Down-regulation of the Tumor Suppressor Protein 14-3-3σ Is a Sporadic Event in Cancer of the Breast

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14-3-3 proteins comprise a family of highly conserved and broadly expressed multifunctional regulatory proteins that are involved in various cellular processes such as cell cycle progression, cell growth, differentiation, and apoptosis. Transcriptional expression of the σ isoform of 14-3-3 is frequently impaired in human cancers, including carcinomas of the breast, which has led to the suggestion that this protein might be involved in the neoplastic transformation of breast epithelial cells. Here we report on the analysis of 14-3-3σ expression in primary breast tumors using a proteomic approach complemented by immunohistochemical analysis by means of specific antibodies against this isoform. We show that the levels of expression of 14-3-3σ were similar in non-malignant breast epithelial tissue and matched malignant tissue with only sporadic loss of expression observed in 3 of the 68 tumors examined. Moreover we show that 14-3-3σ immunoreactivity was restricted to epithelial cells and significantly stronger in the myoepithelial cells that line the mammary ducts and lobules. The lack of expression of 14-3-3σ in the three breast carcinomas was not associated with high levels of expression of the dominant-negative transcriptional regulator ΔNp63 or with increased expression of estrogen-responsive finger protein, a ubiquitin-protein ligase (E3) that targets 14-3-3σ for proteolysis. Validation of the results was performed retrospectively on an independent clinical tumor sample set using a tissue microarray containing 65 primary tumors. Our data suggest that, contrary to what was previously thought, loss of expression of 14-3-3σ protein is not a frequent event in breast tumorigenesis. Molecular & Cellular Proteomics 4: 555–569, 2005.

There were an estimated 10 million new cases, 6 million deaths, and 22 million persons living with cancer in the year 2000. Breast cancer, with 1.05 million new cases, is one of the most common malignancies accounting for 18% of all cancers in women (1). In Denmark, ~3800 women develop breast cancer per annum, and an estimated 1200 die from the disease (2). To date few serum tumor markers are available for detection of breast cancer, and the most commonly used such as serum levels of CA 15-3, mucinous-like cancer antigen, carcinoembryonic antigen, tissue polypeptide antigen, Neu oncoprotein, and tissue polypeptide-specific antigen are of limited usefulness for detecting breast cancer at the early stage of disease (3, 4), underscoring the need for discovering new and more specific biomarkers. Our laboratory has recently embarked on a long term proteomic study of breast cancer as part of a large translational research project aiming at biomarker and target discovery in breast malignancy. We have only just completed the first major milestones in this project: a feasibility study involving 26 high risk patients (5) and the description of the protein composition of the fluid that bathes the tumor microenvironment (6). Given its scope and experimental setup, this project provides an excellent framework in which, in addition to implementing discovery-driven research, to evaluate previously reported breast cancer-associated markers.

Recently others have identified 14-3-3σ as a gene whose expression is lost during neoplastic transformation of breast epithelial cells (7, 8). 14-3-3σ is a member of a multifunctional protein family comprising seven mammalian isoforms (β, γ, η, τ, σ, ε, and ζ). The 14-3-3 proteins are involved in a plethora of biological processes and were suggested to play a regulatory role in many of these processes, such as apoptotic cell death, mitogenic signal transduction, and cell cycle control (9). The σ isoform of 14-3-3 inhibits G1/S cell cycle progression in a p53-regulated manner and is critical to uphold G2 arrest upon DNA damage in colorectal cancer cells (10, 11). Additionally 14-3-3σ associates with CDK2 and CDK4, suggesting that it may also regulate G1/S progression (12). Moreover in primary human epidermal keratinocytes down-regulation of 14-3-3σ results in evasion from senescence (13). Overall these lines of evidence suggest that functional inactivation of 14-3-3σ may be linked to carcinogenesis, a hypothesis that is supported by the discovery of 14-3-3σ down-regulation in various human malignancies, including cancer of the breast, stomach, colon, lung, liver, prostate, oral cavity, and vulva (7, 8, 14–19), attributed to hypermethylation of the CpG island present in the promoter area of the gene. However, expression of 14-3-3σ is also reportedly up-regulated in
lung (20), head and neck (21), gastric (22), and pancreatic cancer (23–25) suggesting that this protein might play a key role in carcinogenesis through multiple mechanisms.

Hypermethylation of the 14-3-3-σ promoter is an early and frequent event in breast neoplastic transformation (7, 8, 26, 27), leading to the suggestion that silencing of 14-3-3-σ may be an important event in tumor progression and particularly in breast carcinogenesis. Hypermethylation and loss of σ isoform expression are the most consistent molecular alterations in breast cancer identified so far.

Here we show that down-regulation of 14-3-3-σ at the protein level was sporadic in the primary breast carcinomas analyzed, and no clear correlation between protein levels of 14-3-3-σ and breast epithelia neoplastic transformation could be established, challenging the current view that 14-3-3-σ is a tumor suppressor for breast malignancies.

MATERIALS AND METHODS

Breast Tissue Biopsies

A series of 65 breast tumors were collected at the Copenhagen University Hospital, Copenhagen, Denmark as part of a large translational breast cancer research project involving high risk breast cancer patients (the criteria for high risk cancer applied are: age below 35 years old, and/or tumor diameter of more than 20 mm, and/or histological malignancy grade 2 or 3, and/or negative estrogen and/or progesterone receptor status, and/or positive axillary lymph node status). After dicing the biopsies with a scalpel into 1–2-mm³ pieces, samples were homogenized in 0.3–0.4 ml of lysis solution (8 m urea, 100 mM DTT, 2% Nonidet P-40, 2% carrier ampholytes pH 7–9) with the aid of a hand glass homogenizer and subsequently stored at −20 °C until use. Fresh tissue samples were obtained both from the resected tumor and from distant non-malignant areas of the surgical specimen and, when available, from axillary nodal metastasis from women with primary operable high risk invasive breast cancer. All patients had had no previous surgery to the breast and did not receive preoperative treatment. They presented a unifocal tumor of an estimated size of more than 20 mm, and all patients, except one, had mastectomy with axillary dissection. Patients’ characteristics are presented in Table I. Normal tissue was also obtained from reduction mammoplasty specimens with informed consent from the patients. The project was approved by the Scientific and Ethical Committee of the Copenhagen and Frederiksborg Municipalities (KF 01-069/03).

Carboplatin and gemcitabine were the standard treatments given to patients with operable tumors.

Cell Cultures

Human breast cell lines MCF-10A, MCF-7, MDA-MB-231, and MDA-MB-435 were cultured according to the guidelines of the ATCC.

Western Blot Analysis

Proteomic Analysis

Two-dimensional (2D) PAGE was performed as described previously (29). Gels were stained with silver nitrate. Proteins were identified using a combination of procedures that included matrix-assisted laser desorption ionization time-of-flight (Biflex, Bruker Daltonics Inc., Billerica, MA); 2D-PAGE Western immunoblotting, and comparison with the master 2D gel images of human keratinocytes and urothelial carcinoma proteins (proteomics.cancer.dk). For quantitation, 2D gel autoradiographs were scanned using a Molecular Imager device (Bio-Rad) and were analyzed using PDQuest 7.1 analysis software (Bio-Rad). Only gels presenting well focused spots and limited amount of protein remaining at the origin were selected for quantitation.

One-dimensional Analysis—Protein extracts for one-dimensional Western blotting were prepared by either lysis in Laemmli buffer of subconfluent dishes of cultured cells or by a 10-fold dilution in Laemmli buffer of 2–3 μl of breast tissue samples homogenized in 2D-PAGE lysis solution. Extracts were resolved on NuPAGE 4–12% gels (Invitrogen), blotted onto Immobilon-P PVDF membranes (Millipore), and detected with either the 14-3-3-σ-specific rabbit polyclonal antibody raised against a C-terminal peptide of the protein (Eurogentec, Brussels, Belgium).

Immunohistochemistry

Tissue blocks obtained from breast cancer patients and dissected from the tumor, axillary nodal metastasis, or non-malignant breast...
epithelium were immediately placed in formalin fixative in the operating theater and paraffin-embedded for archival use. For each case, a representative set of paraffin blocks was selected that contained well matched tumor and benign breast tissue as well as axillary nodal metastasis.

Five-micrometer sections were cut from the paraffin blocks of breast tumor, matched benign tissue, and the axillary nodal metastasis; mounted on Super Frost Plus slides (Menzel-Gläser, Braunschweig, Germany); baked at 60 °C for 60 min; deparaffinized; and rehydrated through graded alcohol rinses. Heat-induced antigen retrieval was performed by immersing slides in 10 mM citrate buffer (pH 6.0) and microwaving in a 750-watt microwave oven for 10 min. The slides were then cooled at room temperature for 20 min and rinsed abundantly in tap water. Nonspecific staining of slides was blocked (10% normal goat serum in PBS buffer) for 15 min, and endogenous peroxidase activity was quenched using 0.3% H2O2 in methanol for 30 min. Antigen was detected by overnight incubation at 4 °C with a primary antibody at the appropriate dilution followed by a secondary antibody conjugated to a peroxidase complex (horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody, DakoCytomation, Glostrup, Denmark). Finally, color development was done with 3,3’-diaminobenzidine (Pierce) as a chromogen to detect bound antibody complex. When relevant, slides were counterstained with hematoxylin. Standardization of the incubation and development times allowed an accurate comparison of expression levels in all cases. Primary antibodies used were: anti-14-3-3-α rabbit polyclonal antibody (Eurogentec, San Diego, CA) and mouse monoclonal antibodies against p53 (clone DO-7), HER-2/neu (HerceptestTM), estrogen receptor (clone 1D5), progesterone receptor (clone 1A6), smooth muscle actin (clone 1A4), vimentin (clone Vim3B4) (DakoCytomation), or estrogen-responsive finger protein (Efp) (Santa Cruz Biotechnology, Santa Cruz, CA); also used were anti-phosphohistone H3 (Ser-9), anti-phospho-p38 MAPK (Thr-180/Thr-182), anti-cleaved caspase-3 (Asp-175), and anti-phospho-β-catenin (Ser-78) antibodies, all from Cell Signaling Technologies (Beverly, MA).

**Immunohistochemical Staining of Tissue Microarrays**

T-BO-1 breast and ovarian cancer tissue microarray slides were obtained from the Cooperative Human Tissue Network and the Tissue Array Research Program (TARP) of the NCI, National Institutes of Health, Bethesda, MD. The slides were stained as above using an appropriate primary antibody. For detection of immune complexes we used a horseradish peroxidase-labeled polymer (Envision+ detection kit, DakoCytomation) as a secondary antibody.

**Indirect Immunofluorescence**

All tissue specimens were frozen immediately upon arrival to our laboratory and stored frozen in liquid nitrogen. Immunohistochemical analysis was performed on 6-μm-thick sections of frozen tissue obtained from high risk breast cancer patients and dissected from the same tumor, axillary nodal metastasis, or non-malignant breast epithelium. Immunostaining was performed according to standard methods using a rabbit polyclonal antibody raised against a C-terminal peptide specific for 14-3-3-α (Eurogentec, San Diego, CA), and a mouse monoclonal antibody against p63 or vimentin (p63 Ab-4, Neo-markers, Lab Vision Corp.; vimentin clone Vim3B4, DakoCytomation). Briefly formaldehyde-fixed sections (3.6% formaldehyde for 4 min) were immersed for 15 min in normal fetal calf serum to block nonspecific staining and then were incubated with the relevant primary antibodies overnight at 4 °C. The sections were washed three times with cold PBS between incubations. Normal goat or mouse serum instead of primary antibody was used as a negative control. Double staining, using appropriate Alexa Fluor® 488- and Alexa Fluor 594-labeled secondary antibodies (Molecular Probes, Eugene, OR) were performed to investigate the relative localization of 14-3-3-α and p63. To examine the possible co-localization of p63 and 14-3-3-α we used isoform-specific rabbit antibodies reactive against Tap63α or ΔNp63α (kindly provided by Dr. Karin Nylander) and 14-3-3-α using the ZenonTM tricolor rabbit IgG labeling kit (Molecular Probes). Sections were imaged using either standard epiluminescence fluorescence microscopy (Leica DMRB) or laser scanning microscopy (Zeiss 510LSM).

**RESULTS**

**Quantification and Characterization of 14-3-3-α Protein Expression in Non-malignant and Malignant Breast Epithelia**

Immunohistochemical Demonstration of the Presence of 14-3-3-α Protein in Normal and Cancerous Breast Tissue—To study changes in 14-3-3-α expression that might take place during the progression from normal breast epithelium to invasive breast cancer, we analyzed “normal” tissue (i.e. non-malignant epithelia collected from sites distal to the carcinoma and judged histologically normal) and malignant tissue obtained from the same patient by immunohistochemistry. Analysis of specimens was performed using paraffin-embedded matched malignant and non-malignant specimens analyzed in parallel by 2D-PAGE and Western blot analysis (Table II). Samples were considered positive if more than 10% of the specimen was stained and were classified as exhibiting high (+++), moderate (+), low (+), or no (−) immunoreactivity for 14-3-3-α. We observed that breast malignancy does not correlate with loss of 14-3-3-α expression with only three tumors (patients 10, 19, and 34) showing loss of 14-3-3-α expression.

To ascertain the distribution of the 14-3-3-α protein in normal breast tissue we used samples derived from reduction mammoplasty in patients without any clinical history of cancer. Hence, in normal epithelia, 14-3-3-α immunoreactivity is seen in mammary glands (Fig. 1, A and B; two separate patients) with the strongest staining observed at periductal or periglandular locations similar to myoepithelial cells (Fig. 1C, arrowhead). The majority of the tumors examined showed homogeneous 14-3-3-α immunoreactivity (Fig. 1, D and E; malignant tissue from patients 56 and 14, respectively) with little difference to matched non-malignant tissue. In some lesions, however, we could observe areas containing entrapped normal looking ducts with stronger 14-3-3-α staining (Fig. 1F, black arrowheads; patient 3) than that of adjacent malignant cells (Fig. 1F, white arrowheads; patient 3). Nevertheless the majority of the lesions examined showed even staining of malignant cells (Fig. 1G) with occasional stronger immunoreactivity in clusters of tumor cells in basal layers corresponding to myoepithelial cells (black arrowhead). Three tumors (patients 10, 19, and 34) showed complete loss of immunoreactivity to 14-3-3-α antibody (Fig. 1H, patient 34), and one tumor (patient 53) showed focal loss of immunoreactivity.
Abrogation of Protein 14-3-3σ in Breast Cancer Is Sporadic

Immunohistochemical analysis of 14-3-3σ in primary breast carcinomas

Tumors negative for 14-3-3σ are marked with an asterisk.

| Patient | Histological type | IHC* | Non-malignant | Malignant | Nodal metastasis |
|---------|-------------------|------|---------------|-----------|-----------------|
| 1       | Ductal            | +    | +             | N/A       |                 |
| 2       | Ductal            | ++   | +             | N/A       |                 |
| 3       | Ductal            | N/A  | +             | N/A       |                 |
| 4       | Ductal            | +    | ++            | N/A       |                 |
| 5       | Ductal            | +    | +             | N/A       |                 |
| 6       | Ductal            | ++   | +             | N/A       |                 |
| 7       | Ductal            | ++   | ++            | N/A       |                 |
| 8       | Ductal            | ++   | ++            | N/A       |                 |
| 9       | Ductal            | ++   | ++            | N/A       |                 |
| 10*     | Lobular           | +    | +             | N/A       |                 |
| 11      | Lobular           | +    | +             | N/A       |                 |
| 12      | Ductal            | ++   | +             | N/A       |                 |
| 13      | Ductal            | ++   | ++            | N/A       |                 |
| 14      | Ductal            | ++   | ++            | N/A       |                 |
| 15      | Ductal            | ++   | ++            | N/A       |                 |
| 16      | Ductal            | +    | ++            | N/A       |                 |
| 17      | Ductal            | +    | ++            | N/A       |                 |
| 18      | Ductal            | +    | ++            | N/A       |                 |
| 19*     | Ductal            | +    | ++            | N/A       |                 |
| 20      | Ductal            | ++   | ++            | N/A       |                 |
| 21      | Ductal            | ++   | +             | N/A       |                 |
| 22      | Ductal            | ++   | +             | N/A       |                 |
| 23      | Ductal            | +    | +             | N/A       |                 |
| 24      | Ductal            | +    | +             | N/A       |                 |
| 25      | Ductal            | +    | +             | N/A       |                 |
| 26      | Mucinous          | ++   | +             | N/A       |                 |
| 27      | Ductal            | ++   | +             | N/A       |                 |
| 28      | Ductal            | +    | +             | N/A       |                 |
| 29      | Ductal            | +    | +             | N/A       |                 |
| 30      | Ductal            | +    | +             | N/A       |                 |
| 31      | Ductal            | ++   | +             | N/A       |                 |
| 32      | Ductal            | +    | +             | N/A       |                 |
| 33*     | Ductal            | +    | N/A           |           |                 |
| 34*     | Ductal            | +    | N/A           |           |                 |
| 35      | Lobular           | +    | +             | N/A       |                 |
| 36      | Ductal            | ++   | +             | N/A       |                 |
| 37      | Ductal            | +    | +             | N/A       |                 |
| 38      | Ductal            | +    | +             | N/A       |                 |
| 39      | Ductal            | +    | +             | N/A       |                 |
| 40      | Ductal            | ++   | +             | N/A       |                 |
| 41      | Ductal            | ++   | +             | N/A       |                 |
| 42      | Ductal            | ++   | +             | N/A       |                 |
| 43      | Lobular           | ++   | +             | N/A       |                 |
| 44      | Ductal            | ++   | +             | N/A       |                 |
| 45      | Ductal            | ++   | +             | N/A       |                 |
| 46      | Ductal            | ++   | +             | N/A       |                 |
| 47      | Ductal            | ++   | +             | N/A       |                 |
| 48      | Ductal            | ++   | +             | N/A       |                 |
| 49      | Ductal            | ++   | +             | N/A       |                 |
| 50      | Ductal            | ++   | +             | N/A       |                 |
| 51      | Ductal            | ++   | +             | N/A       |                 |
| 52      | Lobular           | ++   | +             | N/A       |                 |
| 53*     | Ductal            | ++   | +             | N/A       |                 |
| 54      | Ductal            | +    | +             | N/A       |                 |
| 55      | Ductal            | +    | +             | N/A       |                 |
| 56      | Lobular           | ++   | +             | N/A       |                 |
| 57      | Ductal            | ++   | +             | N/A       |                 |
| 58      | Ductal            | ++   | +             | N/A       |                 |
| 59      | Ductal            | ++   | +             | N/A       |                 |
| 60      | Ductal            | +    | +             | N/A       |                 |
| 61      | Ductal            | N/A  | +             | N/A       |                 |
| 62      | Ductal            | +    | +             | N/A       |                 |
| 63      | Ductal            | ++   | +             | N/A       |                 |
| 64      | Ductal            | ++   | +             | N/A       |                 |
| 65      | Lobular           | ++   | +             | N/A       |                 |

* Samples were considered positive if more than 10% of the specimen was stained and were classified as exhibiting high (+++), moderate (++), low (+), or no (--) immunoreactivity for 14-3-3σ. N/A denotes samples that are either absent or with insufficient tissue morphology/IEF gel quality to allow reproducible scoring.

We have also examined eight ductal carcinoma in situ specimens. These premalignant lesions showed highly heterogeneous patterns of staining with areas of strong immunoreactivity adjacent to areas with weak or moderate staining. Importantly we did not observe any significant loss of 14-3-3σ expression in these lesions that could support a role for 14-3-3σ in early carcinogenesis of the breast as illustrated in Fig. 1.

The differences in 14-3-3σ immunoreactivity between non-malignant breast epithelium and adjacent cancer cells are well evident in Fig. 2. This figure was constructed by stitching together (Photostitch 3.1, Canon) three topologically consecutive overlapping images acquired from the same specimen (patient 13), which contains histologically normal ducts adjacent to cancerous tissue. As can be seen, staining of myoepithelial cells (Fig. 2, **black arrowhead**) is stronger than the immunoreactivity for 14-3-3σ of the suprabasal epithelial layer (Fig. 2, **white arrowhead**) and malignant epithelia (Fig. 2, **gray arrowhead**) with the latter two showing very similar immunoreactivities for most of the specimen.

It should be noted that, although our antibody was tested extensively for cross-reactivity by 2D-PAGE (illustrated in Fig. 3), we cannot rule out the possibility that an unrelated structural neoepitope is recognized in paraffin-embedded breast specimens, masking the loss of 14-3-3σ in the tumor samples. To address this point, we replicated some of the experiments presented in this report using a commercially available mouse monoclonal antibody specific for the σ isoform of 14-3-3 (Neomarkers, Lab Vision Corp.) and obtained identical results (data not shown).

Proteomic Analysis of 14-3-3σ in Mammary Carcinomas—in all cases, immunohistochemical analysis was complemented by 2D-PAGE-based proteomic profiling to confirm correct isoform expression and exclude possible antibody-dependent effects. Specimens containing low numbers of myoepithelial cells, judged by immunohistochemical analysis, showed very similar 14-3-3σ patterns of expression. Fig. 4A shows a representative set of 2D gels for a well matched set of samples of non-malignant, malignant, and metastatic ori-
gin, respectively. As can be seen, expression of 14-3-3α was similar in all three specimens paralleling the results obtained by immunohistochemistry (Fig. 4A and Table II, respectively). Moreover we analyzed serial sections of frozen single specimens by 2D-PAGE and immunostaining (Fig. 4B). Samples that were negative for myoepithelial cells, judged by the absence of reactivity to cell type-specific markers (CK14 and smooth muscle actin) showed expression of 14-3-3α by immunohistochemistry and 2D-PAGE (Fig. 4B), further substantiating the presence of this protein in malignant cells of non-myoepithelial character. Correspondingly, for specimens displaying lack of reactivity in immunohistochemical analysis, we could not detect 14-3-3α protein in the 2D gel analysis (Figs. 1H and 4C, respectively). More heterogeneous specimens made interpretation of the obtained expression patterns problematic.

**Tissue Distribution of 14-3-3α Protein**—To make certain that the difference in cell lineage-dependent immunoreactivity we observed was due to a cell type-specific differential expression pattern, we performed double immunostaining analysis of snap frozen tissue samples from breast carcinomas, their paired non-neoplastic tissues, and corresponding lymph node metastases. To distinguish myoepithelial cells we used an antibody against p63, a recently identified member of the p53 gene family, that is expressed in the vast majority of mammary myoepithelial cells, thus being a reliable marker for these cells both in normal and pathologic conditions of the
human breast (30). Double immunostaining of 14-3-3-α/H9268 and p63 in normal breast samples (Fig. 5, A and B) clearly shows that the strongest expression of 14-3-3-α occurs in myoepithelial cells (p63-immunoreactive cells). This is also the case for carcinomas where one can detect myoepithelial cells either in normal looking ducts occurring within malignant areas.
(Fig. 5C, patient 23) or within clusters of cells (Fig. 5D, patient 15). However, myoepithelial lineage is not an absolute requirement for high level expression of 14-3-3\(\alpha\). One can also observe strong immunoreactivity in scattered malignant non-myoeopithelial cells at levels comparable to those observed in myoepithelial cells (Fig. 5, E and F; patients 14 and 2, respectively).

The differential immunoreactivity for 14-3-3\(\alpha\) protein in luminal versus myoepithelial cells, with myoepithelial cells showing on average 10-fold higher immunoreactivity than luminal cells, shows that cell lineage plays a major role in determining the expression level of this protein, increasing the complexity and difficulty in interpreting expression analysis data obtained from patient specimens. This is clearly demonstrated in Fig. 6. Western blot analysis of the levels of expression of 14-3-3\(\alpha\) normalized to the amount of CK19 present in the samples (Fig. 6B) showed that malignant tissue from patient 9 had increased ratios of expression of 14-3-3\(\alpha\) as compared with matched non-malignant tissue. However, immunohistochemistry analysis of the same samples attributed identical staining scores to the two specimens (Table II). Additional stainings of these samples showed that the malignant tissue specimen had highly heterogenous expression of 14-3-3\(\alpha\) with numerous cells displaying low level expression of this protein (Fig. 7A, black arrowheads). Furthermore this specimen contained large areas that stained with myoepithelial cell markers (Fig. 7B, black arrowheads). This combination contributed to skewing the ratio of 14-3-3\(\alpha\)/CK19 leading to an overestimation of the protein levels.

Western Analysis of 14-3-3\(\alpha\) Protein Expression Patterns—
The ratio of glands to connective tissue varies widely between patients in non-malignant breast epithelia as well as between different locations within the breast of the same patient, and as a result the relative contribution of epithelial cell proteins to the total protein extract varies accordingly. Thus, to assess the relative amount of epithelium present in a given sample we used expression of CK19, an epithelium-specific marker expressed by most of the tumors at similar levels, to normalize the results of the Western analysis. Fig. 6 shows a Western blot analysis of a representative collection of non-malignant and malignant breast epithelia. Tissue lysates were analyzed for 14-3-3\(\alpha\) expression by Western blot analysis using antibodies against CK19 and 14-3-3\(\alpha\). Levels of CK19 are used as a normalizing factor for the relative contribution of epithelial cells to the total amount of protein loaded. Lanes are numbered according to patient number and sample classification, non-malignant (N) and tumor (T).
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Fig. 7. Immunohistochemistry analysis of a tumor specimen from patient 9: staining (arrowheads) for CK19 (A) and smooth muscle actin (SMA; B). Magnifications are ×200.

non-correlated fashion to CK19. Hence patients 16 and 20 have positive ratios of \(\sigma\) to CK19 of 2.4 and 11, respectively, whereas patients 3 and 22 have ratios of 0.64 and 0.37, respectively. In tumors where most of the specimen consists of malignant epithelial cells, more consistent ratios of \(\sigma\) to CK19 were seen (Fig. 6B) with one exception, patient 23, where expression of CK19 was lost in the malignant epithelia (loss of CK19 expression was confirmed by immunohistochemistry and 2D-PAGE analysis; data not shown). Notwithstanding we only observed abrogation of 14-3-3\(\alpha\) expression in 3 out the 65 samples examined by a combination of complementary technologies (immunohistochemical analysis, Western blotting, and 2D-PAGE), showing that loss of 14-3-3\(\alpha\) expression is not a hallmark of breast malignancy but rather an infrequent event.

Retrospective Study of 14-3-3\(\alpha\) Expression in Tissue Microarrays

To validate retrospectively in an independent set of specimens the results obtained with the 65 tumor specimens included in our pilot study, we examined the expression of 14-3-3\(\alpha\) in a further 65 breast tumor specimens present on the T-BO-1 TARP tissue microarray (TMA). Tumors were considered positive if more than 10% of the cells were stained. Immunostaining yielded an interpretable result in 37 of the 65 arrayed tumors (56.9%). The intensity of the immunostaining was divided into four groups: negative (−), weak (+), moderate (++), and strong (+++) (Table III; tumors negative for 14-3-3\(\alpha\) are marked with an asterisk). Representative examples of microarray immunostaining are displayed in Fig. 8. Immunohistochemical TMA analysis of 14-3-3\(\alpha\) expression shows that only 6 of the 37 classified specimens were negative. The remaining were divided evenly between weak (13 of 37), moderate (9 of 37), and strong (9 of 37) staining. These data substantiate our observation that abrogation of 14-3-3\(\alpha\) is a sporadic event in primary breast carcinomas.

Analysis of Molecular Mechanisms Involved in the Regulation of 14-3-3\(\alpha\)

We tried to identify the molecular mechanism(s) that could be responsible for impairment of 14-3-3\(\alpha\) expression. Induction of 14-3-3\(\alpha\) in colorectal cancer cells by \(\gamma\) irradiation and

| Block identifier | Diagnosis | 14-3-3\(\alpha\) | p53 | Vimentin | Efp |
|------------------|-----------|----------------|-----|----------|-----|
| YY-00-0056       | IDC       | +              | −   | −        | −   |
| YY-00-0063       | IDC       | ++             | −   | +        | −   |
| YY-00-0064\*     | IDC       | −              | −   | −        | −   |
| YY-00-0066       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0069       | IDC       | +              | −   | −        | −   |
| YY-00-0175       | IDC       | ++             | −   | −        | −   |
| YY-00-0177       | DCIS      | ++             | +   | −        | −   |
| YY-00-0189       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0192       | IDC       | ++             | ++  | −        | −   |
| YY-00-0193       | IDC       | +              | −   | −        | −   |
| YY-00-0196       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0197       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0199       | IDC       | +              | −   | −        | −   |
| YY-00-0209       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0210\*     | IDC       | −              | −   | −        | −   |
| YY-00-0211       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0212       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0218       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0219       | IDC       | +              | −   | −        | −   |
| YY-00-0332\*     | IDC       | −              | −   | −        | −   |
| YY-00-0337       | IDC       | +              | −   | −        | −   |
| YY-00-0343       | IDC       | ++             | −   | −        | −   |
| YY-00-0346       | IDC       | ++             | −   | −        | −   |
| YY-00-0349       | IDC       | −              | −   | −        | −   |
| YY-00-0351       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0352       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0353       | IDC       | +              | −   | −        | −   |
| YY-00-0354       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0355       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0356       | IDC       | +              | −   | −        | −   |
| YY-00-0365       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0366       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0074       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0078       | IDC       | +              | −   | −        | −   |
| YY-00-0145       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0147       | IDC       | ++             | +   | −        | −   |
| YY-00-0151       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0152       | IDC       | +              | −   | −        | −   |
| YY-00-0153       | IDC       | ++             | +   | −        | −   |
| YY-00-0154\*     | IDC       | −              | −   | −        | −   |
| YY-00-0155       | IDC       | +              | −   | −        | −   |
| YY-00-0156       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0158       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0160       | IDC       | +              | −   | −        | −   |
| YY-00-0163       | IDC       | +              | −   | −        | −   |
| YY-00-0165\*     | IDC       | −              | −   | −        | −   |
| YY-00-0167       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0170       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0173       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0223       | IDC       | +              | −   | −        | −   |
| YY-00-0224       | IDC       | +              | −   | −        | −   |
| YY-00-0225       | IDC       | +              | −   | −        | −   |
| YY-00-0255       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0313       | IDC       | +              | −   | −        | −   |
| YY-00-0314       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0315       | IDC       | +              | −   | −        | −   |
| YY-00-0317       | IDC       | N/A            | N/A | N/A      | N/A |
| YY-00-0318\*     | IDC       | −              | −   | −        | −   |
| YY-00-0319       | IDC       | +              | −   | −        | −   |
| YY-00-0410       | IDC       | +              | −   | −        | −   |
| YY-00-0550       | IDC       | N/A            | N/A | N/A      | N/A |

\* ILC, invasive lobular carcinoma; IDC, invasive ductal carcinoma; DCIS, ductal carcinoma in situ.

\(\alpha\) Samples were considered positive if more than 10% of the specimen was stained and were classified as exhibiting high (+++), moderate (++), low (+), or no (−) immunoreactivity for 14-3-3\(\alpha\). N/A denotes cores that are either absent or present but with insufficient malignant tissue to allow scoring.
other DNA-damaging agents is mediated by a p53-responsive element (11). Additionally, besides being a target gene of p53, 14-3-3-σ appears to have a positive feedback effect on p53 activity (31). Given the functional link between 14-3-3-σ activities and p53 regulation (11, 31, 32) we decided to investigate the possibility that the tumor specimens we had analyzed had abnormally increased levels of p53. Immunohistochemical analysis of all 65 tumor samples showed that only 14 of these had detectable levels of p53 (Table II), ruling out abnormally increased levels of p53 as the molecular explanation for the persistent expression of 14-3-3-σ in the malignant breast samples examined.

p63 encodes two types of isoforms that can either function to transactivate p53-responsive genes (TAp63 isoforms) or act as a dominant-negative transcription factor (ΔNp63 isoforms) (33–35). The dominant-negative splice variant ΔNp63α has transcriptional repressor activity and can bind to the 14-3-3σ promoter in vivo (36). As several studies on human tumors have suggested an oncogenic function for p63 isoforms (33, 37–40) we investigated a possible correlation between expression of p63α isoforms and 14-3-3σ in breast carcinomas. We performed double immunostainings to characterize the differential distribution of p63 and 14-3-3σ in malignant epithelia using a TAp63α or ΔNp63α isoform-specific antibody (34) and our 14-3-3σ-specific antibody. In all cases examined the expression of 14-3-3σ did not show any particular correlation with p63 isoforms (data not shown). We observed malignant epithelial cells concomitantly express the dominant-negative ΔNp63α and 14-3-3σ at high levels, ruling out potential inhibitory effects of this isoform on 14-3-3σ transcription. Conversely we also ascertained that expression of the transactivating isoform TAp63α is not required for high expression of 14-3-3σ to occur as cells devoid of TAp63α can express 14-3-3σ even at levels comparable to TAp63α-expressing cells (data not shown).

The Efp, one of the estrogen receptor target genes, has recently been shown to be a RING finger-dependent ubiquitin ligase (E3) that directly targets 14-3-3σ for proteolysis thus modulating 14-3-3σ protein turnover (19). Consequently cellular levels of 14-3-3σ are ultimately dependent on Efp function, suggesting that the loss of 14-3-3σ expression we observed in some tumors could be the result of drastically increased expression of Efp. However, immunohistochemical analysis of Efp expression in the breast carcinoma samples we had previously examined for 14-3-3σ expression (Fig. 9) showed that Efp was not overexpressed in specimens devoid of 14-3-3σ protein (Fig. 9, A and B, tumors 10 and 34, respectively). Interestingly expression of Efp was lower in the malignant specimens that have no 14-3-3σ protein than in the other samples examined (Fig. 9, compare A and B with C).

We have recently shown that 14-3-3σ is down-regulated in invasive transitional cell carcinomas of the urinary bladder undergoing epithelial-to-mesenchymal transition (EMT) (41). To determine whether the loss of expression of 14-3-3σ we had observed in breast carcinomas (3 of the 65 patients examined) correlated with EMT events. Immunohistochemical analysis of the tumor samples showed that five of these were undergoing cell conversions (Table II; tumors 25, 39, 41, 42, and 46 were immunoreactive for vimentin). However, these tumors were still immunoreactive for 14-3-3σ. We extended this analysis to the T-BO-1 TARP tissue microarray and obtained identical results (Table III).

Double immunostainings using a vimentin-specific antibody and our 14-3-3σ-specific antibody showed that malignant
cells concomitantly expressed both markers at high levels (Fig. 10, A–C), ruling out potential inhibitory effects of EMT on 14-3-3α expression. Furthermore we also established that loss of expression of 14-3-3α occurs independently of EMT events as cells devoid of 14-3-3α can be undergoing conversion to a mesenchymal phenotype (Fig. 10B, encircled area of heterogeneity showing loss of 14-3-3α with expression of vimentin) or not (Table II; tumors 25, 39, 41, 42, and 46 are vimentin-positive and 14-3-3α-positive). Furthermore we also examined expression in these tumors of another mesenchymal specific marker, protein gene product 9.5, a marker associated with mesenchymal neoplasms, which is not expressed in connective tissue (42). As with vimentin, we found that cells positive for protein gene product 9.5 had not lost expression of 14-3-3α verifying that loss of 14-3-3α was not due to EMT events (data not shown). These data show that impaired expression of 14-3-3α is not associated with altered p53, p63, or Efp expression or with epithelial-to-mesenchymal cell transitions but with another yet unknown mechanism.

**Physiological Consequences of the Loss of 14-3-3α in Mammary Epithelium**

The 14-3-3 proteins are implicated in the regulation of the cell cycle machinery at several key points (for a review, see Ref. 43). One pivotal role of 14-3-3α is its control of the G2 cell cycle checkpoint (11). Normally in the G2 phase of the cell cycle, the CDC2-cyclin B1 complex enters the nucleus to initiate mitosis. In response to DNA damage, 14-3-3α is induced via a p53-responsive element, sequestering the CDC2-cyclin B1 complex and preventing it from entering the nucleus (10). To evaluate the biological consequences of loss of 14-3-3α expression in mammary epithelium, we determined the levels and localization of cyclin B1 in the breast specimens that we showed have lost α expression. We observed increased nuclear localization of cyclin B1 in two (patients 19 and 34; data not shown and Fig. 11B, respectively) of the three carcinomas devoid of 14-3-3α (Fig. 11, compare A and B; tumors 10 and 34, respectively). Some tumor samples with high levels of 14-3-3α protein also showed nuclear localization of cyclin B1 (Fig. 11C, tumor 46) albeit in a focal manner and not to the extent seen in tumors 19 and 34. Nuclear localization of cyclin B1 was not observed in the matched non-malignant tissue of patients 19 and 34 (Fig. 13D, non-malignant breast tissue of patient 19 and data not shown).

Conceivably loss of the 14-3-3α protein could lead to extraneous mitotic activation. To determine the number of mitotic cells in the specimens examined, we performed immunohistochemical analysis of paraffin-embedded samples using a phosphohistone H3 (Ser-10) antibody. As can be seen in Fig. 14, tumors lacking 14-3-3α protein (Fig. 12, A, B, and C; tumors 10, 19, and 34, respectively) did not show abnormally high numbers of mitotic cells compared with samples with moderate to elevated levels of 14-3-3α protein (Fig. 12, D, E, and F) as judged by the staining with phosphohistone H3 (Ser-10) antibody. Thus, loss of 14-3-3α does not result in uncontrolled cell cycle or untimely G2/M progression.

Another major role of 14-3-3 proteins is the regulation of numerous cellular signaling pathways that are implicated in tumorigenesis. Binding to cognate phosphorylated serine/threonine motifs allows for modulation of key signaling pro-
Expression Levels of 14-3-3α Are Not Correlated with Breast Neoplastic Transformation—Stratifin or 14-3-3α was originally identified as an epithelium-specific marker, HME1, whose expression is down-regulated in immortalized mammary cells (44). Subsequent studies showed that overexpression of 14-3-3α blocks cell cycle entry by inhibiting cyclin-CDK activity in many breast cancer cell lines, establishing 14-3-3α as a negative regulator of cell cycle progression (12). Expression of 14-3-3α is also reduced in primary breast carcinomas primarily by methylation-mediated silencing (7, 8), and hypermethylation of 14-3-3α has been established as an early event in breast neoplastic transformation (7, 26) suggesting that 14-3-3α has an important function in preventing breast tumor cell growth (18).

Here we present a protein profiling study of 65 breast carcinomas analyzed by Western blotting, 2D-PAGE, and immunohistochemistry and provide evidence that 14-3-3α down-regulation at the protein level is not a frequent event in breast cancer. We found that in most cases, matched malignant, non-malignant, and nodal metastatic tissue displayed only minor differences in the expression of this protein. Consequently the results we obtained do not support the contention that 14-3-3α represents a valid marker for the early detection of breast lesions.

But how to explain the discrepancy between our observations and the strong correlation between hypermethylation status of α and breast malignancy reported? Clearly previous results demonstrating overall concordance levels of 91% (75 of 82) and 80% (8 of 10) between α hypermethylation and breast malignancy (7, 8) do not easily apply to protein expression levels. Ferguson and colleagues (8) reported that Northern blot analysis of 26 of 43 samples from primary breast lesions, which were partially or completely methylated, showed that all 26 tumors lacked detectable gene expression. This is in contrast with our own results where only in three cases (patients 10, 19, and 34) did we observe abrogation of 14-3-3α expression in malignant tissue. The prospective study we performed on 65 breast tumor specimens present on the T-BO-1 TARP TMA largely substantiates these data with just 6 of 37 tumors examined revealing loss of 14-3-3α expression. Another important piece of evidence comes from a recent report on the functional role of Efp, one of the estrogen receptor target genes (19). Efp is a RING finger-dependent ubiquitin ligase (E3) that directly targets 14-3-3α for proteolysis thus modulating protein turnover. Consequently cellular levels of 14-3-3α are ultimately dependent on proteosome function making it paramount to use protein-based analytical methods to be able to correlate 14-3-3α protein...
expression with breast epithelial cell tumorigenesis on a functional level.

Loss of Expression 14-3-3α Is Evident Only for a Subset of Breast Carcinomas—But even if our results do not support the claim that loss of expression of 14-3-3α is a frequent event in breast carcinomas, we did observe a preponderance in our 2D-PAGE and Western blot analysis toward lower levels of expression in malignant compared with matched non-malignant samples. However, we have also unambiguously shown that cell lineage is an important determinant of 14-3-3α expression in human primary breast carcinomas, we did observe a preponderance in our 2D-PAGE or Western blot analysis either by 2D-PAGE or Western blot will inevitably reflect the ratio of myoepithelial to luminal epithelial cells present in the specimen.

It is believed that most breast carcinomas arise from luminal epithelial cells since malignant epithelium generally exhibits a luminal epithelial phenotype as established on the basis of their cytokeratin profile and other cell type-specific markers such as MUC-1 and smooth muscle actin among others (45–47). Hence as 14-3-3α expression is stronger in myoepithelial cells as compared with luminal epithelial cells and tumors contain proportionally larger numbers of luminal epithelium-derived cells, breast neoplasms specimens when analyzed by averaging methods (such as Western blot and 2D-PAGE) will necessarily show lower levels of α expression. Immunohistochemical analysis of tissue samples confirms this with expression of 14-3-3α being heterogeneous in nature with predominantly myoepithelial cells showing stronger immunoreactivity than adjacent luminal epithelial cells, malignant or non-malignant (Figs. 1, 2, and 5).

Recent work by Vercoutter-Edouart and colleagues (48) showed down-regulation of 14-3-3α in human primary breast carcinomas by comparing levels of this protein in 35 primary breast carcinomas with an averaged value obtained from eight different cultured normal HMEC samples. Interestingly these authors observed that, in all of the breast carcinomas studied, the amount of 14-3-3α detected was down-regulated by an average of 10-fold compared with the value obtained for cultured HMECs. In contrast, we observed that in the majority of the lesions examined, levels of 14-3-3α in matched malignant, non-malignant, and nodal metastasis tissue were similar. Nonetheless the only discordant observation between the two studies is the fact that the values obtained by Vercoutter-Edouart and colleagues (48) for the levels of 14-3-3α present in cultured normal HMECs were much higher than the values observed in malignant tissue samples. From patient 41 we isolated and cultured HMECs from non-malignant epithelium, which displayed low levels of expression of 14-3-3α. Expression levels of 14-3-3α in cultured HMECs (passage 6) were similar to those in the non-malignant cell line MCF-10A and about 3-fold higher than in MCF-7 (Fig. 14); values comparable to those obtained by Vercoutter-Edouart et al. (48). Several explanations are possible for this observation. First, it was shown that cultured HMECs lose definition of cell lineage unless grown on selective media (49), and as expression of 14-3-3α is much higher in myoepithelial cells than in luminal epithelial cells, cultures consisting of predominantly myoepithelial-like cells would always present higher levels of expression of this protein. Second, one of the phenotypic changes associated with finite life span HMEC culturing is the differentiation toward squamous epithelia (50, 51). We and others have shown that expression of 14-3-3α is highest in cells of the squamous cell lineage (51, 52); thus, squamous differentiation of cultured HMECs would result in abnormally in-

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**Fig. 13.** Analysis of the activation state of various signaling pathways in tumor 34, a 14-3-3α-negative carcinoma. Immunohistochemical staining of cleaved caspase-3 (A) and phosphorylated GSK-3β (B), AKT (C), p38 MAPK (D), p44/42 MAPK (E), and SAPK/JNK (F) showing focal activation of the various pathways (black arrowheads). Magnification, ×100 except for ×200 in F.

**Fig. 14.** Levels of expression of 14-3-3α in various breast cell lines and cultured primary human mammary epithelial cells. Cell lysates were analyzed for 14-3-3α expression by Western blot analysis. Levels of β-actin are used as a normalizing factor for total amount of protein loaded.
creased levels of 14-3-3-σ protein compared with the tissue biopsy from which these cells were derived.

It is worthwhile noting that the analysis of 35 primary breast carcinomas reported by Vercoutter-Edouart and colleagues (48) showed that 14-3-3-σ was undetectable in five samples (14%). These results parallel those obtained in our study with respect to the number of tumors showing abrogation of expression of 14-3-3-σ. We observed that 3 of 65 (5%) malignant tissue specimens collected and 6 of 37 (16%) tumors present on the T-BO-1 TMA had no detectable expression of 14-3-3-σ. Unlike in bladder lesions, loss of 14-3-3-σ in this subset of tumors is not due to EMT events (Tables II and III and Fig. 10, A–C). Furthermore we could not observe any significant correlation with other known prognostic markers such as HER-2/neu, histological grade, and lymph node status or with transcription factors known to control expression of 14-3-3-σ such as p53, Efp, or ΔNp63, making it important to clarify the molecular mechanism(s) leading to the loss of expression of this protein as well as of the possible biological and clinical implications. Our data show that at least one of the biological functions carried out by 14-3-3-σ, sequestration of cyclin B1, can become impaired in tumors lacking 14-3-3-σ (Fig. 11). The active cyclin B1-CDK1 complex must translocate to the nucleus to begin phosphorylation of cognate proteins, an event necessary for mitotic onset. However, insofar as mitotic control (Fig. 12) and regulation of signaling cascades (Fig. 13) is concerned we observed no abnormal values in tumors lacking 14-3-3-σ. Moore and colleagues (53) have recently shown that relocating cyclin B1 from the cytoplasm to the nucleus can trigger DNA replication. Nonetheless increased nuclear levels of cyclin B1 in the tumors lacking 14-3-3-σ do not seem to provoke entry into mitosis (compare Fig. 11B with Fig. 12C). Consequently DNA synthesis may be taking place in the absence of mitosis, which would result in genomic instability and aneuploidy, a hallmark of malignant cells.

In conclusion, our data show that loss of expression of 14-3-3-σ at the protein level is a limited event, occurring in only 5–16% of breast tumors. Changes in the level of this protein observed in the remaining lesions are probably inherent to the pathophysiology of the tissue sample examined in particular to the number of myoepithelial cells present (the level of 14-3-3-σ is an average of 5-fold higher in myoepithelial than in luminal epithelial cells) but also to the presence of epithelial cells undergoing squamous differentiation since expression of 14-3-3-σ tends to be highest in epithelial cells of the squamous cell lineage (54).

The cell lineage-dependent difference in expression levels of 14-3-3-σ together with the considerable variation we observed in the levels of σ expression across patients under-score the importance of comparing matched malignant and normal breast epithelium samples by immunohistochemistry. A larger clinical study with a more numerous data set containing matched non-malignant and malignant specimens is warranted to ascertain whether down-regulation of 14-3-3-σ correlates with breast epithelial cell carcinogenesis in a causal manner in a particular subset of tumors.

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