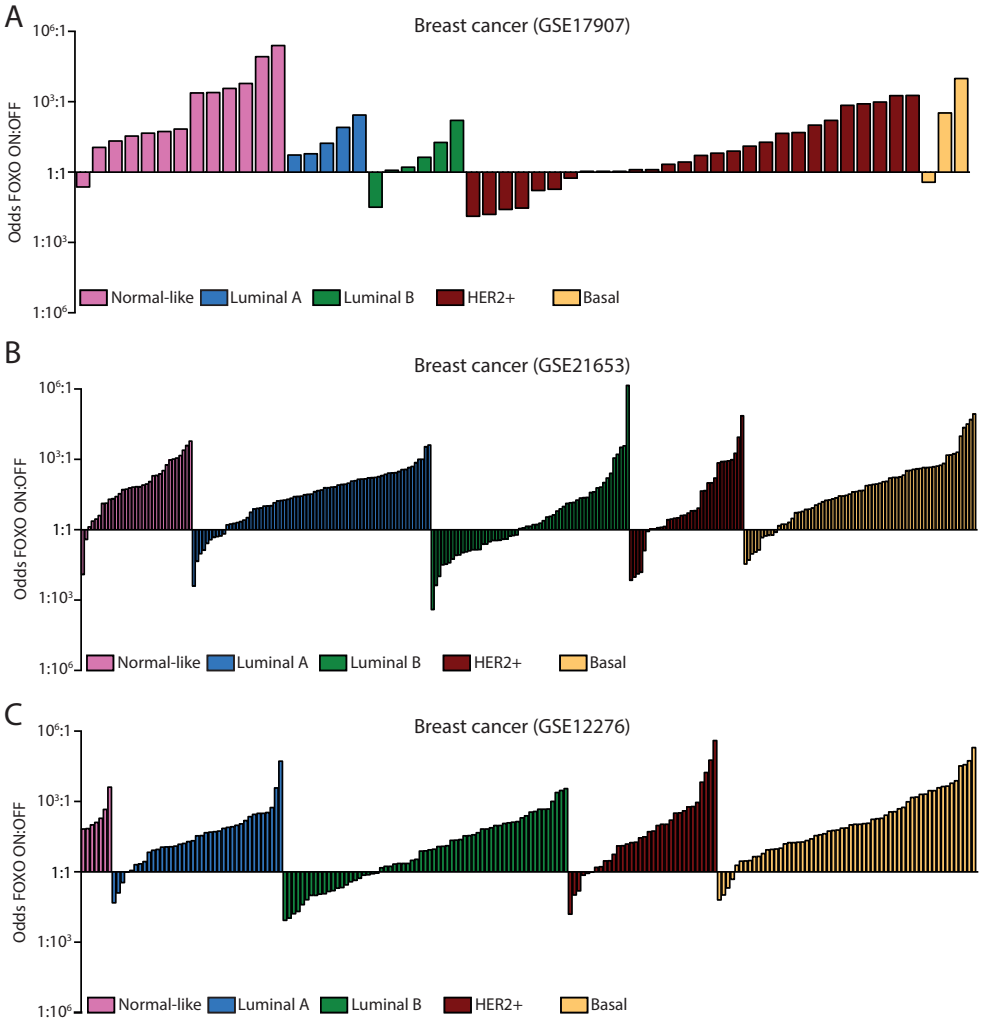


Figure 4: FOXO is predicted to be active in various breast cancer subtypes.
A. Prediction of FOXO activity in normal-like breast cancer, Luminal A, Luminal B, HER2 positive and Basal breast carcinoma patient samples within the GSE17907 dataset. Each bar represents the analysis results of one sample. The vertical axis indicates the probability that the FOXO3 is "active" (values above the horizontal axis) versus "inactive" (values below the horizontal axis). The pink bars represent normal-Like breast, blue bars represent Luminal A breast cancer, green bars represent Luminal B, red bars represent HER2+ and the yellow bars represent Basal breast carcinoma. **B.** Prediction of FOXO activity in normal-like breast cancer, Luminal A, Luminal B, HER2 positive and Basal breast carcinoma patient samples within the GSE21653 dataset. The pink bars represent normal-Like breast, blue bars represent Luminal A breast cancer, green bars represent Luminal B, red bars represent HER2+ and the yellow bars represent Basal breast carcinoma. **C.** Prediction of FOXO activity in in normal-like breast cancer, Luminal A, Luminal B, HER2 positive and Basal breast carcinoma patient samples within the GSE12276 dataset. The pink bars represent normal-Like breast, blue bars represent Luminal A breast cancer, green bars represent Luminal B, red bars represent HER2+ and the yellow bars represent Basal breast carcinoma.

to have low FOXO activity. These observations were also in line with the observed FOXO3 localization in the stained tissue sections. However, in both the colon adenoma and carcinoma data sets multiple tumors were predicted to have active FOXO but a more heterogeneous distribution of FOXO3 localization was found in tissue sections of colon carcinomas. The computational model showed an even more pronounced FOXO activation signature in all different types of breast cancer in multiple breast cancer data sets, including the high-grade tumors. This is unexpected since the PI3K pathway is the most frequently mutated pathway in breast cancer and even though we do not know the exact genetic background of the patient samples within these data sets it is save to assume most of these tumors have active growth factor signaling.
Together these results show that we can model FOXO activity in both cell lines and patient material, but in order to reliably PI3K activity based on FOXO activity the model needs to be further refined. Also, FOXO and PI3K activity yet needs to be characterized in the corresponding cancer tissue samples to ascertain that the model is accurately predicting FOXO, and eventually PI3K, activity. For example, we need to ensure that tissue heterogeneity or differential FOXO activity levels throughout the cancer samples do not cause the predicted outcomes of the model.

Stress signaling could distort the PI3K activity readout

The prediction of active FOXO in high-grade cancers holds two interesting implications that can contribute to a better model. First, FOXOs might be active in the tumor without suppressing cancer progression. Second, FOXOs can be active in tumors in the presence of active growth factor signaling, suggesting activation by other signaling pathways. FOXOs are considered tumor suppressors but also function as regulators of cellular homeostasis and respond to various adverse conditions including, DNA damage, high levels of reactive oxygen species and low nutrient availability (2, 3, 375). Tumors often suffer from these adverse conditions during growth and therefore it is likely that FOXOs become activated stress-signaling pathways (20, 401). As such, cellular stresses interfere with the simple inverse FOXO-PI3K relationship, complicating the deduction of PI3K activity from measured FOXO activity in cancer tissue samples. It is therefore mandatory to distinguish between FOXO3 activity in a growth factor signalling role versus a role in cellular homeostasis. The finding that FOXO3 is mainly localized in the nucleus of MDA-MB-231 cells under normal culture conditions is in line with the finding that triple negative breast cancers are predicted to have active FOXO within the tumor and was also reported previously (71). It is curious however that FOXO activity is predicted to be inactive under normal conditions while FOXO3 is nuclear, implying that there are indeed additional inputs in FOXO activity and separate transcriptional programs that can be executed by FOXO, an observation previously made in *C. elegans* as well (402).
Determination of a subset of FOXO target genes that can discriminate between these two functions should be added to our current model to allow separation of these different transcriptional modes of FOXOs in order to more reliably predict PI3K activity in tumor tissue samples in the future.

Clinical application of computational models

Quick assessment of active signalling within patient tumor samples allows for rapid diagnosis and efficient establishment of a treatment strategy. Computational methods to elucidate prognostic mRNA expression profiles allow narrowing down the amount of parameters that need to be assessed for efficient diagnosis. Development of comprehensive RT-qPCR kits by selecting a subset of diagnostic genes characteristic to a certain signalling pathway can avoid elaborate sequencing and histopathological methods for this purpose. Models similar to our FOXO activity model predicting WNT and

ER pathway activity are already put to practise (388). Although FOXOs are implicated in major diseases like cancer and diabetes, using FOXO activity as a prognostic marker has been proven difficult. Over the years, studies that aimed to correlate FOXO expression or localization to disease outcome in cancer patients came to opposing conclusions. On one hand various studies correlated high FOXO expression and/or nuclear localization to good prognosis (48-57, 403). On the other hand several studies correlated high FOXO levels and/or activity to poor prognosis (68-72, 404). Determination of FOXO levels or localization is clearly insufficient to address whether in a specific patient FOXOs are active or inactive. Therefore, our computational model based on a robust set of FOXO target genes could be used to address the question if FOXOs are active. FOXO can be expected to be active in different types and stages of cancer based on predictions made by our computational model, suggesting FOXO can be active even in the presence of growth factor signalling. Separation of FOXO activity regulated by growth factor signalling from FOXO activity induced by stress signalling might provide insight on the vulnerabilities of the tumor. For example, if FOXOs are found inactive due to high PI3K activity, treating tumors with PI3K inhibitors might induce tumor suppressive FOXO activity. The other way around, if FOXOs are found active in the context of cellular stress, targeting cellular metabolism might be more suitable as FOXOs are already active and no additional tumor suppressive effect can be expected from FOXO. To this end we are currently assessing whether there are FOXO target genes that can distinguish between FOXO activation in the context of growth factor or stress signalling.

The focus now lies on developing models for single signaling pathways, but when established a combined model for multiple pathways could be generated and allows simultaneous assessment of signalling pathways relevant to cancer diagnosis, patient prognosis and treatment strategy determination (van Ooijen 2016, submitted). To uncover a tumor's vulnerabilities and anticipate adapting to targeted therapies we need to understand how a tumor cell is wired and responds, the models as proposed in this chapter provide a feasible tool to provide this understanding. The use of these models does not need to be restricted to cancer of course as they can be applied to a wide variety of diseases in which the corresponding pathways are function.

Chapter 6:

FOXO3 activation in S- or G2-phase induces a pre-mitotic cell cycle exit by repressing *EMI1* expression

M. Hornsveld[1], A.M.M. Smits[1], M. Rodriguez-Colman M. L. Krenning2, Meerlo[1], L. Faber[1], A. Stokkermans[1], T.B. Dansen[1], B.M.T. Burgering[1]

[1]Department of Molecular Cancer Research, University Medical Center Utrecht, Utrecht
[2]Hubrecht Institute, The Royal Netherlands Academy of Arts and Sciences (KNAW) and University Medical Center Utrecht

Manuscript in preparation

6

Abstract
Forkhead Box O (FOXO) transcription factors are key players in cell cycle regulation. Activation of FOXO leads to expression of cyclin dependent kinase inhibitors (CDKis) that subsequently repress cell cycle progression from G1 to S or G2 to M phase of the cell cycle. How the decision arises to arrest either in G1 or in G2 following FOXO activation is however unclear. We used untransformed Retinal Pigment Epithelial (RPE) cells carrying the Fluorescent Ubiquitination-based Cell Cycle Indicator system (FUCCI) to examine the FOXO mediated effects on the cell cycle in detail. We found that FOXOs induce a cell cycle exit from G2 phase, leaving the cells in a tetraploid G0/G1 like state. Further elucidation of the mechanism that underlies this FOXO induced G2 exit revealed that FOXOs repress *EMI1* expression, which is a key inhibitor of APC/c activity in S,G2 and early mitosis. We find that FOXOs drive the expression of p27, MXI1 and FBXW7 and thereby can inhibit MYC and E2F1, the main transcription factors for *EMI1* expression. Combined our results unveil a novel mechanism by which FOXOs can repress the cell cycle

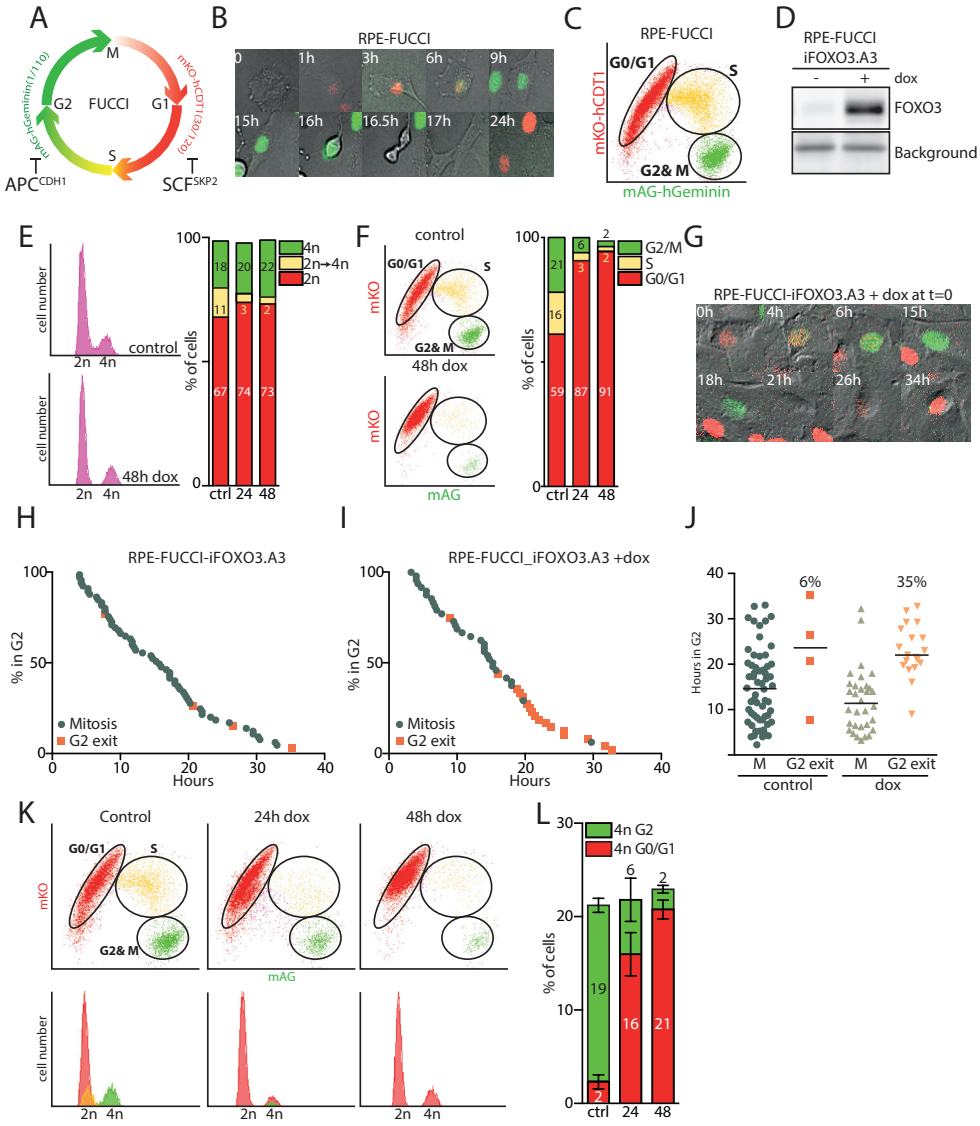
Introduction

FOXO transcription factors are key players in regulating the cell cycle. FOXO activity is restrained in the presence of growth factors through PI3K-AKT signaling and FOXOs become activated in response to reduced growth factor signaling or adverse conditions like increased levels of reactive oxygen species. Upon activation FOXO can arrest the cell cycle or even steer the cell into a permanent withdrawal from the cell cycle by means of senescence (7, 8, 38).

G1 to S phase transition depends on inactivation of APC^{CDH1} ubiquitin ligases and activation of E2F transcription factors, which are repressed by the retinoblastoma family of proteins (RB, p107 and p130) in non-proliferating cells. During G1, expression of Cyclin D (CCND1/2/3) increases in response to mitogenic signaling. The active Cyclin D-CDK4/6 complexes phosphorylate and thereby inhibit RB, resulting in release of E2Fs in order to start transcription of S-phase proteins and Cyclins (37). From S-phase on Cyclin-E/A-CDK2 complexes and SCF^{SKP2} ubiquitin ligases take over and ensure correct DNA replication and cellular growth before entering mitosis. Cyclin B/CDK1 complexes become active at the end of G2 and drive the cells into mitosis. At the onset of anaphase Cyclin B is quickly degraded by APC^{CDC20} in order to ensure the end of the cell cycle (434-436).

The cell cycle arrest induction by FOXO is mediated through transcription of multiple cell cycle kinase inhibitors (CKI). The canonical CDKi downstream of FOXO is p27 (CDKN1B) (35). Additional to CDKN1B FOXO can also be a transcriptional regulator of p21 (CDKN1A), p57 (CDKN1C), p15 (CDKN2B) and p19 (CDKN2D) (420, 437-439). FOXO mediated induction of CDKi expression leads to inhibition of the Cyclin/CDK complexes responsible for progression through the different phases of the cell cycle and results in a robust cell cycle arrest in G0/G1,G2 or even senescence (35, 38-40, 425, 426).

Figure 1: FOXO3 activation in late G1 or early S/G2 induces a cell cycle exit
A. Schematic representation of the different mKO-hCDT1 and mAG-hGeminin degradation cycles during the cell cycle (FUCCI). **B.** Live cell imaging stills of an example RPE-FUCCI cell over the course of 24 hours. Showing accumulation of mKO-hCDT1 prior to mAG-hGeminin accumulation and followed by rapid degradation of mAG-hGeminin at the end of mitosis after which mKO-hCDT1 accumulates again. **C.** Flow cytometric analysis of RPE-FUCCI cells allows separation of cell in distinct cell cycle phases. **D.** Western blot analysis of FOXO3 levels after 16 hours of doxycycline (dox) treatment. Equal concentrations of protein are loaded and aspecific background staining is used to visualize equal protein content. **E.** Flow cytometric analysis of RPE-FUCCI-iFOXO3.A3 cell DNA profiles stained with Hoechst after 48 hours of doxycycline treatment. Bar graph represents the average percentage of cells with 2n (red), between 2n and 4n (yellow) or 4n (green) after 24 and 48 hours. N=3, numbers represent the average percentage. **F.** Flow cytometric analysis of RPE-FUCCI-iFOXO3.A3 cell cycle distribution after 48h doxycycline. Bar graph represents the average percentage of cells in G1 (red), S (yellow) and G2/M (Green) (as indicated by the gates) after 24 or 48 hours of doxycycline. N=3, numbers represent the average percentage **G.** Live cell imaging stills of an example RPE-FUCCI-iFOXO3. A3 cell treated with doxycycline over the course of 34 hours, showing mAG-hGeminin degradation without entering mitosis and subsequently accumulating mKO-hCDT1. **H&I.** Live cell imaging quantification of the time spend in G2 of RPE-FUCCI-iFOXO3.A3 cells. Each dot represents a cell and corresponds to the time spend in G2 without or after FOXO3.A3 expression. Green indicates the cell exits G2 by mitosis, orange indicates the cells exits G2 skipping mitosis. **J.** Alternative representation of the live cell imaging quantification of the time spend in G2 of RPE-FUCCI-iFOXO3.A3 cells. Black bars represent the median time spend in G2 and the total percentages of G2 exit by skipping mitosis are indicated. **K.** Flow cytometric analysis of RPE-FUCCI-iFOXO3.A3 cells stained for DNA content with Hoechst. The upper panels show the populations of cells expressing mKO-hCDT1 (red) and mAG-hGeminin (Green). The lower panel represents the DNA content of the corresponding mKO-hCDT1 (red) and mAG-hGeminin (green) and double positive (yellow) populations. **L.** Quantification of the flow cytometric analysis of RPE-FUCCI-iFOXO3.A3 cells stained with Hoechst. Graph represents the average percentage of 4n mKO-hCDT1 (red)(G1) and 4n mAG-hGeminin (green)(G2) positive cells after 24 and 48 ours of doxycycline. N=3, numbers represent the average percentage.



The mechanisms underlying FOXO induced G1 arrest is well characterized, but the current understanding of the observed FOXO induced G2 arrest is limited. How a cell determines to arrest in G1 or G2 upon FOXO activation in cycling cells is also unclear. To scrutinize the effects of FOXO on the cell cycle with high temporal resolution we generated Retinal Pigment Epithelial cells (RPE) carrying the Fluorescent Ubiquitination-based Cell Cycle Indicator system (FUCCI) and Inducible hyper active FOXO3 (FOXO3.A3). We find FOXO activation in early S/G2 phase does not arrest cells in G2 but rather leads to a cell cycle exit from G2 without mitosis. FOXO does so by repressing transcription of the APC/c inhibitor EMI1, resulting in premature APC^{CDH1} activation in G2 and a subsequent cell cycle exit giving rise to tetraploid cells.

Results

FOXO3 activation in S/G2 results in premature APC^{CDH1} activation and exit from G2

RPE cells expressing mKO-hCDT1(30/120) and mAG-hGeminin(1/110) (also known as FUCCI) were established in order to elucidate FOXO induced effects on the cell cycle (Figure 1A) (440). Live cell imaging and flow cytometric analysis of RPE-FUCCI cells showed gradual accumulation of mKO-hCDT1 after mitosis and SCF^{SKP2} mediated degradation of mKO-CDT1 coincides with the accumulation of mAG-hGeminin prior to mitosis. At the end of mitosis mAG-Geminin is rapidly degraded by APC$^{CDC20/CDH1}$ activation followed again by gradual accumulation of mKO-CDT1 in cells after mitosis (Figure 1B&C). RPE-FUCCI cells stably transduced with pINDUCER20-iFOXO3.A3 allowed us to conditionally express a hyper active mutant of FOXO3 (iFOXO3.A3) using doxycycline (Figure 1D). RPE-FUCCI-iFOXO3.A3 cells arrest with both 2n and 4n DNA content upon doxycycline treatment for 24 or 48 hours, resulting in 74±4% 2n, 20±7% 4n and 73±1% 2n, 22±2% 4n respectively (Figure 1E). A Striking discrepancy was observed when cell cycle distribution was characterized in the same cells using the FUCCI system. RPE-FUCCI-iFOXO3.A3 cells treated with doxycycline accumulated in G1, as 87±4% and 91±2% of the cells were mKO-hCDT1 positive after 24 and 48 hours respectively (Figure 1F). This observation indicates that cells mainly arrest in a G1 like state but can do so in a diploid and a tetraploid state. We observed cells exit the cell cycle and revert to a G0/G1 state without going through mitosis after 15-35 hours of S/G2 when tracking the fate of S/G2 cells upon FOXO activation by live cell time lapse microscopy (Figure 1G-J). We confirmed this observation by combining the FUCCI system with staining for DNA content, revealing that 16±2% and 21±1% of mKO-hCDT1 positive cells carry 4n DNA after 24 and 48 hours of FOXO3.A3 expression respectively (Figure 1K&L). Together these results unveil that FOXO3.A3 activation in S/G2 can induce a G0/G1 like state with 4n DNA. mAG-Geminin degradation in the absence of mitosis shows that APC^{CDH1} is activated prematurely following FOXO3.A3 expression, leading to direct progression to a G0/G1 like state. As no cells are detected in S phase after FOXO3.A3 activation with either 2n or 4n DNA we can also conclude that cells are arrested in G0/G1. This FOXO induced cell cycle exit represents a mechanism by which FOXOs can regulate cell proliferation that has not been previously described.

FOXO induced cell cycle exit is independent of p53 and p21

G2-exit from the cell cycle has previously been attributed to DNA damage and p53 activation. The subsequent transcription of p21 inhibits Cyclin E/A-CDK2 complexes required for keeping the cell in G2, resulting in premature activation of APC^{CDH1} followed by exiting the cell cycle and senescence (441-443). As FOXOs are known to regulate p53 activity and p21 expression we determined whether FOXO induced G2 exit is mediated by p53 or p21 (38, 373, 420, 438).

Expression of *p21* mRNA was up regulated after 24 hours of FOXO3.A3 expression and *p53* mRNA levels remained stable (Figure 2A&B). No changes in p21 and p53 protein expression were detected on western blot after 24 hours of FOXO3.A3 expression (Figure 2C). The FOXO3 induced cell cycle exit from G2 was unaffected in cells transfected with siRNA smartpools targeting either *p21* or *p53* (Figure 2D-I). Although the time it takes for G2 cells to exit the cell cycle in siP53 treated cells is not changed, the percentage of cells undergoing the FOXO3.A3 induced cell cycle exit is slightly decreased (Figure 2I). This observation suggests that even though p53 does not seem to actively participate in the FOXO induced cell cycle exit, cells are more sensitive to exit from G2 in the presence of p53. As the levels of p21 and p53 remain unchanged after FOXO3.A3 expression and activation of FOXO3.A3 in the absence of p21 and p53 does not impair G2 exit, we conclude that the FOXO3 induced G2 exit is independent of p53 and p21.

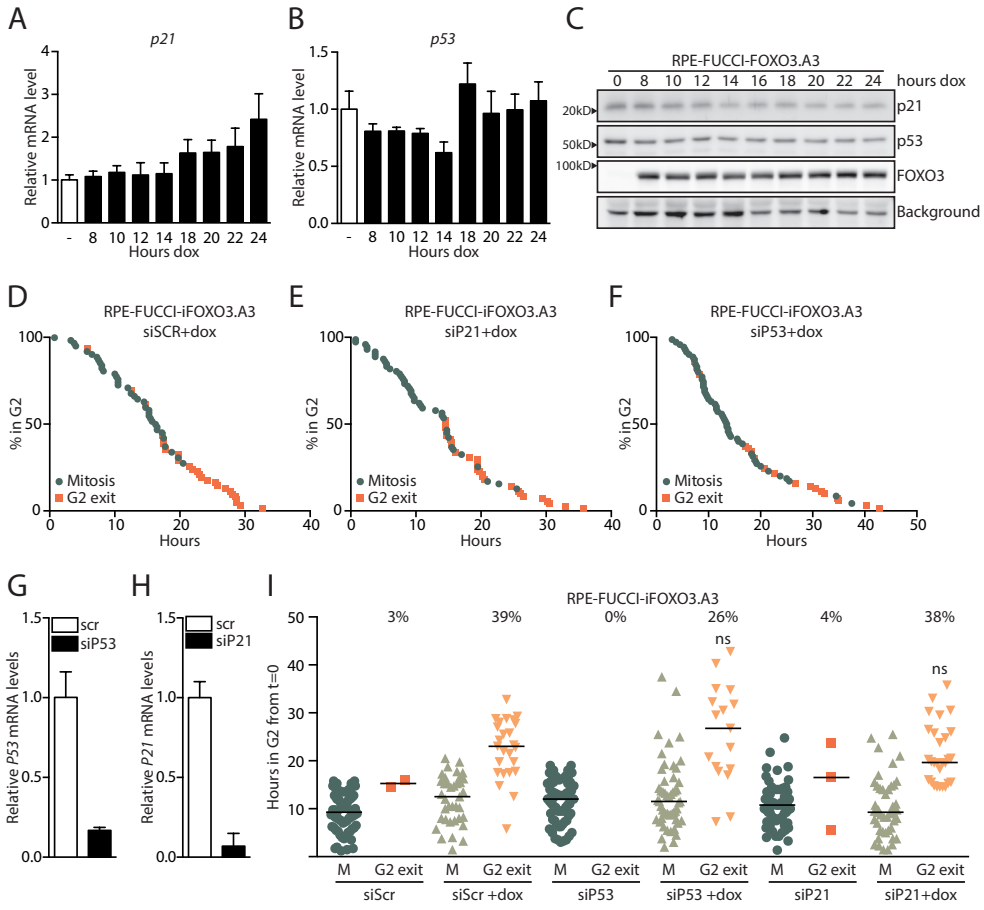


Figure 2: The FOXO3 induced G2 exit is independent of p21 and p53
A&B. RT-qPCR analysis of *P21* and *P53* mRNA expression level changes in RPE-FUCCI-iFOXO3.A3 cells over the course of 24 hours doxycycline (dox) treatment. **C.** Western blot analysis of p21, p53 and FOXO3 protein levels in RPE-FUCCI-iFOXO3.A3 after doxycycline treatment for 24 hours. Equal concentrations of protein are loaded and aspecific background staining is used to visualize equal protein content. **D-F.** Live cell imaging quantification of the time spend in G2 of RPE-FUCCI-iFOXO3.A3 cells treated with siSCR, siP21 or p53 for 72h prior to doxycycline. Each dot represents a cell and corresponds to the time spend in G2 without or after FOXO3.A3 expression. Green indicates the cell exits G2 by mitosis, orange indicates the cells exits G2 skipping mitosis. **G&H.** RT-qPCR analysis of *P21* and *P53* mRNA expression level changes in RPE-FUCCI-iFOXO3.A3 cells treated with Scrambled siRNA (siSCR) siP21 and siP53 for 72 hours. **I.** Alternative representation of the live cell imaging quantification of the time spend in G2 of RPE-FUCCI-iFOXO3.A3 cells. Black bars represent the median time spend in G2 and the total percentages of G2 exit by skipping mitosis are indicated. No significant difference in the average G2 exit time is observed compared to siSCR+dox (ns).

FOXO3 induces a G2 exit by repressing EMI1 transcription

Cells ensure progression through G2 and entry into M phase by preventing $APC^{CDC20/CDH1}$ activation until the cell reaches the end of metaphase. $APC^{CDC20/CDH1}$ activity is inhibited by EMI1 (also known as FBXO5). From the restriction point at the beginning of S phase on, EMI1 binds to APC^{CDH1} and inhibits ubiquitin chain elongation on its substrates (444-447). Previous reports described that EMI1 levels are down regulated in response to DNA damage and this down regulation is essential to establish a G2 arrest or withdraw from the cell cycle to become senescent (441, 448, 449).

In line with this, we observed a rapid decrease in both *EMI1* mRNA and protein levels in response to FOXO3.A3 expression in RPE-FUCCI-iFOXO3.A3 cells (Figure 3A&B), implicating that the FOXO mediated cell cycle exit could be a result of *EMI1* repression. To test this, we induced mTurquoise-EMI1 expression concurrently with FOXO3.A3. Combined FOXO3.A3 and mTurq-EMI1 expression resulted in reduced levels of endogenous EMI1 and high levels of exogenous EMI1 (Figure 3C). Time-lapse microscopy showed that forced expression of EMI1 overcomes the FOXO3.A3 mediated G2 exit, allowing cells to progress normally to metaphase. (figure 3D).

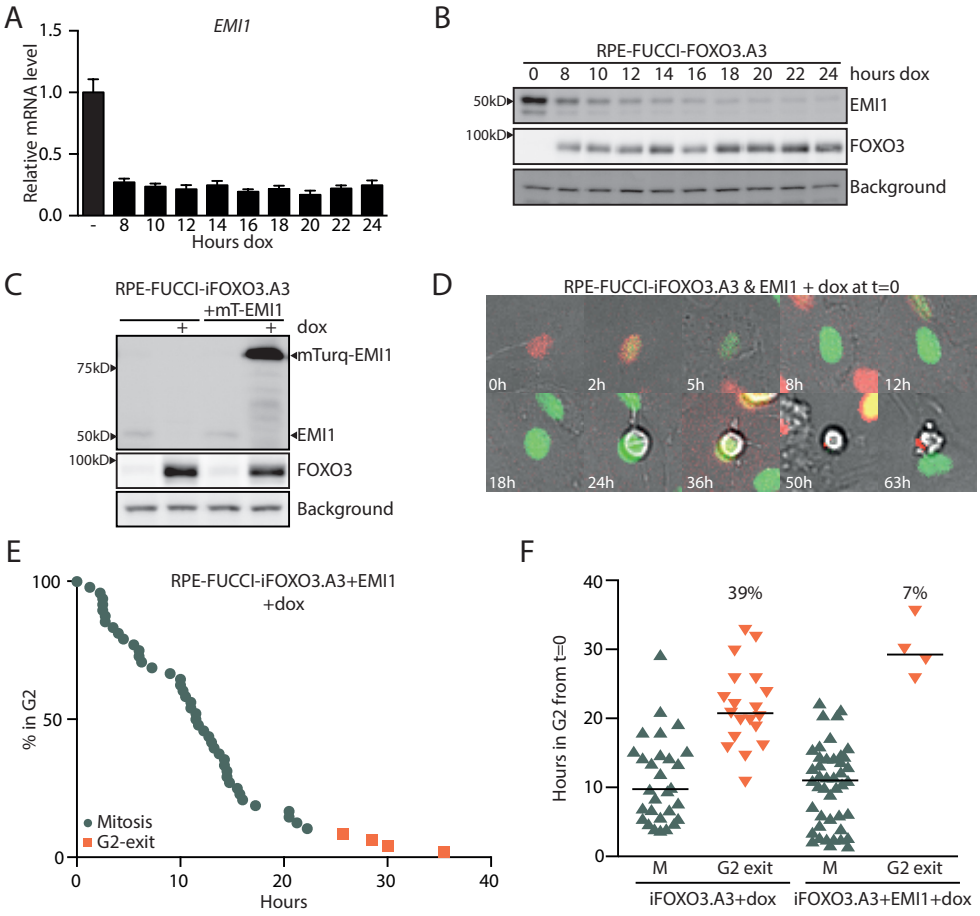


Figure 3: FOXO3 induces a G2 exit by down regulating EMI1
A. RT-qPCR analysis of *EMI1* mRNA expression level changes in RPE-FUCCI-iFOXO3.A3 cells over the course of 24 hours doxycycline (dox) treatment. **B.** Western blot analysis of EMI1 and FOXO3 protein levels in RPE-FUCCI-iFOXO3.A3 after doxycycline treatment for 24 hours. Equal concentrations of protein are loaded and aspecific background staining is used to visualize equal protein content. **C.** Western blot analysis of EMI1 and FOXO3 protein levels in RPE-FUCCI-iFOXO3.A3 and RPE-FUCCI-iFOXO3.A3+pCW-mTurq-EMI1 after doxycycline treatment for 24 hours. Equal concentrations of protein are loaded and aspecific background staining is used to visualize equal protein content. **D.** Live cell imaging stills of an example RPE-FUCCI-iFOXO3+pCW-mTurq-Emi1 cell over the course of 63 hours. Showing entry into mitosis with high levels of mAG-hGeminin expression and cell death after 63 hours of mitotic arrest. **E.** Live cell imaging quantification of the time spend in G2 of RPE-FUCCI-iFOXO3.A3+pCW-mTurq-EMI1 cells. Each dot represents a cell and corresponds to the time spend in G2 without or after FOXO3.A3 expression. Green indicates the cell exits G2 by mitosis, orange indicates the cells exits G2 skipping mitosis. **F.** alternative representation of the live cell imaging quantification of the time spend in G2 of RPE-FUCCI-iFOXO3.A3+pCW-mTurq-EMI1 cells. Black bars represent the median time spend in G2 and the total percentages of G2 exit by skipping mitosis are indicated.

As a result of the induced EMI1 expression cells subsequently arrest at metaphase for many hours due to the inability to activate $APC^{CDC20/CDH1}$ and execute anaphase as indicated by mAG-hGeminin positivity, and eventually die (Figure 3D). Co-expression of EMI1 with FOXO3.A3 abrogated the FOXO3.A3 induced G2 exit, resulting in G2 exit by mitosis (Figure 3E&F). These data establish that the FOXO3 induced G2 exit is the result of decreased EMI1 expression and subsequent APC^{CDH1} activation.

FOXO3 reduces EMI1 expression in multifarious ways

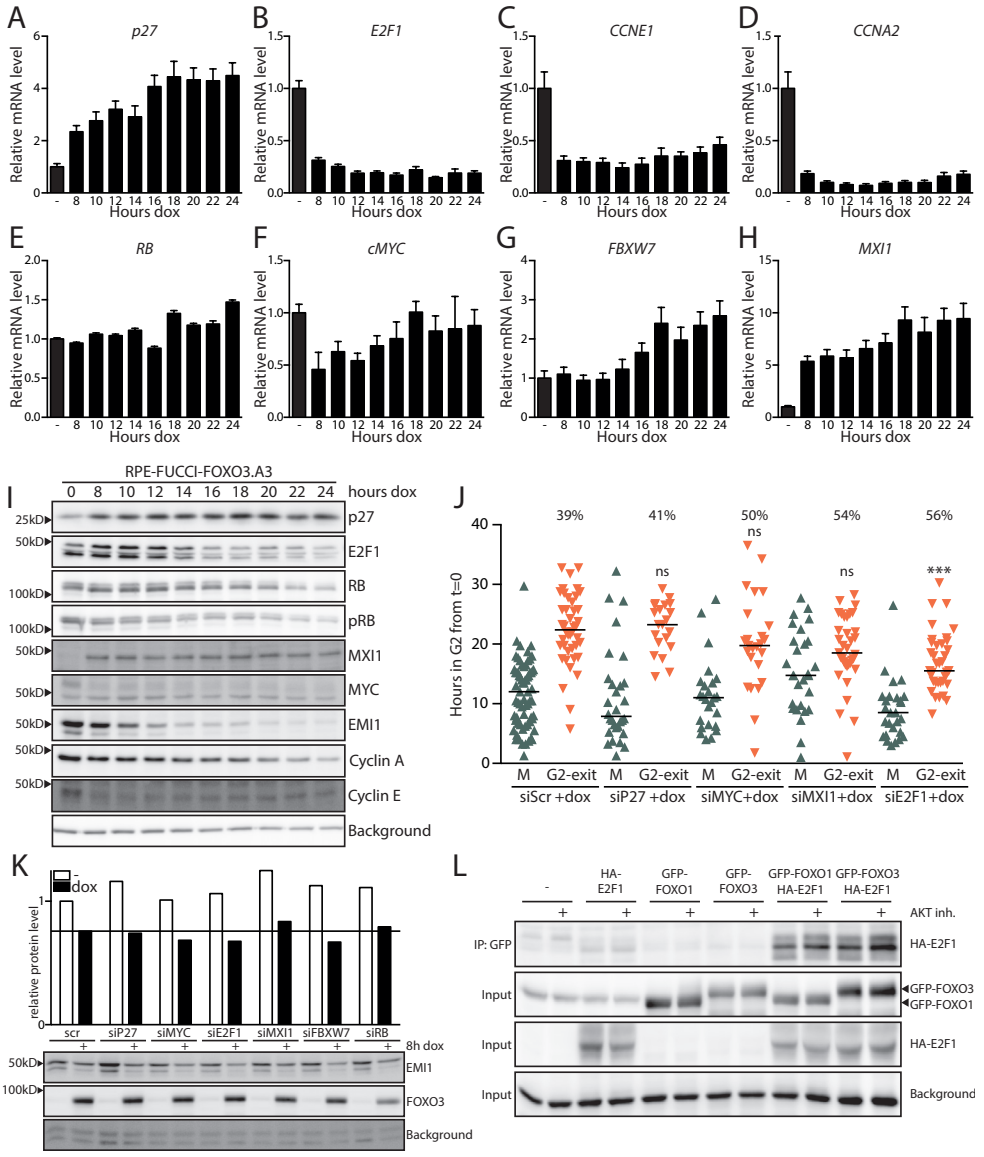
EMI1 expression is driven by the S-phase transcription factor E2F1 (450). Additionally, examination of the *EMI1* promoter using the USCG Genome browser showed that the transcription factor MYC can bind to the *EMI1* promoter and potentially regulates EMI1 expression. FOXOs can influence E2F1 and MYC activity in multiple ways. First FOXOs transcribe CDKis that inhibit Cyclin/CDK complexes required for E2F1 activity and FOXO can directly bind to E2F1 and attenuate its function (451-453). Second, FOXOs drive the expression of the MYC inhibitor MXI1 (11, 427). Finally, FOXO activity correlates with increased levels of *FBXW7* (also known as FBXO30) expression, which is responsible for substrate recognition by the SCF^{FBXW7} ubiquitin ligase complex that degrades E2F1, MYC and S-phase Cyclins (11, 389).

To test how different E2F1 and MYC effectors are changing in response to FOXO activation we looked for changes in p27, E2F1, Cyclin E, Cyclin A RB, MYC, FBXW7 and MXI1 expression levels. Upon FOXO3.A3 expression in RPE-FUCCI-iFOXO3.A3 cells we observed no change in *RB* expression, increased levels of *p27, FBXW7* and *MXI1* and decreased levels of *E2F1, Cyclin E1 (CCNE1), Cyclin E2 (CCNE2)* and *MYC* expression over the course of 24 hours (Figure 4A-H). Protein levels of p27 and MXI1 increase and EMI1 drop rapidly after FOXO3.A3 expression, while E2F1, RB, phospho-RB, MYC, Cyclin E and Cyclin A levels decrease gradually over time (Figure 4I). No significant effects of p27, MYC and MXI1 loss on the timing of FOXO3.A3 induced G2 exit was observed while tracking cells treated with siRNAs targeting p27, MYC, MXI1, or E2F1 using time lapse microscopy (Figure 4J and SFigure 1A, SFigure 2). However, a significant decrease in the average time required to exit G2 in response to FOXO3 activation was observed when E2F1 was knocked down. Also, reducing expression of MYC, E2F1 and unexpectedly MXI1 increases the percentage of cells undergoing a cell cycle exit. Probing for EMI1 protein levels after FOXO3.A3 activation in the absence of p27, MYC, E2F1, MXI1, FBXW7 or RB showed that FOXO mediates EMI1 levels through E2F1 and MYC (Figure 4K, SFigure 1). FOXO activation in the absence of p27 did not influence EMI1 repression but reduction of MYC and E2F1 levels resulted in slightly more efficient reduction of EMI1 levels by FOXO3.A3. In line with the latter, reducing RB or MXI1 levels slightly reduces the efficiency of EMI1 repression by FOXO3.A3. Reducing the levels of FBXW7 expression resulted in slightly increased EMI1 repression efficiency in contrast to the expected role in degrading E2F1, MYC and Cyclin E/A. We also observed that FOXO1 and FOXO3 might be able to influence E2F1 transcriptional activity by direct binding, as shown by Co-immunoprecipitation of HA-E2F1 with GFP-Flag-FOXO1 and GFP-Flag-FOXO3 (Figure 4L). Together these results suggest FOXO3 activation in S/G2 reduces EMI1 expression by modulating E2F1 and MYC transcriptional activity.

Discussion

A new role for FOXOs in cell cycle regulation

The involvement of FOXOs in repressing the cell cycle is well established and many studies elaborated on the induction of a CKI dependent G1 arrest (7, 8, 35, 451). Cell cycle profiles using DNA staining to characterize FOXO induced effects on the cell cycle indicated that FOXOs could also induce a cell cycle arrest with 4n DNA (35, 39, 416, 425, 426).



The mechanism behind this proposed FOXO induced G2 arrest remained unclear to date. Activation of FOXO3.A3 in RPE cells resulted in a robust cell cycle arrest with both 2n and 4n DNA based on classic cell cycle profiling. Strikingly, combining classic cell cycle profiling with the FUCCI system, which monitors the activity of essential cell cycle ubiquitin ligase complex activity, revealed that the cell previously proposed as G2 arrested cells based on 4n DNA content are actually biochemically residing in a G1 or G0 state. Following the fate of cells in G2 when FOXO3 is activated revealed that cells indeed switch form SCF^{SKP2} to APC^{CDH1} activity without passing through mitosis. Switching from S/G2 to a G0/G1 state in response to FOXO3 activity has never been reported and might have been obscured by the use of merely the DNA content as a surrogate cell cycle phase marker.

Figure 4: FOXO3 represses EMI1 in a E2F1 and MYC dependent way
A-H. RT-qPCR analysis of *p27, E2F1, CCNE1, CCNE2, RB, MYC, FBXW7* and *MXI1* mRNA expression level changes in RPE-FUCCI-iFOXO3.A3 cells over the course of 24 hours doxycycline (dox) treatment. **I.** Western blot analysis of p27, E2F1, RB, phospo-RB, MXI1, MYC, EMI1, Cyclin A, and Cyclin E protein levels in RPE-FUCCI-iFOXO3.A3 after doxycycline treatment for 24 hours. Equal concentrations of protein are loaded and aspecific background staining is used to visualize equal protein content. **J.** Representation of live cell imaging quantification of time spend in G2 of RPE-FUCCI-iFOXO3.A3 cells. Black bars represent the median time spend in G2 and the total percentages of G2 exit by skipping mitosis are indicated. t-Test of the average time until G2 exit p<0.005=*** or not significant (ns) compared to siSCR +dox **K.** Western blot analysis of EMI1 protein levels in RPE-FUCCI-iFOXO3.A3 after doxycycline treatment for 24 hours in combination with Scrambled siRNA (siSCR), siP27, siMYC, siE2F1, siMXI1, siFBXW7 or siRB. Equal concentrations of protein are loaded and aspecific background staining is used to visualize equal protein content. Graph represents the quantification of EMI1 chemiluminescent intensity corrected for protein levels and normalized to untreated siSCR. **L.** Co-immunoprecipitation between HA-E2F1 and GFP-FOXO1 & GFP-FOXO3 in 293T cells visualized by western blot analysis of HA and GFP tagged protein levels. HA-E2F1, GFP-FOXO1 and GFP-FOXO3 ware expressed either alone or in combination in the presence or absence of AKT inhibitor and immunoprecipitated (IP) using anti-GFP antibody. The upper lane represents the anti-GFP IP, the lower 3 lanes represent the IP input. Equal concentrations of protein were used and aspecific background staining is used to visualize equal protein content.

With this notion in mind, one might reason that the previously suggested G2 arrest in response to FOXO activity might actually have been a cell cycle exit from G2 to G1 or G0. Whether the G2 exit with 4n DNA observed after FOXO3 activation is a reversible or a definitive retraction from the cell cycle remains to be explored.

Multiple pathways converge on EMI1 expression
EMI1 down regulation in response to DNA damage signaling and consequential P53 and p21 activation is a well-established mechanism to prevent cells from undergoing mitosis with damaged DNA and results in senescence with 4n DNA (441-443, 448-450). We now describe an additional p53 and p21 independent way in which EMI1 levels can be regulated in order to evade mitosis by linking the FOXO induced cell cycle exit to down regulation of EMI1. The mechanism by which FOXOs repress EMI1 levels is complex but culminates on the regulation of E2F1 and potentially MYC (450). We show FOXOs can potentially do so by transcribing CDKis like p27 resulting in inactivation of S-phase Cyclin/CDK complexes followed by RB reactivation. Additionally FOXO3 drives expression of the MYC inhibitor MXI and the SCF substrate recognition protein FBXW7 that degrades MYC, E2F1 and S-phase Cyclins. FOXOs might also repress E2F1 directly as we show FOXOs can bind to E2F1. Activation of FOXOs in the absence of E2F1 or MYC resulted in slightly faster degradation of EMI1, whereas, conversely in the absence of MXI and RB EMI1 reduction is slightly impaired. The observation that EMI1 levels are only mildly affected can be a consequence of insufficient knockdown efficiency of the siRNA targets, or targeting more than a single aspect of EMI1 transcription regulation is required. Further elucidation of the mechanism underlying FOXO mediated EMI1 repression is required in order to determine whether E2F1 is mainly repressed by RB or by direct binding and to what extend MYC contributes to retain S/G2 phase progression.

Are FOXOs guardians of homeostasis?
P53 is involved in many homeostatic processes but most notably in monitoring the cell for DNA damage and is therefore regularly referred to as a guardian of the genome (144). When cells encounter DNA damage P53 becomes activated and subsequently influences cell fate depending on if the cell can recover from DNA damage or not.. We now show that FOXOs might fulfill a similar or additive role in S and G2 as FOXOs are capable to abort the cell cycle before entering mitosis. Similar to p53, FOXOs respond to cellular stresses like DNA damage, become activated and participate in the DNA damage response (373, 454-456). Although FOXO and p53 functions are intertwined, we do not observe p53 activation in response to FOXO3.A3 expression, suggesting FOXOs have

parallel functions to DNA damage signaling in S and G2 and might act as sensors of other homeostatic properties that need to be in check before mitosis. If FOXOs influence the p53 mediated G2 exit and the other way around still needs to be fully characterized however, as we do observe decreased sensitivity for the FOXO induced cell cycle exit in the absence of P53. The exit from G2 is observed only after 15-35 hours in G2, suggesting that FOXOs buy the cell time to recover from sudden fluctuations in cellular homeostasis using gradual reduction of EMI1 levels as a clock. If the cell is not recovered when EMI1 levels drop below the threshold to restrain APC^{CDH1} activity, the cell exits the cell cycle and arrests in G0/G1. Taken together we reveal a novel way in which FOXO can prevent cell cycle progression.

Materials & Methods

Cell culture

Retinal Pigment Epithelial cells (RPE) were cultured in DMEM-F12 (Lonza) containing 10% FCS (Lonza), 100U/ml penicillin and 100mg/ml streptomycin (Lonza). HEK293T cells were cultured in DMEM (Lonza) containing 10% FCS (Lonza), 100U/ml penicillin and 100mg/ml streptomycin (lonza). For doxycycline treatment 100 nM Doxycycline was used for the duration of indicated times. Transfecting third generation packaging vectors using Poly-ethylenimine into HEK293T cells generated lentiviral particles (377). Transfection of pCDNA-HA-E2F1, pCDNA-GFP-FOXO1 and pCDNA-GFP-FOXO3 in HEK293T cells was performed using FuGENE HD (Promega).

Constructs, lentiviral transduction and transfections

RPE-FUCCI cells were obtained as a kind gift from the Medema lab (440, 442) Lentiviral cDNA expression vectors expressing FOXO3.A3 were generated using Gateway cloning in the pINDUCER20 (Addgene #44012) doxycycline inducible expression system (371). The lentiviral construct pCW-mTurqouise-EMI1 was obtained as a kind gift from the Medema lab (449).

Immunoblotting & antibodies

For western blot cells are lysed in sample buffer sample buffer containing 0.2%SDS, 10% glycerol, 0.2% b-mercapto ethanol, 60mM Tris pH6.8. Proteins were detected using 6-15% SDS-PAGE gels and subsequent western-blot analysis with primary antibodies recognizing FOXO3 (H144 Santa Cruz), p27 (BD-610241), p21 (SC-471), p53 (SC-126), EMI1 (ThermoFisher 3D2D6), E2F1 (CST-3742), RB (BD-654136) RB-S807/811 (CST-9308), MXI1 (SC-1042), MYC (CST-9402), Cyclin A (AB-16726), Cyclin E (SC-198), HA (SC-12CA5), GFP (AB-6673), used 1:2000. Primary antibodies were detected by secondary HRP conjugated antibodies targeting mouse, rabbit, and rat IgG and visualized using chemiluminescence (Biorad) and a ImageQuant LAS 4000 scanner (GE Healthcare). For immunoprecipitation HEK293T cells transfected with HA-E2F1, GFP-FOXO1, GFP-FOXO3 were lysed in 50mM Tris, 150mM NaCl, 1% Triton-X100. For immunnuprecei[itation Chromotec GFP beads were incubated with cell lysate for 2 hours at 4°C, subsequently washed with lysis buffer and boiled in sample buffer.

RT-qPCR

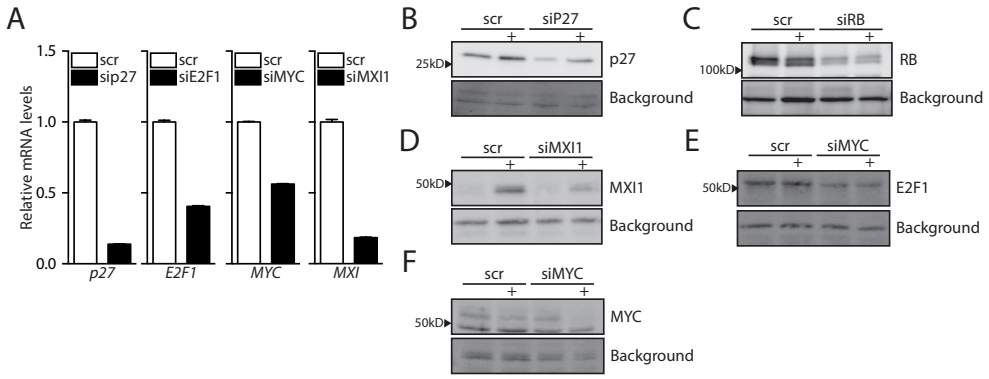
mRNA was isolated from live cells using the Qiagen RNeasy kit (Qiagen) and cDNA synthesis was performed using the iScript cDNA synthesis kit (BioRad). Real-time PCR was performed using SYBR green FastStart master mix (Roche) in the CFX Connect Real-time PCR detection system (BioRad). Target genes were amplified using specific primer pairs (Supplemental table 1) and specificity was confirmed by analysis of the melting curves. Target gene expression levels were normalized to *GAPDH* and *HNRNPA1* levels.

Flow cytometry

For DNA content profiling live cells were incubated with 10 mM Hoechst33342 for 30 min. at 37°C. After incubation cells were trypsinized and transferred to normal culture medium before measuring. mKO-hCDT1, mAG-hGeminin and Hoechst33342 intensity was measured using a BD LSR Fortessa Flow cytometer (BD bioscience).

Live cell imaging and tracking

20.000 RPE cells were cultured in Lab-Tek II 8-well imaging chambers. Prior to imaging normal culture medium is replaced with Leibovitz medium (Lonza) containing 10% FCS (Lonza),2 mM L-Glutamin, 100U/ml penicillin and 100mg/ml streptomycin (Lonza). Imaging was performed on a Zeiss Cell observer Real-Time imaging microscope for 48 hours at 37°C. Cell tracking and quantification was performed using ImageJ. Cells expressing mAG-hGeminin at the moment of doxycycline addition and cell starting to express mAG-hGeminin within 3 hours after doxycycline addition were considered S/G2 in the analysis.

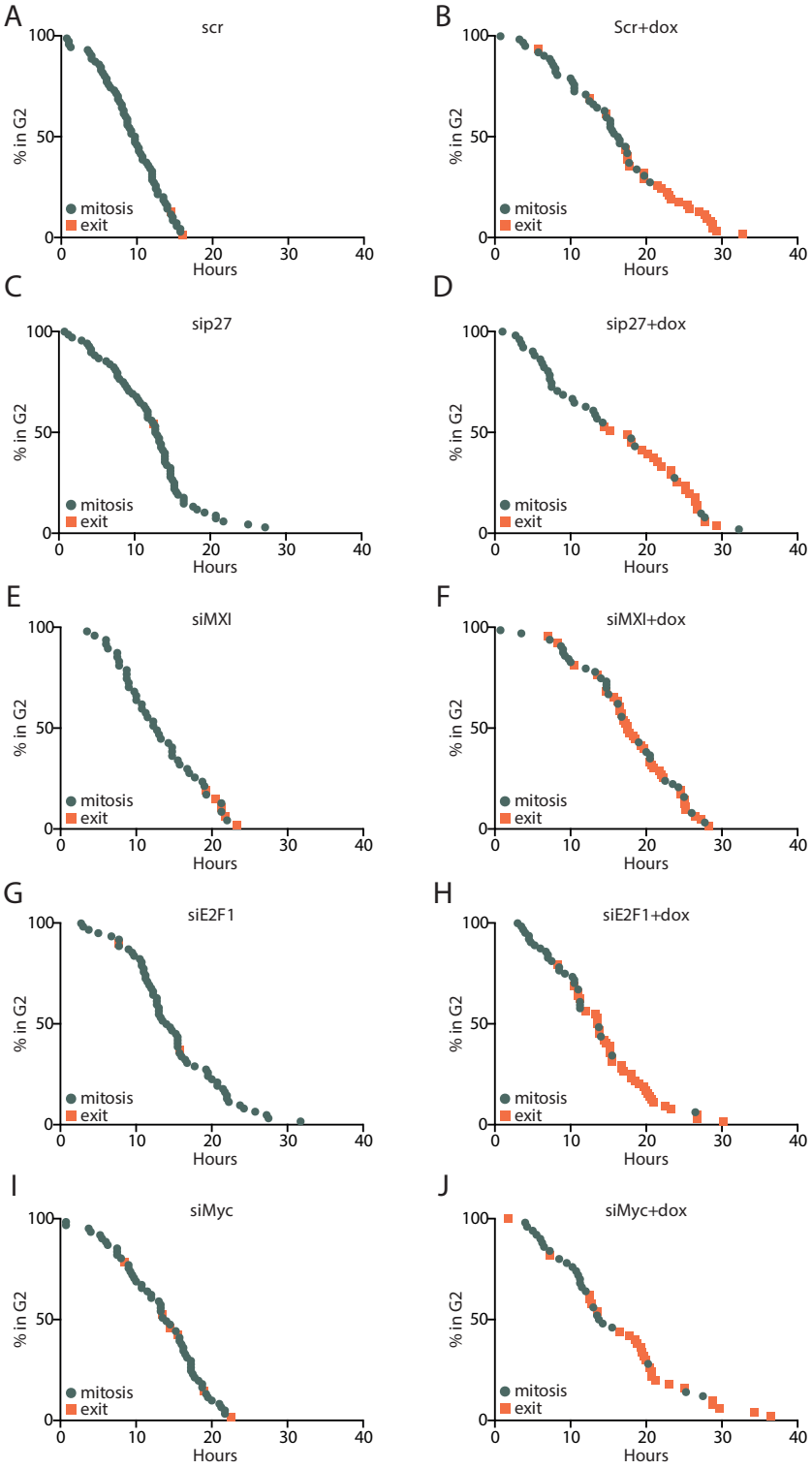


Supplemental Figure 1: siRNA knockdown efficiency for experiments in figure 4

A. RT-qPCR analysis of *p27, E2F1, MYC, MXI1* mRNA expression level changes in RPE-FUCCI-iFOXO3.A3 cells after 72 hours treatment with siSCR, siP27, siE2F1, siMYC or siMXI1. **B.** Western blot analysis of p27 protein levels in RPE-FUCCI-iFOXO3.A3 after 72 hours of siSCR or siP27 in combination with doxycycline treatment for 8 hours. Equal concentrations of protein are loaded and aspecific background staining is used to visualize equal protein content. **C.** Western blot analysis of RB protein levels in RPE-FUCCI-iFOXO3.A3 after 72 hours of siSCR or siRB in combination with doxycycline treatment for 8 hours. Equal concentrations of protein are loaded and aspecific background staining is used to visualize equal protein content. **D.** Western blot analysis of pMXI protein levels in RPE-FUCCI-iFOXO3.A3 after 72 hours of siSCR or siMXI in combination with doxycycline treatment for 8 hours. Equal concentrations of protein are loaded and aspecific background staining is used to visualize equal protein content. **E.** Western blot analysis of E2F1 protein levels in RPE-FUCCI-iFOXO3.A3 after 72 hours of siSCR or siE2F1 in combination with doxycycline treatment for 8 hours. Equal concentrations of protein are loaded and aspecific background staining is used to visualize equal protein content. **F.** Western blot analysis of MYC protein levels in RPE-FUCCI-iFOXO3.A3 after 72 hours of siSCR or siMYC in combination with doxycycline treatment for 8 hours. Equal concentrations of protein are loaded and aspecific background staining is used to visualize equal protein content.

Supplemental figure 2: Live cell imaging quantification of the time spend in G2 corresponding to Figure 4.

A-J. Live cell imaging quantification of the time spend in G2 of RPE-FUCCI-iFOXO3.A3 treated with siSCR, siP27, siMXI1, siE2F1 or siMYC for 72 hours. Each dot represents a cell and corresponds to the time spend in G2 without or after FOXO3.A3 expression. Green indicates the cell exits G2 by mitosis, orange indicates the cells exits G2 skipping mitosis.


A
scr
B
Scr+dox
C
sip27
D
sip27+dox
E
siMXI
F
siMXI+dox
G
siE2F1
H
siE2F1+dox
I
siMyc
J
siMyc+dox
% in G2
Hours
mitosis
exit
0
50
100
10
20
30
40

Chapter 7:

General discussion

Redefining the role of FOXOs in cancer

FOXOs are not tumor suppressors

FOXOs are tumor suppressors. This is the textbook view attributed to FOXOs in the context of their role in cancer. The initial seminal reports identifying FOXOs as the major transcriptional output of PI3K-PKB signaling were soon followed by the observation that FOXO induces either a cell cycle arrest or apoptosis, when activated in response to PI3K-PKB inhibition or when a mutant insensitive to PKB regulation is expressed (7, 35, 36, 39, 407, 416). As the PI3K-PKB pathway is commonly hyper activated in cancer and restricts FOXO activity, the idea that FOXOs could act as tumor suppressors was coined (457, 458). Indeed reduction of primary tumor growth was observed in xenograft experiments with tumor cells expressing hyperactive mutants of FOXO (46, 47). Conventional knockout mouse models for loss of individual *Foxo1*, *Foxo3* or *Foxo4* function did reveal developmental defects (*Foxo1* and *Foxo3*), but did however not indicate a role for individual FOXOs in tumor suppression (58-60). The fact that no tumor suppressive effects were found in single *Foxo* knockout mice proved to be the consequence of redundancy between FOXOs, as only compound loss of *Foxo1,3* and *4* knockout mice present with tumors (61). The observation that PI3K-PKB signaling inhibits FOXO to allow proliferation and avoid apoptosis, combined with the increased tumor incidence in *Foxo* triple knockout mice led to the proposition of FOXOs as bona fide tumor suppressors at least for certain tissue lineages (158). Our observations in **Chapter 3** fall in line with this concept as we show that FOXO inhibition is essential for anchorage independent survival. FOXO needs to be inhibited when cells are cultured in the absence of anchorage to prevent expression of the pro-apoptotic BH3-only proteins *BIM* and *BMF* and the subsequent induction of anoikis. In the case of mouse invasive lobular carcinoma cells, PI3K-PKB signaling is proposed to be active as a consequence of E-cadherin loss, and hence the subsequent inhibition of FOXO could allow cells to survive without anchorage (459). Indeed we find that loss of E-cadherin in anchorage dependent cells results in acquired anoikis resistance and correlates to restrained *BIM* and *BMF* expression (45). Additionally, we show in **Chapter 4** that expression of FOXO3 or FOXO3.A3 inhibits metastasis in mILC, confirming that cancer cells need to inactivate FOXOs in order to successfully disseminate.

The term bona fide tumor suppressor is commonly used for tumor suppressor genes and implies that these genes faithfully repress tumorigenesis. Suggesting that genes like FOXO exist to prevent cancer. However if the biological role of tumor suppressor proteins is truly focused on repressing tumorigenesis or that the tumor suppressive effect of certain genes is just a side effect of its normal function is debatable, certainly for FOXOs. In parallel to the findings that put FOXOs forward as tumor suppressors, various studies were published on the homeostatic function of FOXOs in balancing reactive oxygen species levels, stem cell maintenance and bioenergetics, FOXO functions expected to be predominantly involved in its longevity mediating effects (19, 21, 24, 89) (reviewed in: (2, 3, 10, 14)). There is no reason to assume however that FOXO discriminates between tumor cells and healthy cells in its homeostatic function. The outcome of this function is however context dependent and may be perceived as tumor suppressive functions in tumor cells. For instance, even a mild induction of CDKis or pro-apoptotic factors may be enough to push tumor cells over the apoptotic threshold or into a cell cycle arrest. Once the tumor has reached a stage that it is insensitive to these aspects of FOXO function, it may actually benefit from for instance the ROS scavenging roles downstream of FOXOs. Studies on the consequence of FOXO loss in neuronal and hematopoietic stem cells, CML and AML indeed revealed that both healthy and tumor cells depend on the presence of FOXO in order to function optimally (21, 24, 73, 76). Additionally, FOXO was found to facilitate breast cancer cell motility, stimulate metastasis of

colorectal cancer cells and to participate in development of resistance to PI3K and PKB inhibitors (72, 77, 94-96).

Our current understanding of the role of FOXOs in cancer appears contradictory and suggests that tumor suppression or tumor supportive functions of FOXO are at least context-dependent. Based on the observation summarized above, the suggestion that FOXOs are bona fide tumor suppressors clearly needs revision. In **chapter 3 and 4** we addressed the role of FOXO in cancer by studying the effect of gain and loss of FOXO function in one defined model system of tumorigenesis, namely invasive lobular carcinoma. The experiments demonstrate that tumor cells need to restrain FOXO activity in a PKB-dependent manner. Overexpression of wild-type FOXO3 does not significantly influence primary tumor growth but overexpression of the PKB insensitive FOXO3. A3 mutant inhibits primary tumor growth efficiently. Restriction of FOXO activity is important for tumor development, but losing the ability to activate FOXO was found to be even more detrimental for tumor growth. Similarly, gain or loss of FOXO activity equally well delayed the timing of metastasis formation. This suggests that both hyper activation and loss of FOXO obstructs the extravasation or homing of primary tumor cells. Interestingly, in contrast to the absence of effect on primary tumor growth, over expression of wild-type FOXO3 did influence metastasis onset, indicating that cells tolerate high levels of FOXO expression in the primary tumor but not during the process of metastasis. These observations imply that during tumor progression the regulation of FOXO activity needs to be fine-tuned and different FOXO activity levels are required at distinct stages of tumor progression.

The fact that aggressive types of cancer rely on activated growth factor signaling in one way or another, and consequentially repress FOXO activity, does suggest tumors always need to restrict FOXO activity to a certain extent. It is curious however that FOXOs are rarely lost or mutated in a way that activity is fully reduced. Additionally, we describe in **Chapter 4** that cells isolated from FOXO3.A3 induced tumors still express super-endogenous levels of hyper-active FOXO3.A3 without suffering from reduced proliferation. FOXOs are predicted to be active in cancers of different grades, using the computational model for FOXO activity generated in **chapter 5** on patient cancer samples, suggesting FOXOs are actively participating in tumorigenesis and not solely act as tumor suppressors.

Based on these observations it becomes temping to speculate that endogenous (low) levels of FOXO activity are hardly tumor suppressive when it comes to primary tumor formation, and actually aid in metastatic outgrowth. Whether endogenous FOXOs can confer a tumor suppressive phenotype has not been studied outside the context of PKB inhibition or FOXO overexpression, since these experiments are technically challenging, for example by making a heterozygous endogenous inducible PKB insensitive FOXO mutant, but this question needs to be addressed in the future to understand how FOXO works.

A gene that needs to be inactivated by mutation or lost in order for cancer to arise is the definition of a tumor suppressor gene (460). Based on our findings presented in this thesis and this definition of a tumor suppressor, we conclude that FOXOs are not classical tumor suppressors. Again, it needs to be stressed that oncogenes and tumor suppressor genes are rather non-biological terms for the function of these genes in general. There are no genes that solely exist to cause or suppress cancer, let alone show bona fide or mala fide behavior. Rather the phenotypes caused by mutations in these genes represent an exaggeration of the signaling mechanisms they are normally involved in. Addressing the physiological functions of genes involved in cancer in healthy tissues instead of focusing on their function as oncogenes and tumor suppressors results in understanding of their role in cancer. Since versatile and complex 3D cell culture systems resembling healthy tissues and organs have recently, and finally, become broadly

available for in vitro studies, this will be a strong tool to apply for this purpose (461).

Dependency of cancer on FOXO: primary tumor vs. metastasis

From **Chapter 3, 4 and 5** the picture arises that FOXO needs to be regulated in a flexible manner within the primary tumor, but in addition needs to be strongly inactivated during the metastatic process. The fact that overexpression of wild-type FOXO3 hardly influences primary tumor growth compared to FOXO3.A3 shows that FOXO activity is restricted in a PKB dependent way also within the tumor. However, FOXOs are expected to be active in the majority of breast cancers based on our observations in **Chapter 5**. The *in vitro* data presented in **chapter 3** on the effects of FOXO3 expression in mILC1 shows that FOXO represses proliferation under adherent conditions and switch to inducing apoptosis when mILC1 is cultured in suspension. This indicates that not only the tumor stage but also the space in which the cell resides heavily impacts on the output of FOXO mediated transcription. Whether changes in concurrent signaling upon transfer to anchorage independent growth or changes in the epigenetic landscape are driving FOXO output towards apoptosis remains to be elucidated. In **Chapter 3** we find that FOXO3.A3 binding to the *Bmf* locus is further enhanced upon transfer to suspension. This suggests the *Bmf* locus becomes more permissive for FOXO binding when cells are cultured in suspension. Therefore low levels of FOXO activity in suspension might already be sufficient to drive the cell into anoikis while under adherent conditions low levels of FOXO activity is better tolerated with respect to apoptosis. These observations lead us to draw up a model in which the environmental context of a cancer cell determines whether FOXOs are tumor suppressors or not (Figure 1). The induction of anoikis by FOXO fits well in its broad spectrum of homeostatic functions, as preventing ectopic cell growth is essential to maintain tissue integrity and organ function. But this still does not explain why FOXOs are retained in cancer if they need to be inactivated anyway.

Close examination of how losing endogenous FOXOs affects cancer cells in **Chapter 4** revealed that FOXOs are involved in many different processes required for optimal cancer cell growth. In line with the homeostatic functions of FOXO discussed above we find that FOXO is required for maintaining proliferative signaling, redox homeostasis, glucose uptake and bioenergetics. Losing FOXO in mILC results in high levels of reactive oxygen species, low bioenergetic metabolism and low PI3K-PKB signaling. This shows that although FOXO supposedly is largely inhibited by PKB, FOXO is actually actively contributing to maintaining cellular homeostasis. These findings support the observation in **Chapter 5** that suggest that FOXOs are expected to be active in multiple breast cancers. It is unclear however how much FOXO activity is required for its homeostatic function and how much for its cell cycle and apoptotic regulatory function.

Active growth factor signaling, cell motility and invasion are essential for a cancer cell to metastasize and these are reduced upon FOXO loss. This might explain why mILC1-shFOXOs cells have more difficulty to establish metastasis compared to FOXO3.A3 cells that only need to overcome the anti-proliferative and anoikis effects of FOXO hyper-activation. Also we have to keep in mind that we only observe strong anoikis induction when FOXOs are over expressed or in the context of PKB inhibition: a situation in which FOXO is artificially hyper active and a situation in which PKB is concurrently inhibited. The observation that mILC1 cells also induce *Bim* and *Bmf* and a fraction undergoes anoikis when untreated conditions illustrates that mILC1 cells are already balancing on the edge of anoikis with low levels of active endogenous FOXO. This shows that balancing FOXOs is essential also in metastasizing cells and having a small window FOXO activity might come with the benefit of maintaining glucose uptake, GFR signaling and redox homeostasis: all crucial requirements for anchorage independent survival (292). The role of FOXOs in cancer is clearly complex but based on the results presented in this thesis we propose a model in which the phase of tumorigenesis and the specific spatial context of the cancer cell determines the dependency on and the transcriptional

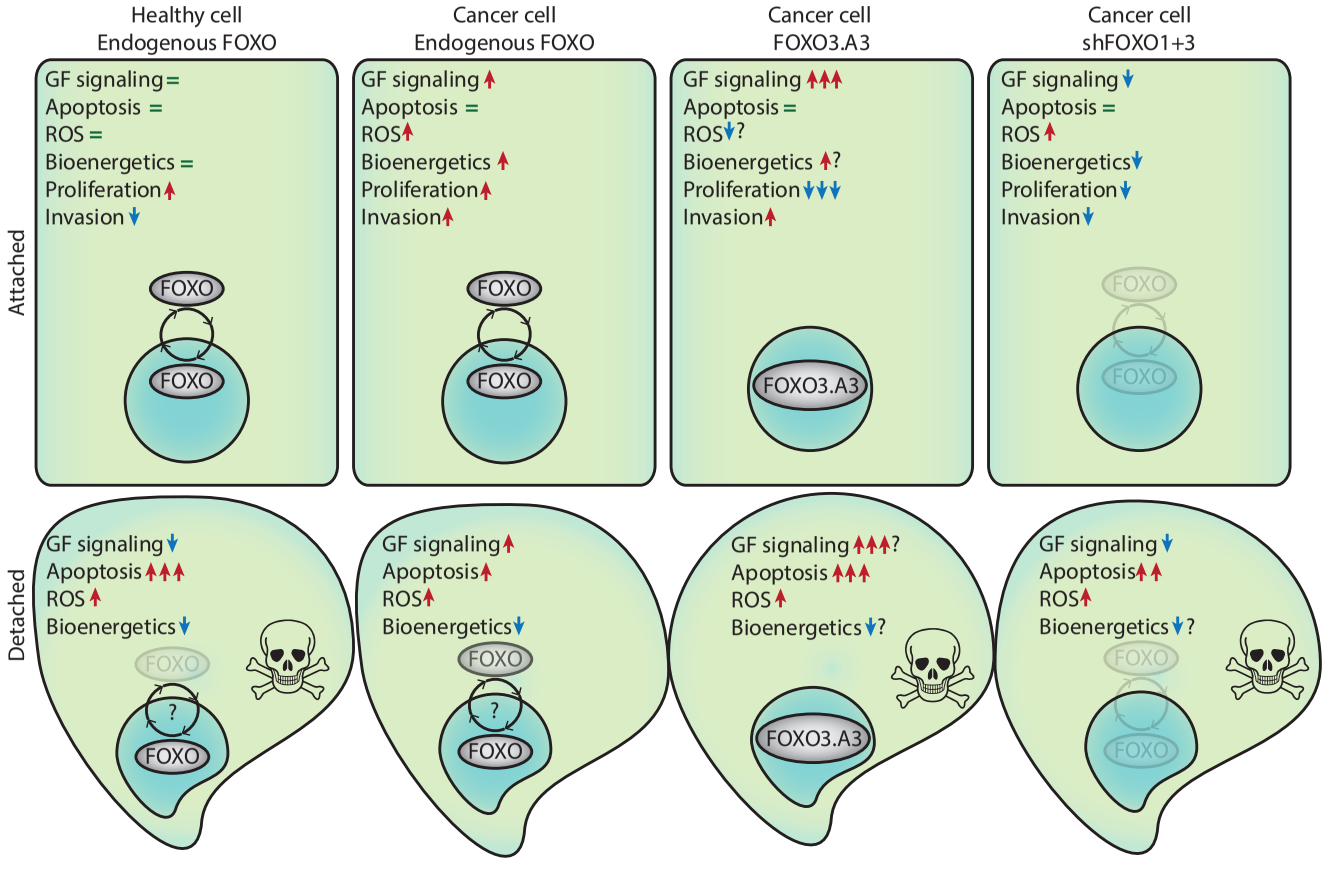


Figure 1: Spatiotemporal dependency of cancer on FOXO: primary tumor vs metastasis.
Illustrated are the effects of different levels of FOXO activity on Growth factor signaling, apoptosis, ROS levels, Bioenergetics, proliferation and invasion in the primary tumor or healthy tissue (attached) and cells that are disseminating or accidentally detached (detached). Arrows indicate if processes are up or down based on this thesis and previously published data. Question marks (?) indicate processes that are not characterized in detail to date.

program of FOXO (Figure 1). Having FOXOs around during the early stages of tumor development is beneficial for tumors to expand, and only during the short period a cancer cell metastasizes FOXO activity needs to be limited.

Maintaining the FOXO balance

The next step in understanding FOXO function is characterizing how FOXO activity is subtly regulated. There are various mechanisms proposed however by which FOXO activity can be modulated and tuned (Reviewed in(2, 3, 16, 20)). Nuclear translocation of FOXOs was found to be a continuous dynamic process as blocking overall nuclear export using CRM1 inhibitors results in nuclear accumulation of FOXOs even in the presence of active growth factor signaling (5, 9, 462). These observations illustrate that FOXO does not operate in a binary "ON" and "OFF" fashion but in equilibrium between nuclear import and export. The rate of nuclear import and export is influenced by a multitude of post-translational modifications that can affect FOXOs reported to date (Chapter 1, Figure 1)(2, 3). Stress kinases, DNA damage, AMPK kinase, redox and growth factor signaling are the best-established routes modulating FOXO transcriptional activity. Next to responding to nutrient availability, ROS levels, DNA damage and other stimuli directly, recently it became clear that FOXOs are part of a continuous growth factor signaling feedback loop that is required for maintaining PI3K signaling (6, 11, 71, 94-96). When growth factor signaling is low, FOXO accumulates in the nucleus and induces transcription and amongst these target genes are those that constitute essential parts of growth factor signaling cascades including various RTKs, RTK adaptor proteins and regulators of the mTOR complex 1 and 2. By transcribing these genes FOXO stimulates growth factor signaling and its own inactivation. Inactivation of FOXOs subsequently results in lowered expression of RTK pathway components and reduction of growth factor signaling (Figure 2). The relevance of this signaling feedback in healthy cells remains to be fully characterized but a likely function is sensitize growth factor signaling during a period of low nutrient and growth factor availability as to optimize the response to future growth factor availability. The discovery of this feed back mechanism is of great importance with respect to cancer therapy as it was found to be a key mechanism behind development of resistance to PI3K-PKB inhibiting drugs (94, 96). Loss of FOXO impaired the development of drug resistance but it remains to be characterized which components of growth factor signaling that are directly regulated by FOXOs are responsible for this. In **chapter 4** we followed up on these findings in mILC1 and show that FOXO loss resulted in decreased PI3K-PKB signaling and expression of FOXO3.A3 increased pathway activity. We found that FOXOs indeed regulate the expression of multiple RTKs, RTK adaptor proteins and regulators of mTOR complex 1 and 2. Showing that FOXOs drive the expression novel and previously described components of RTK signaling and that modulation of FOXO activity in vivo directly impacts on RTK signaling activity. These results emphasize that using PI3K-PKB inhibitors or other strategies that result in strong FOXO activation has dangerous side effects due to this innate growth factor signaling feedback. The mild phenotypes observed in FOXO3 wild type experiments can be explained by this feedback loop, as high levels of FOXOs mediate their own inactivation. Next to the complex phenotype caused by loss of FOXO described in **chapter 4**, the fact that FOXO activity needs to be constantly balanced provides an additional explanation as to why it is hard to rescue the loss of FOXO by only over expressing FOXO or adding back hyper active $PI3K^{H1047R}$ and ^{myr}PKB.

Next to its role in cancer, this feedback loop might also be essential in the onset of diabetes. In **chapter 4** we show that loss of FOXOs results in metabolic defects including impaired glucose uptake and loss of growth factor signaling and therefore insulin sensitivity. In other words, loss of FOXOs induces a "diabetes type II-like phenotype" in cancer cells.

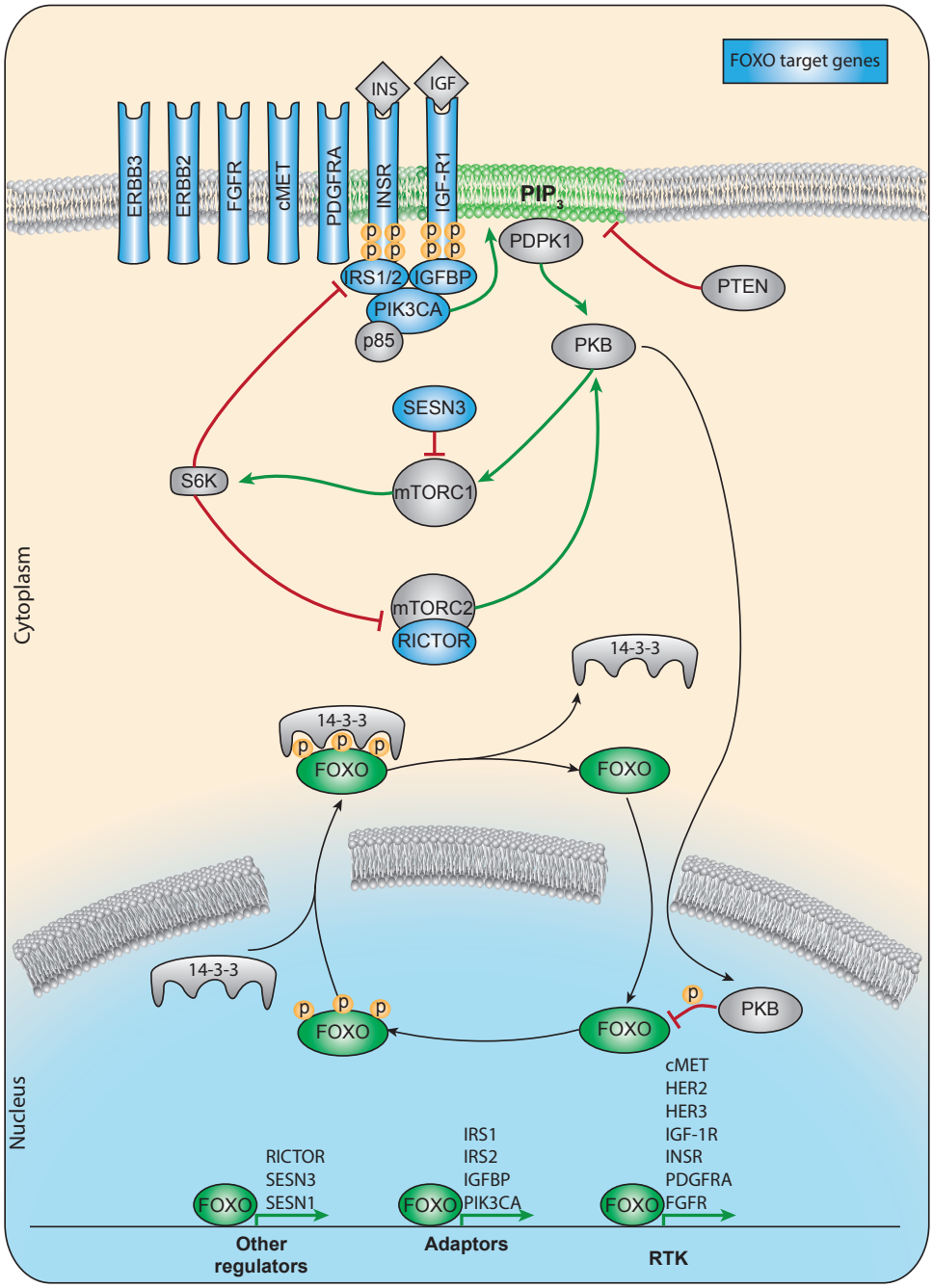


Figure 2: The growth factor signaling balance between RTK and FOXO activity.
The scheme illustrates intrinsic growth factor signaling feedback loop that balances RTK activity with FOXO activity. In blue are direct target genes of FOXO transcribed upon FOXO accumulation in the nucleus.

It is clear that mechanism by which the cell mediates FOXO functions is through balancing nucleo-cytoplasmic shuttling. Experiments scrutinizing the exact endogenous dynamics of FOXO shuttling are lacking to date however, mostly due to technical difficulties. Understanding the dynamics of endogenous FOXO shuttling and intervening with these dynamics might hold interesting insights for both cancer therapy and diabetes treatment. Modern genome editing technologies allowing modulation and tagging of endogenous proteins recently became available and paves the way to elucidate physiological shuttling rates of FOXO under various conditions.

Using FOXO in the clinic

Although FOXO is implicated in major diseases like cancer and diabetes, developing FOXO targeted therapies or using FOXO as a diagnostic marker has been proven difficult. Histopathological studies that focused on correlating FOXO expression and localization to disease outcome in cancer patients yielded contradicting results. On one hand many studies observed a correlation between high FOXO expression and/or nuclear localization and good prognosis (48-57). On the other hand various studies implicated that high FOXO levels and/or activity correlate to poor prognosis (68-72, 404). Although these studies come to contradictory conclusions we noticed that most of these studies did not include sufficient technical and biological IHC controls to validate their observations. We have systematically analyzed a great variety of antibodies that are employed in IHC staining of FOXO and surprisingly for many of these antibodies it is unclear whether they actually stain FOXO in IHC, as they similar signals in FOXO TKO tissue (own unpublished observation). In addition, many papers do not report the antibodies used, making interpretation and reproduction of these studies impossible. Also from a functional perspective as reported in **chapter 4** it becomes apparent that FOXO does not fulfill a role in cancer that adheres to a classical view as being either anti- or pro-tumorigenesis. Using FOXO as a prognostic marker for cancer progression therefore appears at least difficult if it is unclear at what stage of cancer progression FOXO is being analyzed. Due to its role in therapy resistance, correlating *in vivo* FOXO activity prior to and following patient treatment to therapy response might be interesting in light of increasing therapy efficiency or the decision to undergo therapy in the first place. Histopathological examination of FOXO activity in this perspective is not expected to be the suitable approach as we show that even when FOXOs is expected to be inactive in tumors based on the tumors dependency on active growth factor signaling, this is not necessarily the case. Considering the above, determination of the expression level of FOXO target genes is likely more informative to determine what the actual status of FOXO activity is. In **chapter 5** we determined a core set of FOXO transcriptional target genes based on microarray and publicly available data set analysis. We used this core set of genes to predict PI3K activity in patient material. Interestingly, FOXOs are predicted both active and inactive in a variety of cancer patient tissues, including high grade tumors. Additionally, immunohistochemical staining of FOXO3 in colon carcinomas revealed that FOXO activity is heterogeneously spread throughout the tumor, based on the observation that within the same slice there are cells with nuclear and cells with cytoplasmic FOXO3. Together these observations illustrate that FOXO activity is heterogeneous within tumor types but also within tumors it self, explaining why the many pathological studies on FOXOs come to opposing conclusions. This FOXO target gene set can also be applied on data sets and patient material in order to correlate FOXO levels and activity to therapy response in the future. One might expect that tumors with low levels of FOXO expression are less susceptible for developing treatment resistance towards PI3K inhibitors for example. The FOXO core target genes could also be used to uncover unexpected contributions of FOXO to therapy response in more conventional therapies that do not specifically target the PI3K pathway.

In this thesis we showed that multiple aspects of tumorigenesis are dependent on FOXOs. Targeting FOXOs directly and preventing FOXO activity might therefore be a suitable strategy for cancer therapy. Repressing FOXOs using a drug might induce similar effects as we observed using the shFOXOs. Combining these compounds with drugs targeting PI3K might then aid in preventing resistance. Attempts to design FOXO specific inhibitors are limited but have been reported. AS1842856 is the only commercially available small molecule inhibitor targeting FOXOs and should prevent translocation to the nucleus (463). AS1842856 has mainly been tested in the context of diabetes, bone development, adipocyte differentiation and pulmonary hypertension (464-467). In cancer AS1842856 was found to prevent the induction of senescence in progesterone receptor positive ovarian cancer and block MYC driven lapatinib resistance in breast cancer (468, 469). Although the results suggest FOXO inhibition is possible and has promising effects in cancer, we have to note that we could not confirm inhibition of FOXO translocation in mILC1 cells treated with AS1842856. No studies have been published to date that show FOXO translocation is inhibited by AS1842856 so caution with drawing conclusions based on this drug is required. The development of additional pan-FOXO inhibitors is desirable based on the promising effects of inhibiting FOXOs in cancer presented in this thesis.
We also uncovered the next hurdle that should be taken in case FOXO inhibitors would eventually be used in the clinic, namely the adaptation to impaired FOXO function. In **Chapter 4** we show potent inhibition of cancer progression when shFOXOs are expressed in mILC, but also show that tumors adapt to the loss of FOXOs and regain their growth and metastatic capacity. In contrast to negative selection against the FOXO3.A3 construct we show that shFOXO tumors retain the shRNA construct and become FOXO independent. The mechanism that drives this adaptation is unclear, but understanding this mechanism holds a promising strategy for cancer therapy as blocking this adaptation can be deleterious for a tumor.

A new perspective on an old function of FOXO in the cell cycle
Inhibition of the cell cycle was amongst the first functions described for FOXO and through the years the transcription of several CDKis has been linked to FOXO activity. FOXO induced CDKi transcription can be initiated by various stimuli including growth factor withdrawal and adverse conditions like DNA damage, elevated levels of ROS or hypoxia (2, 3, 451). The cell cycle arrest induced by CDKis is proposed to function as a safeguard for tissue, cellular and genomic homeostasis and prevents the cell from dividing untimely or under adverse conditions. The role for FOXO in establishing a G0/G1 arrest is well studied but how FOXO mediates a putative G2 arrest remained to be fully characterized (39, 425, 426). In **chapter 6** we aimed to characterize the FOXO induced cell cycle arrest in more detail and elucidate how FOXO determines to arrest in G0/G1 or G2. By combining classic DNA cell cycle profiling with the newly described FUCCI system we unveiled that FOXO activation in S/G2 results in switching to a G0/G1 like state with 4n DNA (440). This function of FOXO has long been obscured by the way cell cycle phase determination has been performed. We now show that merely looking at DNA content, even in combination with S-phase or mitosis markers, is insufficient to draw conclusions on the cell cycle phase a cell resides in. Although the full mechanism behind the FOXO induced G2 withdrawal needs to be characterized we show that this mechanism involves the down regulation of EMI1 by preventing its transcription. If there is a physiological stimulus that induces this FOXO mediated cell cycle exit is still under investigation and what the purpose of this mechanism is in the cell cycle remains to be defined. This FOXO function might be to guard the cell from dividing under adverse conditions and thus reduce the risk of damaging the genome or vital organelles. By repressing mitotic entry FOXO allows the cell to anticipate and recover from adverse

conditions before entering mitosis. Gradual down regulation of EMI1 by FOXO might function as a timer for how long a cell is allowed to recover from an adverse condition. If FOXO becomes inactivated as a result of recovery before EMI1 levels drop below the APC^{CDH1} inhibiting threshold the cell will undergo mitosis. Conversely, if recovery takes too long and FOXOs remain activated, EMI1 levels drop below the APC^{CDH1} inhibiting threshold and the cell exits the cell cycle. This way EMI1 levels do not only function as the G1/S restriction point but also as a G2/M restriction point (446).
Similar to FOXO, p53 functions to sense adverse conditions in S/G2 and prevents entry into mitosis when the cell is suffering from DNA damage or elevated ROS levels (441-443, 448, 449). Even though we do not find active contribution of p53 to the FOXO induced cell cycle arrest we do observe that cells are less susceptible to undergo a FOXO induced G2 exit in the absence of p53. As FOXO and p53 signaling is heavily intertwined and responds to similar stimuli we expect that under physiological circumstances FOXO and p53 function in overlapping pathways (144, 373). Future characterization of how p53 and FOXO mediated G2 exit is attenuated in the absence of either FOXO or p53 is needed to determine the exact contribution of each to this phenotype.

In this thesis we show that the role of FOXO in cancer, but also in healthy cells, is highly complex. Instead of being a classical tumor suppressor that induces a cell cycle arrest or apoptosis in response to stresses or GF withdrawal, we show that FOXO functions in many processes essential for tumorigenesis. The insight that FOXO plays a key role in tumorigenesis and that balancing FOXO activity is crucial for its cancer supporting function opens many new roads to be explored. Inhibiting FOXO by designing small molecule inhibitors might abrogate development of drug resistance, the current hurdle in cancer therapy. Conversely, finding strategies that fully activate FOXO in disseminated cells might severely hamper cancer progression and increase patient survival. Finding ways to stimulate the FOXO induced G2 cell cycle exit might efficiently inhibit cancer cell proliferation. Understanding and applying FOXO transcriptional output could help in the development of better prognostic tools for cancer patient survival and therapy. All new possibilities sprouting from observations made while characterizing the fundamental properties of FOXO and shifting the paradigm from FOXO as a tumor suppressor to FOXO as a key regulator of homeostasis in both healthy and cancer cells.

&

Addenda:

References

Nederlandse samenvatting

Curriculum Vitae

List of publications

Dankwoord

References:

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
2. Eijkelenboom A, Burgering BM. FOXOs: signalling integrators for homeostasis maintenance. Nat Rev Mol Cell Biol. 2013;14(2):83-97.
3. van der Horst A, Burgering BM. Stressing the role of FoxO proteins in lifespan and disease. Nat Rev Mol Cell Biol. 2007;8(6):440-50.
4. Furuyama T, Nakazawa T, Nakano I, Mori N. Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. The Biochemical journal. 2000;349(Pt 2):629-34.
5. Jacobs FM, van der Heide LP, Wijchers PJ, Burbach JP, Hoekman MF, Smidt MP. FoxO6, a novel member of the FoxO class of transcription factors with distinct shuttling dynamics. J Biol Chem. 2003;278(38):35959-67.
6. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science. 2005;307(5712):1098-101.
7. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell. 1999;96(6):857-68.
8. Kops GJ, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL, Burgering BM. Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature. 1999;398(6728):630-4.
9. Brunet A, Kanai F, Stehn J, Xu J, Sarbassova D, Frangioni JV, et al. 14-3-3 transits to the nucleus and participates in dynamic nucleocytoplasmic transport. J Cell Biol. 2002;156(5):817-28.
10. Kenyon CJ. The genetics of ageing. Nature. 2010;464(7288):504-12.
11. Eijkelenboom A, Mokry M, de Wit E, Smits LM, Polderman PE, van Triest MH, et al. Genome-wide analysis of FOXO3 mediated transcription regulation through RNA polymerase II profiling. Mol Syst Biol. 2013;9:638.
12. Eijkelenboom A, Mokry M, Smits LM, Nieuwenhuis EE, Burgering BM. FOXO3 selectively amplifies enhancer activity to establish target gene regulation. Cell Rep. 2013;5(6):1664-78.
13. van der Vos KE, Coffer PJ. FOXO-binding partners: it takes two to tango. Oncogene. 2008;27(16):2289-99.
14. Storz P. Forkhead homeobox type O transcription factors in the responses to oxidative stress. Antioxidants & redox signaling. 2011;14(4):593-605.
15. Essers MA, Weijzen S, de Vries-Smits AM, Saarloos I, de Ruiter ND, Bos JL, et al. FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. EMBO J. 2004;23(24):4802-12.
16. van den Berg MCW, Burgering BMT. Integrating opposing signals toward forkhead box o. Antioxidants & redox signaling. 2011;14(4):607-21.
17. Putker M, Madl T, Vos HR, de Ruiter H, Visscher M, van den Berg MC, et al. Redox-dependent control of FOXO/DAF-16 by transportin-1. Mol Cell. 2013;49(4):730-42.
18. Putker M, Vos HR, van Dorenmalen K, de Ruiter H, Duran AG, Snel B, et al. Evolutionary acquisition of cysteines determines FOXO paralog-specific redox signaling. Antioxid Redox Signal. 2015;22(1):15-28.
19. Yeo H, Lyssiotis CA, Zhang Y, Ying H, Asara JM, Cantley LC, et al. FoxO3 coordinates metabolic pathways to maintain redox balance in neural stem cells. EMBO J. 2013;32(19):2589-602.
20. Klotz LO, Sanchez-Ramos C, Prieto-Arroyo I, Urbanek P, Steinbrenner H, Monsalve M. Redox regulation of FoxO transcription factors. Redox Biol. 2015;6:51-72.
21. Tothova Z, Kollipara R, Huntly BJ, Lee BH, Castrillon DH, Cullen DE, et al. FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell. 2007;128(2):325-39.
22. Miyamoto K, Araki KY, Naka K, Arai F, Takubo K, Yamazaki S, et al. Foxo3a is essential for maintenance of the hematopoietic stem cell pool. Cell stem cell. 2007;1(1):101-12.
23. Yalcin S, Marinkovic D, Mungamuri SK, Zhang X, Tong W, Sellers R, et al. ROS-mediated amplification of AKT/mTOR signalling pathway leads to myeloproliferative syndrome in Foxo3(-/-) mice. EMBO J. 2010;29(24):4118-31.
24. Paik JH, Ding Z, Narurkar R, Ramkissoon S, Muller F, Kamoun WS, et al. FoxOs cooperatively regulate diverse pathways governing neural stem cell homeostasis. Cell Stem Cell. 2009;5(5):540-53.
25. Kousteni S. FoxO1, the transcriptional chief of staff of energy metabolism. Bone. 2012;50(2):437-43.
26. Greer EL, Oskoui PR, Banko MR, Maniar JM, Gygi MP, Gygi SP, et al. The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription factor. J Biol Chem. 2007;282(41):30107-19.
27. Van Der Heide LP, Hoekman MF, Smidt MP. The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. Biochem J. 2004;380(Pt 2):297-309.
28. Yang Y, Hou H, Haller EM, Nicosia SV, Bai W. Suppression of FOXO1 activity by FHL2 through SIRT1-mediated deacetylation. EMBO J. 2005;24(5):1021-32.
29. Wang YC, Li C. Evolutionarily conserved protein arginine methyltransferases in non-mammalian animal systems. FEBS J. 2012;279(6):932-45.
30. Yamagata K, Daitoku H, Takahashi Y, Namiki K, Hisatake K, Kako K, et al. Arginine methylation of FOXO transcription factors inhibits their phosphorylation by Akt. Mol Cell. 2008;32(2):221-31.
31. Dansen TB, Smits LM, van Triest MH, de Keizer PL, van Leenen D, Koerkamp MG, et al. Redox-sensitive cysteines bridge p300/CBP-mediated acetylation and FoxO4 activity. Nat Chem Biol. 2009;5(9):664-72.
32. Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, et al. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science. 2004;303(5666):2011-5.
33. van der Horst A, Tertoolen LG, de Vries-Smits LM, Frye RA, Medema RH, Burgering BM. FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2(SIRT1). J Biol Chem. 2004;279(28):28873-9.
34. Coomans de Brachene A, Demoulin JB. FOXO transcription factors in cancer development and therapy. Cell Mol Life Sci. 2016;73(6):1159-72.
35. Medema RH, Kops GJ, Bos JL, Burgering BM. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature. 2000;404(6779):782-7.
36. Kops GJPL, Medema RH, Glassford J, Essers MAG, Dijkers PF, Coffer PJ, et al. Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. Mol Cell Biol. 2002;22(7):2025-36.
37. Dick FA, Rubin SM. Molecular mechanisms underlying RB protein function. Nat Rev Mol Cell Biol. 2013;14(5):297-306.

38. de Keizer PLJ, Packer LM, Szypowska AA, Riedl-Polderman PE, van den Broek NJF, de Bruin A, et al. Activation of forkhead box O transcription factors by oncogenic BRAF promotes p21cip1-dependent senescence. Cancer Res. 2010;70(21):8526-36.
39. Alvarez B, Martínez-A C, Burgering BM, Carrera AC. Forkhead transcription factors contribute to execution of the mitotic programme in mammals. Nature. 2001;413(6857):744-7.
40. Courtois-Cox S, Genther Williams SM, Reczek EE, Johnson BW, McGillicuddy LT, Johannessen CM, et al. A negative feedback signaling network underlies oncogene-induced senescence. Cancer Cell. 2006;10(6):459-72.
41. Fu Z, Tindall DJ. FOXOs, cancer and regulation of apoptosis. Oncogene. 2008;27(16):2312-9.
42. Strasser A, Jost PJ, Nagata S. The many roles of FAS receptor signaling in the immune system. Immunity. 2009;30(2):180-92.
43. Ryter SW, Kim HP, Hoetzel A, Park JW, Nakahira K, Wang X, et al. Mechanisms of cell death in oxidative stress. Antioxid Redox Signal. 2007;9(1):49-89.
44. Strasser A, Cory S, Adams JM. Deciphering the rules of programmed cell death to improve therapy of cancer and other diseases. EMBO J. 2011;30(18):3667-83.
45. Hornsveld M, Tenhagen M, van de Ven RA, Smits AM, van Triest MH, van Amersfoort M, et al. Restraining FOXO3-dependent transcriptional BMF activation underpins tumour growth and metastasis of E-cadherin-negative breast cancer. Cell Death Differ. 2016.
46. Yang H, Zhao R, Yang H-Y, Lee M-H. Constitutively active FOXO4 inhibits Akt activity, regulates p27 Kip1 stability, and suppresses HER2-mediated tumorigenicity. Oncogene. 2005;24(11):1924-35.
47. Hu MC-T, Lee D-F, Xia W, Golfman LS, Ou-Yang F, Yang J-Y, et al. IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. Cell. 2004;117(2):225-37.
48. Zhang B, Tomita Y, Ch'ng E, Qiu Y, He J, Jin YF, et al. Prognostic significance of phosphorylated FOXO1 expression in soft tissue sarcoma. Ann Surg Oncol. 2009;16(7):1925-37.
49. Wu Y, Elshimali Y, Sarkissyan M, Mohamed H, Clayton S, Vadgama JV. Expression of FOXO1 is associated with GATA3 and Annexin-1 and predicts disease-free survival in breast cancer. Am J Cancer Res. 2012;2(1):104-15.
50. Cheong JW, Eom JI, Maeng HY, Lee ST, Hahn JS, Ko YW, et al. Constitutive phosphorylation of FKHR transcription factor as a prognostic variable in acute myeloid leukemia. Leuk Res. 2003;27(12):1159-62.
51. Zhang H, Pan Y, Zheng L, Choe C, Lindgren B, Jensen ED, et al. FOXO1 inhibits Runx2 transcriptional activity and prostate cancer cell migration and invasion. Cancer Res. 2011;71(9):3257-67.
52. Bullock MD, Bruce A, Sreekumar R, Curtis N, Cheung T, Reading I, et al. FOXO3 expression during colorectal cancer progression: biomarker potential reflects a tumour suppressor role. Br J Cancer. 2013;109(2):387-94.
53. Ni D, Ma X, Li HZ, Gao Y, Li XT, Zhang Y, et al. Downregulation of FOXO3a promotes tumor metastasis and is associated with metastasis-free survival of patients with clear cell renal cell carcinoma. Clin Cancer Res. 2014;20(7):1779-90.
54. Smit L, Berns K, Spence K, Ryder WD, Zeps N, Madiredjo M, et al. An integrated genomic approach identifies that the PI3K/AKT/FOXO pathway is involved in breast cancer tumor initiation. Oncotarget. 2016;7(3):2596-610.
55. Shiota M, Song Y, Yokomizo A, Kiyoshima K, Tada Y, Uchino H, et al. Foxo3a suppression of urothelial cancer invasiveness through Twist1, Y-box-binding protein 1, and E-cadherin regulation. Clin Cancer Res. 2010;16(23):5654-63.
56. Habashy HO, Rakha EA, Aleskandarany M, Ahmed MA, Green AR, Ellis IO, et al. FOXO3a nuclear localisation is associated with good prognosis in luminal-like breast cancer. Breast Cancer Res Treat. 2011;129(1):11-21.
57. Su B, Gao L, Baranowski C, Gillard B, Wang J, Ransom R, et al. A genome-wide RNAi screen identifies FOXO4 as a metastasis-suppressor through counteracting PI3K/AKT signal pathway in prostate cancer. PLoS One. 2014;9(7):e101411.
58. Furuyama T, Kitayama K, Shimoda Y, Ogawa M, Sone K, Yoshida-Araki K, et al. Abnormal angiogenesis in Foxo1 (Fkhr)-deficient mice. The Journal of biological chemistry. 2004;279(33):34741-9.
59. Hosaka T, Biggs WH, Tieu D, Boyer AD, Varki NM, Cavenee WK, et al. Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. Proc Natl Acad Sci U S A. 2004;101(9):2975-80.
60. Castrillon DH, Miao L, Kollipara R, Horner JW, DePinho RA. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science. 2003;301(5630):215-8.
61. Paik JH, Kollipara R, Chu G, Ji H, Xiao Y, Ding Z, et al. FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell. 2007;128(2):309-23.
62. Galili N, Davis RJ, Fredericks WJ, Mukhopadhyay S, Rauscher FJ, 3rd, Emanuel BS, et al. Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma. Nat Genet. 1993;5(3):230-5.
63. Mercado GE, Barr FG. Fusions involving PAX and FOX genes in the molecular pathogenesis of alveolar rhabdomyosarcoma: recent advances. Curr Mol Med. 2007;7(1):47-61.
64. Hillion J, Le Coniat M, Jonveaux P, Berger R, Bernard OA. AF6q21, a novel partner of the MLL gene in t(6;11)(q21;q23), defines a forkhead transcriptional factor subfamily. Blood. 1997;90(9):3714-9.
65. So CW, Cleary ML. Common mechanism for oncogenic activation of MLL by forkhead family proteins. Blood. 2003;101(2):633-9.
66. Morin RD, Mendez-Lago M, Mungall AJ, Goya R, Mungall KL, Corbett RD, et al. Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. Nature. 2011;476(7360):298-303.
67. Trinh DL, Scott DW, Morin RD, Mendez-Lago M, An J, Jones SJ, et al. Analysis of FOXO1 mutations in diffuse large B-cell lymphoma. Blood. 2013;121(18):3666-74.
68. Kim JH, Kim MK, Lee HE, Cho SJ, Cho YJ, Lee BL, et al. Constitutive phosphorylation of the FOXO1A transcription factor as a prognostic variable in gastric cancer. Mod Pathol. 2007;20(8):835-42.
69. Kim SY, Yoon J, Ko YS, Chang MS, Park JW, Lee HE, et al. Constitutive phosphorylation of the FOXO1 transcription factor in gastric cancer cells correlates with microvessel area and the expressions of angiogenesis-related molecules. BMC Cancer. 2011;11:264.
70. Santamaria CM, Chillon MC, Garcia-Sanz R, Perez C, Caballero MD, Ramos F, et al. High FOXO3a expression is associated with a poorer prognosis in AML with normal cytogenetics. Leuk Res. 2009;33(12):1706-9.
71. Chen J, Gomes AR, Monteiro LJ, Wong SY, Wu LH, Ng T-T, et al. Constitutively nuclear FOXO3a localization predicts poor survival and promotes Akt phosphorylation in breast cancer. PLoS One. 2010;5(8):e12293.
72. Tenbaum SP, Ordóñez-Morán P, Puig I, Chicote I, Arqués O, Landolfi S, et al. β-catenin confers resistance to PI3K

and AKT inhibitors and subverts FOXO3a to promote metastasis in colon cancer. Nat Med. 2012.
73. Naka K, Hoshii T, Muraguchi T, Tadokoro Y, Ooshio T, Kondo Y, et al. TGF-beta-FOXO signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia. Nature. 2010;463(7281):676-80.
74. Hurtz C, Hatzi K, Cerchietti L, Braig M, Park E, Kim YM, et al. BCL6-mediated repression of p53 is critical for leukemia stem cell survival in chronic myeloid leukemia. J Exp Med. 2011;208(11):2163-74.
75. Fernandez de Mattos S, Essafi A, Soeiro I, Pietersen AM, Birkenkamp KU, Edwards CS, et al. FoxO3a and BCR-ABL regulate cyclin D2 transcription through a STAT5/BCL6-dependent mechanism. Mol Cell Biol. 2004;24(22):10058-71.
76. Sykes SM, Lane SW, Bullinger L, Kalaitzidis D, Yusuf R, Saez B, et al. AKT/FOXO Signaling Enforces Reversible Differentiation Blockade in Myeloid Leukemias. Cell. 2011;146(5):697-708.
77. Storz P, Döppler H, Copland JA, Simpson KJ, Toker A. FOXO3a promotes tumor cell invasion through the induction of matrix metalloproteinases. Mol Cell Biol. 2009;29(18):4906-17.
78. Feng X, Wu Z, Wu Y, Hankey W, Prior TW, Li L, et al. Cdc25A regulates matrix metalloprotease 1 through Foxo1 and mediates metastasis of breast cancer cells. Mol Cell Biol. 2011;31(16):3457-71.
79. Arques O, Chicote I, Puig I, Tenbaum SP, Argiles G, Dienstmann R, et al. Tankyrase Inhibition Blocks Wnt/beta-Catenin Pathway and Reverts Resistance to PI3K and AKT Inhibitors in the Treatment of Colorectal Cancer. Clin Cancer Res. 2016;22(3):644-56.
80. Muranen T, Selfors LM, Worster DT, Iwanicki MP, Song L, Morales FC, et al. Inhibition of PI3K/mTOR leads to adaptive resistance in matrix-attached cancer cells. Cancer Cell. 2012;21(2):227-39.
81. Sisci D, Maris P, Cesario MG, Anselmo W, Coroniti R, Trombino GE, et al. The estrogen receptor alpha is the key regulator of the bifunctional role of FoxO3a transcription factor in breast cancer motility and invasiveness. Cell Cycle. 2013;12(21):3405-20.
82. Sunters A, Madureira PA, Pomeranz KM, Aubert M, Brosens JJ, Cook SJ, et al. Paclitaxel-induced nuclear translocation of FOXO3a in breast cancer cells is mediated by c-Jun NH2-terminal kinase and Akt. Cancer Res. 2006;66(1):212-20.
83. Essafi A, Fernandez de Mattos S, Hassen YAM, Soeiro I, Mufti GJ, Thomas NSB, et al. Direct transcriptional regulation of Bim by FoxO3a mediates STI571-induced apoptosis in Bcr-Abl-expressing cells. Oncogene. 2005;24(14):2317-29.
84. Chen Q, Ganapathy S, Singh KP, Shankar S, Srivastava RK. Resveratrol induces growth arrest and apoptosis through activation of FOXO transcription factors in prostate cancer cells. PLoS One. 2010;5(12):e15288.
85. Roy SK, Srivastava RK, Shankar S. Inhibition of PI3K/AKT and MAPK/ERK pathways causes activation of FOXO transcription factor, leading to cell cycle arrest and apoptosis in pancreatic cancer. J Mol Signal. 2010;5:10.
86. Li Y, Yu J, Du D, Fu S, Chen Y, Yu F, et al. Involvement of post-transcriptional regulation of FOXO1 by HuR in 5-FU-induced apoptosis in breast cancer cells. Oncol Lett. 2013;6(1):156-60.
87. Han CY, Cho KB, Choi HS, Han HK, Kang KW. Role of FoxO1 activation in MDR1 expression in adriamycin-resistant breast cancer cells. Carcinogenesis. 2008;29(9):1837-44.
88. Hui RC, Francis RE, Guest SK, Costa JR, Gomes AR, Myatt SS, et al. Doxorubicin activates FOXO3a to induce the expression of multidrug resistance gene ABCB1 (MDR1) in K562 leukemic cells. Mol Cancer Ther. 2008;7(3):670-8.
89. Kops GJPL, Dansen TB, Polderman PE, Saarloos I, Wirtz KWA, Coffer PJ, et al. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature. 2002;419(6904):316-21.
90. Goto T, Takano M, Hirata J, Tsuda H. The involvement of FOXO1 in cytotoxic stress and drug-resistance induced by paclitaxel in ovarian cancers. Br J Cancer. 2008;98(6):1068-75.
91. Liu P, Cheng H, Roberts TM, Zhao JJ. Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov. 2009;8(8):627-44.
92. Puig O, Tjian R. Transcriptional feedback control of insulin receptor by dFOXO/FOXO1. Genes & development. 2005;19(20):2435-46.
93. Hui RC, Gomes AR, Constantinidou D, Costa JR, Karadedou CT, Fernandez de Mattos S, et al. The forkhead transcription factor FOXO3a increases phosphoinositide-3 kinase/Akt activity in drug-resistant leukemic cells through induction of PIK3CA expression. Mol Cell Biol. 2008;28(19):5886-98.
94. Lin A, Piao HL, Zhuang L, Sarbassov dos D, Ma L, Gan B. FoxO transcription factors promote AKT Ser473 phosphorylation and renal tumor growth in response to pharmacologic inhibition of the PI3K-AKT pathway. Cancer Res. 2014;74(6):1682-93.
95. Chen CC, Jeon SM, Bhaskar PT, Nogueira V, Sundararajan D, Tonic I, et al. FoxOs inhibit mTORC1 and activate Akt by inducing the expression of Sestrin3 and Rictor. Dev Cell. 2010;18(4):592-604.
96. Chandarlapaty S, Sawai A, Scaltriti M, Rodrik-Outmezguine V, Grbovic-Huezo O, Serra V, et al. AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity. Cancer Cell. 2011;19(1):58-71.
97. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57-70.
98. Holmstrom KM, Finkel T. Cellular mechanisms and physiological consequences of redox-dependent signalling. Nat Rev Mol Cell Biol. 2014;15(6):411-21.
99. D'Autreaux B, Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. Nat Rev Mol Cell Biol. 2007;8(10):813-24.
100. Murphy MP. Mitochondrial thiols in antioxidant protection and redox signaling: distinct roles for glutathionylation and other thiol modifications. Antioxid Redox Signal. 2012;16(6):476-95.
101. Murphy MP. How mitochondria produce reactive oxygen species. Biochem J. 2009;417(1):1-13.
102. Sabharwal SS, Schumacker PT. Mitochondrial ROS in cancer: initiators, amplifiers or an Achilles' heel? Nat Rev Cancer. 2014;14(11):709-21.
103. Fisher AB. Redox signaling across cell membranes. Antioxid Redox Signal. 2009;11(6):1349-56.
104. Sobotta MC, Liou W, Stocker S, Talwar D, Oehler M, Ruppert T, et al. Peroxiredoxin-2 and STAT3 form a redox relay for H2O2 signaling. Nat Chem Biol. 2015;11(1):64-70.
105. Wang Y, Yang J, Yi J. Redox sensing by proteins: oxidative modifications on cysteines and the consequent events. Antioxid Redox Signal. 2012;16(7):649-57.
106. Lennicke C, Rahn J, Heimer N, Lichtenfels R, Wessjohann LA, Seliger B. Redox proteomics: Methods for the

identification and enrichment of redox-modified proteins and their applications. Proteomics. 2016;16(2):197-213.
107. Xiong Y, Uys JD, Tew KD, Townsend DM. S-glutathionylation: from molecular mechanisms to health outcomes. Antioxid Redox Signal. 2011;15(1):233-70.
108. Gallogly MM, Mieyal JJ. Mechanisms of reversible protein glutathionylation in redox signaling and oxidative stress. Curr Opin Pharmacol. 2007;7(4):381-91.
109. Winterbourn CC. Reconciling the chemistry and biology of reactive oxygen species. Nat Chem Biol. 2008;4(5):278-86.
110. Day AM, Brown JD, Taylor SR, Rand JD, Morgan BA, Veal EA. Inactivation of a peroxiredoxin by hydrogen peroxide is critical for thioredoxin-mediated repair of oxidized proteins and cell survival. Mol Cell. 2012;45(3):398-408.
111. Jarvis RM, Hughes SM, Ledgerwood EC. Peroxiredoxin 1 functions as a signal peroxidase to receive, transduce, and transmit peroxide signals in mammalian cells. Free Radic Biol Med. 2012;53(7):1522-30.
112. Jones DP, Sies H. The Redox Code. Antioxid Redox Signal. 2015;23(9):734-46.
113. Glasauer A, Chandel NS. Targeting antioxidants for cancer therapy. Biochem Pharmacol. 2014;92(1):90-101.
114. Burch PM, Heintz NH. Redox regulation of cell-cycle re-entry: cyclin D1 as a primary target for the mitogenic effects of reactive oxygen and nitrogen species. Antioxid Redox Signal. 2005;7(5-6):741-51.
115. Burhans WC, Heintz NH. The cell cycle is a redox cycle: linking phase-specific targets to cell fate. Free Radic Biol Med. 2009;47(9):1282-93.
116. Karisch R, Fernandez M, Taylor P, Virtanen C, St-Germain JR, Jin LL, et al. Global proteomic assessment of the classical protein-tyrosine phosphatome and "Redoxome". Cell. 2011;146(5):826-40.
117. Park HS, Park D, Bae YS. Molecular interaction of NADPH oxidase 1 with betaPix and Nox Organizer 1. Biochem Biophys Res Commun. 2006;339(3):985-90.
118. Kwon J, Lee SR, Yang KS, Ahn Y, Kim YJ, Stadtman ER, et al. Reversible oxidation and inactivation of the tumor suppressor PTEN in cells stimulated with peptide growth factors. Proc Natl Acad Sci U S A. 2004;101(47):16419-24.
119. Boivin B, Zhang S, Arbiser JL, Zhang ZY, Tonks NK. A modified cysteinyl-labeling assay reveals reversible oxidation of protein tyrosine phosphatases in angiomyolipoma cells. Proc Natl Acad Sci U S A. 2008;105(29):9959-64.
120. Lee SR, Yang KS, Kwon J, Lee C, Jeong W, Rhee SG. Reversible inactivation of the tumor suppressor PTEN by H2O2. J Biol Chem. 2002;277(23):20336-42.
121. Leslie NR, Batty IH, Maccario H, Davidson L, Downes CP. Understanding PTEN regulation: PIP2, polarity and protein stability. Oncogene. 2008;27(41):5464-76.
122. Leslie NR, Bennett D, Lindsay YE, Stewart H, Gray A, Downes CP. Redox regulation of PI 3-kinase signalling via inactivation of PTEN. EMBO J. 2003;22(20):5501-10.
123. Adachi Y, Shibai Y, Mitsushita J, Shang WH, Hirose K, Kamata T. Oncogenic Ras upregulates NADPH oxidase 1 gene expression through MEK-ERK-dependent phosphorylation of GATA-6. Oncogene. 2008;27(36):4921-32.
124. Mahadev K, Motoshima H, Wu X, Ruddy JM, Arnold RS, Cheng G, et al. The NAD(P)H oxidase homolog Nox4 modulates insulin-stimulated generation of H2O2 and plays an integral role in insulin signal transduction. Mol Cell Biol. 2004;24(5):1844-54.
125. Antico Arciuch VG, Galli S, Franco MC, Lam PY, Cadenas E, Carreras MC, et al. Akt1 intramitochondrial cycling is a crucial step in the redox modulation of cell cycle progression. PLoS One. 2009;4(10):e7523.
126. Huang X, Begley M, Morgenstern KA, Gu Y, Rose P, Zhao H, et al. Crystal structure of an inactive Akt2 kinase domain. Structure. 2003;11(1):21-30.
127. Murata H, Ihara Y, Nakamura H, Yodoi J, Sumikawa K, Kondo T. Glutaredoxin exerts an antiapoptotic effect by regulating the redox state of Akt. J Biol Chem. 2003;278(50):50226-33.
128. Wani R, Qian J, Yin L, Bechtold E, King SB, Poole LB, et al. Isoform-specific regulation of Akt by PDGF-induced reactive oxygen species. Proc Natl Acad Sci U S A. 2011;108(26):10550-5.
129. Leonard SE, Reddie KG, Carroll KS. Mining the thiol proteome for sulfenic acid modifications reveals new targets for oxidation in cells. ACS Chem Biol. 2009;4(9):783-99.
130. Cheung EC, Lee P, Ceteci F, Nixon C, Blyth K, Sansom OJ, et al. Opposing effects of TIGAR- and RAC1-derived ROS on Wnt-driven proliferation in the mouse intestine. Genes Dev. 2016;30(1):52-63.
131. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell. 1997;88(5):593-602.
132. Macleod KF. The role of the RB tumour suppressor pathway in oxidative stress responses in the haematopoietic system. Nat Rev Cancer. 2008;8(10):769-81.
133. Burkhart DL, Sage J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. Nat Rev Cancer. 2008;8(9):671-82.
134. van den Heuvel S, Dyson NJ. Conserved functions of the pRB and E2F families. Nat Rev Mol Cell Biol. 2008;9(9):713-24.
135. Abbas T, Dutta A. p21 in cancer: intricate networks and multiple activities. Nat Rev Cancer. 2009;9(6):400-14.
136. Cicchillitti L, Fasanaro P, Biglioli P, Capogrossi MC, Martelli F. Oxidative stress induces protein phosphatase 2A-dependent dephosphorylation of the pocket proteins pRb, p107, and p130. J Biol Chem. 2003;278(21):19509-17.
137. Magenta A, Fasanaro P, Romani S, Di Stefano V, Capogrossi MC, Martelli F. Protein phosphatase 2A subunit PR70 interacts with pRb and mediates its dephosphorylation. Mol Cell Biol. 2008;28(2):873-82.
138. Matsuzawa A, Ichijo H. Redox control of cell fate by MAP kinase: physiological roles of ASK1-MAP kinase pathway in stress signaling. Biochim Biophys Acta. 2008;1780(11):1325-36.
139. Fujikawa M, Katagiri T, Tugores A, Nakamura Y, Ishikawa F. ESE-3, an Ets family transcription factor, is up-regulated in cellular senescence. Cancer Sci. 2007;98(9):1468-75.
140. Kranc KR, Schepers H, Rodrigues NP, Bamforth S, Villadsen E, Ferry H, et al. Cited2 is an essential regulator of adult hematopoietic stem cells. Cell Stem Cell. 2009;5(6):659-65.
141. Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M. The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. Nature. 1999;397(6715):164-8.
142. Stein GH, Drullinger LF, Soulard A, Dulic V. Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. Mol Cell Biol. 1999;19(3):2109-17.

143. Childs BG, Baker DJ, Kirkland JL, Campisi J, van Deursen JM. Senescence and apoptosis: dueling or complementary cell fates? EMBO Rep. 2014;15(11):1139-53.
144. Kruiswijk F, Labuschagne CF, Vousden KH. p53 in survival, death and metabolic health: a lifeguard with a licence to kill. Nat Rev Mol Cell Biol. 2015;16(7):393-405.
145. Vurusaner B, Poli G, Basaga H. Tumor suppressor genes and ROS: complex networks of interactions. Free Radic Biol Med. 2012;52(1):7-18.
146. Maillet A, Pervaiz S. Redox regulation of p53, redox effectors regulated by p53: a subtle balance. Antioxid Redox Signal. 2012;16(11):1285-94.
147. Held JM, Danielson SR, Behring JB, Atsriku C, Britton DJ, Puckett RL, et al. Targeted quantitation of site-specific cysteine oxidation in endogenous proteins using a differential alkylation and multiple reaction monitoring mass spectrometry approach. Mol Cell Proteomics. 2010;9(7):1400-10.
148. Rainwater R, Parks D, Anderson ME, Tegtmeyer P, Mann K. Role of cysteine residues in regulation of p53 function. Mol Cell Biol. 1995;15(7):3892-903.
149. Hainaut P, Milner J. Redox modulation of p53 conformation and sequence-specific DNA binding in vitro. Cancer Res. 1993;53(19):4469-73.
150. Buzek J, Latonen L, Kurki S, Peltonen K, Laiho M. Redox state of tumor suppressor p53 regulates its sequence-specific DNA binding in DNA-damaged cells by cysteine 277. Nucleic Acids Res. 2002;30(11):2340-8.
151. Parks D, Bolinger R, Mann K. Redox state regulates binding of p53 to sequence-specific DNA, but not to non-specific or mismatched DNA. Nucleic Acids Res. 1997;25(6):1289-95.
152. Liu B, Chen Y, St Clair DK. ROS and p53: a versatile partnership. Free Radic Biol Med. 2008;44(8):1529-35.
153. Sun XZ, Vinci C, Makmura L, Han S, Tran D, Nguyen J, et al. Formation of disulfide bond in p53 correlates with inhibition of DNA binding and tetramerization. Antioxid Redox Signal. 2003;5(5):655-65.
154. Velu CS, Niture SK, Doneanu CE, Pattabiraman N, Srivenugopal KS. Human p53 is inhibited by glutathionylation of cysteines present in the proximal DNA-binding domain during oxidative stress. Biochemistry. 2007;46(26):7765-80.
155. Sporn MB, Liby KT. NRF2 and cancer: the good, the bad and the importance of context. Nat Rev Cancer. 2012;12(8):564-71.
156. Chen W, Sun Z, Wang XJ, Jiang T, Huang Z, Fang D, et al. Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response. Mol Cell. 2009;34(6):663-73.
157. Yamamoto T, Suzuki T, Kobayashi A, Wakabayashi J, Maher J, Motohashi H, et al. Physiological significance of reactive cysteine residues of Keap1 in determining Nrf2 activity. Mol Cell Biol. 2008;28(8):2758-70.
158. Dansen TB, Burgering BM. Unravelling the tumor-suppressive functions of FOXO proteins. Trends Cell Biol. 2008;18(9):421-9.
159. Yalcin S, Zhang X, Luciano JP, Mungamuri SK, Marinkovic D, Vercherat C, et al. Foxo3 is essential for the regulation of ataxia telangiectasia mutated and oxidative stress-mediated homeostasis of hematopoietic stem cells. J Biol Chem. 2008;283(37):25692-705.
160. Nogueira V, Park Y, Chen CC, Xu PZ, Chen ML, Tonic I, et al. Akt determines replicative senescence and oxidative or oncogenic premature senescence and sensitizes cells to oxidative apoptosis. Cancer Cell. 2008;14(6):458-70.
161. Tsai KL, Sun YJ, Huang CY, Yang JY, Hung MC, Hsiao CD. Crystal structure of the human FOXO3a-DBD/DNA complex suggests the effects of post-translational modification. Nucleic Acids Res. 2007;35(20):6984-94.
162. Motta MC, Divecha N, Lemieux M, Kamel C, Chen D, Gu W, et al. Mammalian SIRT1 represses forkhead transcription factors. Cell. 2004;116(4):551-63.
163. de Lange T. How telomeres solve the end-protection problem. Science. 2009;326(5955):948-52.
164. Hayflick L. Living forever and dying in the attempt. Exp Gerontol. 2003;38(11-12):1231-41.
165. Lazzerini-Denchi E, Sfeir A. Stop pulling my strings - what telomeres taught us about the DNA damage response. Nat Rev Mol Cell Biol. 2016;17(6):364-78.
166. von Zglinicki T. Role of oxidative stress in telomere length regulation and replicative senescence. Ann N Y Acad Sci. 2000;908:99-110.
167. Richter T, Proctor C. The role of intracellular peroxide levels on the development and maintenance of telomere-dependent senescence. Exp Gerontol. 2007;42(11):1043-52.
168. Indran IR, Hande MP, Pervaiz S. hTERT overexpression alleviates intracellular ROS production, improves mitochondrial function, and inhibits ROS-mediated apoptosis in cancer cells. Cancer Res. 2011;71(1):266-76.
169. Madlener S, Strobel T, Vose S, Saydam O, Price BD, Demple B, et al. Essential role for mammalian apurinic/apyrimidinic (AP) endonuclease Ape1/Ref-1 in telomere maintenance. Proc Natl Acad Sci U S A. 2013;110(44):17844-9.
170. Stephens PJ, Tarpey PS, Davies H, Van Loo P, Greenman C, Wedge DC, et al. The landscape of cancer genes and mutational processes in breast cancer. Nature. 2012;486(7403):400-4.
171. Luanpitpong S, Chanvorachote P, Stehlik C, Tse W, Callery PS, Wang L, et al. Regulation of apoptosis by Bcl-2 cysteine oxidation in human lung epithelial cells. Mol Biol Cell. 2013;24(6):858-69.
172. Westphal D, Dewson G, Czabotar PE, Kluck RM. Molecular biology of Bax and Bak activation and action. Biochim Biophys Acta. 2011;1813(4):521-31.
173. Klamt F, Zdanov S, Levine RL, Pariser A, Zhang Y, Zhang B, et al. Oxidant-induced apoptosis is mediated by oxidation of the actin-regulatory protein cofilin. Nat Cell Biol. 2009;11(10):1241-6.
174. Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B. A model for p53-induced apoptosis. Nature. 1997;389(6648):300-5.
175. Macip S, Igarashi M, Berggren P, Yu J, Lee SW, Aaronson SA. Influence of induced reactive oxygen species in p53-mediated cell fate decisions. Mol Cell Biol. 2003;23(23):8576-85.
176. Giorgio M, Migliaccio E, Orsini F, Paolucci D, Moroni M, Contursi C, et al. Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. Cell. 2005;122(2):221-33.
177. Trinei M, Giorgio M, Cicalese A, Barozzi S, Ventura A, Migliaccio E, et al. A p53-p66Shc signalling pathway controls intracellular redox status, levels of oxidation-damaged DNA and oxidative stress-induced apoptosis. Oncogene. 2002;21(24):3872-8.
178. Khanday FA, Santhanam L, Kasuno K, Yamamori T, Naqvi A, Dericco J, et al. Sos-mediated activation of rac1 by p66shc. J Cell Biol. 2006;172(6):817-22.

179. Khanday FA, Yamamori T, Mattagajasingh I, Zhang Z, Bugayenko A, Naqvi A, et al. Rac1 leads to phosphorylation-dependent increase in stability of the p66shc adaptor protein: role in Rac1-induced oxidative stress. Mol Biol Cell. 2006;17(1):122-9.
180. Juin P, Geneste O, Gautier F, Depil S, Campone M. Decoding and unlocking the BCL-2 dependency of cancer cells. Nat Rev Cancer. 2013;13(7):455-65.
181. Marani M, Hancock D, Lopes R, Tenev T, Downward J, Lemoine NR. Role of Bim in the survival pathway induced by Raf in epithelial cells. Oncogene. 2004;23(14):2431-41.
182. Shao Y, Aplin AE. ERK2 phosphorylation of serine 77 regulates Bmf pro-apoptotic activity. Cell Death Dis. 2012;3:e253.
183. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell. 1997;91(2):231-41.
184. Gardai SJ, Hildeman DA, Frankel SK, Whitlock BB, Frasch SC, Borregaard N, et al. Phosphorylation of Bax Ser184 by Akt regulates its activity and apoptosis in neutrophils. J Biol Chem. 2004;279(20):21085-95.
185. Stahl M, Dijkers PF, Kops GJ, Lens SM, Coffer PJ, Burgering BM, et al. The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2. J Immunol. 2002;168(10):5024-31.
186. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. Nat Rev Cancer. 2011;11(2):85-95.
187. Alberghina L, Gaglio D. Redox control of glutamine utilization in cancer. Cell Death Dis. 2014;5:e1561.
188. Locasale JW. Serine, glycine and one-carbon units: cancer metabolism in full circle. Nat Rev Cancer. 2013;13(8):572-83.
189. Franco R, Cidlowski JA. Apoptosis and glutathione: beyond an antioxidant. Cell Death Differ. 2009;16(10):1303-14.
190. Anastasiou D, Poulogiannis G, Asara JM, Boxer MB, Jiang JK, Shen M, et al. Inhibition of pyruvate kinase M2 by reactive oxygen species contributes to cellular antioxidant responses. Science. 2011;334(6060):1278-83.
191. Nakajima H, Amano W, Fujita A, Fukuhara A, Azuma YT, Hata F, et al. The active site cysteine of the proapoptotic protein glyceraldehyde-3-phosphate dehydrogenase is essential in oxidative stress-induced aggregation and cell death. J Biol Chem. 2007;282(36):26562-74.
192. Peralta D, Bronowska AK, Morgan B, Doka E, Van Laer K, Nagy P, et al. A proton relay enhances H2O2 sensitivity of GAPDH to facilitate metabolic adaptation. Nat Chem Biol. 2015;11(2):156-63.
193. Colussi C, Albertini MC, Coppola S, Rovidati S, Galli F, Ghibelli L. H2O2-induced block of glycolysis as an active ADP-ribosylation reaction protecting cells from apoptosis. FASEB J. 2000;14(14):2266-76.
194. Warburg O. On respiratory impairment in cancer cells. Science. 1956;124(3215):269-70.
195. Warburg O. On the origin of cancer cells. Science. 1956;123(3191):309-14.
196. Ying H, Kimmelman AC, Lyssiotis CA, Hua S, Chu GC, Fletcher-Sananikone E, et al. Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. Cell. 2012;149(3):656-70.
197. Boroughs LK, DeBerardinis RJ. Metabolic pathways promoting cancer cell survival and growth. Nat Cell Biol. 2015;17(4):351-9.
198. Elstrom RL, Bauer DE, Buzzai M, Karnauskas R, Harris MH, Plas DR, et al. Akt stimulates aerobic glycolysis in cancer cells. Cancer Res. 2004;64(11):3892-9.
199. Weinberg F, Hamanaka R, Wheaton WW, Weinberg S, Joseph J, Lopez M, et al. Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. Proc Natl Acad Sci U S A. 2010;107(19):8788-93.
200. Gaglio D, Metallo CM, Gameiro PA, Hiller K, Danna LS, Balestrieri C, et al. Oncogenic K-Ras decouples glucose and glutamine metabolism to support cancer cell growth. Mol Syst Biol. 2011;7:523.
201. Zhang C, Liu J, Liang Y, Wu R, Zhao Y, Hong X, et al. Tumour-associated mutant p53 drives the Warburg effect. Nat Commun. 2013;4:2935.
202. Kawauchi K, Araki K, Tobiume K, Tanaka N. p53 regulates glucose metabolism through an IKK-NF-kappaB pathway and inhibits cell transformation. Nat Cell Biol. 2008;10(5):611-8.
203. Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, Bartrons R, et al. TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell. 2006;126(1):107-20.
204. Bensaad K, Cheung EC, Vousden KH. Modulation of intracellular ROS levels by TIGAR controls autophagy. EMBO J. 2009;28(19):3015-26.
205. Kim HR, Roe JS, Lee JE, Cho EJ, Youn HD. p53 regulates glucose metabolism by miR-34a. Biochem Biophys Res Commun. 2013;437(2):225-31.
206. Contractor T, Harris CR. p53 negatively regulates transcription of the pyruvate dehydrogenase kinase Pdk2. Cancer Res. 2012;72(2):560-7.
207. Hu W, Zhang C, Wu R, Sun Y, Levine A, Feng Z. Glutaminase 2, a novel p53 target gene regulating energy metabolism and antioxidant function. Proc Natl Acad Sci U S A. 2010;107(16):7455-60.
208. Suzuki S, Tanaka T, Poyurovsky MV, Nagano H, Mayama T, Ohkubo S, et al. Phosphate-activated glutaminase (GLS2), a p53-inducible regulator of glutamine metabolism and reactive oxygen species. Proc Natl Acad Sci U S A. 2010;107(16):7461-6.
209. Sanchez-Macedo N, Feng J, Faubert B, Chang N, Elia A, Rushing EJ, et al. Depletion of the novel p53-target gene carnitine palmitoyltransferase 1C delays tumor growth in the neurofibromatosis type I tumor model. Cell Death Differ. 2013;20(4):659-68.
210. Assaily W, Rubinger DA, Wheaton K, Lin Y, Ma W, Xuan W, et al. ROS-mediated p53 induction of Lpin1 regulates fatty acid oxidation in response to nutritional stress. Mol Cell. 2011;44(3):491-501.
211. Ruiz-Lozano P, Hixon ML, Wagner MW, Flores AI, Ikawa S, Baldwin AS, Jr., et al. p53 is a transcriptional activator of the muscle-specific phosphoglycerate mutase gene and contributes in vivo to the control of its cardiac expression. Cell Growth Differ. 1999;10(5):295-306.
212. Mathupala SP, Heese C, Pedersen PL. Glucose catabolism in cancer cells. The type II hexokinase promoter contains functionally active response elements for the tumor suppressor p53. J Biol Chem. 1997;272(36):22776-80.
213. Jiang F, Zhang Y, Dusting GJ. NADPH oxidase-mediated redox signaling: roles in cellular stress response, stress tolerance, and tissue repair. Pharmacol Rev. 2011;63(1):218-42.
214. Jiang P, Du W, Wang X, Mancuso A, Gao X, Wu M, et al. p53 regulates biosynthesis through direct inactivation of

glucose-6-phosphate dehydrogenase. Nat Cell Biol. 2011;13(3):310-6.
215. Labuschagne CF, van den Broek NJ, Mackay GM, Vousden KH, Maddocks OD. Serine, but not glycine, supports one-carbon metabolism and proliferation of cancer cells. Cell Rep. 2014;7(4):1248-58.
216. Maddocks OD, Berkers CR, Mason SM, Zheng L, Blyth K, Gottlieb E, et al. Serine starvation induces stress and p53-dependent metabolic remodelling in cancer cells. Nature. 2013;493(7433):542-6.
217. Chaneton B, Gottlieb E. Rocking cell metabolism: revised functions of the key glycolytic regulator PKM2 in cancer. Trends Biochem Sci. 2012;37(8):309-16.
218. Chaneton B, Hillmann P, Zheng L, Martin AC, Maddocks OD, Chokkathukalam A, et al. Serine is a natural ligand and allosteric activator of pyruvate kinase M2. Nature. 2012;491(7424):458-62.
219. Nakae J, Kitamura T, Silver DL, Accili D. The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. J Clin Invest. 2001;108(9):1359-67.
220. Altomonte J, Richter A, Harbaran S, Suriawinata J, Nakae J, Thung SN, et al. Inhibition of Foxo1 function is associated with improved fasting glycemia in diabetic mice. Am J Physiol Endocrinol Metab. 2003;285(4):E718-28.
221. van der Vos KE, Eliasson P, Proikas-Cezanne T, Vervoort SJ, van Boxtel R, Putker M, et al. Modulation of glutamine metabolism by the PI(3)K-PKB-FOXO network regulates autophagy. Nat Cell Biol. 2012;14(8):829-37.
222. Charitou P, Rodriguez-Colman M, Gerrits J, van Triest M, Groot Koerkamp M, Hornsveld M, et al. FOXOs support the metabolic requirements of normal and tumor cells by promoting IDH1 expression. EMBO Rep. 2015;16(4):456-66.
223. Tennant DA, Durán RV, Gottlieb E. Targeting metabolic transformation for cancer therapy. Nature reviews Cancer. 2010;10(4):267-77.
224. Gorrini C, Harris IS, Mak TW. Modulation of oxidative stress as an anticancer strategy. Nat Rev Drug Discov. 2013;12(12):931-47.
225. Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol. 1956;11(3):298-300.
226. Hekimi S, Lapointe J, Wen Y. Taking a "good" look at free radicals in the aging process. Trends Cell Biol. 2011;21(10):569-76.
227. Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. Cell. 2013;153(6):1194-217.
228. Swenberg JA, Lu K, Moeller BC, Gao L, Upton PB, Nakamura J, et al. Endogenous versus exogenous DNA adducts: their role in carcinogenesis, epidemiology, and risk assessment. Toxicol Sci. 2011;120 Suppl 1:S130-45.
229. Hoeijmakers JH. Genome maintenance mechanisms for preventing cancer. Nature. 2001;411(6835):366-74.
230. Jackson SP, Bartek J. The DNA-damage response in human biology and disease. Nature. 2009;461(7267):1071-8.
231. Russo MT, Blasi MF, Chiera F, Fortini P, Degan P, Macpherson P, et al. The oxidized deoxynucleoside triphosphate pool is a significant contributor to genetic instability in mismatch repair-deficient cells. Mol Cell Biol. 2004;24(1):465-74.
232. Gad H, Koolmeister T, Jemth AS, Eshtad S, Jacques SA, Strom CE, et al. MTH1 inhibition eradicates cancer by preventing sanitation of the dNTP pool. Nature. 2014;508(7495):215-21.
233. Luo M, He H, Kelley MR, Georgiadis MM. Redox regulation of DNA repair: implications for human health and cancer therapeutic development. Antioxid Redox Signal. 2010;12(11):1247-69.
234. Kelley MR, Georgiadis MM, Fishel ML. APE1/Ref-1 role in redox signaling: translational applications of targeting the redox function of the DNA repair/redox protein APE1/Ref-1. Curr Mol Pharmacol. 2012;5(1):36-53.
235. Jayaraman L, Murthy KG, Zhu C, Curran T, Xanthoudakis S, Prives C. Identification of redox/repair protein Ref-1 as a potent activator of p53. Genes Dev. 1997;11(5):558-70.
236. Abate C, Patel L, Rauscher FJ, 3rd, Curran T. Redox regulation of fos and jun DNA-binding activity in vitro. Science. 1990;249(4973):1157-61.
237. Huang LE, Arany Z, Livingston DM, Bunn HF. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. J Biol Chem. 1996;271(50):32253-9.
238. Saha T, Rih JK, Roy R, Ballal R, Rosen EM. Transcriptional regulation of the base excision repair pathway by BRCA1. J Biol Chem. 2010;285(25):19092-105.
239. Gorrini C, Baniasadi PS, Harris IS, Silvester J, Inoue S, Snow B, et al. BRCA1 interacts with Nrf2 to regulate antioxidant signaling and cell survival. J Exp Med. 2013;210(8):1529-44.
240. Bae I, Fan S, Meng Q, Rih JK, Kim HJ, Kang HJ, et al. BRCA1 induces antioxidant gene expression and resistance to oxidative stress. Cancer Res. 2004;64(21):7893-909.
241. Le Page F, Randrianarison V, Marot D, Cabannes J, Perricaudet M, Feunteun J, et al. BRCA1 and BRCA2 are necessary for the transcription-coupled repair of the oxidative 8-oxoguanine lesion in human cells. Cancer Res. 2000;60(19):5548-52.
242. Lieber MR. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annu Rev Biochem. 2010;79:181-211.
243. San Filippo J, Sung P, Klein H. Mechanism of eukaryotic homologous recombination. Annu Rev Biochem. 2008;77:229-57.
244. Ditch S, Paull TT. The ATM protein kinase and cellular redox signaling: beyond the DNA damage response. Trends Biochem Sci. 2012;37(1):15-22.
245. Guo Z, Kozlov S, Lavin MF, Person MD, Paull TT. ATM activation by oxidative stress. Science. 2010;330(6003):517-21.
246. Shiloh Y, Ziv Y. The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. Nat Rev Mol Cell Biol. 2013;14(4):197-210.
247. Cosentino C, Grieco D, Costanzo V. ATM activates the pentose phosphate pathway promoting anti-oxidant defence and DNA repair. EMBO J. 2011;30(3):546-55.
248. Semenza GL. Oxygen sensing, homeostasis, and disease. N Engl J Med. 2011;365(6):537-47.
249. Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, et al. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell. 2001;107(1):43-54.
250. Denko NC. Hypoxia, HIF1 and glucose metabolism in the solid tumour. Nat Rev Cancer. 2008;8(9):705-13.
251. Ema M, Hirota K, Mimura J, Abe H, Yodoi J, Sogawa K, et al. Molecular mechanisms of transcription activation by HLF and HIF1alpha in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. EMBO J. 1999;18(7):1905-14.
252. Yang J, Gupta V, Carroll KS, Liebler DC. Site-specific mapping and quantification of protein S-sulphenylation in cells. Nat Commun. 2014;5:4776.

253. Chen C, Pore N, Behrooz A, Ismail-Beigi F, Maity A. Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. J Biol Chem. 2001;276(12):9519-25.
254. Mathupala SP, Rempel A, Pedersen PL. Glucose catabolism in cancer cells: identification and characterization of a marked activation response of the type II hexokinase gene to hypoxic conditions. J Biol Chem. 2001;276(46):43407-12.
255. Ullah MS, Davies AJ, Halestrap AP. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism. J Biol Chem. 2006;281(14):9030-7.
256. Semenza GL, Roth PH, Fang HM, Wang GL. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem. 1994;269(38):23757-63.
257. Maltepe E, Schmidt JV, Baunoch D, Bradfield CA, Simon MC. Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. Nature. 1997;386(6623):403-7.
258. Minchenko A, Leshchinsky I, Opentanova I, Sang N, Srinivas V, Armstead V, et al. Hypoxia-inducible factor-1-mediated expression of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) gene. Its possible role in the Warburg effect. J Biol Chem. 2002;277(8):6183-7.
259. Firth JD, Ebert BL, Ratcliffe PJ. Hypoxic regulation of lactate dehydrogenase A. Interaction between hypoxia-inducible factor 1 and cAMP response elements. J Biol Chem. 1995;270(36):21021-7.
260. Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab. 2006;3(3):177-85.
261. Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab. 2006;3(3):187-97.
262. Semenza GL. Molecular mechanisms mediating metastasis of hypoxic breast cancer cells. Trends Mol Med. 2012;18(9):534-43.
263. Rey S, Semenza GL. Hypoxia-inducible factor-1-dependent mechanisms of vascularization and vascular remodelling. Cardiovasc Res. 2010;86(2):236-42.
264. Gossage L, Eisen T, Maher ER. VHL, the story of a tumour suppressor gene. Nat Rev Cancer. 2015;15(1):55-64.
265. Bardos JI, Ashcroft M. Hypoxia-inducible factor-1 and oncogenic signalling. Bioessays. 2004;26(3):262-9.
266. Konior A, Schramm A, Czesnikiewicz-Guzik M, Guzik TJ. NADPH oxidases in vascular pathology. Antioxid Redox Signal. 2014;20(17):2794-814.
267. Al-Shabrawey M, Bartoli M, El-Remessy AB, Platt DH, Matragoon S, Behzadian MA, et al. Inhibition of NAD(P)H oxidase activity blocks vascular endothelial growth factor overexpression and neovascularization during ischemic retinopathy. Am J Pathol. 2005;167(2):599-607.
268. Cai T, Fassina G, Morini M, Aluigi MG, Masiello L, Fontanini G, et al. N-acetylcysteine inhibits endothelial cell invasion and angiogenesis. Lab Invest. 1999;79(9):1151-9.
269. Wheeler MD, Smutney OM, Samulski RJ. Secretion of extracellular superoxide dismutase from muscle transduced with recombinant adenovirus inhibits the growth of B16 melanomas in mice. Mol Cancer Res. 2003;1(12):871-81.
270. Tojo T, Ushio-Fukai M, Yamaoka-Tojo M, Ikeda S, Patrushev N, Alexander RW. Role of gp91phox (Nox2)-containing NAD(P)H oxidase in angiogenesis in response to hindlimb ischemia. Circulation. 2005;111(18):2347-55.
271. Kim YM, Kim KE, Koh GY, Ho YS, Lee KJ. Hydrogen peroxide produced by angiopoietin-1 mediates angiogenesis. Cancer Res. 2006;66(12):6167-74.
272. Garrido-Urbani S, Jemelin S, Deffert C, Carnesecchi S, Basset O, Szyndralewiez C, et al. Targeting vascular NADPH oxidase 1 blocks tumor angiogenesis through a PPARalpha mediated mechanism. PLoS One. 2011;6(2):e14665.
273. Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT. Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc Natl Acad Sci U S A. 1998;95(20):11715-20.
274. Kim YW, Byzova TV. Oxidative stress in angiogenesis and vascular disease. Blood. 2014;123(5):625-31.
275. Arbiser JL, Petros J, Klafter R, Govindajaran B, McLaughlin ER, Brown LF, et al. Reactive oxygen generated by Nox1 triggers the angiogenic switch. Proc Natl Acad Sci U S A. 2002;99(2):715-20.
276. Colavitti R, Pani G, Bedogni B, Anzevino R, Borrello S, Waltenberger J, et al. Reactive oxygen species as downstream mediators of angiogenic signaling by vascular endothelial growth factor receptor-2/KDR. J Biol Chem. 2002;277(5):3101-8.
277. Bochkov VN, Philippova M, Oskolkova O, Kadl A, Furnkranz A, Karabeg E, et al. Oxidized phospholipids stimulate angiogenesis via autocrine mechanisms, implicating a novel role for lipid oxidation in the evolution of atherosclerotic lesions. Circ Res. 2006;99(8):900-8.
278. Shatrov VA, Sumbayev VV, Zhou J, Brune B. Oxidized low-density lipoprotein (oxLDL) triggers hypoxia-inducible factor-1alpha (HIF-1alpha) accumulation via redox-dependent mechanisms. Blood. 2003;101(12):4847-9.
279. Birukova AA, Lee S, Starosta V, Wu T, Ho T, Kim J, et al. A role for VEGFR2 activation in endothelial responses caused by barrier disruptive OxPAPC concentrations. PLoS One. 2012;7(1):e30957.
280. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell. 2010;140(6):883-99.
281. Teng MW, Swann JB, Koebel CM, Schreiber RD, Smyth MJ. Immune-mediated dormancy: an equilibrium with cancer. J Leukoc Biol. 2008;84(4):988-93.
282. Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. Immunology. 2007;121(1):1-14.
283. Kitamura T, Qian BZ, Pollard JW. Immune cell promotion of metastasis. Nat Rev Immunol. 2015;15(2):73-86.
284. Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. Oxidative stress, inflammation, and cancer: how are they linked? Free Radic Biol Med. 2010;49(11):1603-16.
285. Monte M, Davel LE, de Lustig ES. Inhibition of lymphocyte-induced angiogenesis by free radical scavengers. Free Radic Biol Med. 1994;17(3):259-66.
286. Monte M, Davel LE, Sacerdote de Lustig E. Hydrogen peroxide is involved in lymphocyte activation mechanisms to induce angiogenesis. Eur J Cancer. 1997;33(4):676-82.
287. Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis. 2009;30(7):1073-81.
288. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol. 2014;15(3):178-96.
289. Giannoni E, Parri M, Chiarugi P. EMT and oxidative stress: a bidirectional interplay affecting tumor malignancy. Antioxid Redox Signal. 2012;16(11):1248-63.

290. Schafer ZT, Grassian AR, Song L, Jiang Z, Gerhart-Hines Z, Irie HY, et al. Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment. Nature. 2009;461(7260):109-13.
291. Davison CA, Durbin SM, Thau MR, Zellmer VR, Chapman SE, Diener J, et al. Antioxidant enzymes mediate survival of breast cancer cells deprived of extracellular matrix. Cancer Res. 2013;73(12):3704-15.
292. Buchheit CL, Weigel KJ, Schafer ZT. Cancer cell survival during detachment from the ECM: multiple barriers to tumour progression. Nat Rev Cancer. 2014;14(9):632-41.
293. Debnath J, Mills KR, Collins NL, Reginato MJ, Muthuswamy SK, Brugge JS. The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. Cell. 2002;111(1):29-40.
294. Mailleux AA, Overholtzer M, Schmelzle T, Bouillet P, Strasser A, Brugge JS. BIM regulates apoptosis during mammary ductal morphogenesis, and its absence reveals alternative cell death mechanisms. Dev Cell. 2007;12(2):221-34.
295. Zhu P, Tan MJ, Huang RL, Tan CK, Chong HC, Pal M, et al. Angiopoietin-like 4 protein elevates the prosurvival intracellular O2(-):H2O2 ratio and confers anoikis resistance to tumors. Cancer Cell. 2011;19(3):401-15.
296. Giannoni E, Buricchi F, Raugei G, Ramponi G, Chiarugi P. Intracellular reactive oxygen species activate Src tyrosine kinase during cell adhesion and anchorage-dependent cell growth. Mol Cell Biol. 2005;25(15):6391-403.
297. Giannoni E, Buricchi F, Grimaldi G, Parri M, Cialdai F, Taddei ML, et al. Redox regulation of anoikis: reactive oxygen species as essential mediators of cell survival. Cell Death Differ. 2008;15(5):867-78.
298. Giannoni E, Fiaschi T, Ramponi G, Chiarugi P. Redox regulation of anoikis resistance of metastatic prostate cancer cells: key role for Src and EGFR-mediated pro-survival signals. Oncogene. 2009;28(20):2074-86.
299. Schmelzle T, Mailleux AA, Overholtzer M, Carroll JS, Solimini NL, Lightcap ES, et al. Functional role and oncogene-regulated expression of the BH3-only factor Bmf in mammary epithelial anoikis and morphogenesis. Proc Natl Acad Sci U S A. 2007;104(10):3787-92.
300. Leung CT, Brugge JS. Outgrowth of single oncogene-expressing cells from suppressive epithelial environments. Nature. 2012;482(7385):410-3.
301. Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat Rev Drug Discov. 2009;8(7):579-91.
302. Wondrak GT. Redox-directed cancer therapeutics: molecular mechanisms and opportunities. Antioxid Redox Signal. 2009;11(12):3013-69.
303. Blot WJ, Li JY, Taylor PR, Guo W, Dawsey S, Wang GQ, et al. Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population. J Natl Cancer Inst. 1993;85(18):1483-92.
304. Zhang W, Shu XO, Li H, Yang G, Cai H, Ji BT, et al. Vitamin intake and liver cancer risk: a report from two cohort studies in China. J Natl Cancer Inst. 2012;104(15):1173-81.
305. Qiao YL, Dawsey SM, Kamangar F, Fan JH, Abnet CC, Sun XD, et al. Total and cancer mortality after supplementation with vitamins and minerals: follow-up of the Linxian General Population Nutrition Intervention Trial. J Natl Cancer Inst. 2009;101(7):507-18.
306. Hurst R, Hooper L, Norat T, Lau R, Aune D, Greenwood DC, et al. Selenium and prostate cancer: systematic review and meta-analysis. Am J Clin Nutr. 2012;96(1):111-22.
307. Richman EL, Chan JM. Selenium and prostate cancer: the puzzle isn't finished yet. Am J Clin Nutr. 2012;96(1):1-2.
308. Klein EA, Thompson IM, Jr., Tangen CM, Crowley JJ, Lucia MS, Goodman PJ, et al. Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). JAMA. 2011;306(14):1549-56.
309. Fuchs-Tarlovsky V. Role of antioxidants in cancer therapy. Nutrition. 2013;29(1):15-21.
310. Yoshida T, Goto S, Kawakatsu M, Urata Y, Li TS. Mitochondrial dysfunction, a probable cause of persistent oxidative stress after exposure to ionizing radiation. Free Radic Res. 2012;46(2):147-53.
311. Wang Y, Liu L, Pazhanisamy SK, Li H, Meng A, Zhou D. Total body irradiation causes residual bone marrow injury by induction of persistent oxidative stress in murine hematopoietic stem cells. Free Radic Biol Med. 2010;48(2):348-56.
312. Conklin KA. Coenzyme q10 for prevention of anthracycline-induced cardiotoxicity. Integr Cancer Ther. 2005;4(2):110-30.
313. Berndtsson M, Hagg M, Panaretakis T, Havelka AM, Shoshan MC, Linder S. Acute apoptosis by cisplatin requires induction of reactive oxygen species but is not associated with damage to nuclear DNA. Int J Cancer. 2007;120(1):175-80.
314. Hwang PM, Bunz F, Yu J, Rago C, Chan TA, Murphy MP, et al. Ferredoxin reductase affects p53-dependent, 5-fluorouracil-induced apoptosis in colorectal cancer cells. Nat Med. 2001;7(10):1111-7.
315. Somwar R, Erdjument-Bromage H, Larsson E, Shum D, Lockwood WW, Yang G, et al. Superoxide dismutase 1 (SOD1) is a target for a small molecule identified in a screen for inhibitors of the growth of lung adenocarcinoma cell lines. Proc Natl Acad Sci U S A. 2011;108(39):16375-80.
316. Sweeney C, Liu G, Yiannoutsos C, Kolesar J, Horvath D, Staab MJ, et al. A phase II multicenter, randomized, double-blind, safety trial assessing the pharmacokinetics, pharmacodynamics, and efficacy of oral 2-methoxyestradiol capsules in hormone-refractory prostate cancer. Clin Cancer Res. 2005;11(18):6625-33.
317. Donate F, Juarez JC, Burnett ME, Manuia MM, Guan X, Shaw DE, et al. Identification of biomarkers for the antiangiogenic and antitumour activity of the superoxide dismutase 1 (SOD1) inhibitor tetrathiomolybdate (ATN-224). Br J Cancer. 2008;98(4):776-83.
318. Tai DJ, Jin WS, Wu CS, Si HW, Cao XD, Guo AJ, et al. Changes in intracellular redox status influence multidrug resistance in gastric adenocarcinoma cells. Exp Ther Med. 2012;4(2):291-6.
319. Ryu CS, Kwak HC, Lee JY, Oh SJ, Phuong NT, Kang KW, et al. Elevation of cysteine consumption in tamoxifen-resistant MCF-7 cells. Biochem Pharmacol. 2013;85(2):197-206.
320. Griffith OW. Mechanism of action, metabolism, and toxicity of buthionine sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. J Biol Chem. 1982;257(22):13704-12.
321. Davison K, Cote S, Mader S, Miller WH. Glutathione depletion overcomes resistance to arsenic trioxide in arsenic-resistant cell lines. Leukemia. 2003;17(5):931-40.
322. Maeda H, Hori S, Ohizumi H, Segawa T, Kakehi Y, Ogawa O, et al. Effective treatment of advanced solid tumors by the combination of arsenic trioxide and L-buthionine-sulfoximine. Cell Death Differ. 2004;11(7):737-46.
323. Engel RH, Evens AM. Oxidative stress and apoptosis: a new treatment paradigm in cancer. Front Biosci. 2006;11:300-12.

324. Trachootham D, Zhou Y, Zhang H, Demizu Y, Chen Z, Pelicano H, et al. Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by beta-phenylethyl isothiocyanate. Cancer Cell. 2006;10(3):241-52.
325. Raj L, Ide T, Gurkar AU, Foley M, Schenone M, Li X, et al. Selective killing of cancer cells by a small molecule targeting the stress response to ROS. Nature. 2011;475(7355):231-4.
326. Townsend DM, He L, Hutchens S, Garrett TE, Pazoles CJ, Tew KD. NOV-002, a glutathione disulfide mimetic, as a modulator of cellular redox balance. Cancer Res. 2008;68(8):2870-7.
327. Gout PW, Buckley AR, Simms CR, Bruchovsky N. Sulfasalazine, a potent suppressor of lymphoma growth by inhibition of the x(c)- cystine transporter: a new action for an old drug. Leukemia. 2001;15(10):1633-40.
328. Guan J, Lo M, Dockery P, Mahon S, Karp CM, Buckley AR, et al. The xc- cystine/glutamate antiporter as a potential therapeutic target for small-cell lung cancer: use of sulfasalazine. Cancer Chemother Pharmacol. 2009;64(3):463-72.
329. Timmerman LA, Holton T, Yuneva M, Louie RJ, Padro M, Daemen A, et al. Glutamine sensitivity analysis identifies the xCT antiporter as a common triple-negative breast tumor therapeutic target. Cancer Cell. 2013;24(4):450-65.
330. Sobhakumari A, Love-Homan L, Fletcher EV, Martin SM, Parsons AD, Spitz DR, et al. Susceptibility of human head and neck cancer cells to combined inhibition of glutathione and thioredoxin metabolism. PLoS One. 2012;7(10):e48175.
331. Marzano C, Gandin V, Folda A, Scutari G, Bindoli A, Rigobello MP. Inhibition of thioredoxin reductase by auranofin induces apoptosis in cisplatin-resistant human ovarian cancer cells. Free Radic Biol Med. 2007;42(6):872-81.
332. Wang JB, Erickson JW, Fuji R, Ramachandran S, Gao P, Dinavahi R, et al. Targeting mitochondrial glutaminase activity inhibits oncogenic transformation. Cancer Cell. 2010;18(3):207-19.
333. Le A, Lane AN, Hamaker M, Bose S, Gouw A, Barbi J, et al. Glucose-independent glutamine metabolism via TCA cycling for proliferation and survival in B cells. Cell Metab. 2012;15(1):110-21.
334. Vaughn AE, Deshmukh M. Glucose metabolism inhibits apoptosis in neurons and cancer cells by redox inactivation of cytochrome c. Nat Cell Biol. 2008;10(12):1477-83.
335. Polimeni M, Voena C, Kopecka J, Riganti C, Pescarmona G, Bosia A, et al. Modulation of doxorubicin resistance by the glucose-6-phosphate dehydrogenase activity. Biochem J. 2011;439(1):141-9.
336. Rudolph J, Stokoe D. Selective inhibition of mutant Ras protein through covalent binding. Angew Chem Int Ed Engl. 2014;53(15):3777-9.
337. Visscher M, Arkin MR, Dansen TB. Covalent targeting of acquired cysteines in cancer. Curr Opin Chem Biol. 2016;30:61-7.
338. Frisch SM, Screaton RA. Anoikis mechanisms. Curr Opin Cell Biol. 2001;13(5):555-62.
339. Schwartz MA. Integrins, oncogenes, and anchorage independence. J Cell Biol. 1997;139(3):575-8.
340. Humphreys RC, Krajewska M, Krnacik S, Jaeger R, Weiher H, Krajewski S, et al. Apoptosis in the terminal endbud of the murine mammary gland: a mechanism of ductal morphogenesis. Development. 1996;122(12):4013-22.
341. Hausmann M, Leucht K, Ploner C, Kiessling S, Villunger A, Becker H, et al. BCL-2 modifying factor (BMF) is a central regulator of anoikis in human intestinal epithelial cells. The Journal of biological chemistry. 2011;286(30):26533-40.
342. Puthalakath H, Villunger A, O'Reilly LA, Beaumont JG, Coultas L, Cheney RE, et al. Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. Science. 2001;293(5536):1829-32.
343. Chiarugi P, Giannoni E. Anoikis: a necessary death program for anchorage-dependent cells. Biochem Pharmacol. 2008;76(11):1352-64.
344. Muthuswamy SK, Li D, Lelievre S, Bissell MJ, Brugge JS. ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. Nat Cell Biol. 2001;3(9):785-92.
345. Danes CG, Wyszomierski SL, Lu J, Neal CL, Yang W, Yu D. 14-3-3 zeta down-regulates p53 in mammary epithelial cells and confers luminal filling. Cancer Res. 2008;68(6):1760-7.
346. Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, et al. High frequency of mutations of the PIK3CA gene in human cancers. Science. 2004;304(5670):554.
347. Qian X, Karpova T, Sheppard AM, McNally J, Lowy DR. E-cadherin-mediated adhesion inhibits ligand-dependent activation of diverse receptor tyrosine kinases. EMBO J. 2004;23(8):1739-48.
348. Schackmann RCJ, Klarenbeek S, Vlug EJ, Stelloo S, van Amersfoort M, Tenhagen M, et al. Loss of p120-Catenin Induces Metastatic Progression of Breast Cancer by Inducing Anoikis Resistance and Augmenting Growth Factor Receptor Signaling. Cancer Res. 2013;73(15):4937-49.
349. van Roy F, Berx G. The cell-cell adhesion molecule E-cadherin. Cell Mol Life Sci. 2008;65(23):3756-88.
350. Derksen PW, Braumuller TM, van der Burg E, Hornsveld M, Mesman E, Wesseling J, et al. Mammary-specific inactivation of E-cadherin and p53 impairs functional gland development and leads to pleomorphic invasive lobular carcinoma in mice. Dis Model Mech. 2011;4(3):347-58.
351. Boussadia O, Kutsch S, Hierholzer A, Delmas V, Kemler R. E-cadherin is a survival factor for the lactating mouse mammary gland. Mech Dev. 2002;115(1-2):53-62.
352. Berx G, van Roy F. Involvement of members of the cadherin superfamily in cancer. Cold Spring Harb Perspect Biol. 2009;1(6):a003129.
353. Derksen PWB, Liu X, Saridin F, van der Gulden H, Zevenhoven J, Evers B, et al. Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. Cancer Cell. 2006;10(5):437-49.
354. Vaillant F, Merino D, Lee L, Breslin K, Pal B, Ritchie ME, et al. Targeting BCL-2 with the BH3 Mimetic ABT-199 in Estrogen Receptor-Positive Breast Cancer. Cancer Cell. 2013;24(1):120-9.
355. Schackmann RCJ, van Amersfoort M, Haarhuis JHI, Vlug EJ, Halim VA, Roodhart JML, et al. Cytosolic p120-catenin regulates growth of metastatic lobular carcinoma through Rock1-mediated anoikis resistance. J Clin Invest. 2011;121(8):3176-88.
356. Reginato MJ, Mills KR, Becker EB, Lynch DK, Bonni A, Muthuswamy SK, et al. Bim regulation of lumen formation in cultured mammary epithelial acini is targeted by oncogenes. Mol Cell Biol. 2005;25(11):4591-601.
357. Curto M, Cole BK, Lallemand D, Liu CH, McClatchey AI. Contact-dependent inhibition of EGFR signaling by Nf2/Merlin. J Cell Biol. 2007;177(5):893-903.
358. Zhang Y, Adachi M, Kawamura R, Imai K. Bmf is a possible mediator in histone deacetylase inhibitors FK228 and CBHA-induced apoptosis. Cell Death Differ. 2006;13(1):129-40.

359. Shao Y, Aplin AE. BH3-only protein silencing contributes to acquired resistance to PLX4720 in human melanoma. Cell Death Differ. 2012;19(12):2029-39.
360. VanBrocklin MW, Verhaegen M, Soengas MS, Holmen SL. Mitogen-activated protein kinase inhibition induces translocation of Bmf to promote apoptosis in melanoma. Cancer Res. 2009;69(5):1985-94.
361. Bolden JE, Shi W, Jankowski K, Kan CY, Cluse L, Martin BP, et al. HDAC inhibitors induce tumor-cell-selective pro-apoptotic transcriptional responses. Cell Death Dis. 2013;4:e519.
362. Piñon JD, Labi V, Egle A, Villunger A. Bim and Bmf in tissue homeostasis and malignant disease. Oncogene. 2008;27 Suppl 1:S41-52.
363. Labi V, Erlacher M, Kiessling S, Manzl C, Frenzel A, O'Reilly L, et al. Loss of the BH3-only protein Bmf impairs B cell homeostasis and accelerates gamma irradiation-induced thymic lymphoma development. The Journal of experimental medicine. 2008;205(3):641-55.
364. Chonghaile TN, Roderick JE, Glenfield C, Ryan J, Sallan SE, Silverman LB, et al. Maturation stage of T-cell acute lymphoblastic leukemia determines BCL-2 versus BCL-XL dependence and sensitivity to ABT-199. Cancer Discov. 2014;4(9):1074-87.
365. Miller LA, Goldstein NB, Johannes WU, Walton CH, Fujita M, Norris DA, et al. BH3 mimetic ABT-737 and a proteasome inhibitor synergistically kill melanomas through Noxa-dependent apoptosis. J Invest Dermatol. 2009;129(4):964-71.
366. Choudhary GS, Al-Harbi S, Mazumder S, Hill BT, Smith MR, Bodo J, et al. MCL-1 and BCL-xL-dependent resistance to the BCL-2 inhibitor ABT-199 can be overcome by preventing PI3K/AKT/mTOR activation in lymphoid malignancies. Cell Death Dis. 2015;6:e1593.
367. Reuland SN, Goldstein NB, Partyka KA, Cooper DA, Fujita M, Norris DA, et al. The combination of BH3-mimetic ABT-737 with the alkylating agent temozolomide induces strong synergistic killing of melanoma cells independent of p53. PLoS One. 2011;6(8):e24294.
368. Oakes SR, Vaillant F, Lim E, Lee L, Breslin K, Feleppa F, et al. Sensitization of BCL-2-expressing breast tumors to chemotherapy by the BH3 mimetic ABT-737. Proc Natl Acad Sci U S A. 2012;109(8):2766-71.
369. Kutuk O, Letai A. Displacement of Bim by Bmf and Puma rather than increase in Bim level mediates paclitaxel-induced apoptosis in breast cancer cells. Cell Death Differ. 2010;17(10):1624-35.
370. Mukherjee N, Reuland SN, Lu Y, Luo Y, Lambert K, Fujita M, et al. Combining a BCL2 inhibitor with the retinoid derivative fenretinide targets melanoma cells including melanoma initiating cells. J Invest Dermatol. 2015;135(3):842-50.
371. Meerbrey KL, Hu G, Kessler JD, Roarty K, Li MZ, Fang JE, et al. The pINDUCER lentiviral toolkit for inducible RNA interference in vitro and in vivo. Proc Natl Acad Sci U S A. 2011;108(9):3665-70.
372. van de Weijer ML, Bassik MC, Luteijn RD, Voorburg CM, Lohuis MA, Kremmer E, et al. A high-coverage shRNA screen identifies TMEM129 as an E3 ligase involved in ER-associated protein degradation. Nat Commun. 2014;5:3832.
373. Renault VM, Thekkat PU, Hoang KL, White JL, Brady CA, Kenzelmann Broz D, et al. The pro-longevity gene FoxO3 is a direct target of the p53 tumor suppressor. Oncogene. 2011.
374. Tothova Z, Gilliland DG. FoxO transcription factors and stem cell homeostasis: insights from the hematopoietic system. Cell stem cell. 2007;1(2):140-52.
375. Webb AE, Brunet A. FOXO transcription factors: key regulators of cellular quality control. Trends Biochem Sci. 2014;39(4):159-69.
376. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat Rev Cancer. 2009;9(8):550-62.
377. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, et al. A third-generation lentivirus vector with a conditional packaging system. J Virol. 1998;72(11):8463-71.
378. Herold MJ, van den Brandt J, Seibler J, Reichardt HM. Inducible and reversible gene silencing by stable integration of an shRNA-encoding lentivirus in transgenic rats. Proc Natl Acad Sci U S A. 2008;105(47):18507-12.
379. Burgering BM, Coffer PJ. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature. 1995;376(6541):599-602.
380. Ashley EA. Towards precision medicine. Nat Rev Genet. 2016;17(9):507-22.
381. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Jr., Kinzler KW. Cancer genome landscapes. Science. 2013;339(6127):1546-58.
382. van de Stolpe A. On the origin and destination of cancer stem cells: a conceptual evaluation. Am J Cancer Res. 2013;3(1):107-16.
383. Fruman DA, Rommel C. PI3K and cancer: lessons, challenges and opportunities. Nat Rev Drug Discov. 2014;13(2):140-56.
384. Arnedos M, Vicier C, Loi S, Lefebvre C, Michiels S, Bonnefoi H, et al. Precision medicine for metastatic breast cancer-limitations and solutions. Nature reviews Clinical oncology. 2015.
385. Rodon J, Dienstmann R, Serra V, Tabernero J. Development of PI3K inhibitors: lessons learned from early clinical trials. Nat Rev Clin Oncol. 2013;10(3):143-53.
386. Kwiatkowski DJ, Wagle N. mTOR Inhibitors in Cancer: What Can We Learn from Exceptional Responses? EBioMedicine. 2015;2(1):2-4.
387. Verhaegh W, Van de Stolpe A. Knowledge-based computational models. Oncotarget. 2014;5(14):5196-7.
388. Verhaegh W, van Ooijen H, Inda MA, Hatzis P, Versteeg R, Smid M, et al. Selection of personalized patient therapy through the use of knowledge-based computational models that identify tumor-driving signal transduction pathways. Cancer Res. 2014;74(11):2936-45.
389. Webb AE, Kundaje A, Brunet A. Characterization of the direct targets of FOXO transcription factors throughout evolution. Aging Cell. 2016;15(4):673-85.
390. Czymai T, Viemann D, Sticht C, Molema G, Goebeler M, Schmidt M. FOXO3 modulates endothelial gene expression and function by classical and alternative mechanisms. J Biol Chem. 2010;285(14):10163-78.
391. Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol. 2009;27(8):1160-7.
392. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000;406(6797):747-52.

393. Sabatier R, Finetti P, Cervera N, Lambaudie E, Esterni B, Mamessier E, et al. A gene expression signature identifies two prognostic subgroups of basal breast cancer. Breast Cancer Res Treat. 2011;126(2):407-20.
394. Shmelkov E, Tang Z, Aifantis I, Statnikov A. Assessing quality and completeness of human transcriptional regulatory pathways on a genome-wide scale. Biol Direct. 2011;6:15.
395. van der Vos KE, Coffer PJ. The extending network of FOXO transcriptional target genes. Antioxid Redox Signal. 2011;14(4):579-92.
396. Skrzypczak M, Goryca K, Rubel T, Paziewska A, Mikula M, Jarosz D, et al. Modeling oncogenic signaling in colon tumors by multidirectional analyses of microarray data directed for maximization of analytical reliability. PLoS One. 2010;5(10).
397. Sabates-Bellver J, Van der Flier LG, de Palo M, Cattaneo E, Maake C, Rehrauer H, et al. Transcriptome profile of human colorectal adenomas. Mol Cancer Res. 2007;5(12):1263-75.
398. Sircoulomb F, Bekhouche I, Finetti P, Adelaide J, Ben Hamida A, Bonansea J, et al. Genome profiling of ERBB2-amplified breast cancers. BMC Cancer. 2010;10:539.
399. Bos PD, Zhang XH, Nadal C, Shu W, Gomis RR, Nguyen DX, et al. Genes that mediate breast cancer metastasis to the brain. Nature. 2009;459(7249):1005-9.
400. Sabatier R, Finetti P, Adelaide J, Guille A, Borg JP, Chaffanet M, et al. Down-regulation of ECRG4, a candidate tumor suppressor gene, in human breast cancer. PLoS One. 2011;6(11):e27656.
401. Hornsveld M, Dansen TB. The Hallmarks of Cancer from a Redox Perspective. Antioxid Redox Signal. 2016;25(6):300-25.
402. Lin K, Hsin H, Libina N, Kenyon C. Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. Nat Genet. 2001;28(2):139-45.
403. Kornblau SM, Singh N, Qiu Y, Chen W, Zhang N, Coombes KR. Highly phosphorylated FOXO3A is an adverse prognostic factor in acute myeloid leukemia. Clin Cancer Res. 2010;16(6):1865-74.
404. Hagenbuchner J, Rupp M, Salvador C, Meister B, Kiechl-Kohlendorfer U, Muller T, et al. Nuclear FOXO3 predicts adverse clinical outcome and promotes tumor angiogenesis in neuroblastoma. Oncotarget. 2016.
405. Kim MS, Pak YK, Jang PG, Namkoong C, Choi YS, Won JC, et al. Role of hypothalamic Foxo1 in the regulation of food intake and energy homeostasis. Nat Neurosci. 2006;9(7):901-6.
406. Kitamura T, Feng Y, Kitamura YI, Chua SC, Jr., Xu AW, Barsh GS, et al. Forkhead protein FoxO1 mediates Agrp-dependent effects of leptin on food intake. Nat Med. 2006;12(5):534-40.
407. Dijkers PF, Medema RH, Lammers JW, Koenderman L, Coffer PJ. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Current biology : CB. 2000;10(19):1201-4.
408. Gilley J, Coffer PJ, Ham J. FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons. J Cell Biol. 2003;162(4):613-22.
409. Tang TT, Dowbenko D, Jackson A, Toney L, Lewin DA, Dent AL, et al. The forkhead transcription factor AFX activates apoptosis by induction of the BCL-6 transcriptional repressor. J Biol Chem. 2002;277(16):14255-65.
410. Mammucari C, Milan G, Romanello V, Masiero E, Rudolf R, Del Piccolo P, et al. FoxO3 controls autophagy in skeletal muscle in vivo. Cell Metab. 2007;6(6):458-71.
411. Zhao J, Brault JJ, Schild A, Cao P, Sandri M, Schiaffino S, et al. FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells. Cell Metab. 2007;6(6):472-83.
412. Bakker WJ, Blazquez-Domingo M, Kolbus A, Besooyen J, Steinlein P, Beug H, et al. FoxO3a regulates erythroid differentiation and induces BTG1, an activator of protein arginine methyl transferase 1. J Cell Biol. 2004;164(2):175-84.
413. Nemoto S, Finkel T. Redox regulation of forkhead proteins through a p66shc-dependent signaling pathway. Science. 2002;295(5564):2450-2.
414. Roy UK, Henkhaus RS, Ignatenko NA, Mora J, Fultz KE, Gerner EW. Wild-type APC regulates caveolin-1 expression in human colon adenocarcinoma cell lines via FOXO1a and C-myc. Mol Carcinog. 2008;47(12):947-55.
415. van den Heuvel APJ, Schulze A, Burgering BMT. Direct control of caveolin-1 expression by FOXO transcription factors. The Biochemical journal. 2005;385(Pt 3):795-802.
416. Schmidt M, Fernandez de Mattos S, van der Horst A, Klompmaker R, Kops GJ, Lam EW, et al. Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. Mol Cell Biol. 2002;22(22):7842-52.
417. Chen J, Yusuf I, Andersen HM, Fruman DA. FOXO transcription factors cooperate with delta EF1 to activate growth suppressive genes in B lymphocytes. J Immunol. 2006;176(5):2711-21.
418. Martinez-Gac L, Marques M, Garcia Z, Campanero MR, Carrera AC. Control of cyclin G2 mRNA expression by forkhead transcription factors: novel mechanism for cell cycle control by phosphoinositide 3-kinase and forkhead. Mol Cell Biol. 2004;24(5):2181-9.
419. Nakae J, Kitamura T, Kitamura Y, Biggs WH, 3rd, Arden KC, Accili D. The forkhead transcription factor Foxo1 regulates adipocyte differentiation. Dev Cell. 2003;4(1):119-29.
420. Seoane J, Le HV, Shen L, Anderson SA, Massague J. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell. 2004;117(2):211-23.
421. Dijkers PF, Medema RH, Pals C, Banerji L, Thomas NS, Lam EW, et al. Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). Mol Cell Biol. 2000;20(24):9138-48.
422. Guo S, Sonenshein GE. Forkhead box transcription factor FOXO3a regulates estrogen receptor alpha expression and is repressed by the Her-2/neu/phosphatidylinositol 3-kinase/Akt signaling pathway. Mol Cell Biol. 2004;24(19):8681-90.
423. Ciechomska I, Pyrzynska B, Kazmierczak P, Kaminska B. Inhibition of Akt kinase signalling and activation of Forkhead are indispensable for upregulation of FasL expression in apoptosis of glioma cells. Oncogene. 2003;22(48):7617-27.
424. Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, et al. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell. 2004;117(3):399-412.
425. Furukawa-Hibi Y. FOXO Forkhead Transcription Factors Induce G2-M Checkpoint in Response to Oxidative Stress. J Biol Chem. 2002;277(30):26729-32.
426. Tran H, Brunet A, Grenier JM, Datta SR, Fornace AJ, Jr., DiStefano PS, et al. DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. Science. 2002;296(5567):530-4.

427. Delpuech O, Griffiths B, East P, Essafi A, Lam EW, Burgering B, et al. Induction of Mxi1-SR alpha by FOXO3a contributes to repression of Myc-dependent gene expression. Mol Cell Biol. 2007;27(13):4917-30.
428. Potente M, Urbich C, Sasaki K, Hofmann WK, Heeschen C, Aicher A, et al. Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. J Clin Invest. 2005;115(9):2382-92.
429. Sekine K, Chen YR, Kojima N, Ogata K, Fukamizu A, Miyajima A. Foxo1 links insulin signaling to C/EBPalpha and regulates gluconeogenesis during liver development. EMBO J. 2007;26(15):3607-15.
430. Harada M, Magara-Koyanagi K, Watarai H, Nagata Y, Ishii Y, Kojo S, et al. IL-21-induced Bepsilon cell apoptosis mediated by natural killer T cells suppresses IgE responses. The Journal of experimental medicine. 2006;203(13):2929-37.
431. Daitoku H, Yamagata K, Matsuzaki H, Hatta M, Fukamizu A. Regulation of PGC-1 promoter activity by protein kinase B and the forkhead transcription factor FKHR. Diabetes. 2003;52(3):642-9.
432. Chiribau CB, Cheng L, Cucoranu IC, Yu Y-S, Clempus RE, Sorescu D. FOXO3A regulates peroxiredoxin III expression in human cardiac fibroblasts. The Journal of biological chemistry. 2008;283(13):8211-7.
433. Modur V, Nagarajan R, Evers BM, Milbrandt J. FOXO proteins regulate tumor necrosis factor-related apoptosis inducing ligand expression. Implications for PTEN mutation in prostate cancer. J Biol Chem. 2002;277(49):47928-37.
434. Frescas D, Pagano M. Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer. Nat Rev Cancer. 2008;8(6):438-49.
435. Nakayama KI, Nakayama K. Ubiquitin ligases: cell-cycle control and cancer. Nat Rev Cancer. 2006;6(5):369-81.
436. Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. Nat Rev Cancer. 2009;9(3):153-66.
437. Katayama K, Nakamura A, Sugimoto Y, Tsuruo T, Fujita N. FOXO transcription factor-dependent p15(INK4b) and p19(INK4d) expression. Oncogene. 2008;27(12):1677-86.
438. Gomis RR, Alarcon C, He W, Wang Q, Seoane J, Lash A, et al. A FoxO-Smad synexpression group in human keratinocytes. Proc Natl Acad Sci U S A. 2006;103(34):12747-52.
439. Pellicano F, Scott MT, Helgason GV, Hopcroft LE, Allan EK, Aspinall-O'Dea M, et al. The antiproliferative activity of kinase inhibitors in chronic myeloid leukemia cells is mediated by FOXO transcription factors. Stem Cells. 2014;32(9):2324-37.
440. Sakaue-Sawano A, Kurokawa H, Morimura T, Hanyu A, Hama H, Osawa H, et al. Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. Cell. 2008;132(3):487-98.
441. Wiebusch L, Hagemeier C. p53- and p21-dependent premature APC/C-Cdh1 activation in G2 is part of the long-term response to genotoxic stress. Oncogene. 2010;29(24):3477-89.
442. Krenning L, Feringa FM, Shaltiel IA, van den Berg J, Medema RH. Transient activation of p53 in G2 phase is sufficient to induce senescence. Mol Cell. 2014;55(1):59-72.
443. Johmura Y, Shimada M, Misaki T, Naiki-Ito A, Miyoshi H, Motoyama N, et al. Necessary and sufficient role for a mitosis skip in senescence induction. Mol Cell. 2014;55(1):73-84.
444. Frye JJ, Brown NG, Petzold G, Watson ER, Grace CR, Nourse A, et al. Electron microscopy structure of human APC/C(CDH1)-EMI1 reveals multimodal mechanism of E3 ligase shutdown. Nat Struct Mol Biol. 2013;20(7):827-35.
445. Wang W, Kirschner MW. Emi1 preferentially inhibits ubiquitin chain elongation by the anaphase-promoting complex. Nat Cell Biol. 2013;15(7):797-806.
446. Cappell SD, Chung M, Jaimovich A, Spencer SL, Meyer T. Irreversible APC(Cdh1) Inactivation Underlies the Point of No Return for Cell-Cycle Entry. Cell. 2016;166(1):167-80.
447. Chang L, Zhang Z, Yang J, McLaughlin SH, Barford D. Atomic structure of the APC/C and its mechanism of protein ubiquitination. Nature. 2015;522(7557):450-4.
448. Lee J, Kim JA, Barbier V, Fotedar A, Fotedar R. DNA damage triggers p21WAF1-dependent Emi1 down-regulation that maintains G2 arrest. Mol Biol Cell. 2009;20(7):1891-902.
449. Feringa FM, Krenning L, Koch A, van den Berg J, van den Broek B, Jalink K, et al. Hypersensitivity to DNA damage in antephase as a safeguard for genome stability. Nat Commun. 2016;7:12618.
450. Hsu JY, Reimann JD, Sorensen CS, Lukas J, Jackson PK. E2F-dependent accumulation of hEmi1 regulates S phase entry by inhibiting APC(Cdh1). Nat Cell Biol. 2002;4(5):358-66.
451. Ho KK, Myatt SS, Lam EW-F. Many forks in the path: cycling with FoxO. Oncogene. 2008;27(16):2300-11.
452. Shats I, Gatza ML, Liu B, Angus SP, You L, Nevins JR. FOXO transcription factors control E2F1 transcriptional specificity and apoptotic function. Cancer Res. 2013;73(19):6056-67.
453. Xie Q, Peng S, Tao L, Ruan H, Yang Y, Li TM, et al. E2F transcription factor 1 regulates cellular and organismal senescence by inhibiting Forkhead box O transcription factors. J Biol Chem. 2014;289(49):34205-13.
454. Huang H, Regan KM, Lou Z, Chen J, Tindall DJ. CDK2-dependent phosphorylation of FOXO1 as an apoptotic response to DNA damage. Science (New York, NY). 2006;314(5797):294-7.
455. Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER, 3rd, Hurov KE, Luo J, et al. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science. 2007;316(5828):1160-6.
456. Tsai WB, Chung YM, Takahashi Y, Xu Z, Hu MC. Functional interaction between FOXO3a and ATM regulates DNA damage response. Nat Cell Biol. 2008;10(4):460-7.
457. Burgering BMT, Kops GJPL. Cell cycle and death control: long live Forkheads. Trends Biochem Sci. 2002;27(7):352-60.
458. Burgering BMT, Medema RH. Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty. J Leukoc Biol. 2003;73(6):689-701.
459. Jeanes A, Gottardi CJ, Yap AS. Cadherins and cancer: how does cadherin dysfunction promote tumor progression? Oncogene. 2008;27(55):6920-9.
460. Sherr CJ. Principles of tumor suppression. Cell. 2004;116(2):235-46.
461. Fatehullah A, Tan SH, Barker N. Organoids as an in vitro model of human development and disease. Nat Cell Biol. 2016;18(3):246-54.
462. Brownawell AM, Kops GJ, Macara IG, Burgering BM. Inhibition of nuclear import by protein kinase B (Akt) regulates the subcellular distribution and activity of the forkhead transcription factor AFX. Mol Cell Biol. 2001;21(10):3534-46.
463. Nagashima T, Shigematsu N, Maruki R, Urano Y, Tanaka H, Shimaya A, et al. Discovery of novel forkhead box O1 inhibitors for treating type 2 diabetes: improvement of fasting glycemia in diabetic db/db mice. Mol Pharmacol.

2010;78(5):961-70.
464. Savai R, Al-Tamari HM, Sedding D, Kojonazarov B, Muecke C, Teske R, et al. Pro-proliferative and inflammatory signaling converge on FoxO1 transcription factor in pulmonary hypertension. Nat Med. 2014;20(11):1289-300.
465. Zou P, Liu L, Zheng L, Liu L, Stoneman RE, Cho A, et al. Targeting FoxO1 with AS1842856 suppresses adipogenesis. Cell Cycle. 2014;13(23):3759-67.
466. Karki S, Farb MG, Ngo DT, Myers S, Puri V, Hamburg NM, et al. Forkhead box O-1 modulation improves endothelial insulin resistance in human obesity. Arterioscler Thromb Vasc Biol. 2015;35(6):1498-506.
467. Tan P, Guan H, Xie L, Mi B, Fang Z, Li J, et al. FOXO1 inhibits osteoclastogenesis partially by antagnozing MYC. Sci Rep. 2015;5:16835.
468. Matkar S, Sharma P, Gao S, Gurung B, Katona BW, Liao J, et al. An Epigenetic Pathway Regulates Sensitivity of Breast Cancer Cells to HER2 Inhibition via FOXO/c-Myc Axis. Cancer Cell. 2015;28(4):472-85.
469. Diep CH, Charles NJ, Gilks CB, Kalloger SE, Argenta PA, Lange CA. Progesterone receptors induce FOXO1-dependent senescence in ovarian cancer cells. Cell Cycle. 2013;12(9):1433-49.

Nederlandse samenvatting

Alle organismen bestaan uit cellen, sommige functioneren als eencelligen, andere als een georganiseerde klont van aan elkaar klevende en samenwerkende cellen. Een volwassen mens bestaat uit miljarden cellen, allemaal ooit gevormd uit één bevruchte eicel. Na bevruchting ondergaat een eicel celdeling. Tijdens dit proces wordt het DNA in de cel gekopieerd en precies in tweeën verdeeld over de twee dochtercellen. Het DNA bevat alle genetische informatie van de cel en bestaat uit de genen die coderen voor alle eiwitten die een cel kan maken. Dit celdelingsproces zorgt voor de vermeerdering van het aantal cellen en daarmee de groei en ontwikkeling van het lichaam. Het uiteindelijke resultaat is een volwassen lichaam met miljarden cellen die samen o.a. organen, ledematen, botten en het immuunsysteem vormen. Ook in een volwassen lichaam vindt nog celdeling plaats. Dit proces is essentieel om organen te onderhouden, wonden te helen en kapotte cellen te vervangen door verse, functionele cellen.
Het is van groot belang om celdeling streng te reguleren. Te weinig celdeling leidt tot aftakeling van het lichaam, maar te veel celdeling kan het normaal functioneren van organen en het lichaam verstoren. Een bekende ziekte die voortkomt uit ongeremde celdeling is kanker. In kankercellen is de controle over het besluit om te delen verloren gegaan, wat resulteert in snelle ongecontroleerde celdeling en overwoekering van gezonde weefsels door een teveel aan cellen.
Het reguleren van celdeling gebeurt op moleculair niveau in de cel. Op de buitenkant van de cel zitten receptoren (dit zijn eiwitten die in staat zijn andere eiwitten in de omgeving van de cel te detecteren) die gevoelig zijn voor groeihormonen. Op het moment dat een cel groeihormonen detecteert wordt aan de binnenkant van de cel een signaal afgegeven dat de cel instrueert om zich te gaan delen. Dit signaal wordt doorgeven door specifieke eiwitten (kinases) die doelgericht binden aan weer andere eiwitten die celdeling stimuleren en deze hiermee activeren. Onder deze laatstgenoemde eiwitten bevinden zich transcriptiefactoren. Die binden aan genen in het DNA betrokken bij de celcyclus en kunnen deze activeren of deactiveren. Kankercellen hebben vaak mutaties in genen die betrokken zijn bij het reguleren van celdeling. Deze mutaties kunnen resulteren in het onomkeerbaar activeren van celdeling-stimulerende genen (oncogenen), of in het verlies van celdeling-remmende genen (tumorsuppressorgenen).
Om gepaste behandelingen te ontwerpen die kanker bestrijden is het essentieel om te begrijpen hoe moleculaire signalen worden doorgegeven en welke van deze signalen belangrijk zijn voor het ontstaan en de ontwikkeling van kanker. In de afgelopen decennia zijn er vele mutaties ontdekt en in kaart gebracht. Vanwege de grote complexiteit waarmee moleculaire signalen worden doorgegeven en geïnterpreteerd in de cel, is het echter nog altijd niet precies duidelijk hoe dit exact werkt en hoe we hier het beste in de kliniek op kunnen ingrijpen.

&

Insulinesignalering in kankercellen
In dit proefschrift ligt de focus op de insulinesignaleringsroute van de cel. De bij insulinesignalering betrokken eiwitten reguleren een transcriptiefactor genaamd Forkhead Box O (FOXO). Bij afwezigheid van insuline in het bloed, bijvoorbeeld voorafgaand aan een maaltijd, is de insulinesignaleringsroute in cellen inactief. Onder deze omstandigheden zijn FOXO transcriptiefactoren actief en remmen zij celdeling. Tegelijkertijd zorgt FOXO ervoor dat elke cel zuinig omgaat met zijn energie en gezond blijft. Na een maaltijd is er veel suiker in het bloed aanwezig en genereert de alvleesklier insuline. Insuline in het bloed stimuleert cellen in het lichaam om suiker op te nemen door te binden aan de insulinereceptoren aan de buitenkant van een cel. Aan de binnenkant van de cel worden dan verschillende kinases geactiveerd, waaronder PI3-Kinase en Protein Kinase B (PKB). Deze kinases stimuleren tegelijkertijd de opname van suiker

door de cel en de celdeling. Een belangrijke stap in het proces van insulinesignalering is het inactiveren van FOXO door PKB. Dit zorgt ervoor dat de cel inderdaad kan gaan delen en veel suiker gaat gebruiken om hieruit energie en bouwstenen te winnen. Bij insulinesignalering betrokken eiwitten zijn regelmatig gemuteerd in kankercellen. Hierdoor is insulinesignalering vaak hyperactief in kankercellen en daarmee wordt het celdelingsremmende effect van FOXO geblokkeerd. Hierom bestaat de verwachting dat het omgekeerde ook opgaat, namelijk dat FOXO tumorgroei kan remmen.
Door de jaren heen is er veel onderzoek gedaan naar de effecten van activatie en verlies van FOXO in cellen. Hieruit kunnen we concluderen dat het artificieel activeren van FOXO inderdaad celdeling kan remmen en celdood kan stimuleren in veel verschillende types cellen. Daarnaast is beschreven dat muizen zonder FOXO-genen specifieke types kanker ontwikkelen. Op basis van deze bevindingen is het idee ontstaan dat FOXO-transcriptiefactoren mogelijk een belangrijke tumorgroei-onderdrukkende rol spelen in de ontwikkeling van kanker. Hoe FOXO verschillende aspecten van de tumorgroei kan beïnvloeden in kankercellen en wat de exacte rol van FOXO's in kankercellen is, is echter nog niet in detail bekend. Uit ander onderzoek rijst het beeld dat de rol van FOXO complexer is. FOXO-genen zijn namelijk bijna nooit gemuteerd in kankercellen en dus altijd functioneel aanwezig. Bovendien blijkt uit histopathologische onderzoeken dat FOXO-activiteit gecorreleerd is aan zowel een goede als slechte prognose voor kankerpatiënten. Dit proefschrift richt zich daarom op de vraag wat de exacte rol van FOXO in kankercellen is.

FOXO onderdrukt uitzaaiingen
In **hoofdstuk 3** van dit proefschrift hebben we de rol van FOXO in uitzaaiende borstkankercellen onderzocht. Het moment dat een tumor begint met uitzaaien is een onomkeerbaar moment voor een kankerpatiënt. Nadat de tumor is uitgezaaid daalt de overlevingskans van de patiënt aanzienlijk doordat de tumor niet alleen plaatselijk schade toebrengt maar nu ook in de rest van het lichaam. Door te begrijpen hoe uitzaaien werkt op celniveau wordt het mogelijk om in de toekomst behandelingen te ontwikkelen die uitzaaien tegengaan. Hiermee kunnen de overlevingskansen van patiënten verbeterd worden.
Uitzaaien is voor een kankercel totaal niet vanzelfsprekend. Van de vele cellen die uit de primaire tumor loskomen overleeft slechts een klein deel de reis door het lichaam en hiervan is op zijn beurt maar een fractie in staat een nieuwe tumor te vormen. Een belangrijk verdedigingsmechanisme van het lichaam tegen uitzaaien is celdood die specifiek geïnduceerd wordt door het loslaten van een cel uit het weefsel waar deze thuishoort (anoikis). Deze gereguleerde celdood weerhoudt cellen ervan zich buiten hun originele weefsel te nestelen. In **hoofdstuk 3** ontdekken we dat FOXO een belangrijke schakel is in dit anoikisproces. Op het moment dat cellen losraken uit een tumor wordt FOXO actief en doodt het de kankercel door middel van anoikis. Alleen als FOXO inactief blijft via onderdrukking door PKB kan een cel uitzaaien. Deze observatie kan nu gebruikt worden om nieuwe behandelingsstrategieën uit te proberen, bijvoorbeeld het farmacologisch onderdrukken van PKB-activiteit in combinatie met het verlagen van de drempel om celdood te ondergaan.

De rol van FOXO in kanker herzien
In **hoofdstuk 4** onderzoeken we in detail wat de effecten zijn van FOXO-activatie en -inactivatie op de ontwikkeling van borstkanker. We bevestigen allereerst onze bevindingen in hoofdstuk 3 dat FOXO-activatie in borstkanker inderdaad tumorgroei kan remmen en uitzaaiing van de tumorcellen belemmert. Ook het verlies van FOXO-activiteit in tumorcellen remt tumorgroei efficiënt en blokkeert het uitzaaien. We beschrijven dat FOXO belangrijk is tijdens verschillende aspecten van tumorontwikkeling, inclusief

celmigratie, het onderhouden van het energiemetabolisme, het beschermen tegen vrije zuurstofradicalen en het in stand houden van actieve groeifactorsignalering. In tegenstelling tot de verwachting dat FOXO-verlies tumorgroei bevordert, ontdekken we hiermee dat de aanwezigheid van FOXO in kankercellen juist essentieel is voor tumorontwikkeling. Hieruit concluderen we dat het uitbalanceren van FOXO-activiteit een essentiële voorwaarde is voor tumorgroei en dat FOXO alleen in het proces van uitzaaien tijdelijk inactief moet zijn om dit voor een kankercel mogelijk te maken. Hiermee herdefiniëren we de rol van FOXO in kanker, namelijk dat de rol van FOXO niet tumorgroei-onderdrukkend is maar dat FOXO juist tumorgroei ondersteunt. Met deze conclusie in het achterhoofd kunnen we in de toekomst proberen FOXO tijdens de tumorgroei te remmen met specifieke medicatie en daarmee de groei van tumoren te stoppen.

In **hoofdstuk 5** ontwikkelen we een wiskundig model waarmee we op basis van observatie van door FOXO gereguleerde genexpressie kunnen voorspellen of FOXO actief is. Door dit model toe te passen op genexpressieprofielen van tumormateriaal van kankerpatiënten voorspellen we de mate van FOXO-activiteit en correleren deze aan de status van de insulinesignaleringsactiviteit. Met dit model kan snel bepaald worden welke signaleringsroutes actief zijn in tumoren en op basis hiervan kan een specifieke behandelingsstrategie ontworpen worden. Daarnaast voorspelt het model dat FOXO regelmatig actief is in tumoren waarin groeifactorsignalering ook actief is. In de toekomst kunnen we deze kennis gebruiken om te weten te komen in hoeverre FOXO-activiteit correleert met de uitkomst van behandelingsstrategieën die zich richten op het remmen van o.a. insulinesignalering.

Een nieuwe rol voor FOXO in celdeling

Om beter te begrijpen hoe FOXO celdeling kan remmen bekijken we in **hoofdstuk 6** in detail wat de effecten van FOXO-activatie op celdeling zijn. Met behulp van fluorescerende eiwitten die uitsluitend in specifieke fases van de celcyclus zichtbaar zijn, volgen we met live-videomicroscopie wat het effect van FOXO-activatie is in deze verschillende celcyclus-fases. FOXO-activatie in het begin van de celcyclus of vlak voor het einde van celcyclus beëindigt de celcyclus in de G0/G1-fase. FOXO-activatie tijdens de S/G2-fase, het moment dat het DNA gekopieerd wordt, induceert een voorheen voor FOXO onbeschreven remming van de celdeling. Cellen stoppen de celcyclus in S/G2-fase van de celcyclus met gekopieerd DNA, en verlaten de celcyclus zonder door mitose te gaan, om vervolgens in een G0/G1 toestand te stoppen. FOXO realiseert dit door middel van het onderdrukken van het EMI1-gen. EMI1 is een belangrijk gen dat ervoor zorgt dat de cel niet kan delen voordat het DNA volledig is gekopieerd en de cel voldoende is gegroeid om deling te ondergaan.

Het feit dat FOXO niet alleen een belangrijke speler is in insulinesignalering, maar ook betrokken is bij het detecteren van ongunstige omstandigheden voor een cel, zoals DNA-schade, verhoogde concentraties vrije zuurstofradicalen en een laag energieniveau, zou onze observaties kunnen verklaren. De verwachting is dat FOXO-activatie in één of meerdere van bovengenoemde omstandigheden tijdens het kopiëren van DNA de voortgang van de celcyclus remt, zodat de cel eerst goed kan herstellen alvorens te delen. Als het niet lukt om de problemen op te lossen blijft FOXO actief, waardoor de EMI1-activiteit in de tijd afneemt en de cel uiteindelijk de celcyclus verlaat. EMI1 functioneert op deze manier als een klok voor de cel die de maximale tijd bepaalt waarin een cel mag herstellen. Of FOXO activatie door ongunstige omstandigheden inderdaad leidt tot het verlaten van de celcyclus vanuit S-G2-fase is de volgende stap in dit onderzoek.

Conclusie

De hoofdstukken in dit proefschrift laten samen zien dat de rol van FOXO in kankercellen complex is. Het belangrijkste inzicht dat verkregen is, is dat FOXO een cruciale rol speelt bij het goed kunnen groeien en uitzaaien van kankercellen. We vinden dus dat de realiteit het tegenovergestelde is van de voorheen veronderstelde tumorgroei-onderdrukkende rol van FOXO. Nu we weten dat FOXO essentieel is voor kankercellen om te kunnen functioneren, biedt dit nieuwe mogelijkheden om behandelingsstrategieën te ontwikkelen tegen kanker. Is het bijvoorbeeld mogelijk om met specifieke medicijnen FOXO-activiteit te onderdrukken in tumoren? Kunnen we de ontwikkeling van medicijnresistentie stoppen door FOXO inactief te houden in kankercellen? Gaan andere beschikbare medicijnen beter werken wanneer FOXO inactief gehouden wordt? Allemaal vragen die in de toekomst beantwoord gaan worden en mogelijk leiden tot verbeterde kankerbehandeling.

Curriculum Vitae

Marten Hornsveld werd geboren op 14 december 1984 te Naarden. In 2003 behaalde hij zijn HAVO diploma aan de Huizermaat te Huizen. In 2003 begon Marten met de Bachelor of Applied Science in Biotechnologie aan de Hogeschool Utrecht. De eerste stage van de bachelor werd gelopen in het lab van prof. Jos Jonkers in het Nederlands Kanker Instituut te Amsterdam. Onder begeleiding van dr. Patrick Derksen deed Marten onderzoek naar de effecten van E-cadherineverlies op de ontwikkeling van borstkanker in muizen. Hieropvolgend heeft hij 3 maanden als analist doorgewerkt op dit project. De tweede stage van de bachelor liep Marten in de groep van Dr. Jacqueline Deschamps in het Hubrecht Instituut te Utrecht. Tijdens deze stage is onderzocht hoe CDX & HOXA genenclusters de ontwikkeling van muizenembryo's beïnvloeden. Hierna heeft hij 3 maanden gewerkt als dierverzorger bij het Hubrecht Instituut. Na succesvolle afronding van de Bachelor in 2007 is Marten aansluitend begonnen aan het prestige masterprogramma Cancer, Genomics & Developmental Biology aan de Universiteit Utrecht. De eerste stage van het masterprogramma werd gelopen in de groep van prof. Susanne Lens, waarbij onderzoek gedaan werd naar nieuwe medicinale remmers van Aurora kinases in kanker. Na afronding van de stage in 2008 heeft Marten nog 4 maanden doorgewerkt aan het project als research analist. De tweede stage voor het masterprogramma werd gelopen in het lab van dr. Helder Maiato in het IBMC (Instituto de Biologia Molecular e Cellular) in Porto, te Portugal. Hier deed hij onderzoek naar de bijdrage van centrosomen aan het vormen van de mitotische spoel tijdens celdeling in *Drosophila melanogaster*. Na succesvolle afronding van de stage in 2009 heeft Marten een stage van 2 maanden gelopen in het lab van dr. Benjamin Rowland in het UMC Utrecht waar hij onderzoek deed naar bindingspartners van cohesinringen in gist. Zijn masterscriptie getiteld "Putative Roles of Planar Cell Polarity Proteins in the Establishment and Regulation of Asymmetric Cell Division" is geschreven onder begeleiding van Prof. Sander van den Heuvel aan de Universiteit Utrecht. In 2010 heeft Marten zijn master succesvol afgerond en het diploma in ontvangst genomen. In januari 2011 begon Marten met zijn promotietraject onder begeleiding van promotor prof. Boudewijn Burgering en copromotor dr. Tobias Dansen. De resultaten van dit onderzoek staan beschreven in dit proefschrift en worden op 23 februari 2017 verdedigd. Aansluitend op het promotietraject zal Marten zijn onderzoek nog 6 maanden voortzetten in het lab van prof. Boudewijn Burgering.

List of publications:

Functional activity of signal transduction pathways differs across breast cancer subtypes and predicts relapse-free survival based on a multi-pathway score.
*Henk van Ooijen, Márcia Alves de Inda, Paul van de Wiel, **Marten Hornsveld**, Kalyan Dulla, Dianne van Strijp, Boudewijn Burgering, Ralf Hoffmann, Anja van de Stolpe & Wim Verhaegh*
Submitted

HDAC inhibitors sensitize colon cancer stem cells (CSCs) to apoptosis via FOXO4 regulation.
*Selcuk Colak, Cheryl Zimberlin, Maria Serena Roca, Kate Cameron, Evan Santo, Sander Van Hooff, Hans Rodermond, **Marten Hornsveld**, Salvatore Simmini, Michael Bots, Catarina M. Grandela, Boudewijn Burgering, and Jan Paul Medema*
Submitted

Interplay between metabolic identities in the intestinal crypt supports stem cell function.
Maria J. Rodriguez Colman, Matthias Schewe, Maaike Meerlo, Edwin Stigter, Johan Gerrits, Mia Pras-Raves, Andrea Sacchetti, ***Marten Hornsveld****, Koen C. Oost, Hugo J. Snippert, Nanda Verhoeven-Duif, Riccardo Fodde, and Boudewijn M.T. Burgering*
Nature 2017

The hallmarks of cancer from a redox perspective.
Hornsveld M*, Dansen TB.*
Antioxidants & Redox Signaling 2016 Aug 20;25(6):300-25.
PMID: 27353526

Restraining FOXO3-dependent transcriptional BMF activation underpins tumour growth and metastasis of E-cadherin-negative breast cancer.
Hornsveld M*, Tenhagen M, van de Ven RA, Smits AM, van Triest MH, van Amersfoort M, Kloet DE, Dansen TB, Burgering BM, Derksen PW*.
Cell Death & Differentiation. 2016 Sep 1;23(9):1483-92.
PMID: 27035620

FOXOs support the metabolic requirements of normal and tumor cells by promoting IDH1 expression.
Charitou P, Rodriguez-Colman M, Gerrits J, van Triest M, Groot Koerkamp M, ***Hornsveld M****, Holstege F, Verhoeven-Duif NM, Burgering BM.*
EMBO Reports 2015 Apr;16(4):456-66.
PMID: 25648147

Mammary-specific inactivation of E-cadherin and p53 impairs functional gland development and leads to pleomorphic invasive lobular carcinoma in mice.
Derksen PW, Braumuller TM, van der Burg E, ***Hornsveld M****, Mesman E, Wesseling J, Krimpenfort P, Jonkers J*.
Disease Models & Mechanisms 2011 May;4(3):347-58.
PMID: 21282721

Genes involved in centrosome-independent mitotic spindle assembly in Drosophila S2 cells.
Moutinho-Pereira S, Stuurman N, Afonso O, ***Hornsveld M****, Aguiar P, Goshima G, Vale RD, Maiato H.*
PNAS 2013 Dec 3;110(49):19808-13.
PMID: 24255106

Dankwoord

Iedereen bedankt!

Boudewijn, wat zalig om te mogen promoveren in jouw lab! De vrijheid die je creëert voor mensen om zich te ontwikkelen is uniek en het garant staan door weer en wind is iets waar vele hoogleraren en PI's wat van kunnen leren. Onze gedeelde vogelpassie gaat er voor zorgen dat we nog vaak op pad gaan in de toekomst. Onze bezoeken aan India en China waren onvergetelijk, aan Argentinië wordt gewerkt en wie weet waar we nog terecht komen!

Tobias, ik heb het enorm gewaardeerd om je als copromotor te mogen hebben! Je kritische blik en onuitputtelijke discussie-uithoudingsvermogen hebben de hoofdstukken veel goed gedaan. Tussen het discussiëren door, of al discussiërend een paar mooie ringslangen gezien en je bent me altijd te slim af wat betreft goede Uithof soorten! Je bent een van de weinigen die ik blindelings vertrouw op een goed recept, een goede reden om nog eens goed te gaan eten en een Witte Dakhaos te drinken.

Lydia, labmoeder, hier ligt weer een heel boekwerk vol figuren die door jou gepipetteerd zijn. Je hebt me enorm geholpen de laatste jaren om het allemaal te bolwerken en daarmee een gigantische bijdrage geleverd aan dit boek. De hoeveelste OIO ben ik die je al over de streep geholpen hebt eigenlijk?! Ik heb erg genoten van onze samenwerking en ben benieuwd of ik nog wel zindelijk genoeg ben om nog op mezelf te functioneren ;) Op naar de volgende promovendus!

En dan Familie Burgering. **Maria,** my paranymph and coffee/beer drinking partner. It is great to have you around, your drive, enthusiasm and indestructability are invigorating for everybody around you! Good luck with all the Nature papers and let's take over the rest of the world while we're at it! **Maaike,** super dat je me wilde helpen om door de eindeloze muizenproeven heen te komen in het GDL! Nu rammen we lekker alles eruit tijdens het kickbaggen, heerlijk, dit houden we er in! **Sabina,** next in line ;). Heel veel succes met het afronden van de promotie. Geen slechte filmsuggesties meer van mijn kant voor de movie-nights, the floor is yours! **Miranda**, bedankt voor alle hulp en gezelligheid op het lab en doe de groeten aan Henk als je hem nog eens ziet! **Paulien,** stoere brandweervrouw, houd die mannen maar flink onder de duim! **Cristina**, bedankt voor het worstelen met alle burocratie die komt kijken bij een PhD en natuurlijk de visumaanvragen voor de mooie reisjes die gemaakt zijn. **Robert,** succes met al het mass-spec werk en het begeleiden van Harmjan! **Yvonne,** leuk om je nog even mee te maken in het lab. De CyTOF is een gruwelijk apparaat dus daar ga ik zeker nog een keer voor langskomen in de toekomst! **Tianshu & Tao,** good luck with your PhD! **Runyue "John",** a.k.a. Beast from the East**,** great to meet you. I will never forget our visit to Guangzhou, thank you for taking such good care of us! Good luck with finishing your work in the Netherlands and all the best in the future as honorable super boss ;)

Man cave members: Harmjan, Loes, Sasha, fijne kamergenoten, nooit is het saai, maar er kan dan ook nooit langer dan 5 min aaneengesloten gewerkt worden. Veel gelachen, veel geborreld en veel gekonkeld! **Harmjan**, bedankt voor de vele input, zorg goed voor onze loden vriend "the cure". **Sasha** "Beest van België", succes met het binnenslepen van beurzen en het afmaken van je wormenstuk! **Loes**, aan jou de nobele taak om iedereen aan het bier te houden de komende jaren en heel veel succes met het afronden van je promotie!

Oude labgenootjes **Marrit, Astrid, Maaike, Marieke, Evi, Hesther** het lijkt alweer eeuwen geleden. Dank voor jullie enthousiasme en hulp tijdens het opstarten van mijn PhD! Veel succes met jullie verdere carrières binnen en buiten de wetenschap! **David,** wat hebben wij geouwehoerd over van alles en nog wat de afgelopen jaren. Fijn om lief en leed te delen en vooral veel te sparren over hoe het allemaal beter kan met onze wetenschap. Het was een eer om je paranimf te mogen zijn, nu is het voor mij tijd voor

het hoofdpodium! Inmiddels heb ik wel een ander sportmaatje gevonden ;) Heel veel succes bij het CBS en vooral tot snel!
Familie Derksen. **Patrick**, ouwe, ooit kwam ik de wetenschap binnen als HLO-student bij jou en Jos in het NKI. Nu inmiddels 10 jaar later werken we nog altijd samen, hebben we een mooie paper gepubliceerd ("the one that says BMF") en zit jij straks achter de tafel als ik sta te zweten tijdens mijn verdediging. Hopelijk kunnen we in de toekomst blijven samenwerken en ideeën uitwisselen. Succes met proeven rammen! **Milou,** partner in crime, we hebben samen goed werk geleverd met onze gezamenlijke paper, iets waar we trots op kunnen zijn! Heel veel succes met het opbouwen van je carrière in de consultancy business! **Miranda,** ooit samen op de HLO gezeten en tijdens mijn PhD ben je van onmisbare waarde geweest in het helpen opzetten en uitvoeren van mijn muizenproeven. Je behulpzaamheid, bereidwilligheid, gezelligheid en geduld worden erg gewaardeerd! Je bent de pilaar van het Derksen lab. **Ron & Robert**, gezellige knorrepotten langs de zijlijn van dit alles. Het waren mooie tijden! Hopelijk doet de VS jullie veel goed en wie weet drinken we als jullie terug zijn weer eens een biertje!

Anja, Henk & Wim, wat een ontzettend mooie kans en goede ervaring om met een bedrijf als Philips aan de slag te gaan. Het is een zeer interessant kijkje in de keuken van het bedrijfsleven dat ik heb gekregen en het stuk als resultaat van onze samenwerking wordt echt mooi! De laatste loodjes gaan we in de komende tijd nog even verwerken, waarna het met succes afgerond kan worden.

Lucas, UUUUuuuUUUuUuUU my wiggah! **WJ,** UUUUUuuuuUUUuuUU! Wat waardeer ik jou enorm, je bent de hart en ziel van het Stratenum! Wat wordt ons volgende concert? Houd je taai! **Mitchell,** net als bij WJ, wat wordt ons volgende concert?! Daarnaast snel een keertje gamen! Succes met het vinden van een mooie Post-doc plek! **Vittoria**, congratulations with your PhD! We had a great time together, with a lot of interesting conversations and opinions about this world. Good luck in the future and for the time being enjoy the beautiful Italian countryside and its birds! **Alex,** the violent discussions were great, hope you are doing well in the UK! **Maria K,** borrelmaatje door dik en dun. Het was altijd ontzettend gezellig om met je te kletsen over van alles en nog wat, bovenal de politiek. Hopelijk heb je je draai gevonden in de wereld van de regelgeving en beleidsvoering!
Susanne & Martijn, bij jullie kwam ik in aanraking met het Stratenum tijdens mijn eerste masterstage. Jullie enthousiasme en gedrevenheid hebben enorm stimulerend gewerkt en me definitief overtuigd om door te zetten en via de stage in Porto aan mijn promotie te beginnen. Een keuze waar ik nooit spijt van zal hebben! Mijn oude studenten, **Anniek, Nur, Hashmat & Luke,** het was leuk om jullie te mogen begeleiden, wellicht ook andersom. Ik hoop dat jullie een mooie toekomst staat te wachten! Goed in het boek kijken, want van iedereen staat er wel een experiment in! And of course the many many other people from the Stratenum, **Snipperts (Hugo,** je wordt nog een keer een vogelaar!**), Bos Gang, Gloerichs, Holsteges, Timmersen, Kopsen, Lenskes, Cuppens, Kalkhovens, van Mils, Vermeulens, Medema's,** thanks for all the discussions and most importantly the jolly good borrels!

Helder, Sara and the rest of the Maiato family, I had a great time while visiting your lab, an experience I will never forget! The enthusiastic and inspiring environment of the lab and of course the good life in Portugal were impressive. I hope you are all doing well and wish you all the best for the future!

Wat is het leven van een promovendus zonder leven? Vele uren buiten het lab heb ik besteed op de mooiste plekken van de wereld in gezelschap van de leukste vrienden van de wereld! **Wouter, Assia, Maartje, Peter, Rob, Helen, Janne, Sander, Henk, Anne, Martijn, Rinske, Bas, Blanca, Jelmer, Sjoerd, Nienke, Martje, Teize, Lodewijk, Thomas, Niels, Ferry, Frank, KKSjes van de Birding Basterds, DTers,** het vogelen, karnen, zeerapen, wereldreizen en bieren was een welkome afwisseling met het werken op het lab. Vele onvergetelijke momenten, grappen, discussies en vakanties zijn er de afgelopen jaren voorbij gekomen en blijven nog vele jaren komen! Ik hou van jullie. **Abbath**, I thank you for your music, it helped to Triumph Against the tide and write my chapters One by one.

Familie als vrienden, beter kan je het niet wensen. **Ank, Hans, Frank, Batsheva** bedankt voor alle gezelligheid en het onvoorwaardelijke vertrouwen in ons kunnen en steun in donkere dagen. Vrienden als familie, **Paul & Lies**, jullie vriendschap en steun de afgelopen jaren zijn van onschatbare waarde geweest voor zowel mij als de rest van de familie. Mijn dank is groot en dat gaan we vieren tijdens de vele borrels en BBQs die nog komen gaan!

Alice, moeder, **Josse**, broeder, de toekomst lacht ons toe! Ik hou ontzettend veel van jullie! Zonder jullie steun, onbegrensde vertrouwen en geloof in mijn kunnen had ik het nooit tot hier geschopt in het leven. We hebben zeer zware jaren achter de rug en ik hoop dat dit proefschrift bijdraagt aan een toekomst waarin andere mensen dit leed wordt bespaard.

Pa, ik mis je.

Vivian, elkaars paranimfen voor het leven. Bedankt dat je er altijd voor me bent, voor al je hulp, steun en liefde door de jaren heen. Ik hou van je!