



BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Assessment of Functional Phosphatidylinositol 3-Kinase Pathway Activity in Cancer Tissue Using Forkhead Box-O Target Gene Expression in a Knowledge-Based Computational Model



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The phosphatidylinositol 3-kinase (PI3K) pathway is commonly activated in cancer. Tumors are potentially sensitive to PI3K pathway inhibitors, but reliable diagnostic tests that assess functional PI3K activity are lacking. Because PI3K pathway activity negatively regulates forkhead box-O (FOXO) transcription factor activity, FOXO target gene expression is inversely correlated with PI3K activity. A knowledge-based Bayesian computational model was developed to infer PI3K activity in cancer tissue samples from FOXO target gene mRNA levels and validated in cancer cell lines treated with PI3K inhibitors. However, applied to patient tissue samples, FOXO was often active in cancer types with expected active PI3K. *SOD2* was differentially expressed between FOXO-active healthy and cancer tissue samples, indicating that cancer-associated cellular oxidative stress alternatively activated FOXO. To enable correct interpretation of active FOXO in cancer tissue, threshold levels for normal *SOD2* expression in healthy tissue were defined above which FOXO activity is oxidative stress induced and below which PI3K regulated. In slow-growing luminal A breast cancer and low Gleason score prostate cancer, FOXO was active in a PI3K-regulated manner, indicating inactive PI3K. In aggressive luminal B, HER2, and basal breast cancer, FOXO was increasingly inactive or actively induced by oxidative stress, indicating PI3K activity. We provide a decision tree that facilitates functional PI3K pathway activity assessment in tissue samples from patients with cancer for therapy response prediction and prognosis. (*Am J Pathol* 2018, 188: 1956–1972; <https://doi.org/10.1016/j.ajpath.2018.05.020>)

In the past decade, systemic treatment of cancer moved from conventional chemotherapy toward administration of drugs that target tumor traits chosen on an individual patient basis. This precision medicine approach requires biomarkers that reliably predict response to targeted drugs.¹ Cancer growth and metastasis are driven by roughly 10 to 12 cellular signal transduction pathways, relatively independent of the cancer cell type of origin.^{2–4} The phosphatidylinositol 3-kinase (PI3K) pathway is one of the main cellular growth factor signaling pathways frequently hyperactivated in cancer.²

The PI3K pathway inhibitors are used in cancer treatment alone or in combinations with other targeted strategies or conventional chemotherapy.^{5–7} Despite selection of

potentially responsive patients based on PI3K pathway mutation analysis, only a subpopulation of patients respond adequately to the drugs.^{8,9} To improve prediction of drug

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response and monitor therapy efficacy or emerging resistance, tests that measure functional PI3K activity are needed.

We have previously described a knowledge-based computational approach to assess signal transduction pathway activity in cancer tissue samples based on measuring target gene mRNA levels of the pathway-specific transcription factor.^{10,11} We report development of an mRNA-based assay for quantitative measurement of PI3K pathway activity that uses forkhead box-O (FOXO) transcription factor–induced RNA transcription as readout based on the well-known inverse association between FOXO and PI3K pathway activity.^{12–16} The model was biologically validated, using breast and lung cancer cell lines treated with PI3K pathway inhibitors or carrying doxycycline-inducible active FOXO3 constructs.

In cancer tissue, FOXO can be alternatively activated by cellular oxidative stress, a common trait in cancer that interferes with the inverse association with PI3K pathway activity. The level of *SOD2/MnSOD*, a FOXO target gene, was used to distinguish between the two functional states of FOXO activity, resulting in a robust approach to assess PI3K pathway activity in individual patient cancer samples.

Materials and Methods

Cell Culture–Based Model System for FOXO3 Activity

MCF7 and MDA-MB-231 breast cancer cell lines were cultured in Dulbecco's modified Eagle's medium F12 that contained 10% fetal bovine serum (FBS) (Lonza Group, Basel, Switzerland), 100 U/mL of penicillin, and 100 µg/mL of streptomycin (Lonza Group). Transfecting third-generation packaging vectors using polyethylenimine into HEK293T cells generated lentiviral particles.¹⁷ MCF7 and MDA-MB-231 cells were stably transduced with lentivirus that contained pINDUCER20-FOXO3.A3, allowing doxycycline-induced expression of constitutively active FOXO3 (FOXO3.A3).^{13,18,19} Cells were treated with 20% FBS or 10 µmol/L of the PI3K inhibitor LY294002 (Selleckchem, Houston, TX) for 16 hours to activate and inactivate the endogenous PI3K pathway, respectively. FOXO3.A3 expression was induced by 16 hours treatment with 10 ng/mL of doxycycline.

RNA Isolation and Affymetrix Microarray Hybridization

Treated cells were harvested after 16 hours of the respective incubations as indicated; RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany) and hybridized on Affymetrix HT HG-U133+PM Array Plate by ServiceXS (GenomeScan BV, Leiden, the Netherlands) and AROS (Eurofins, Aarhus, Denmark). Affymetrix data are available in the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>; accession number GSE113479).

Microarray Data Processing and Quality Control on Affymetrix Microarray Data

All microarrays that were used were from Affymetrix HG-U133Plus2.0 or Affymetrix HG-U133+PM microarrays (Supplemental Table S1), which have been processed with Frozen Robust Multiarray Analysis (fRMA) with random-effect summarization.²⁰ All microarray data sets can be found in the GEO database and Array express (<https://www.ebi.ac.uk/arrayexpress>). In principle, the Affymetrix HG-U133+PM platform contains all the perfect match probes of the HG-U133Plus2.0 platform, albeit with some minor reselections. To make the processed data comparable from both microarray types, a chip description file that contains only the shared probes was used, and the processing parameters of this subset were taken from the HG-U133Plus2.0 frmavecs to process data from both platforms. On all Affymetrix microarray data, from experiments performed for this study and data sets from the public GEO database, an extensive quality control was performed. Quality control of the microarray samples has been performed using several quality checks. These checks include the mean value of all Perfect Match probes intensities, negative or extreme (>16-bit) intensity values, poly-A RNA (sample preparation spike-ins) and labeled cRNA (hybridization spike-ins) controls, β-actin and glyceraldehyde-3-phosphate dehydrogenase 3'/5' ratio, values and center of intensity of the positive and negative border controls determined by the affyQCReport package, and an RNA degradation value determined by the AffyRNAdeg function from the affy package. Samples from the breast, colon, and prostate data sets that did not pass the quality criteria were removed from further analyses.

Western Blotting

Western blot analysis was performed using standard 6% to 15% SDS-PAGE. Proteins were detected with primary rabbit antibodies (1:2000) for FOXO3 (H144, Santa Cruz Biotechnology, Santa Cruz, CA). Blots were incubated with horseradish peroxidase–conjugated secondary antibodies at 4°C for 16 hours. Proteins were visualized with enhanced chemiluminescent (Bio-Rad Laboratories, Hercules, CA) using an ImageQuant LAS 4000 scanner (GE Healthcare, Chicago, IL).

Immunofluorescence and IHC

For immunofluorescent staining, cells were grown on glass coverslips, fixed using 4% paraformaldehyde, and blocked with phosphate-buffered saline that contained 2% bovine serum albumin (Invitrogen, Carlsbad, CA) and 0.1% normal goat serum (Invitrogen). Cells were incubated with FOXO3 antibody (Foxo3A rabbit monoclonal antibody, 1:500 CST-75D8), secondary Alexa563 conjugated antibodies, and

DAPI (Sigma-Aldrich, St. Louis, MO). Slides were imaged on a Zeiss LSM710 confocal microscope.

For FOXO3 immunohistochemistry (IHC) staining, 4- μ m sections of formalin-fixed, paraffin-embedded tissue samples were deparaffinized and rehydrated. After blocking of endogenous peroxidase activity, antigen retrieval was performed with TE buffer at pH 9.0 (Dako, Santa Clara, CA) in a water bath at 95°C to 96°C for 25 minutes. After cooling for at least 15 minutes and washing steps with phosphate-buffered saline, the samples were blocked with 1% bovine serum albumin in phosphate-buffered saline for 15 minutes. Then the sections were incubated with FOXO3 antibody (1:50, CST-75D8) for 1 hour at room temperature. Visualization was achieved using the Dako Envision+™ System anti-rabbit horseradish peroxidase. As a counterstain, Gill's 2-hematoxylin was used. Images were generated with a three-dimensional Histech scanner. Negative controls consisted of sections that underwent similar staining procedures without addition of the primary antibody. As a positive control, nonmalignant tonsil tissue was used.

Development of the Computational Model for Predicting FOXO Activity

The signal transduction pathway modeling approach is based on inferring pathway activity from the measured mRNA levels of target genes of the FOXO transcription factor, as readout for PI3K pathway activity, using probabilistic Bayesian network inference. Development of the computational model for FOXO transcriptional activity is based on probabilistic Bayesian network inference as described earlier.¹¹ In brief, the parameters that describe the relation between the FOXO transcription complex (TC) and a target gene represent the probability of the respective gene being up-regulated or not, depending on whether the TC is active or not. Furthermore, the parameters that describe the relation between each target gene and its respective Affymetrix probe sets represent the probability of the probe set having a high or low intensity, depending on whether the target gene is up or down (some probe sets may be more discriminating than others). Finally, for each probe set, a threshold was set above which it was called a high intensity and below which it was called low. Parameters that enforce the associations between the FOXO TC and target genes were manually set to improve generalization behavior of the model across different tissue types.¹¹ Parameters that describe associations between target genes and their respective probe sets were calibrated on a public data set of human umbilical vein endothelial cells (HUVECs) carrying inducible constitutively active FOXO3.A3—estrogen receptor (ER) (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE16573). A threshold for FOXO activity was set using three samples in which constitutively active FOXO.A3 was induced for 12 hours as active FOXO calibration samples, whereas three controls, without

induction, were used as inactive FOXO calibration samples.²¹ The model was frozen and applied to Affymetrix microarray data of cell line and tissue samples by entering probe set measurements as observations into the model, inferring backwards FOXO transcription factor activity score as the \log_2 value of the FOXO transcription factor odds ($p/(1 - p)$).

Samples are classified as FOXO active if pathway activity exceeds an activity score above 0, corresponding with an odds active to inactive ratio above 1:1 that the pathway is active, and inactive in case the activity score is <0 . For validation purposes, FOXO activity analysis was always performed on independent fRMA (unless otherwise indicated) preprocessed Affymetrix HG-U133Plus2.0 microarray data from described experiments and from public GEO data sets.

Identification of Direct Target Genes for FOXO

For optimal performance, across multiple different tissue types, the mathematical model should contain direct target genes of the FOXO transcription factor. Target genes were manually selected based on extensive scientific evidence for each gene being a direct target gene of the respective TC, including promoter region enhancer motif analysis, transcription factor—binding experiments (electrophoretic mobility shift assay and chromatin immunoprecipitation), gene promoter luciferase reporter experiments, and differential mRNA expression analysis. Available literature retrieved from the MEDLINE database was extensively evaluated using PubMed for FOXO target genes. In addition, literature on FOXO target genes was 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 method as described earlier.¹¹ Only the highest ranked target genes, also included in the list published by van der Vos and Coffey,²² were selected as bona fide target genes (Table 1).

SOD2 Level Associated with Oxidative Stress

To investigate differences in individual FOXO target gene expression levels between FOXO-active samples of healthy and cancer tissue, publicly available microarray data sets were used from healthy and corresponding premalignant or malignant tumor tissue samples (Supplemental Table S1). fRMA-normalized intensities on a \log_2 scale measured on the different gene-specific probe sets on the microarray reflect gene expression levels. FOXO gene and FOXO target gene expression levels were compared between FOXO-active tumor and healthy tissue samples.

To establish a threshold for *SOD2* expression levels in the absence of oxidative stress, means \pm 2 SDs were determined for *SOD2* Affymetrix U133 Plus 2.0 probe set values

Table 1 Selected Forkhead Box-O Target Genes

Gene	Probe set	References
<i>AGRP</i>	207193_at	23,24
<i>BCL2L11</i>	1553096_s_at, 1555372_at, 1558143_a_at, 208536_s_at, 222343_at, 225606_at	25,26
<i>BCL6</i>	203140_at, 215990_s_at	27,28
<i>BNIP3</i>	201848_s_at, 201849_at	29,30
<i>BTG1</i>	1559975_at, 200920_s_at, 200921_s_at	31
<i>CAT</i>	201432_at, 211922_s_at, 215573_at	32
<i>CAV1</i>	203065_s_at, 212097_at	33,34
<i>CCND1</i>	208711_s_at, 208712_at, 214019_at	35
<i>CCND2</i>	200951_s_at, 200952_s_at, 200953_s_at, 231259_s_at	35
<i>CCNG2</i>	1555056_at, 202769_at, 202770_s_at, 211559_s_at, 228081_at	36,37
<i>CDKN1A</i>	1555186_at, 202284_s_at	38,39
<i>CDKN1B</i>	209112_at	40,41
<i>ESR1</i>	205225_at, 211233_x_at, 211234_x_at, 211235_s_at, 211627_x_at, 215551_at, 215552_s_at, 217190_x_at, 207672_at	42
<i>FASLG</i>	210865_at, 211333_s_at	13,43
<i>FBXO32</i>	225801_at, 225803_at, 225345_s_at, 225328_at	44
<i>GADD45A</i>	203725_at	45,46
<i>INSR</i>	207851_s_at, 213792_s_at, 226212_s_at, 226216_at, 226450_at	47
<i>MXI1</i>	202364_at	48
<i>NOS3</i>	205581_s_at	49
<i>PCK1</i>	208383_s_at	50
<i>POMC</i>	205720_at	23,51
<i>PPARGC1A</i>	1569141_a_at, 219195_at	52
<i>PRDX3</i>	201619_at, 209766_at	53
<i>RBL2</i>	212331_at, 212332_at	36,54
<i>SOD2</i>	215078_at, 215223_s_at, 216841_s_at, 221477_s_at	55
<i>TNFSF10</i>	202687_s_at, 202688_at, 214329_x_at	56

FOXO target genes selected for model and associated probe sets (Affymetrix HG-U133Plus2.0).

for different types of healthy normal tissue. In FOXO-active samples with *SOD2* mRNA levels that exceeded the threshold level of both probe sets, FOXO activity was considered as oxidative stress induced.

Subtyping Breast Cancer Samples from Public Data Sets According to Perou

Intrinsic breast cancer subtypes of all breast cancer samples were determined from microarray data according to the method described by Parker et al.⁵⁷ and Perou et al.⁵⁸ Intrinsic subtypes were determined from the Affymetrix microarray data using the methods as described by Parker et al.⁵⁷ and the Prosigna Packet Insert (NanoString Technologies, Seattle, WA). fRMA-normalized gene expression of all 50 genes included in PAM50 was extracted from the microarray data using the probe sets associated with the PAM50 genes. The probe set with the highest variance was selected in case more than one probe set was associated with a single gene. Centroids for the luminal A, luminal B, HER2 enriched, basal, and normal-likes were calculated using the samples from GEO database (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE21653) with given subtypes. Next, Pearson correlation coefficients with these centroids were calculated for all samples. Each sample was assigned to the subtype with the highest correlation.

Statistical Analysis

One-sided Wilcoxon signed-rank statistical tests were performed. $P < 0.05$ was considered significant.

Results

Development of a Computational Model for PI3K-FOXO Activity

A Bayesian network-based computational model for FOXO activity was generated, which infers FOXO transcriptional activity from 26 FOXO target gene mRNA levels in a tissue sample (Figure 1A). Although inferring transcription factor activity from the mRNA level of one target gene is not sufficiently specific, inferring activity from expression levels of a larger number of target genes, typically between 20 and 30, appears to be a highly specific way to quantify associated transcription factor activity. For optimal performance across multiple different tissue types, direct target genes are selected. Because pathway databases such as KEGG (www.genome.jp/kegg) and Biocarta (www.biocarta.com) are inconsistent in this aspect,²⁰ genes were manually selected based on scientific evidence from literature (PubMed) and Thomson-Reuters' Metacore.¹¹ On the basis of the 26 highest ranked FOXO target genes

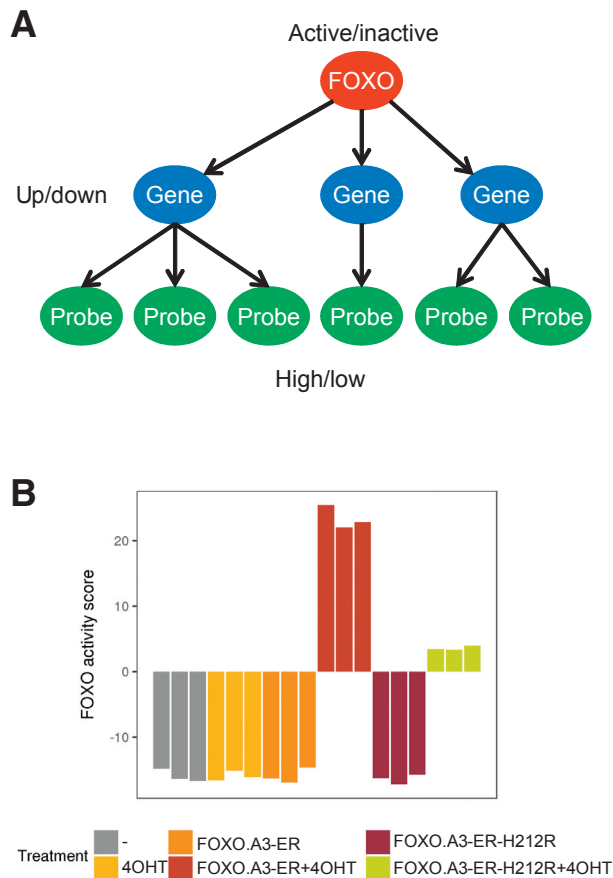


Figure 1 A knowledge-based Bayesian computational model predicting forkhead box-O (FOXO) activity. **A:** The Bayesian network structure used as a basis for our modeling approach shown as a simplified model of the transcriptional program of a cellular signal transduction pathway, consisting of three types of nodes: transcription factor, target gene, and microarray probe sets corresponding to target genes. Adapted with permission.¹¹ **B:** Calibration of the computational FOXO model on a public Gene Expression Omnibus data set (<https://www.ncbi.nlm.nih.gov/geo/>; accession number GSE16573), consisting of Affymetrix microarray Plus2.0 expression data from human umbilical vein endothelial cells containing a 4-hydroxytamoxifen (4OHT)-inducible FOXO3.A3-estrogen receptor (ER) expression construct, with either wild-type or mutated (H212R) FOXO3.A3. Each bar represents the inferred FOXO activity score of an individual sample; FOXO is active (values above the horizontal axis) versus inactive (values below the horizontal axis).

and their corresponding probe sets, the Bayesian network-based computational model inferring FOXO activity was generated (Figure 1A and Table 1).^{16,22}

HUVECs with inducible FOXO activity provided ground truth evidence of FOXO activity status in an untransformed setting and were used to calibrate the model before model freeze. Predictions of the Bayesian model are consistent with known experimental FOXO activity status in the complete HUVEC data set, including independent sample data (Figure 1B). Untreated, 4-hydroxytamoxifen (4OHT)-treated, and untreated HUVEC-FOXO3.A3-ER (4OHT-inducible FOXO3 hyperactive mutant) 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 induction of constitutively active FOXO3.A3. In HUVECs that express FOXO3.A3-ER-H212R, a point mutation in the DNA-binding domain, resulting in reduced DNA-binding capacity, the model predicted FOXO to be inactive in untreated and low in 4OHT-treated cells, respectively.²¹ These observations confirm that the model specifically detects transcriptional changes induced by FOXO and is sensitive to low levels of FOXO activity.

Biological Validation of the PI3K-FOXO Model in Breast Cancer Cell Lines

After calibration, the model was biologically validated in independent breast cancer cell lines, in which ground truth PI3K pathway activity was known. ER-positive, PI3K-CA^{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 protein expression and transcriptional activity on treatment with doxycycline for 16 hours (Figure 2A). Endogenous FOXO3 protein was predominantly detected in the cytoplasm in untreated and 20% FBS-stimulated cells, switching to dominant nuclear localization in cells treated with doxycycline, PI3K inhibitor LY294002, and doxycycline in combination with LY294002 (Figure 2B). This shows that nuclear translocation of FOXO3 was induced in a controlled manner in this experimental cell culture system. On Affymetrix mRNA expression data from this cell model, the FOXO model predicted respectively low FOXO activity in untreated (PI3K pathway active) and high FOXO activity in doxycycline-treated (PI3K pathway inactive) MCF-FOXO3.A3 and MDA-MB-231-FOXO3.A3 cells; low FOXO activity in 20% FBS-treated MCF7 cells (PI3K pathway active) and high activity in doxycycline, LY294002, and combined doxycycline and LY294002-treated cells (all PI3K pathway inactive) (Figure 2, C and D).

To further validate the assay in cells without artificially induced FOXO, two public Affymetrix data sets were available for breast cancer cell line experiments in which the HER2-PI3K pathway activity was actively manipulated, the data being suitable for additional biological validation (Figure 2, E and F). First, the HER2 ligand heregulin- β 1 is known to activate the PI3K pathway and was found to reduce FOXO activity in MCF7 cells after only 2 hours of exposure (Figure 2E). Second, in the BT-474 breast cancer cell line, siRNA-induced knockdown of HER2 resulted in the expected increase in FOXO activity, associated with reduced PI3K pathway activity (Figure 2F). Finally, a few studies using immune cells have been selected for additional biological validation in noncancer cell types (Figure 2, G and H). The cytokine IL-2 is a known inducer of PI3K

pathway activity in T lymphocytes, causing clonal proliferation. Withdrawal of IL-2 resulted in an increase of FOXO activity in T cells (Figure 2G), and stimulation with IL-2 induced a decrease in FOXO activity in natural killer cells (Figure 2H). Finally, activation of CD4⁺ naive T lymphocytes with anti-CD3/anti-CD28 led to the expected reduction in FOXO activity, indicating increased PI3K pathway activity associated with clonal proliferation (Figure 2I).

Together these results confirm that the computational model predicted PI3K-dependent FOXO activity as expected in independent cancer and noncancer cell samples.

Use of the Pathway Model to Predict and Monitor Response to Drugs

On the premise that FOXO activity is inversely related to PI3K activity in cell lines, it was investigated whether the FOXO model was capable of predicting response to drugs that target receptor tyrosine kinase activity in independent cancer cell line data sets (<https://www.ncbi.nlm.nih.gov/geo>; accession numbers GSE51212 and GSE30516).

FOXO activity was scored in an epidermal growth factor receptor (EGFR) mutant HCC827 lung cancer cell line treated with erlotinib (EGFR inhibitor), AZD6244 (selumetinib, MEK inhibitor), or BEZ235 (dactolisib, PI3K/mTOR dual inhibitor). FOXO scored inactive in untreated samples, indicating the active EGFR/PI3K pathway; on treatment with any of the three drugs, FOXO scored active, confirming that all three drugs were effective in directly and/or indirectly reducing PI3K pathway activity (Figure 3A). As expected, the effect was maximal with the EGFR inhibitor specifically targeting the mutant EGFR expressed by the HCC827 cell line. In three breast cancer cell lines representing triple-negative (BT20), ER-positive (MCF7), and HER2-positive breast cancer (MDA-MB-453), the FOXO activity score increased as expected when treated with erlotinib, indicating reduced PI3K pathway activity (Figure 3B). In an interesting preclinical study, a known lapatinib-resistant BT474-J4 breast cancer cell line did not respond with an increase in FOXO activity to single-agent lapatinib and foretinib treatment, in contrast to its sensitive counterpart (BT474), whereas the resistant cell line responded with an increase in FOXO activity to the combination therapy, comparable to that seen after treatment of the sensitive cell line with lapatinib alone (Figure 3C).

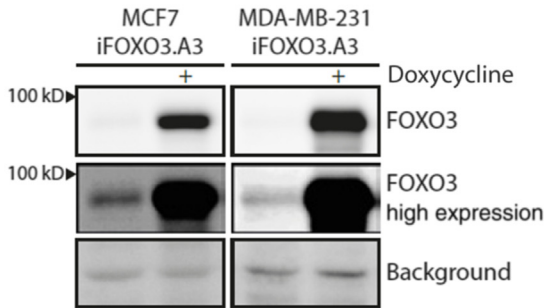
FOXO Activity in Healthy Colon and Colorectal Cancer Tissue Samples

For evaluation of the model when used on patient tissue samples, a number of independent selected public data sets from clinical studies were used. First, the FOXO activity model was applied on tissue samples derived from 32 normal colon and 32 adenoma biopsy specimens (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE8671). A clear difference in FOXO activity score was observed

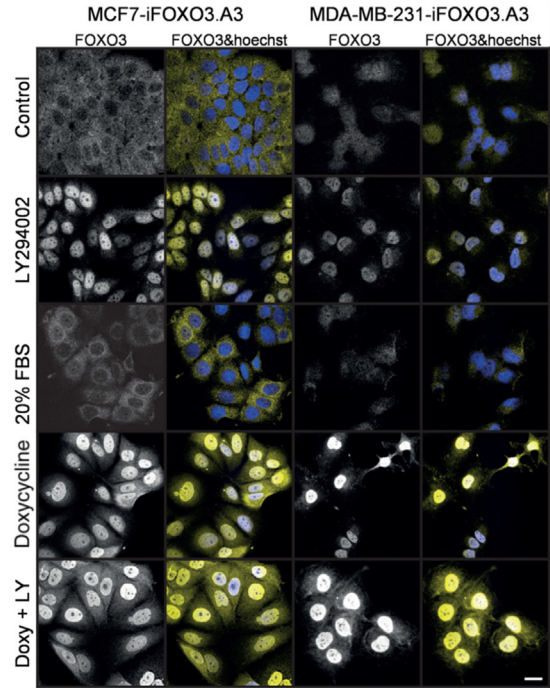
between healthy colon and adenoma tissue, showing FOXO to be active [32 (100%)] and inactive [29 of 32 (90%)], respectively, indicating frequent activation of the PI3K pathway in colon adenoma (Figure 4A). Second, the FOXO model was applied on data from normal colon, colon adenoma, and carcinoma tissues (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE20916) (Figure 4B). In normal colon tissue, FOXO was predicted active [23 of 24 samples (96%)]. In most adenoma tissue samples, FOXO was predicted to be inactive [30 of 45 (67%)], confirming frequent PI3K pathway activity, but less than in the first study (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE8671). In half of the colon carcinoma tissue samples (18 of 36), FOXO was scored inactive, with an inferred percentage of PI3K-active colon cancers lower than in adenoma. In contrast to the benign colon adenoma, colon carcinoma is an invasive cancer, characterized by active PI3K growth factor pathway signaling. This urged us to investigate the possibility of FOXO activity in the presence of PI3K activity. A well-described cause for FOXO activity is cellular oxidative stress, associated with the homeostatic function of FOXO in protection against oxidative damage.^{12,59} This difference in function of FOXO is likely to be reflected in a difference in transcribed target genes. To determine which genes within the used FOXO target gene set would be able to discriminate between FOXO activity in healthy and carcinoma samples, FOXO target gene expression levels were compared between normal colon and colon carcinoma tissue samples, in which FOXO was predicted to be active by the model. *SOD2* and *BNIP3* gene expression was increased in FOXO-active colon carcinoma samples compared with normal colon tissue (Supplemental Table S1). Both genes play a role in cellular response to oxidative stress and are transcribed by FOXO under these circumstances, making them prime candidates to distinguish between the two modes of FOXO activity.^{29,55}

Indeed, comparing *SOD2* and *BNIP* gene expression levels between various FOXO active normal tissues and corresponding premalignant or malignant tumor tissue samples (breast cancer, Barrett esophagus, esophageal cancer, bladder cancer, and gliomas) confirmed increased expression levels of *SOD2* and to a lesser extent *BNIP3* in FOXO-active samples from aggressive cancer types (Supplemental Table S1). In remarkable contrast, in FOXO active samples of two benign hyperproliferative conditions, colon adenoma and Barrett esophagus, FOXO activity was not associated with increased *SOD2* and *BNIP3* expression, indicating that in these benign tumors FOXO was activated in a PI3K-regulated manner to control cell division, comparable to healthy tissue. The *BNIP3* and *SOD2* genes are not unique transcriptional targets of FOXO and can be transcribed by other transcription factors during conditions of oxidative stress.^{60–62}

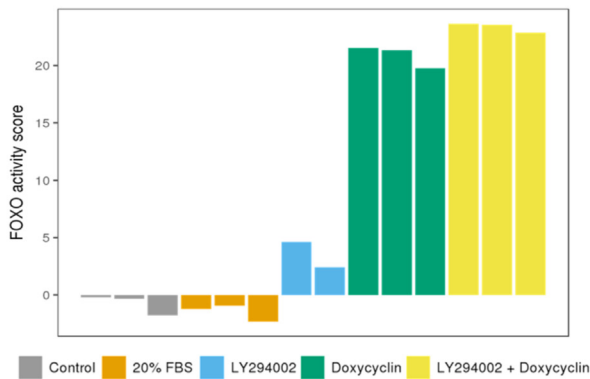
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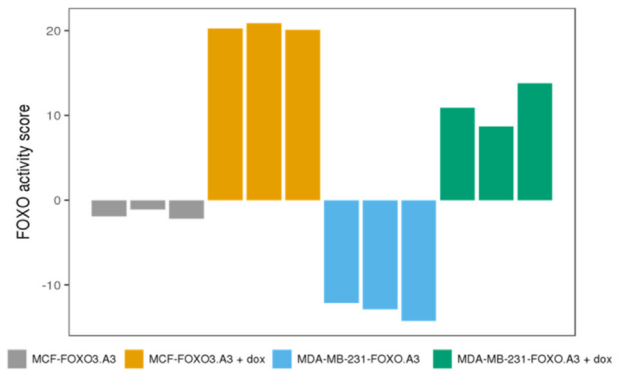
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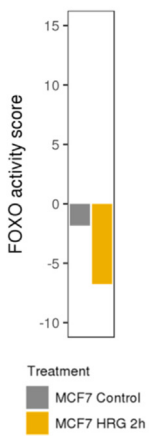
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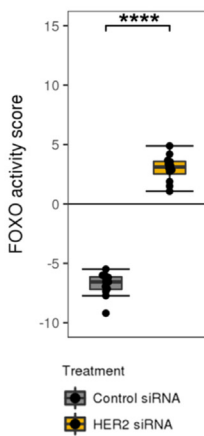
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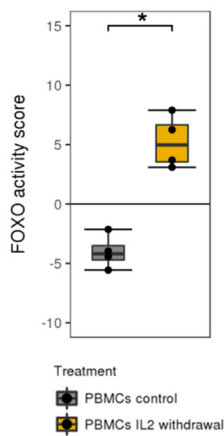
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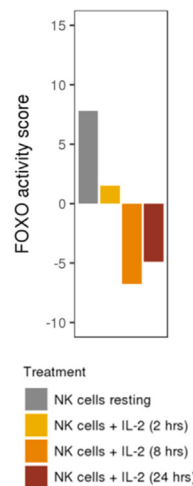
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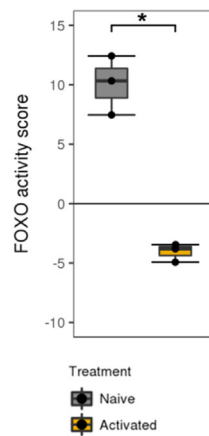
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For reliable use in distinguishing the two modes of FOXO action, expression levels of these target genes should be positively correlated with FOXO activity during oxidative stress. Correlation between FOXO activity and *SOD2*/*BNIP3* mRNA expression levels was studied in a breast cancer data set from a study in which oxidative stress in individual samples was measured in luminal A, HER2, and triple-negative/basal-like breast cancer tissue using expression levels of the H2AX protein.⁶³ In basal-like breast cancer samples, characterized by oxidative stress, FOXO activity was indeed correlated with *SOD2* expression ($P < 0.0001$), whereas this correlation was absent in the luminal A breast cancer samples that typically did not have oxidative stress (Supplemental Figure S1). Such a correlation was absent for the *BNIP3* target gene. These results provide evidence that under conditions of oxidative stress FOXO is the main transcription factor transcribing the *SOD2* gene, whereas regulation of *BNIP3* gene expression is likely to be controlled predominantly by other transcriptional regulators [(eg, NF- κ B and the transcription factor encoded by nuclear factor (erythroid-derived 2)-like 2 (NRF2)]. In agreement with this, *SOD2* showed the most generalized and profound differential expression between FOXO-active normal tissue samples and corresponding FOXO-active cancer types. On the basis of these results, this gene was selected as the most reliable parameter to distinguish between the two modes of FOXO activity. The *SOD2* upper threshold level for PI3K-regulated (nonoxidative stress) FOXO activity was defined as 2 SDs above the mean expression level in normal tissue and was calculated in FOXO-active samples for healthy colon, breast, and prostate tissue separately. Subsequently, the FOXO model in combination with the *SOD2* expression threshold was applied to publicly available data sets with data from individual patients with colon, breast, and prostate cancer. In FOXO-active samples from normal tissue, luminal A and normal-like breast cancer, and prostate cancer, *SOD2* mRNA levels were in general in the normal range; in contrast, *SOD2* was frequently increased in FOXO-active colon cancer, whereas in the more aggressive breast cancer subtypes, *SOD2* levels increased in parallel with tumor aggressiveness (Supplemental Figure S2).

FOXO3 IHC Staining in Tissue Slides

Because FOXO was uniformly scored active in the PI3K-regulated growth control mode on normal tissue samples,

such a FOXO activity score on a cancer slide that contained normal tissue may potentially obscure PI3K pathway activity in cancer cells. FOXO3 is the most ubiquitously expressed FOXO family member with high relevance for cancer and was consistently expressed in most healthy tissues and in all tumor samples from all cancer types that have been analyzed to date, including breast, colon, prostate, bladder, esophagus, and brain (Supplemental Table S1). FOXO3 IHC staining was developed and tested on colon tissue, to enable identification in a mixed healthy and cancer cell tissue sample the cell type(s) that contributed to an active FOXO score of the whole sample. Although nuclear presence of FOXO protein is a prerequisite for its transcriptional activity, it is not sufficient because functional FOXO activity is dependent on its posttranslational status.^{64,65} However, when a high FOXO activity score is measured using the pathway model, nuclear FOXO IHC staining provides complementary information on the cell type(s) causing the high score. In agreement with our analysis of FOXO activity scores in colon tissue, in healthy colon tissue, FOXO3 was localized in the nucleus in mucosa and other nontumor (interstitial) cells and was cytoplasmic in the few (dividing) crypt cells; however, in colon adenoma cells, FOXO3 was localized in the cytoplasm (inactive), and in colon carcinoma tissue, some areas showed nuclear and other areas of cytoplasmic staining (Figure 4C). Total cellular FOXO protein expression levels (nuclear plus cytoplasmic) were observed to vary among stained tissue slides. These differences are most likely attributable to differences in protein turnover because on mRNA level FOXO expression levels were remarkably similar among different tissue types (Supplemental Table S1).

Prediction of FOXO Activity Mode (PI3K Regulated or Oxidative Stress) in Colon Adenoma and Primary Colon, Breast, and Prostate Cancer Tissue

Analysis of a large set of healthy colon tissue samples ($n = 121$) allowed a threshold setting for normal *SOD2* mRNA levels in FOXO-active samples (Figure 5A and Supplemental Figure S2A). Subsequently, an extended independent set of patient colon adenoma and carcinoma sample data were compiled and FOXO activity and *SOD2* expression levels determined. In normal colon samples, only 2.6% of FOXO active samples had *SOD2* expression that

Figure 2 Correct prediction of forkhead box-0 (FOXO) and phosphatidylinositol 3-kinase (PI3K) activity in breast cancer cell lines and other cell types. **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 for 16 hours to induce FOXO3 expression. The lower FOXO3 blot represents a longer exposure of the same blot. **B:** Immunofluorescent staining of FOXO3 (single FOXO staining black and white; overlay in yellow) and DNA (Hoechst blue) in MCF7-FOXO3.A3 and MDA-MB-231-FOXO3.A3 cells treated with 20% fetal bovine serum (FBS), PI3K inhibitor LY294002, doxycycline, or a combination of doxycycline and LY294002 for 16 hours. **C–I:** Biological validation of the computational FOXO model in various cell types. **C:** MCF7-FOXO3.A3 cells treated with 20% FBS, PI3K inhibitor LY294002, doxycycline, or a combination of doxycycline and LY294002 for 16 hours. **D:** MCF7-FOXO3.A3 and MDA-MB-231 cells treated with dox for 16 hours. **E:** GSE8471; breast cancer MCF7 cell line treated with heregulin- β 1 (HRG). **F:** GSE71347; siRNA-induced HER2 knockdown in breast cancer cell line (BT-474). **G:** GSE7345; IL-2 withdrawal from T lymphocytes. **H:** GSE8059; IL-2 stimulation of natural killer (NK) cells. **I:** GSE71566; CD4⁺ T lymphocytes from human umbilical cord blood, naive and activated with anti-CD3/anti-CD28. GSE accession numbers can be found at Gene Expression Omnibus database (<https://www.ncbi.nlm.nih.gov/geo>). * $P < 0.05$, **** $P < 0.0001$ (one-sided Wilcoxon signed-rank statistical tests were performed). Scale bar = 20 μ m.

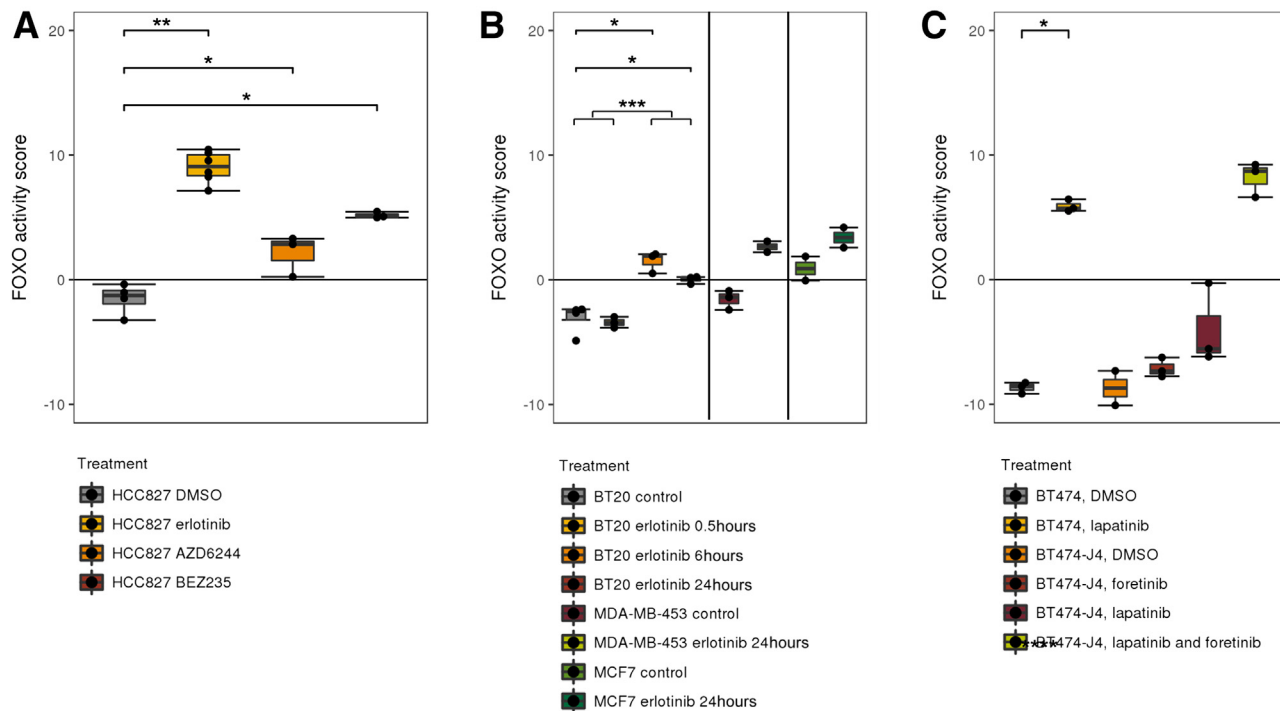


Figure 3 Functional phosphatidylinositol 3-kinase (PI3K) activity prediction correlates with corresponding targeted therapy effects in cancer cell line samples. **A:** The lung cancer cell line HCC827 (<https://www.ncbi.nlm.nih.gov/geo/>; accession number GSE51212) was treated either vehicle [dimethyl sulfoxide (DMSO)] ($n = 4$), erlotinib ($n = 3$), AZD6244 (selumetinib) ($n = 3$), or BEZ235 (dactolisib) ($n = 3$), as indicated. Erlotinib inhibits epidermal growth factor receptor; selumetinib inhibits specifically MEK1/MEK2; dactolisib is a dual inhibitor of PI3K and mechanistic target of rapamycin. **B:** Three breast cancer cell lines (GEO accession number GSE30516) representing triple-negative (BT20), estrogen receptor–positive (MCF7), and HER2-positive breast cancer (MDA-MB-453) were treated with erlotinib (periods indicated in the figure). **C:** Breast cancer cell lines (BT474, HER2-positive and lapatinib-sensitive; BT474-J4, acquired lapatinib-resistance) (GEO accession number GSE16179) treated with lapatinib, foretinib (GSK1363089), lapatinib, and foretinib combined or vehicle. FOXO activity scores plotted in groups using boxplots. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (one-sided Wilcoxon signed-rank statistical tests were performed).

exceeded the threshold level. In the few adenoma samples that were FOXO active ($n = 12$, 16%), *SOD2* levels were above the threshold in half. In the carcinoma samples, one-third of the samples were scored FOXO active, of which 54% with above threshold *SOD2* expression.

Similarly, a compiled set of data from patients with breast cancer was analyzed (Figure 5B and Supplemental Figure S2B). Before FOXO activity analysis, breast cancer tumor subtyping in all cancer data sets was performed using the PAM50 algorithm to ensure that the subtypes in all data sets were similarly determined.⁵⁷ In line with the findings in healthy colon tissue, FOXO was predicted to be generally active in normal breast tissue (90%) and similar in normal-like (96%) and luminal A (89%). In luminal B, HER2, and basal subtypes, 34%, 18%, and 17%, respectively, scored low for FOXO activity, indicating PI3K pathway activity. An increasing percentage of FOXO active samples with elevated *SOD2* (above threshold set in healthy breast tissue) was observed with increasing cancer subtype aggressiveness: from 4.7% in luminal A to 71% in basal breast cancer.

To provide additional evidence of correct differentiation between FOXO activity in growth control versus oxidative stress mode using FOXO activity in combination with

SOD2 mRNA level, the already mentioned study by Grusso et al⁶³ was separately analyzed (Supplemental Figure S3B). The results with respect to the frequency of oxidative stress in luminal A, HER2, and basal-like breast cancer were highly comparable to the results described by the authors.

In normal prostate and prostate cancer samples of patients with lower Gleason score tumors (Gleason score 4 to 7), FOXO was active in 91% and 85%, respectively. Too few samples of patients with high Gleason score tumors (Gleason score 8 to 9) were available to draw any comparative conclusions (Supplemental Figure S3A). Interestingly, in none of the FOXO-active primary prostate cancer samples was *SOD2* expression increased over the threshold level set for normal tissue, indicating that for lower Gleason score prostate cancer PI3K pathway activity can be safely inferred from the FOXO activity score and was inactive in most prostate cancer samples (Supplemental Figure S2C).

Identifying Tumors with an Active PI3K Pathway

To facilitate inference of functional PI3K pathway activity, a simplified decision tree was generated based on two

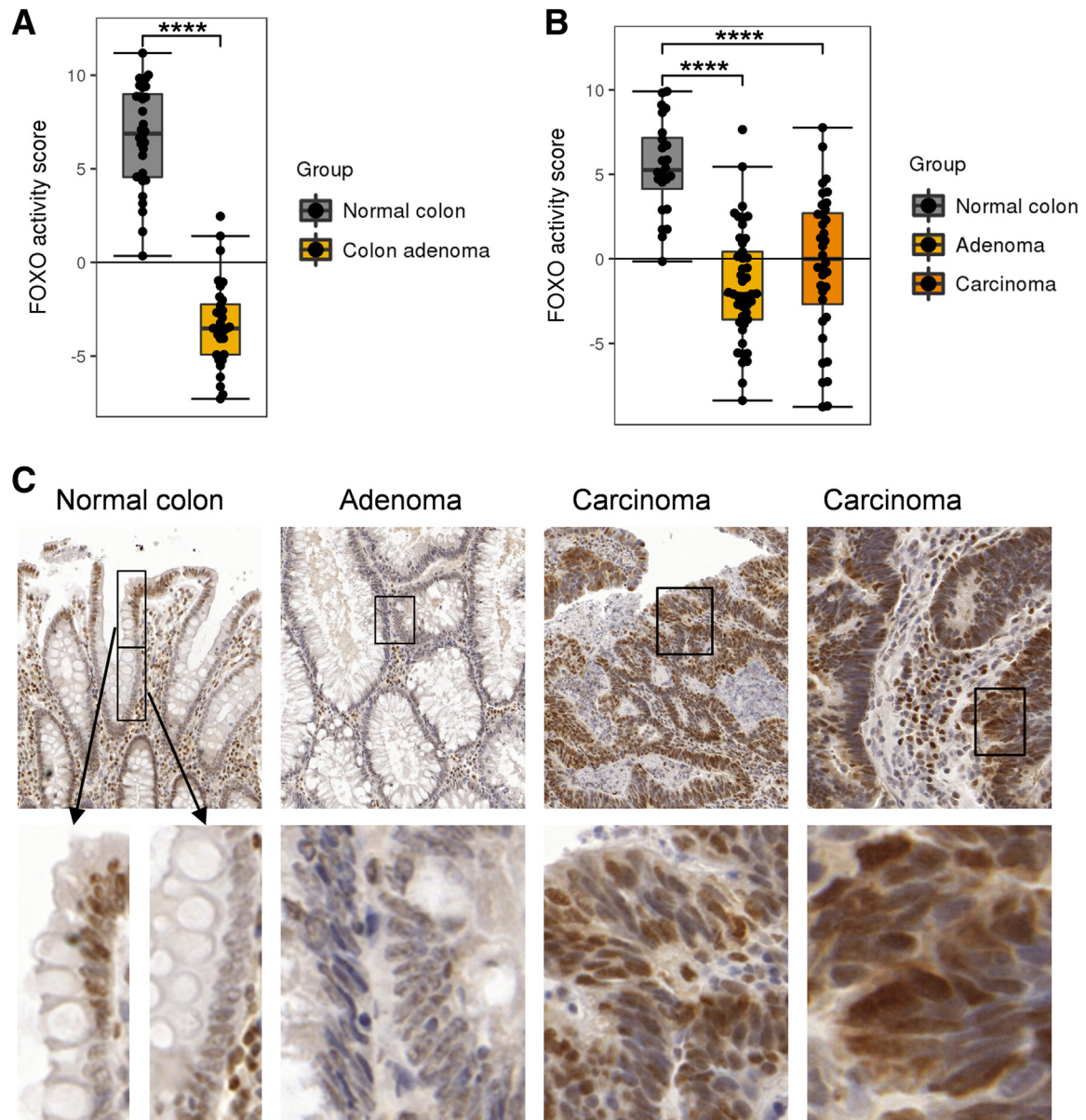
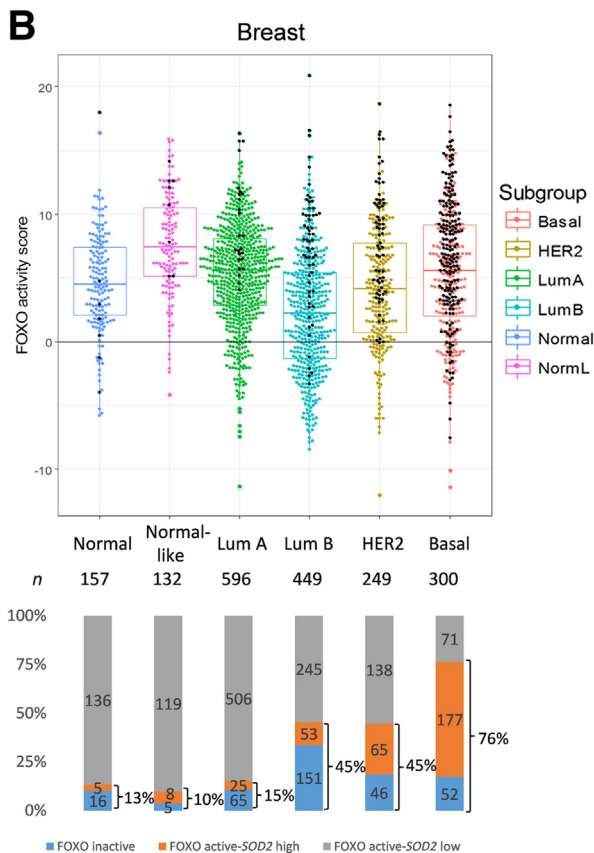
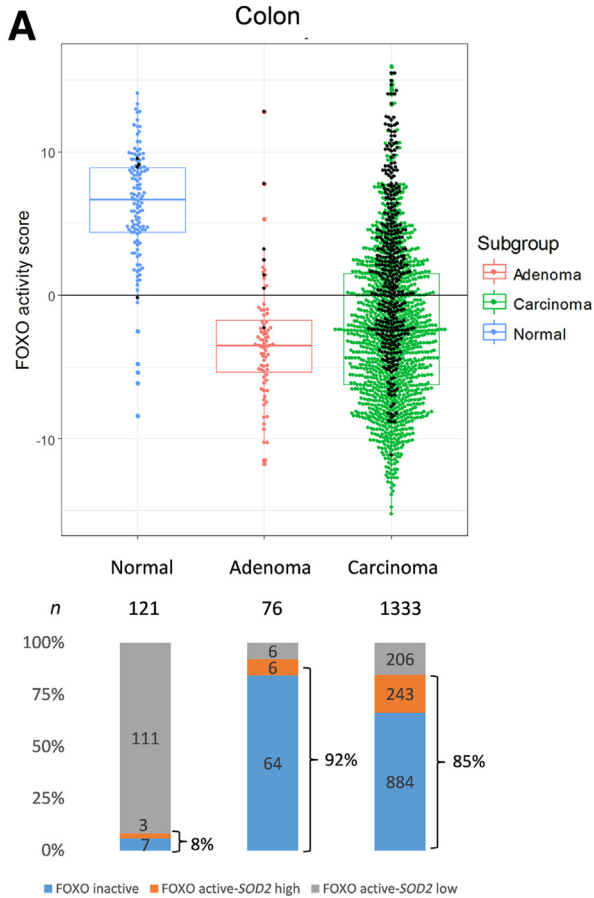


Figure 4 Predicted forkhead box-O (FOXO) activity corresponds to cellular localization in tissue samples from patients with colon cancer. **A:** Biological validation of the computational FOXO model on a public data set (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE8671), with corresponding samples in individuals with normal colon and colon adenoma. FOXO activity scores plotted in groups using boxplots. **B:** Biological validation of the computational FOXO model on a public data set (GEO accession number GSE20916), with samples of healthy colon, colon adenoma, and colon carcinoma tissue. **C:** Immunohistochemical staining of FOXO3 and hematoxylin in normal colon, colon adenoma, and two carcinoma samples (human). **Boxed areas** are shown at higher magnification in the **bottom row**. **** $P < 0.0001$ (one-sided Wilcoxon signed-rank statistical tests were performed). Original magnification: $\times 50$ (C, top row, left three images); $\times 100$ (C, top row, rightmost image).

premises: FOXO is expressed in cancer cells in the sample and measured FOXO activity derives from cancer cells (Figure 6 and Supplemental Figure S4). Assuming the PI3K pathway is active in oxidative stress mode, for each investigated cancer type, these samples can be added (Figure 5) to the number of samples with inactive FOXO (indicating active PI3K pathway) to calculate the total number and percentage of patients likely to have a tumor with an active PI3K pathway. The calculated percentage of PI3K pathway active samples was between 8% and 13% in

normal tissue (colon, breast, prostate) and quite similar in luminal A and normal-like breast cancer and low Gleason score prostate cancer (13%, 15%, and 15%, respectively); in luminal B, HER2, and basal breast cancer, the percentage samples with PI3K pathway activity was much higher (45%, 45%, and 76%, respectively). Interestingly, the percentage of PI3K pathway active samples was already maximal in colon adenoma and did not further increase in carcinoma (92% and 85% of samples, respectively).



Discussion

A Knowledge-Based Bayesian Model for Measuring FOXO and PI3K Pathway Activity in a Tissue Sample

The PI3K pathway is an important oncogenic pathway, and many targeted drugs aim at blocking it at multiple locations. Improving response rates to PI3K pathway inhibitors requires tests that reliably assess functional PI3K pathway activity in cancer samples. FOXO transcription factors are negatively regulated by the PI3K pathway and can in principle be used as an inverse readout for PI3K pathway activity.^{5,12,66} To measure PI3K pathway activity in cancer tissue samples, a computational knowledge-based Bayesian network was developed to infer FOXO transcriptional activity from established FOXO target gene mRNA expression levels to be measured in the tissue sample at hand.¹¹ The different FOXO members are redundant, and a comparative analysis of FOXO1-, FOXO3-, and FOXO4-induced gene regulation indicates a large overlap among the transcriptional profiles of each FOXO member.^{16,22,67} Therefore, our FOXO activity model, which incorporates knowledge on regulation of direct FOXO target genes, performs as a general FOXO activity predictor.

Calibrating and subsequently freezing this Bayesian model on FOXO-inducible HUVECs resulted in a computational FOXO model that predicted FOXO activity as expected in breast cancer cell lines with constitutively active FOXO3, on incubation with PI3K pathway targeting drugs, or on manipulation of HER2 receptor activity. The observation in FOXO-inducible MCF7 cells that FOXO is scored less active in cells treated with the PI3K inhibitor drug LY294002 compared with ectopic FOXO activation is readily explained by higher FOXO3 protein levels induced by ectopic expression and/or as a result of positive growth factor signaling feedback in the case of treatment with LY294002. Pharmacologic inhibition of the PI3K pathway may initiate a growth factor feedback response that can reestablish growth factor signaling, which constitutes a major component of drug resistance development in cancer cells.^{6,7,68}

For extended model validation in other cell types, three data sets from T lymphocytes (including natural killer and CD4⁺ T cells) activated with various stimuli were analyzed. The measured decrease in FOXO score associated with

Figure 5 *SOD2* expression distinguishes phosphatidylinositol 3-kinase (PI3K) and oxidative stress-mediated forkhead box-0 (FOXO) activity in tissue samples from patients with cancer. FOXO-active samples having a *SOD2* expression level that is higher than the threshold of *SOD2* expression (means \pm 2 SDs in corresponding normal tissue) shown as black dots indicate samples with oxidative stress-induced FOXO activity. Other colors indicate samples in which *SOD2* expression is in the normal range, indicating FOXO might not be active in the oxidative stress mode. **A:** Colon cancer. **B:** Breast cancer. Data are expressed as the FOXO activity scores plotted in groups using boxplots as well as dots for every unique sample. Lum, luminal.

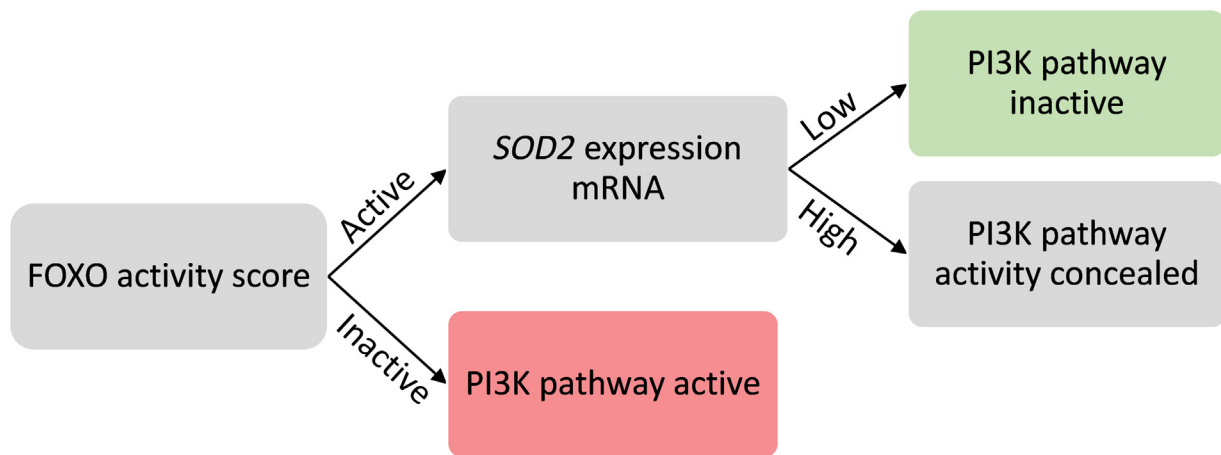
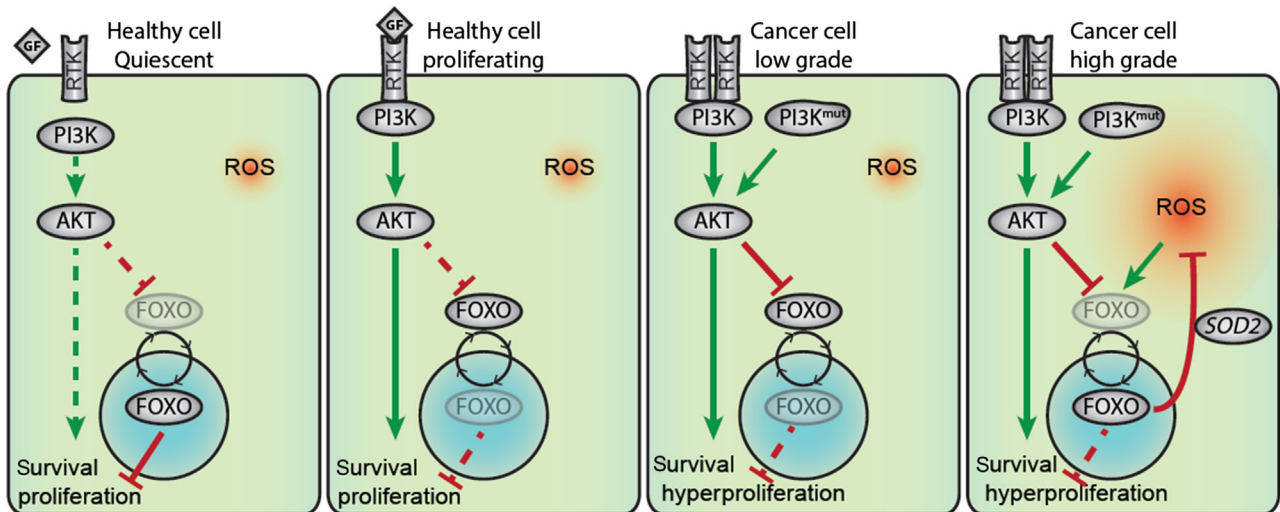


Figure 6 The phosphatidylinositol 3-kinase (PI3K)—forkhead box-0 (-FOXO) pathway and distinction between tumor suppressive and oxidative stress mode of FOXO activity. PI3K-FOXO pathway and relation with *SOD2* target gene expression. In healthy normal tissue, FOXO induces transcription of target genes that control cell division. When the PI3K pathway is activated, by stimuli from the microenvironment (proliferating healthy cell) or by genomic mutations (cancer cell), FOXO activity is blocked and FOXO translocates to the cytoplasm, resulting in loss of control over cell division. Oxidative stress associated with rapid proliferation induces alternative activation of FOXO with the function to protect against reactive oxygen species (ROS); the resultant FOXO target gene expression profile is slightly changed to include *SOD2*. Decision tree to decide on PI3K activity in a tissue sample. This simplified decision tree is based on the assumptions that FOXO is expressed in the cancer cells and there is limited contamination with healthy tissue. A more detailed decision tree can be found in [Supplemental Figure S4](#).

lymphocyte activation reflects well-known induction of PI3K pathway activity necessary for appropriate clonal T-cell expansion.⁶⁹ This provides evidence that the model can also be applied to nonmalignant cells, which was expected because the FOXO target genes were not selected for their role in cancer, but for measurement of FOXO activity irrespective of cell type.

After validation, potential use of the model for drug development purposes was illustrated using data from a number of cancer cell lines. In three breast cancer cell lines and in lung cancer cells with mutated hyperactive EGFR, the model measured an increase in FOXO activity on treatment with the EGFR inhibitor erlotinib, reflecting effective inhibition of PI3K pathway activity. In addition,

treatment of the lung cancer cell line with a dual mechanistic target of rapamycin—PI3K inhibitor (BEZ235, dactolisib) and a MEK1/2 inhibitor (AZD6244, selumetinib) similarly resulted in increased FOXO activity but less than caused by erlotinib. This result illustrates the quantitative nature of the pathway activity score and was as expected because signal transduction initiated by EGFR leads to inactivation of FOXO via the PI3K-AKT and RAS-MEK-ERK-MDM2 pathways.⁷⁰ Finally, the restoration of FOXO activity (and inhibition of PI3K pathway activity) that was observed in a lapatinib-resistant breast cancer cell line when adding the multikinase inhibitor foretinib to lapatinib was in full agreement with the effects on cell growth as described by Liu et al.⁷¹

Thus, the Bayesian model correctly predicted FOXO and PI3K activity in multiple cell cultures, providing biological validation of the model. However, the difference in response of the lung cancer cell line to the three drugs that target different elements of the signaling pathways downstream of EGFR illustrates that to make an optimal choice with respect to a targeted drug, additional analysis (eg, genomic mutation analysis) may be required to establish the underlying cause of PI3K pathway activity.

The reasons the Bayesian approach works well to analyze different cell types has been discussed before.^{10,11} Many earlier approaches to pathway modeling have derived or composed signaling pathway mRNA signatures from genes of which the encoded proteins are known to play a role in a certain signaling pathway.⁷² The major disadvantage of such approaches is that measuring mRNA levels as a surrogate for measuring active signaling proteins is unreliable for predicting pathway activity; however, the approach can be suited for biomarker or drug target discovery purposes. A growth factor pathway modeling approach that merits discussion is the Boolean logic-based model of Kirouac et al,⁷³ which models activity of the growth factor signaling network upstream of the FOXO transcription factor to predict targeted drug resistance, using protein measurements as input. This model may have difficulty in predicting functional activity of FOXO because of missing processes upstream in the growth factor pathway regulating FOXO activity, whereas in the Bayesian model this issue is bypassed by looking only at the result of FOXO transcriptional activity. In addition, application of the model is likely to be tissue type specific because its parameters have been estimated from experimental data, which is not the case in the Bayesian model that can be used across cancer types. On the other hand, as mentioned before, such models are likely to provide complementary biomarker information, especially in experimental settings.

Distinguishing Different Mechanisms That Regulate FOXO Activity

In cancer tissue, interpretation of FOXO activity is more complex. Unexpectedly, in colon carcinoma as well as luminal B, HER2, and basal breast cancer subtypes, where PI3K pathway activity is common, a large portion of the samples were predicted to have an active FOXO transcription factor.^{2,74} This finding suggested that simple inversion of FOXO activity status to infer PI3K pathway activity was not valid in all cancer tissue samples and moreover that FOXOs are not passive bystanders in high-grade cancers. A differentiator was necessary to distinguish between PI3K regulated and putative non-PI3K-regulated FOXO activity. FOXOs function as regulators of cellular homeostasis and respond to various adverse cellular conditions, including DNA damage, high levels of reactive oxygen species, and low nutrient availability.^{12,75,76} Therefore, an active PI3K pathway may be present in combination with active FOXOs.

We hypothesized that the different functional roles of FOXO should be reflected in changes in FOXO target mRNA expression profiles. Comparing FOXO target gene expression levels between multiple FOXO-active cancer samples and corresponding (FOXO-active) healthy tissue samples revealed *SOD2* and *BNIP3* as distinguishing FOXO target genes in all investigated cancer types, typified by increased expression in the FOXO-active cancer samples. Both *SOD2* and *BNIP3* are induced in the presence of cellular oxidative stress.^{29,55,77} However, during cellular oxidative stress, NRF2 and NF- κ B transcription factors may also become activated to transcribe an overlapping subset of oxidative stress target genes, including *SOD2* and *BNIP3*. Although not excluding a contribution of the NF- κ B transcription factor to *SOD2* target gene levels, these results firmly support FOXO-induced *SOD2* transcription during oxidative stress, in contrast to the *BNIP3* gene, which is likely to be preferentially transcribed by other transcription factor(s) under these circumstances.

Because cellular oxidative stress is a common phenomenon in tumors and an alternative inducer of FOXO activation, these findings point toward FOXO activation in tumors suffering from oxidative stress regardless of the PI3K status (Figure 6).^{12,78–80} Adding information on *SOD2* expression level to the FOXO activity score classified FOXO-active luminal A and normal-like breast cancer and lower Gleason score prostate cancer samples in general as having PI3K-regulated FOXO activity, with the PI3K pathway consequently inferred as inactive and FOXO active to control cell division. Indeed, these cancer types are typically more differentiated and slow growing and driven by the ER and androgen receptor pathways, respectively, in the absence of an active PI3K pathway. In contrast, in aggressive breast cancer subtypes, luminal B, HER2, and basal, tissue samples were scored inactive for FOXO (indicating an active PI3K pathway) or FOXO activity was increasingly classified as oxidative stress induced. As shown, these results are in full agreement with the results from a study in which oxidative stress was measured in luminal, HER2, and triple-negative breast cancer samples.⁶³ Summarizing, in good prognosis breast and prostate cancer samples, FOXO transcriptional activity was generally active in the PI3K-regulated growth control mode, whereas in more aggressive cancers, FOXO was inactivated by PI3K or activated in oxidative stress mode. Interestingly, this also reveals that oxidative stress—associated FOXO activity may actually function to support tumor growth by protecting the cell from oxidative damage and simultaneously stimulate the growth factor PI3K-AKT pathway.⁸¹ Indeed, in the study from Gruosso et al,⁶³ the level of oxidative stress in breast cancer samples correlated positively with mitotic index. This study thus nicely complements our results by adding evidence of a higher proliferation rate in tumors with oxidative stress, which is likely to be attributable to increased PI3K pathway activity. In this respect, our observations are fully in line with the recent discovery that

FOXO can suppress and support cancer growth and metastasis and also explain why various clinical studies aimed at correlating FOXO activity to clinical outcome have resulted in contradicting conclusions.^{64,65,81} The mitotic index is predictive of response to chemotherapy, suggesting that the FOXO-*SOD2* model may have value for predicting response to chemotherapy in addition to predicting response to PI3K pathway inhibition.⁸² This observation puts targeting FOXO forward as a potential treatment strategy for selected high-grade cancers. Also of interest, a pathogenetic role of FOXO transcription factors is not limited to cancer, and FOXO1 and 3A inhibitors are being developed by several pharmaceutical companies with an initial focus on treatment of diabetes.^{83–85} This development may conceivably pave the way for the use of FOXO inhibition in cancer.

Use of the Computational FOXO Model to Identify PI3K Pathway Activity in an Individual Cancer Tissue Sample

The intended future use of our computational model is to assess functional PI3K pathway activity in individual cancer tissue samples, to facilitate choice of a targeted drug or other therapy, or to assess therapy response. Using our optimized FOXO computational model to assess FOXO activity, the probability of PI3K pathway activity can be derived using a (simplified) decision tree or the more complex decision tree if significant contamination with normal cells is expected (Figure 6 and Supplemental Figure S4). In the analyzed clinical studies, counting all samples that were scored as FOXO inactive or activated by oxidative stress provided an indication of the total number of patients who might have benefited from PI3K pathway inhibitor treatment. In the low-grade luminal A/normal-like and lower Gleason score prostate cancer groups, the calculated percentage of PI3K-active tumors was close to the percentage of PI3K-active healthy tissue samples (approximately 10% to 15%), whereas in higher-grade breast cancer, this increased to three-quarters in the basal breast cancer group. These results are in perfect agreement with the PI3K pathway being the most frequently activated pathway in cancer, especially in the more aggressive tumors. It cannot be excluded that these results still underestimate the real number of PI3K-active samples because of incidental contamination of the cancer sample with healthy tissue, causing a false-positive (PI3K-regulated) FOXO activity score. Complementary FOXO3 staining may be considered when a cancer sample that contains a significant amount of normal tissue is scored FOXO active to confirm that nuclear FOXO is indeed present in the cancer cells. The developed IHC staining performed well in identifying nuclear, potentially active, FOXO in cells in a tissue slide. The observed differences in total FOXO protein expression between cells are probably attributable to active degradation of cytoplasmic localized FOXOs.^{64,65} This phenomenon does not interfere with the intended use of the staining, which is to identify the

presence of nuclear staining to help interpret an active FOXO activity score obtained on a tissue sample.

Taking this into account, we expect our FOXO model to provide a robust method for quantitative determination of functional PI3K pathway activity in tissue samples and to improve decision making on the use of drugs that target the receptor tyrosine kinase—PI3K—AKT pathway or chemotherapy, aiming at increased treatment efficacy rates. Complementary genomic analysis to identify a mutational cause for abnormal PI3K pathway activity will be helpful to decide which PI3K pathway targeted drug is likely to be most effective, depending on its point of action in the pathway. The model is expected to be applicable to many different tumor types. To enable use on formalin-fixed, tissue-embedded tissue slides, the model has been adapted for real-time quantitative PCR mRNA measurements as input. Monitoring of therapy response and resistance, for example in a neoadjuvant or window-of-opportunity setting, is another envisioned as well as quantitative assessment of drug response during drug development.

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H.v.O. analyzed data, studied biology of FOXO pathway, developed the model and graphics, and wrote the manuscript; M.H. did cell modeling, performed cell culture fluorescent staining, provided expertise in FOXO experiments, and contributed to discussions and writing; C.D.-d.V. developed and performed IHC FOXO staining; R.V. researched data for discovery of oxidative stress—associated FOXO target genes; M.D. performed *SOD2* analysis and generated graphics; W.V. performed computational Bayesian modeling and wrote the manuscript; B.B. provided expertise in FOXO biology experiments and contributed toward discussions and writing; A.v.d.S. conceived clinical scientific concept, researched data, provided insights on biology of pathway model development and clinical relevance/context, and wrote the manuscript.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.05.020>.

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