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The high protein expression of FOXO3, but not that of FOXO1, is associated with markers of good prognosis

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To better define the role of FOXO1 and FOXO3 transcriptional factors in breast carcinogenesis, we performed a comparative study of their expression at both the RNA and protein levels in a series of human breast tumors. We used qRT-PCR assay to quantify mRNA expression and Reverse Phase Protein Arrays (RPPA) to quantify protein expression in 218 breast tumors from patients with known clinical/pathological status and outcome. Weak correlations were observed between mRNA and protein expressions for both FOXO1 and FOXO3 genes. High expression of FOXO3 protein, but not FOXO1 protein, was a good prognostic marker, negatively correlated with KI67 and markers of activity of the PI3K/AKT/mTOR oncogenic pathway, and positively correlated with p53, a marker of apoptosis. Moreover, FOXO3 protein expression, but not FOXO1 protein expression, was also negatively correlated with various proteins involved in different DNA repair mechanisms. FOXO3 protein, but not FOXO1 protein, appears to be a tumor suppressor that inhibits breast cancer by altering DNA damage response (DDR), thereby inducing p53-dependent apoptosis. This antitumor effect appears to be suppressed by excessive activity of the PI3K/AKT/mTOR pathway. High FOXO3 protein expression could be a biomarker of deficient DDR in breast tumors.

Breast cancer is the most common solid malignancy in women in both developed and developing countries1. Based on gene expression profiling, this pathology have been classified into four subtypes: luminal, human epidermal growth factor receptor 2 (HER2/ERBB2)-enriched, basal-like and normal-like2. Most breast tumors of the basal-like subtype are triple-negative (TN), which means that they do not express estrogen and progesterone receptors, and lack ERBB2 overexpression3.

The forkhead box O (FOXO) family is a subclass of the forkhead family of transcription factors and consists of four members: FOXO1, FOXO3, FOXO4 and FOXO64. These four proteins possess the conserved DNA-binding domain named forkhead domain or winged-helix domain5,6. They are involved in the regulation of various cellular processes such as cell cycle, apoptosis, metabolism, and DNA repair4,7,8. Their transcriptional activity is modulated notably by acetylation, ubiquitination, and phosphorylation1,9. Once activated, the PI3K/AKT/mTOR

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cellular pathway induces the phosphorylation of FOXO proteins by AKT. This phosphorylation leads to the exclusion of FOXO proteins from the nucleus, inhibiting therefore their capacity to modulate the transcription of their target genes. This phosphorylation has also been shown to promote the degradation of FOXO3 protein via the proteasome. In human tumors, the PI3K/AKT/mTOR cellular pathway is frequently found over activated leading therefore to inhibition of the transcriptional activity of the FOXO proteins.

Various studies strongly suggest that FOXO proteins are tumor suppressors. The conditional deletion of all FOXO1, FOXO3 alleles in adult mouse tissues induces the development of lymphoblastic thymic lymphomas and hemangiomast. Overexpression of FOXO1 and FOXO3 proteins in breast cancer has been shown to inhibit the growth of breast cancer cells. Is-B kinase and ERK promote breast carcinogenesis via inhibition of FOXO3 protein expression. The cytoplasmic expression of the FOXO3 protein is positively correlated with poor survival in breast cancer. Low expression of FOXO1 or FOXO3 protein in breast tumors is correlated with poor clinical outcome. Altogether, these results strongly suggest that FOXO1 and FOXO3 proteins act as tumor suppressors in breast cancer. However, other studies have described unexpected functions for these two FOXO proteins in resistance to breast cancer treatment and breast cancer promotion. Notably, FOXO1 and FOXO3 proteins have been implicated in the promotion of breast tumor cell invasion. FOXO3 protein expression has also been associated with poor survival in breast cancer.

In order to better define the role of FOXO1 and FOXO3 proteins in breast cancer, we performed a comparative study of their RNA and protein expressions in 218 breast tumors by using real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and Reverse Phase Protein Arrays (RPPA) methods, respectively. We also determined the correlations between FOXO1 and FOXO3 protein expressions and classical clinical biological parameters, as well as the expression of proteins involved in the PI3K/AKT/mTOR pathway, DNA damage response (DDR), apoptosis, cell cycle, and/or cell proliferation.

Results

FOXO1 and FOXO3 RNA and protein expressions in breast cancer. To better define the role of FOXO genes in breast cancer, we analysed their RNA and protein expression in a series of 218 breast tumors (clinical parameters presented in Table S1).

In keeping with our previous work, we found that the expressions of FOXO1, FOXO4, and FOXO6 at the RNA level varied widely in breast tumors compared to normal breast samples (Table S2). In general, FOXO1 and FOXO4 mRNA were significantly underexpressed (expression values < 0.33), whereas FOXO6 mRNA was overexpressed (expression values ≥ 3), compared to normal breast tissue (see Material and Methods page 11). FOXO4 expression in the HR + ERBB2 + subtype and FOXO6 expression in the HR-ERBB2- subtype were similar in tumors and normal breast tissue. FOXO3 gene expression was similar in tumors and normal breast tissue except in the HR + ERBB2 + subtype, in which it was slightly, but significantly overexpressed.

The RPPA method demonstrated a wide range of FOXO1 protein expression (ranging from 0.07 to 1.91) and FOXO3 protein expression (ranging from 0.19 to 3.98) in breast tumors (Table 1). FOXO4 and FOXO6 protein expression was not studied due to the lack of an appropriate specific antibody for these two proteins.

We examined the correlation between protein and mRNA expressions for FOXO1 and FOXO3 genes. We observed weak positive correlation for the FOXO1 gene (r = +0.342, p < 10⁻⁶) and a weak positive correlation for the FOXO3 gene (r = +0.150, p = 0.027), strongly suggesting that the expression of these two genes in breast tumors is regulated by molecular mechanisms independent of transcription and RNA stability (Fig. 1). These results also highlight the importance of considering protein expression when studying the role of FOXO1 and FOXO3 genes in breast cancer.

Relationship between FOXO1 and FOXO3 protein expressions and clinical biological parameters of breast cancer. We investigated the relationships between FOXO1 and FOXO3 protein expressions and several classical clinical biological parameters (Tables 2 and 3). Marked differences were observed between these two FOXO proteins, as high FOXO1 protein expression was associated with negative estrogen receptor (ER) and progesterone receptor (PR) status, while low FOXO3 protein expression was weakly associated with these two biological parameters. We also showed that high FOXO3 protein expression was associated with low SBR histological grade, and, surprisingly, with high level of lymph node status. However, no association was observed between FOXO1 protein expression and these two clinical parameters.

To investigate in more detail the role of FOXO1 and FOXO3 genes in breast cancer, we also performed a log rank test to analyse the relationship between FOXO1 and FOXO3 protein expressions and metastasis-free survival (MFS). Patients with breast tumors expressing high levels of FOXO3 protein had better MFS than patients with breast tumors expressing lower levels of FOXO3 protein (p = 4.1.10⁻⁵), which is consistent with a tumor suppressor role (Fig. 2A). Such an association was not observed for FOXO1 protein. Multivariate analysis using a Cox proportional hazards model assessed the predictive value for MFS of the parameters found to be significant in univariate analysis (Table S1), i.e. lymph node status and macroscopic tumor size, and FOXO3 protein expression (p = 5.5.10⁻³) (Table S3). The prognostic significance of these three parameters persisted in multivariate analysis, indicating that FOXO3 protein expression is an independent prognostic factor in breast cancer. Interestingly, no correlation was demonstrated between FOXO1 or FOXO3 mRNA expression and MFS in this series of 218 breast tumors (Fig. 2B,C), highlighting once again the importance of studying FOXO1 and FOXO3 protein expressions in order to assess their role in breast cancer.

Altogether, these observations suggest that FOXO3 protein, but not FOXO1 protein, may act as a tumor suppressor in breast cancer.

Relationship between the levels of FOXO1, FOXO3, and other proteins involved in the PI3K/AKT/mTOR pathway, DDR, apoptosis, cell cycle, and cell proliferation. In order to better define
473, is essential for its kinase activity. However, because of the lack of appropriate pAKT-T308 antibody for pathway-dependent phosphorylation of AKT to its threonine 308 (pAKT-T308) and, to a lesser extent, its serine as a key multi-protein complex crucial for DNA repair by homologous recombination and NHEJ. Only weakly breast tumors would induce degradation of FOXO3 protein, but not FOXO1 protein.

Of various components of this pathway: AKT (r = −0.317, p < 10−4), PDK1 (r = −0.181, p = 7.3.10−4), mTOR (r = −0.178, p = 8.6.10−3), S6 (r = −0.446, p < 10−4), and IRS1 (r = −0.172, p = 1.1.10−2). We did not detect significant correlation between FOXO3 protein expression and the protein expression of one of the most important negative regulators of the PI3K/AKT/mTOR pathway, PTEN. The weak PI3K/AKT/mTOR pathway activity found in the breast tumors expressing low level of FOXO3 protein would be therefore due to a low level of various components of this pathway but not to a high level of PTEN. Regarding FOXO1, we detected a negative correlation only with PDK1 (r = −0.186, p = 6.10−3). Akt activation has been shown to promote degradation of FOXP3 protein by proteasomes. Therefore, our results suggest that high activity of the PI3K/AKT/mTOR pathway in breast tumors would induce degradation of FOXO3 protein, but not FOXO1 protein.

Interestingly, we also demonstrated negative correlations between FOXO3 protein expression and the protein expression of various factors involved in different DNA repair mechanisms: Ku80 (r = −0.327, p < 10−4) and DNA-PK (r = −0.144, p = 3.4.10−2) involved in non-homologous end joining (NHEJ), PARP (r = −0.401, p < 10−4) crucial for alternative NHEJ and base excision repair, and the three components of the MRN complex: NBS1 (r = −0.303, p < 10−4), RAD50 (r = −0.181, p = 7.2.10−3), and Mre11 (r = −0.203, p = 2.6.10−3), described as a key multi-protein complex crucial for DNA repair by homologous recombination and NHEJ. Only weakly significant negative correlations were demonstrated between FOXO3 protein expression and Ku80, NBS1, and Mre11 (0.05 > p > 0.01) (Table 4). Strong FOXP3 protein expression in breast tumor cells therefore appears to inhibit DDR, which would lead to accumulation of genetic alterations, thereby causing cell cycle arrest and/or p53-dependent apoptosis. Consistent with this hypothesis, we found that the FOXP3 protein level was negatively correlated with the Ki67 protein level, a marker of proliferation (r = −0.460, p < 10−4), and was positively correlated with the cell cycle inhibitor p15 protein level (r = +0.392, p < 10−4), as well as the levels of p53 (r = +0.299, |
| Median (range) | Low level (%)a | High level (%)a |
|----------------|----------------|----------------|
| Median (range) | 218            | 1.0 (0.07–1.91) |
| Low level (%)a | 23 (10.6)      | 16 (7.3)        |
| High level (%)a| 0 (0)          | 13 (6.0)        |
| HR – ERBB2−   | 1.03 (0.11–1.91) | 0.84 (0.39–2.52) |
| Low level (%)a | 3 (6.8)        | 4 (9.1)         |
| High level (%)a| 0 (0)          | 1 (2.3)         |
| HR – ERBB2+   | 1.11 (0.46–1.81) | 0.98 (0.41–2.77) |
| Low level (%)a | 1 (2.4)        | 2 (4.8)         |
| High level (%)a| 0 (0)          | 3 (7.1)         |
| HR – ERBB2−   | 0.95 (0.12–1.76) | 1.08 (0.19–3.98) |
| Low level (%)a | 16 (14.3)      | 9 (8.0)         |
| High level (%)a| 0 (0)          | 8 (7.1)         |
| HR – ERBB2+   | 1.03 (0.07–1.66) | 1.00 (0.45–2.67) |
| Low level (%)a | 3 (15.0)       | 5 (5.0)         |
| High level (%)a| 0 (0)          | 1 (5.0)         |

Table 1. Protein levels of FOXO1 and FOXO3 in the series of 218 breast tumours. Protein levels were normalized so that the median of values in the 218 breast tumours was 1. *Low and high protein levels were defined as twofold variations of level, relative to the median level of the series of 218 breast tumours.
Phospho-p53 (Ser-15) \( (r = +0.320, p < 10^{-4}) \), and Phospho-p53 (Ser-392) \( (r = +0.387, p < 10^{-4}) \) (phosphorylation of p53 at these two sites triggers its apoptotic activity\(^{32}\)), whereas FOXO1 protein level was very slightly positively correlated with Ki67 \( (r = +0.134, p = 4.9 \times 10^{-2}) \) and not correlated with cell cycle and apoptosis protein expressions.

In order to check that FOXO3 is functional in breast tumors, we performed a western blot analysis to visualize Phospho-FOXO3 (pSer-253), the inactive form of FOXO3, of several breast tumors of our series\(^{10}\). We detected at least 8 samples with negative or low levels of phospho-FOXO3 (pSer-253) expression among 12 breast tumor samples expressing high levels of FOXO3, suggesting that this FOXO3 protein may be functional in a majority of these tumors (Fig. S1).

Overall, our results suggest that FOXO3 protein, but not FOXO1 protein, acts as a tumor suppressor in breast cancer, at least in part by DDR inhibition and subsequent induction of p53-dependent apoptosis. They also suggest that the antitumor effect of FOXO3 is abolished by high activity of the PI3K/AKT/mTOR pathway.

**Figure 1.** Scatter plots and Spearman correlation coefficients \( (r) \) between FOXO1 (A) and FOXO3 (B) protein and mRNA levels in a series of 218 breast tumors.
Discussion

Many studies designed to examine the role of genes in carcinogenesis determine the correlations between their RNA expression and classical clinical biological parameters, survival, and the expression of others genes linked to cancer. However, due to post-transcriptional regulations, weak correlations are commonly observed between RNA expression and protein expression. In our study, we found weak correlations between FOXO1 and FOXO3 RNA and protein expressions in breast cancer. The absence of correlation between protein and mRNA expressions for FOXO1 and FOXO3 genes can be fully explain by the fact that these FOXO proteins undergo posttranslational modifications, such as acetylation, ubiquitination, and phosphorylation, modulating their subcellular localization and stability. To investigate the role of FOXO1 and FOXO3 genes in breast carcinogenesis, we therefore used the RPPA method to perform a comparative study of the protein expression of these two FOXO genes in a series of 218 breast tumors.

Our results strongly suggest that FOXO3 protein, but not FOXO1 protein, acts as a tumor suppressor in breast cancer. In particular, we found that patients with breast tumors expressing high levels of FOXO3 protein had better survival rates than patients with breast tumors expressing lower levels of this protein. We also showed that FOXO3 protein expression, but not FOXO1 protein expression, was negatively correlated with the expression of the KI67 marker of proliferation. The role of FOXO3 protein in breast carcinogenesis may therefore depend on the subtype of breast cancer and the stage of disease. Further protein expression studies based on larger series of breast cancers are necessary to determine the precise role of FOXO3 protein in the various subtypes of breast cancer.

| Total population (%) | FOXO1 protein levels | p-value* |
|----------------------|----------------------|----------|
| Total 218 (100)      | 1.0 (0.07–1.91)      |          |
| Age                  |                      |          |
| ≤50 54 (24.8)        | 1.02 (0.32–1.81)     | 0.71 (NS) |
| >50 164 (75.2)       | 0.99 (0.07–1.91)     |          |
| SBR histological gradea,b | |          |
| I 22 (10.4)          | 0.93 (0.43–1.66)     | 0.21 (NS) |
| II 86 (40.8)         | 0.99 (0.07–1.65)     |          |
| III 103 (48.8)       | 1.03 (0.11–1.91)     |          |
| Lymph node statusc   |                      |          |
| 0 69 (31.9)          | 1.00 (0.11–1.69)     | 0.77 (NS) |
| 1–3 84 (38.9)        | 0.98 (0.07–1.81)     |          |
| >3 63 (29.2)         | 1.01 (0.15–1.91)     |          |
| Macroscopic tumor sized | |          |
| ≤25 mm 82 (38.5)     | 0.99 (0.11–1.80)     | 0.97 (NS) |
| >25 mm 131 (61.5)    | 1.00 (0.07–1.91)     |          |
| ERα status           |                      |          |
| Negative 89 (40.8)   | 1.06 (0.11–1.91)     | **0.0047** |
| Positive 129 (59.2)  | 0.95 (0.07–1.76)     |          |
| PR status            |                      |          |
| Negative 117 (53.7)  | 1.03 (0.07–1.91)     | **0.0037** |
| Positive 101 (46.3)  | 0.95 (0.15–1.76)     |          |
| ERBB2 status         |                      |          |
| Negative 156 (71.6)  | 0.98 (0.11–1.91)     | **0.071** |
| Positive 62 (28.4)   | 1.09 (0.07–1.81)     |          |
| Molecular subtypes   |                      |          |
| HR – ERBB2− 44 (20.2) | 1.03 (0.11–1.91) | **0.022** |
| HR – ERBB2+ 42 (19.3) | 1.11 (0.46–1.81) |          |
| HR = ERBB2− 112 (51.4) | 0.95 (0.12–1.76) |          |
| HR = ERBB2+ 20 (9.2) | 1.03 (0.07–1.66) |          |

Table 2. Relationship between FOXO1 protein levels and classical clinical biological parameters in the series of 218 breast tumours. NS: not significant. aMann-Whitney (2 groups) or Kruskal Wallis (more than 2 groups) test. bScarff Bloom Richardson classification. cInformation available for 211 patients. dInformation available for 216 patients. eInformation available for 213 patients.
Several studies suggest that FOXO3 protein acts as a tumor suppressor in breast cancer by inducing the expression of cyclin-dependent kinase inhibitors (CDK inhibitors) and proapoptotic proteins. In line with these findings, we showed that FOXO3 protein expression was positively correlated with expression of the p15 CDK inhibitor, p53 and two active forms of p53 phosphorylated at position S15 and S392 (Table 4). Surprisingly, we also demonstrated negative correlations between FOXO3 protein expression and the expression of numerous proteins involved in various DNA repair mechanisms, suggesting that high FOXO3 protein expression in breast tumors impairs DDR. Inhibition of DDR by FOXO3 protein could induce accumulation of DNA damage, thereby inducing p53-dependent apoptosis. FOXO3 was recently shown to negatively regulate the expression and activity of FOXM1, a forkhead protein activating the transcription of numerous genes involved in various DNA repair mechanisms and genotoxic agent resistance. FOXO3 protein competes with FOXM1 for the binding to the same DNA motifs in target promoters and produces opposing transcriptional outputs. Therefore, one of the mechanisms by which FOXO3 protein could inhibit DDR in breast cancer, would be the inhibition of the transcription of DDR-genes induced by FOXM1.

PARP inhibitors have been shown to be highly lethal to tumor cells with a defect in DNA repair by homologous recombination called “BRCAness”. The activity of these inhibitors is based on the principle of synthetic lethality, which consists of targeting two separate molecular pathways that are nonlethal when disrupted individually, but are lethal when inhibited simultaneously. We found negative correlations between the expression of FOXO3 protein and that of the three components of the MRN complex (NBS1, RAD50, and Mre11) crucial for DNA repair by homologous recombination (Table 4). High expression of FOXO3 protein could therefore be an attractive predictive biomarker of favourable response to treatment with PARP inhibitors in breast tumors.

### Materials and Methods

#### Patients and samples

The conditions of patient’s selection and sample collection were as previously described [19].

The treatment of the 218 patients (mean age: 61.3 years, range: 29–87 years) consisted of modified radical mastectomy in 140 cases (64.2%) and breast-conserving surgery plus locoregional radiotherapy in 77 cases (35.3%) (information available for 217 patients). 171 patients received adjuvant therapy: chemotherapy alone in

| Total population (%) | FOXO3 protein levels | p-value |
|----------------------|----------------------|---------|
| Total                | 218 (100)            | 1.0 (0.19–3.98) | |
| Age                  |                      |         | |
| ≤50                  | 54 (24.8)            | 1.06 (0.39–2.27) | 0.77 (NS) |
| >50                  | 164 (75.2)           | 0.99 (0.19–3.98) | |
| SBR histological grade |                     |         | |
| I                    | 22 (10.4)            | 1.33 (0.43–3.56) | **0.010** |
| II                   | 86 (40.8)            | 1.02 (0.19–3.98) | |
| III                  | 103 (48.8)           | 0.91 (0.28–3.48) | |
| Lymph node status    |                      |         | |
| 0                    | 69 (31.9)            | 0.87 (0.19–2.77) | **0.0010** |
| 1–3                  | 84 (38.9)            | 1.01 (0.28–3.98) | |
| >3                   | 63 (29.2)            | 1.08 (0.31–2.67) | |
| Macroscopic tumor size|                     |         | |
| ≤25 mm               | 82 (38.5)            | 1.07 (0.28–3.48) | 0.22 (NS) |
| >25 mm               | 131 (61.5)           | 0.98 (0.19–3.98) | |
| ERα status           |                      |         | |
| Negative             | 89 (40.8)            | 0.91 (0.39–2.77) | **0.029** |
| Positive             | 129 (59.2)           | 1.08 (0.19–3.98) | |
| PR status            |                      |         | |
| Negative             | 117 (53.7)           | 0.94 (0.31–2.77) | **0.013** |
| Positive             | 101 (46.3)           | 1.08 (0.19–3.98) | |
| ERBB2 status         |                      |         | |
| Negative             | 156 (71.6)           | 1.02 (0.19–3.98) | 0.89 (NS) |
| Positive             | 62 (28.4)            | 0.99 (0.41–2.77) | |
| Molecular subtypes   |                      |         | |
| HR – ERBB2−          | 44 (20.2)            | 0.84 (0.39–2.52) | **0.044** |
| HR – ERBB2+          | 42 (19.3)            | 0.98 (0.41–2.77) | |
| HR− ERBB2−           | 112 (51.4)           | 1.08 (0.19–3.98) | |
| HR+ ERBB2+           | 20 (9.2)             | 1.00 (0.45–2.67) | |

Table 3. Relationship between FOXO3 protein levels and classical clinical biological parameters in the series of 218 breast tumours. NS: not significant. *Mann-Whitney (2 groups) or Kruskal Wallis (more than 2 groups) test. bScarff Bloom Richardson classification. cInformation available for 211 patients. dInformation available for 216 patients. eInformation available for 213 patients.
63 cases, hormone therapy alone in 75 cases and both treatments in 33 cases. The population was divided into four groups according to HR and ERBB2 status, as follows: two luminal subtypes (HR+ ERBB2+ (ERα+ and/or PR+, and ERBB2+, n = 20) and HR+ ERBB2− (ERα+ and/or PR+, and ERBB2−, n = 112)); an ERBB2+ subtype (ERα−, PR−, and ERBB2+, n = 42)) and a triple-negative (TN) subtype (ERα−, PR−, and ERBB2−, n = 44)). The median follow-up is 9.1 years (range: 1 month to 27 years); 100 patients metastasized. Standard prognostic factors are shown in Table S1. The median follow-up was 9.1 years (range 1 month to 27 years); 100 patients developed metastasis. Fifteen specimens of adjacent normal breast tissue from breast cancer patients or normal breast tissue from women undergoing cosmetic breast surgery were used as sources of normal mRNA.

**Real-time qRT-PCR.** The theoretical basis, RNA extraction, cDNA synthesis, design of primers and qRT-PCR conditions have been previously described in detail [33]. The FOXO1 and FOXO3 expression values of the samples were normalized such that the median value for the 15 normal breast tissues was 1. Variation in

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**Figure 2.** Kaplan-Meier metastasis-free survival curves for FOXO3 and FOXO1 genes, according to protein levels (A) for FOXO3 and mRNA levels (B,C) for FOXO1 and FOXO3 in a series of 218 breast tumors. P-values are estimated using the log-rank test. Patients with breast tumors expressing high levels of FOXO3 protein had significantly better MFS than patients with breast tumors expressing lower levels of this protein ($p=4.1 \times 10^{-2}$) (A). FOXO3 and FOXO1 mRNA expressions have no prognostic value (B and C respectively).
FOXO1 and FOXO3 expression values from one sample to another of the 15 normal breast are small (FOXO1 ARNm median = 1.0, min = 0.51, max = 1.85. FOXO3 ARNm median = 1.0, min = 0.71, max = 1.86.), indicating that these expressions are representative. The nucleotide sequences of the primers used were as follows: TBP-U (5′-TGCACAGGAGCCAAGAGTGAA-3′) and TBP-L (5′-CACATCACAGCTCCCCACCA-3′) for TBP gene (132 bp PCR product); FOXO1-U (5′-GTCAAGAGCGTGCCCTACTTCA-3′) and FOXO1-L (5′-TGAACTTGCTGTGTAGGGACAGATTAT-3′) for FOXO1 gene (101 bp PCR product); FOXO3-U (5′-CCTACTTCAAGGATAAGGGCGACAG-3′) and FOXO3-L (5′-GTGCCGGATGGAGTTCTTCCAG-3′) for FOXO3 gene (62 bp PCR product); FOXO4-U (5′-TGGTCCGTACTGTACCCTACTTCA-3′).

Over- and under-expressions were defined as threefold variations of expression relative to the median expression of normal samples. We have previously used the same approach to determine cut-off points for tumor gene altered expression. RPPA. RPPA technology was used for quantifying the relative abundance of total protein expression as previously described. Antibody references are available in Table S4. Low and high protein expressions were defined as twofold variations of expression relative to the median expression of the series of 218 breast tumors.

Western blot. Proteins from breast tumors were extracted with buffer A (50 mM Tris pH = 6.8, 2% SDS, 5% glycerol, 2 mM DTT, 2.5 mM EDTA, 2.5 mM EGTA, 4 mM sodium orthovanadate, 20 mM sodium fluoride, 1 mM PMSF). The antibodies used in this study were: anti-FOXO3 (9467, Cell signalling, Beverly, MA, USA), anti-Phospho-FOXO3A (pSer-253) (ab47285, abcam, Cambridge, MA), and anti-GAPDH used as internal control (sc-20357, Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were detected by the ECL Western Blotting Analysis System procedure (GE Healthcare, Buckinghamshire, UK).

Statistical analysis. Statistical analyses were done as previously described [19]. The Cox proportional hazards regression model was used to assess prognostic significance and the results are presented as hazard ratios (HR) and 95% confidence intervals (CIs).

Compliance with ethical standards. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All patients who entered

| PI3K/AKT/mTOR pathway | FOXO1 protein | FOXO3 protein |
|-----------------------|--------------|--------------|
| Phospho-S6K (Thr-421/Ser-424) | NS | −0.252 0.0002 |
| Phospho-S6K (Thr-389) | NS | −0.163 0.016 |
| Phospho-S6 (Ser-235/236) | NS | −0.186 0.0058 |
| Phospho-S6 (Ser-24) | +0.141 0.038 | −0.301 <0.0001 |
| AKT | NS | −0.317 <0.0001 |
| mTor | NS | −0.178 0.0086 |
| S6 Rib | NS | −0.446 <0.0001 |
| IRS1 | NS | −0.172 0.011 |
| FOXO1 protein | FOXO3 protein |
| r | p-value | r | p-value |
| DNA repair |
| Ku80 | −0.137 0.043 | −0.327 <0.0001 |
| DNA-PK | NS | −0.144 0.034 |
| PARP | NS | −0.401 <0.0001 |
| NBS1 | −0.156 0.021 | −0.303 <0.0001 |
| RAD50 | NS | −0.181 0.0072 |
| Mre11 | −0.137 0.043 | −0.203 0.0026 |
| Apoptosis |
| P53 | NS | +0.299 <0.0001 |
| Phospho-p53 (Ser-15) | NS | +0.320 <0.0001 |
| Phospho-p53 (Ser-392) | NS | +0.387 <0.0001 |
| Cell cycle |
| p15 | NS | +0.392 <0.0001 |
| Proliferation |
| Ki67 | +0.134 0.049 | −0.46 <0.0001 |

Table 4. Spearman rank correlation coefficients (r) and p-values between FOXO1 and FOXO3 protein levels and other proteins of different pathways in the series of 218 breast tumours.
our institution before 2007 were informed that their tumor samples might be used for scientific purposes and were given the opportunity to decline. Since 2007, patients entering our institution have also provided their approval by signing an informed consent form. This study was approved by the local ethics committee (René Huguenin Hospital Breast Group). Informed consent: Informed consent was obtained from all individual participants included in the study.

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References
1. Ferlay, J. et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int. J. Cancer 136, E339–386 (2015).
2. Sortle, T. et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc. Natl. Acad. Sci. USA 100, 8418–8423 (2003).
3. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature 490, 61–70 (2012).
4. Eijkelenboom, A. & Burgering, B. M. T. FOXOs: signalling integrators for homeostasis maintenance. Nat. Rev. Mol. Cell Biol. 14, 83–97 (2013).
5. Bullock, M. FOXO factors and breast cancer: outfoxing endocrine resistance. Endocr. Relat. Cancer 23, R113–130 (2016).
6. Coomans de Brachè, A. & Demoulin, J.-B. FOXO transcription factors in cancer development and therapy. Cell. Mol. Life Sci. CMLS 73, 1159–1172 (2016).
7. Link, W. Introduction to FOXO Biology. Methods Mol. Biol. Clifton NJ 1890, 1–9 (2019).
8. Link, W. & Fernandez-Marcos, P. J. FOXO transcription factors at the interface of metabolism and cancer. Int. J. Cancer 141, 2379–2391 (2017).
9. Wang, Z., Yu, T. & Huang, P. Post-translational modifications of FOXO family proteins (Review). Mol. Med. Rep. 14, 4931–4941 (2016).
10. Brunet, A. et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 96, 857–868 (1999).
11. Jacobs, F. M. J. et al. FoxO6, a novel member of the FoxO class of transcription factors with distinct shuttling dynamics. J. Biol. Chem. 278, 33959–33967 (2003).
12. Plas, D. R. & Thompson, C. B. Akt activation promotes degradation of tuberin and FOXO3a via the proteasome. J. Biol. Chem. 278, 12361–12366 (2003).
13. Shaw, R. J. & Cantley, L. C. Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature 441, 424–430 (2006).
14. Paik, J.-H. et al. FOXOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell 128, 309–323 (2007).
15. Hu, M. C.-T. et al. Ipkapβ2 kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. Cell 117, 225–237 (2004).
16. Zou, Y. et al. Forkhead box transcription factor FOXO3a suppresses estrogen-dependent breast cancer cell proliferation and tumorigenesis. Breast Cancer Res. BCR 10, R21 (2008).
17. Yang, J.-Y. et al. ERK promotes tumorigenesis by inhibiting FOXO3a via IMD2-mediated degradation. Nat. Cell Biol. 10, 138–148 (2008).
18. Guttilla, I. K. & White, B. A. Coordinate regulation of FOXO1 by miR-27a, miR-96, and miR-182 in breast cancer cells. J. Biol. Chem. 284, 23204–23216 (2009).
19. Wu, Y. et al. Expression of FOXO1 is associated with GATA3 and Annexin-1 and predicts disease-free survival in breast cancer. Am. J. Cancer Res. 2, 104–115 (2012).
20. Jiang, Y., Zou, L., Lu, W.-Q., Zhang, Y. & Shen, A.-G. Foxo1a expression is a prognostic marker in breast cancer. PLoS ONE 8, e70746 (2013).
21. Feng, X. et al. Cdc25A regulates matrix metalloprotease 1 through Foxo1 and mediates metastasis of breast cancer cells. Mol. Cell. Biol. 31, 3457–3471 (2011).
22. Storz, P., Doppler, H., Copland, J. A., Simpson, K. J. & Toker, A. FOXO3a promotes tumor cell invasion through the induction of matrix metalloproteinases. Mol. Cell. Biol. 29, 4906–4917 (2009).
23. Rehman, A. et al. FOXO3a expression is associated with lymph node metastasis and poor disease-free survival in triple-negative breast cancer. J. Clin. Pathol. https://doi.org/10.1136/jclinpath-2018-205052 (2018).
24. Chen, J. et al. Constitutively nuclear FOXO3a localization predicts poor survival and promotes Akt phosphorylation in breast cancer. PloS one 5, e12923 (2010).
25. Lallemand, F. et al. Involvement of the FOXO6 transcriptional factor in breast carcinogenesis. Oncotarget 9, 7464–7475 (2018).
26. Laplante, M. & Sabatini, D. M. mTOR signaling in growth control and disease. Cell 149, 274–293 (2012).
27. Yudushkin, I. Getting the Akt Together: Guiding Intracellular Akt Activity by PI3K. Biomolecules 9, (2019).
28. Ciruelos Gil, E. M. Targeting the PI3K/AKT/mTOR pathway in estrogen receptor-positive breast cancer. Cancer Treat. Rev. 40, 862–871 (2014).
29. Yu, J.-L. & Cui, W. Proliferation, survival and metabolism: the role of PI3K/AKT/mTOR signalling in pluripotency and cell fate determination. Dev. Camb. Engl. 143, 3050–3060 (2016).
30. Blackford, A. N. & Jackson, S. P. ATM, ATR, and DNA-PK: The Trinity at the Heart of the DNA Damage Response. Mol. Cell. 66, 801–817 (2017).
31. Nickoloff, J. A., Jones, D., Lee, S.-H., Williamson, E. A. & Hromas, R. Drugging the Cancers Addicted to DNA Repair. J. Natl. Cancer Inst. 109 (2017).
32. Yogosawa, S. & Yoshida, K. Tumor suppressive role for kinases phosphorylating p53 in DNA damage-induced apoptosis. Cancer Sci. 109, 3376–3382 (2018).
33. de Sousa Abreu, R., Penalva, L. O., Marcotte, E. M. & Vogel, C. Global signatures of protein and mRNA expression levels. Mol. Biosyst. 5, 1512–1526 (2009).
34. Sicil, D. et al. The estrogen receptor α is the key regulator of the bifunctional role of FoxO3a transcription factor in breast cancer motility and invasiveness. Cell Cycle Georget. Tex 12, 3405–3420 (2013).
35. Sunters, A. et al. FoxO3a transcriptional regulation of Bim controls apoptosis in paclitaxel-treated breast cancer cell lines. J. Biol. Chem. 278, 49795–49805 (2003).
36. Nestal de Moraes, G., Bella, L., Zona, S., Burton, M. J. & Lam, E. W.-F. Insights into a Critical Role of the FOXO3α-FOXO1 Axis in DNA Damage Response and Genotoxic Drug Resistance. Cancer Drug Targets 17, 164–177 (2016).
37. Finak, G. et al. Gene expression signatures of morphologically normal breast tissue identify basal-like tumors. Breast Cancer Res. BCR 8, R58 (2006).
38. Meseure, D. et al. Expression of ANRIL–Polycyst Complexes-CDKN2A/B/ARF Genes in Breast Tumors: Identification of a Two-Gene (EZH2/CBX7) Signature with Independent Prognostic Value. Mol. Cancer Res. MCR 14, 623–633 (2016).
39. Le Goux, C. et al. mRNA Expression levels of genes involved in antitumor immunity: Identification of a 3-gene signature associated with prognosis of muscle-invasive bladder cancer. *Oncoimmunology* 6, e1358330 (2017).

40. Awadelkarim, K. D. et al. Quantification of PKC family genes in sporadic breast cancer by qRT-PCR: evidence that PKC/λ overexpression is an independent prognostic factor. *Int. J. Cancer* 131, 2852–2862 (2012).

41. Rondeau, S. et al. ATM has a major role in the double-strand break repair pathway dysregulation in sporadic breast carcinomas and is an independent prognostic marker at both mRNA and protein levels. *Br. J. Cancer* 112, 1059–1066 (2015).

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**Author contributions**

L.d.K., S.V., A.B., F.L., A.S., C.L., F.O.B., A.B., R.L., I.B.: acquisition of data, F.L., A.P., K.D., L.d.K., S.V., R.L., I.B., S.M.C. analyse and interpret data, F.L., K.D., B.S.L., C.C., A.V., S.Z.J., R.L., S.M.C., I.B.: wrote and/or reviewed the manuscript; Study supervision: S.M.C., R.L. and I.B.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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