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PROTEOME PROFILING OF HUMAN BREAST CANCER

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For "cancer fighters"

*"Devil in the details, what
about cancer?"*

*"Keep away from those
who try to belittle your
ambitions. Small people always
do that, but the **really** great
make you **believe** that you too
can become great."*

Mark Twain

ABSTRACT

Breast cancer (BC) takes thousands of woman's lives yearly. Several factors have been found to influence initiation and development of breast cancer, and to affect prognosis and treatment of this disease. This thesis focuses on opening-out this complexity and search for approaches that may lead to individualized treatment of breast cancer patients.

We studied clinical samples of breast tumors and adjacent normal tissues using protein-based proteomics. By studying each patient individually, we identified proteins that changed expression during carcinogenesis (p53, Smad2, etc). We observed significant differences in the lists of cancer-related proteins between individual patients. We demonstrated that meta-data analysis of the identified proteins is the most efficient way to describe common and individual features of tumors from different patients. Our validation study by immunohistochemistry analysis of identified molecules (PYK, Smad2, CK2 α) confirmed the changed expressions between tumor and normal tissue, and thereby confirmed the conclusions obtained with proteomics analysis. Thus, we found that meta-data analysis approach is suitable for improved and individualized diagnostics and selection of treatment.

Transforming growth factor- β (TGF β) is a potent regulator of tumorigenesis. In our study of the clinical cases, we demonstrated that TGF β signaling might be influenced in breast tumorigenesis. Phosphoproteomics analysis of TGF β action on MCF10A human breast epithelial cells showed a complex regulation of cell signaling, with strong representation of functional domains such as metabolism. One of the targets of TGF β is 14-3-3 σ protein, and we found that 14-3-3 σ was of a crucial importance for the cross-talk between TGF β and p53 signaling.

We reported also proteins identified by expression proteomics, which are regulated by TGF β in human breast epithelial cells that have phenotype similar to normal breast epithelial cells. We found more than 100 proteins that were regulated by TGF β . Among them, Casein Kinase 2 α (CK2 α), Structure-Specific Recognition Protein-1 (SSRP1) and protein convertase-4 (PC4) may be involved in TGF β -dependent inhibition of cell proliferation by modulating p53 phosphorylation.

Therefore, presented here study describes development of tools for individualized treatment of patients, and provides insights in the complexity of cancer related signaling in breast epithelial cells.

LIST OF PUBLICATIONS

- I. **Olena Zakharchenko**^{*}, Christina Greenwood, Louise Alldridge, Serhiy Souchelnytskyi.

Optimized protocol for protein extraction from the breast tissue that is compatible with two-dimensional gel electrophoresis.

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- II. **Olena Zakharchenko**, Christina Greenwood, Louise Alldridge, Serhiy Souchelnytskyi.

Meta-data Analysis as a Strategy to Evaluate Individual and Common Features of Proteome Changes in Breast Cancer.

Cancer Genomics Proteomics. 2011 Jan-Feb;8(1):1-14.

- III. Anna Dubrovská[#], **Olena Zakharchenko**[#], Serhiy Souchelnytskyi

Phosphoproteomic analysis of TGFβ1 signaling revealed importance of 14-3-3σ phosphorylation for TGFβ1/Smad3-regulated transcription and CDK2/pRb-dependent cell proliferation.

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- IV. Hanna Woksepp[#], **Olena Zakharchenko**[#], Aude Gautier[#], Nazariy Souchelnytskyi, Ulf Hellman, Serhiy Souchelnytskyi.

Proteomics-based network signaling by TGFβ1 in 184A1 non-tumorigenic human breast epithelial cells, and its role in phosphorylation of p53 at Ser392 and regulation of cell proliferation

Manuscript

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LIST OF ABBREVIATIONS

14-3-3 σ or SFN	Stratifin
2D-GE	Two-dimension gel electrophoresis
ALK1	Activin receptor-like kinase1
ALK5	Type I TGF β receptor
BMP	Bone morphogenetic protein
BRCA	Breast cancer associated protein
CDK2	Cyclin dependent kinase 2
CK	Cytokeratin
CK2 α	Casein kinase 2 alpha
DCIS	Ductal carcinoma <i>in situ</i>
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGFR or HER1	Epidermal growth factor receptor 1
EGFR2 or Her2 ^{neu}	Human Epidermal growth factor Receptor 2
EMT	Epithelial to mesenchimal transition
ER	Estrogene receptor
HPLC	High-performance liquid chromatography
IBC	Invasive breast cancer
IDC	Infiltrating ductal carcinoma
IHC	Immunohistochemistry
ILC	Infiltrating lobular carcinoma
IP	Immunoprecipitation
IPG	Immobilized pH gradient
LC	Liquid chromatography
LOH	Loss of heterozygosity
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
PAGE	Polyacrylamid gel electrophoresis
PDGF	Platelet-derived growth factor
pH	Power hydrogen
pI	Isoelectric point
PR	Progesterone receptor
pRB	Retinoblastoma
PTM	Post-translational modification
R-Smad	Receptor-activated Smad
RNA	Ribonucleic acid
Rpm	Reverse per minute
SDS	Sodium dodecyl sulphate
SSRP1	Structure-Specific Recognition Protein
T β R-I	Transforming growth factor β receptor type I

TβR-II	Transforming growth factor β receptor type II
TGFβ	Transforming growth factor β
TNM	Tumor, metastasis, lymph node
TOF	Time of flight
V	Voltage
Vh	Volt/hours
WB	Western blot

I INTRODUCTION

1.1 BREAST CANCER

The first mentioning about breast cancer is dated 1500 B.C. Since that time many discoveries have been made to improve diagnostics and treatment of this disease (Donegan, 1984). Unfortunately, breast cancer today is still the second most common type of cancer among woman and the fifth most common cause of cancer-associated death.

Breast cancer is a complex disease, whose development is influenced by many different factors that include single gene mutation, hormonal status, life style and environment.

Different approaches have been proposed to classify and grade types of the breast cancer and to suggest optimal treatment. Unfortunately, the heterogeneity of the breast neoplasia is high, and there is a need for more methods and markers for improved diagnostics as well as for treatment (Cianchetti, E., R. Cotellesse, 1985; Morrison, A. S., C. R. Lowe, 1972). The switch to the more individualized analysis of each BC patients and tumor, focusing on each patient, using the personalized medicine approach, will improve treatment outcomes.

1.1.1 The Breast

The human female breast is a bilateral organ with a complicated anatomical structure. The breast contains the lobules and ducts that form unique system for production and delivery of milk during the lactation period (Lynch, Cariaty, & Purushotham, 2006). Network of ducts and lobules are surrounded by stromal tissue and adipocytes (Figure 1 reproduced from Lynch, 2006).

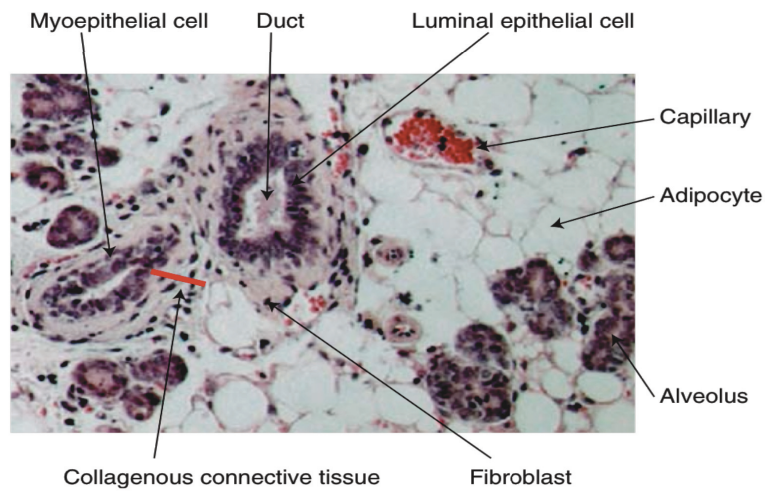


Figure 1. Histological image of human breast.

Arrows indicated histological structures.

Mammary glands are composed of mesenchymal and epithelial elements. The epithelial cells form ducts and lobules. The inner layer of lobules and ducts consists of the luminal cells, which are covered with myoepithelial cells, underlying the basement membranes (Figure 2) (Visvader, 2009). Ducts and lobules are surrounded by fibroblasts and adipocytes.

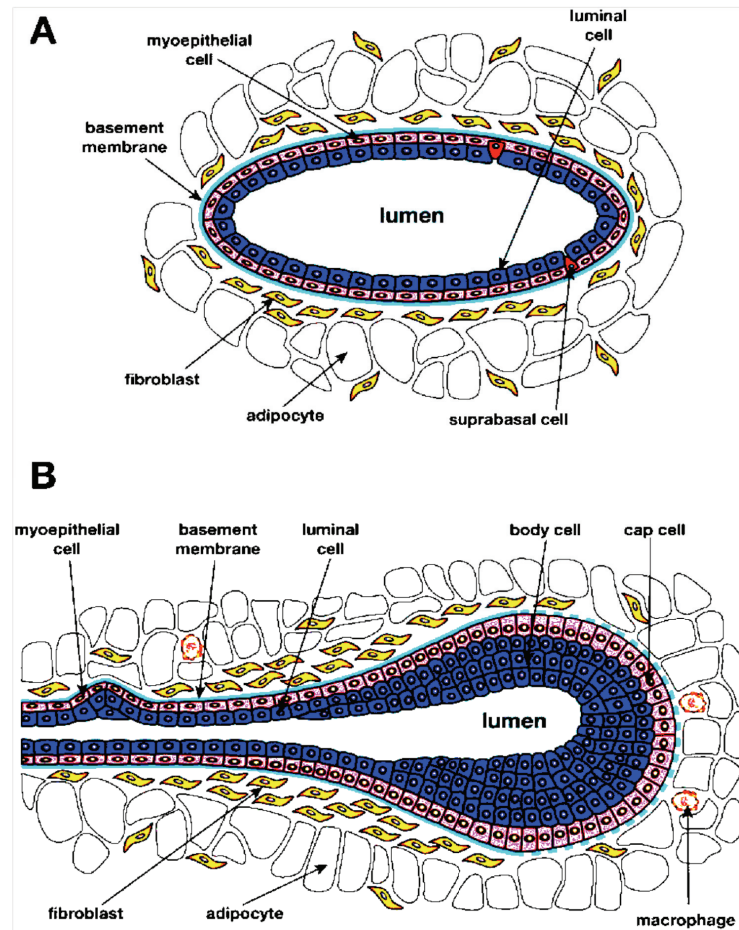


Figure 2. Structure a duct (A) and lobula (B).

Lanes annotate cells and structures respectively. For details see the text.

(Modified from J. E. Visvader, *Genes Dev.* 2009)

1.1.2 Risk factors for developing breast cancer

Several factors are associated with increased risk for development of breast cancer. These include mutations in genes of tumor promoting or tumor suppressing types; family history of breast cancer (genetic predisposition), hormonal status, dietary, stress, drugs intakes (hormone replacement therapy) and life-style (Pascual, 1982) and (Coyle, 2004; Moysich, Beehler, Zirpoli, Choi, & Baker, 2008).

Genetic factors have a strong impact on the development of the breast cancer. Many studies have shown an association between mutations in the BRCA1 and BRCA2 genes and the enhanced risk for cancer development (Proia et al., 2011). That includes also family history of the breast cancer that is characterized by specific frequent mutations of these genes (Cianchetti, E., R. Cotellesse, 1985) and (Wolff, 2006).

For women who do not have a genetic predisposition for BC development hormonal status is an important risk factor. Notably, an increased expression of the estrogen receptors (ER) is associated with a high risk for development of the breast neoplasia.

Psychological stress is another factor, which may suppress immune system allowing BC development by changing the status of the hormonal system. Perceived stress was proposed as one of the factors for breast cancer initiation, by chronic impairment of estrogen synthesis (Nielsen & Grønbaek, 2006).

It has been shown that a regular intake of antibiotics increased risk of incidence of breast cancer (Moysich, Beehler, Zirpoli, Choi, & Baker, 2008). One of the explanations is that this risk is due to suppression of the immune systems and increased expression of the prostaglandins upon exposure to antibiotic (Wei, Wolin, & G. Colditz, 2010).

There are reports that antidepressants also may significantly increase risk of having cancer, due to similarities of antidepressant chemical structure to the natural cellular regulators of growth (Moysich, Beehler, Zirpoli, Choi, & Baker, 2008). Antidepressants may in some cases suppress the immune system and increase level of the intracellular estrogen.

Higher numbers of the incidents of breast cancer appear in developed countries in Northern America (mostly in USA) and Europe (especially Northern Europe countries), as compared to other regions (Cianchetti, E., R. Cotellesse, 1985). Factors that decrease risk of the breast cancer are first pregnancy before age 20, low to moderate food intake, regular physical activity, menopause before age 45 and ovariectomy before age 35 (Kelsey, 1993).

Taking into account that the lifestyle in the modern world is full of persistent stress and increasing pollution, the number of cancer cases is expected to grow.

1.1.3 Clinical classification of breast cancer

Clinical classifications of breast cancer are based on the anatomical and histological features of the BC neoplasia. The TNM classification, which is characterized by the origin and behavior of the cancer such as the tumor size, lymph node involvement and the presence of distal metastasis is the most commonly used classification (Table 1). TNM considers the size of neoplasia, metastasis to lymph node and describes the tumor aggressiveness by presence of the distal metastasis in other parts of the body (Edge, 2010).

T, N and M categories for classification of the breast cancer

Primary Tumor (T):

TX: Primary tumor cannot be assessed
T0: No evidence of primary tumor
Tis: Carcinoma in situ (DCIS, LCIS, or Paget's disease of the nipple with no tumor mass)
T1: Tumor is ≤ 2 cm
T2: Tumor is >2 cm but <5 cm
T3: Tumor is >5 cm
T4: Tumor of any size growing into the chest wall or skin

Lymph Node Status (N):

NX: Nearby lymph nodes cannot be assessed
N0: Cancer has not spread to nearby lymph nodes
N1: Cancer has spread to 1 to 3 axillary lymph nodes, and/or tiny amounts of cancer are found in internal mammary lymph nodes on sentinel lymph node biopsy
N2: Cancer has spread to 4 to 9 axillary lymph nodes under the arm, or cancer has enlarged the internal mammary lymph nodes
N3: One of the following applies:

- Cancer has spread to 10 or more axillary lymph nodes
- Cancer has spread to the lymph nodes under the clavicle
- Cancer has spread to the lymph nodes above the clavicle
- Cancer involves axillary lymph nodes and has enlarged the internal mammary lymph nodes
- Cancer involves 4 or more axillary lymph nodes, and tiny amounts of cancer are found in internal mammary lymph nodes on sentinel lymph node biopsy

Metastases (M):

MX: Presence of distant metastases cannot be assessed
M0: No distant spread
M1: Spread to distant organs is present

Table 1. Breast neoplasia classification according to the tumor size, lymph node involvement, and presence of metastases (Modified from Hayes D, *TNM staging classification for breast cancer*. In: ed. *UpToDate*. Waltham, 2008.)

Another classification system that is used in the clinic is the Bloom-Richardson grade system. The factors are: gland-formation (tubularity), degree of variation in nuclear size and shape (pleomorphism), and 'hyperchromatic figures' as an estimate of proliferation (J. S. Meyer et al., 2005). When cells become differentiated, they acquire different shapes and forms to function as part of an organ. Cancerous cells lose that differentiation. In Bloom-Richardson cancer grading, tumor cells are generally classified as well differentiated (low grade), moderately differentiated (intermediate grade), and poorly differentiated (high grade). Poorly differentiated cancers have a worse prognosis. This grading system has been effective for making prognosis of tumor development.

A third classification scale is the Nottingham Prognostic Index (NPI), which consider histological grade, tumor size and presence of lymph node infiltrations. , In essence, NPI combines TNM classification and Bloom-Richardson gradation system.

Breast cancer: staging, prognosis and survival results

Stage	TNM	Relative Survival (5 y)
0	Tis N0 M0	99%
I	T1 N0 M0	92%
IIA	T0 N1 M0, T1 N1 M0, T2 N0 M0	82%
IIB	T2 N1 M0, T3 N0 M0	65%
IIIA	T0 N2 M0, T1 N2 M0, T2 N2 M0, T3 N1-N2 M0	47%
IIIB	T4 Any N M0, Any T N3 M0	44%
IV	Any T Any N M1	14%

Table 2. Five years survival rate for the patients diagnosed with a breast cancer at the different stages, regarding to the TNM classification.

(Modified from www.cancermonthly.com)

With respect to cell of origin, two major types of the breast cancer have been classified by origin from the lobules (LC) or ducts (DC).

Infiltrative ductal carcinoma (IDC) and infiltrative lobular carcinoma (ILC) account for more than 75% of the total cases of diagnosed breast cancer. IDC that arises in the duct of

the breast has a worse rate of survival of patients, as compared to ILC, which develops in the cells that line the milk-producing lobules.

Ductal Carcinoma In Situ (DCIS) is characterized by the presence of microcalcification and is believed to be a precursor for a future invasive breast cancer (IBC) with potential to metastasize. In more than 50% of cases of DCIS, without treatment, patients will develop infiltrating breast cancer (R. Axelrod, D.E. Axelrod, & Pienta, 2006) and (Sontag & David E Axelrod, 2005).

Several models were proposed to describe breast cancer carcinogenesis from normal tissue to DCIS to IDC. In 1995, Sontag and Axelrod analyzed four different pathways for development of neoplasia: “linear”, “non-linear”, “parallel” and “branched”. The results suggest the possibility that 2 components may diverge from a common progenitor in some tumors with both DCIS and IDC (Erbas, Provenzano, Armes, & Gertig, 2006).

All existing classifications of the breast cancer may be used in combination to achieve more accurate diagnostics and suggest relevant treatment.

1.1.4 Current treatment

There are several treatment options for patients presenting with breast cancer, these include surgery, radiation therapy, chemotherapy and (neo)adjuvant therapy.

During the surgery, malignant tumors would be removed with a part of the breast, including lymph nodes or whole breast, according to the volume and spread of the tumor. Surgery includes lumpectomy, quadrantectomy and mastectomy (Sharabi, Bullocks, Dempsey, & Singletary, 2010).

There are two primary types of radiation therapy: external beam radiation therapy and internal beam radiation, also called brachytherapy. In breast cancer, external beam radiation is much more common than internal beam radiation (Matsunaga et al., 2010).

Chemotherapy is mostly used to treat cancer and to prevent relapse of the neoplasia after surgery. Chemotherapy is more effective to treat breast cancer on the early stages. Chemotherapy drugs have been developed via trials of various compounds, and may have more general actions on the cell functions, as compared to targeting therapies. As an example, DNA damage-inducing drugs would act on proliferating cells via multiple

mechanisms initiated by DNA damage (Chuthapisith, J. Eremin, El-Sheemey, & O. Eremin, 2010). Targeting drugs, in contrary, would address a specific enzyme. Therefore chemotherapy has generally higher propensity to heavy side effects, as compared to targeting drugs. However, chemotherapy has been applied with certain success since years, and continues to be used in clinics.

Targeting therapies are often called for (neo)adjuvant therapy. For treatment of breast cancer have been developed drugs targeting ERs, EGF signaling and a number of intracellular kinases (Di Cosimo & Baselga, 2008). Estrogen receptors maybe blocked by removing the ovary in premenopausal woman with ER+ cancer, or can be blocked by drugs. Selective estrogen receptor modulators (SERM) are hormonal treatment used in women with estrogen receptor positive breast cancer. SERMs work by preventing natural estrogen from sending signals to the estrogen receptor. An example of SERMs is tamoxifen. Another way to block estrogen activity is drugs classified as aromatase inhibitors and used to block conversion of the androgens to estrogen (Bilynskyj, 2010; Lin et al., 2010).

Neoadjuvant therapy may be applied to the patient before main treatment (adjuvant therapy), to reduce the tumor size.

Often treatment comprises a combination of several therapies, and the goal of the treatment is to block the growth of the primary tumor and to prevent metastasis. In cases of metastasis, tumors may spread to the bones, liver and brain through accessing blood vessels. In the case of metastasis, a patient would require chemotherapy and the whole body irradiation. Drawbacks with conventional treatment are that they often rely on a higher dose of drugs and radiation. That may lead to enhance side effects, toxicity and may affect the whole body physiology.

1.1.5 Molecular mechanism of breast tumorigenesis

Tumors can arise from a single cell, accumulated and passing mutation from a generation to a generation of daughter cells. These mutations affect oncogenes and tumor-suppressor genes. Many of genomic changes occur in genes associated with cellular adhesion, motility, signal transduction, transcriptional regulation, cellular metabolism and DNA\RNA processing (Chittenden et al., 2008).

The genome condition of a neoplasia has a significant impact on tumor behavior. In breast cancer tumors, aneuploid and triploid tumors were associated with higher grade and bigger size in comparison to the diploid tumors (Kronenwett, 2006). Aneuploid and triploid tumors show an increased level of the genomic instability and accumulated mutations. They also have a significant potential to develop metastasis. Several genes and their products are involved in processes of tumorigenesis. The most explored genes are BRCA1, BRCA2, TP53, ER and PR.

The mutations for genes BRCA1 and BRCA2 are associated with higher risk for the lifetime developing breast or ovarian cancer (Moorman et al., 2010). Both BRCA1 and BRCA2 are involved in upholding of the genome stability. BRCA1 and BRCA2 are controlling the DNA damage repair. BRCA2 is associated with tumor suppressor genes, as tumors with BRCA2 mutations generally exhibit loss of heterozygosity (LOH) of the wild-type allele.

The BRCA1 protein interacts with other tumor suppressors, DNA damage sensors, and signal transducers and forms a large multi-subunit protein complex known as the BRCA1-associated genome surveillance complex (BASC). BRCA1 protein is involved in regulation of transcription, DNA repair of double-stranded breaks, and recombination. BRCA2 protein contains several copies of a 70aa motif called the BRC motif, and these motifs mediate binding to the RAD51 recombinase which functions in DNA repair. Mutations in this gene are found in more than 80% of the hereditary breast and ovarian cancers. Alternative splicing of BRCA2 plays a role in modulating its subcellular localization and physiological functions (Bertwistle & Ashworth, 1998; E. Y. H. P. Lee & Muller, 2010).

1.1.5.1 Molecular diagnostics

Molecular diagnostics rely on expression and activities of a number of genes and proteins. These genes and proteins have been described as potent regulators of breast tumorigenesis, and therefore have been found to correlate with development of breast cancer. Potential predictor markers are the expression of Her2/neu oncogene product, p53 protein, estrogen (ER) and progesterone (PR) receptors, estrogen-regulated protein, Cathepsin-D (cath-D), CD44 and Ki67. Also, a positive correlation was found between overexpression Her2/neu and p53 (Taneja et al., 2010).

Expression of the ER and PR has been correlated with type of the breast cancer and is used to choose treatment. A negative correlation between Her2/neu overexpression and ER has been shown. The Her2/neu overexpression was significantly associated with comedotype carcinoma. This indicates that tumor cells from a subset of DCIS, which includes comedotype carcinoma (Perin et al., 1996).

A correlation was observed between the expression of p53, Her2/neu, estrogen receptor (ER) and progesterone receptor (PR) in breast cancers (de Roos, de Bock, de Vries, van der Vegt, & Wesseling, 2007). It has also been shown that the overexpression of Her2/neu and p53 in patients with DCIS and IDC was associated with local recurrence (Esteve & Hortobagyi, 2004).

1.1.5.2 Molecular classification

Molecular classification of the neoplasias is based on the expression of specific molecules and on the status of the specific markers. For classification of the breast cancers several molecules have been chosen to monitor the development of neoplasia. The most commonly used in clinic markers for molecular classification are p53, Ki67, ER, PR, HER2/neu.

There are five types of the invasive breast cancers, based on gene expression profiling. That is including luminal (subtypes A and B), basal and null types. Expression of the ER, PR, HER2 and CK6 may be used to define molecular phenotype of cancer. Specifically, definition of the types is as follow: 1) Luminal A type: ER+, and/or PR+ and HER2-; 2) Luminal B: ER+, and/or PR+ and HER2+, 3) HER2 type: ER-, PR-, HER2+, 4) Basal-like type ER, PR, and HER2 are not expressed but CK5/6 and EGFR are positive, and 5) null type is characterized by no detection of any of the markers (Tamimi et al., 2008).

Invasive tumors were considered to be more of the luminal A type, compare to the DCIS that mostly belong to the luminal B type and are characterized by HER2/neu expressions. The basal-like tumors are often detected with more than 2 cm of the size, of the high grade and with the nodal involvement.

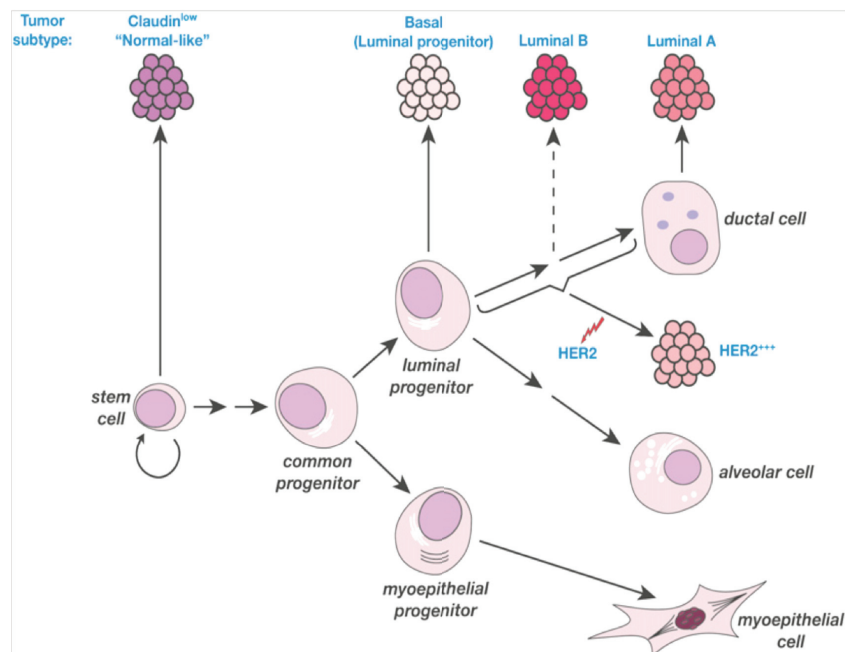


Figure 3. Molecular classification of the breast tumors subtypes that reflects the cells origin and related mutations.

This classification is based on the mRNA expression profiling
(from Jane E. Visvader, *Genes Dev.* 2009)

Human breast consists of several different types of the cells: luminal, basal and myoepithelial (Figure 3). To identify the cell origin of the tumor antibodies to the specific proteins have been used. Two major groups of breast cancers were proposed on the basis on the expression of the epithelial cytokeratins. For example, CK5 and CK14 are expressed in the basal cells of the stratified epithelium and CK8 and CK18 in the luminal cells (Morrison, C. W. Schmidt, Lakhani, Reynolds, & Lopez, 2008). Some authors classified breast cancer according to the expression of the CK-5 and CK17 at the RNA and proteins level (Gusterson, Ross, Heath, & T. Stein, 2005).

Several other genes and their products are involved in the process of the breast carcinogenesis. Expression level of the p53 protein has a strong impact on the neoplasia appearing and development. The p53 gene is classified as a tumor suppressor gene. The

p53 protein acts like a guard for the stability of genome by inducing the cell cycle arrest or apoptosis in case of damage of genomic DNA (Mills, 2005). Mutations in one allele of the p53 gene can lead to the functional inactivation of p53. This mutation presents in approximately 20% of the breast carcinomas, and was found in many human cancers (Jerry, Dunphy, & Hagen, 2010). Normal p53 protein has a short half-life and is present in the cell in low amount. Some of the mutations of p53 gene lead to the stabilization of nonfunctional form of p53 (Lane & Benchimol, 1990).

Bonin S. et al. (2008) described that retinoblastoma (pRB), cyclin-dependent kinase 2 (CDK2), cytokeratin 8 (CK8) and epidermal growth factor receptor 2 (HER2/neu) may affect clinical progression of the disease in 56 % of patients (Bonin et al., 2008). The high incidence of death within 5 years interval among patients with low pRB, high CDK2 level of expression and also with low CK8 and high HER2/neu was demonstrated (Bonin et al., 2008). A positive outcome of treatment against HER2 or CDK2 for patients with higher risk of early relapse was suggested.

HER1 protein is a transmembrane glycoprotein, a member of the EGFR protein kinase superfamily, and a receptor for members of the epidermal growth factor family ligands (Sassen et al., 2008).

EGFR2 (Her2/neu) gene encodes another member of the epidermal growth factor (EGF) receptor family of receptor tyrosine kinases. This protein has no ligand-binding domain of its own and therefore cannot bind growth factors. Her2/neu binds to other members of ligand-bound EGF receptor family to form a heterodimer. That leads to the stabilization of the ligand binding and enhancement of the kinase-mediated activation of downstream signaling pathways, such as mitogen-activated protein kinase and phosphatidylinositol-3 kinase. Overexpression of the Her2/neu gene has been found in many cancers, including breast and ovarian tumors (Taneja et al., 2010) and (Sassen et al., 2008).

Platelet-Derived Growth Factor (PDGF) family contains mitogenic factors for cells of mesenchymal origin. Mutations in this gene are associated with meningioma and skin tumor. PDGF expression has been found to positively correlate with tumor angiogenesis.

Therefore, there is a number of genes associated with development of breast cancer. Knowledge about functions of these genes has significantly improved management of breast cancer, including prediction, diagnostics and treatment. However, these genes and their products do not explain all clinical appearances of breast cancer, neither provide sufficient basis for therapeutic interventions.

1.2 TGF β -SIGNALING

1.2.1 Introduction into the TGF β -signaling

TGF β (transforming growth factor β) is a multifunctional peptide that controls proliferation, differentiation, and other functions in cells. TGF β 's mediate cells and tissue response to the injury, wound healing, controls carcinogenesis and immune system. It also acts as a negative autocrine growth factor in regulation of cell proliferation. Deregulation of TGF β activation and signaling may result in apoptosis and cancer promotion.

TGF β ligand binds to the type II receptor on the cell surface, that form a complex and phosphorylates type I receptor. The heterotetrameric complex of T β R-I and T β R-II phosphorylates R-Smads, which bind co-Smad4, relocates to the nucleus and activates transcription, by binding to the promoters of targeted genes (Derynck & Zhang, 2003; Shi & Joan Massagué, 2003).

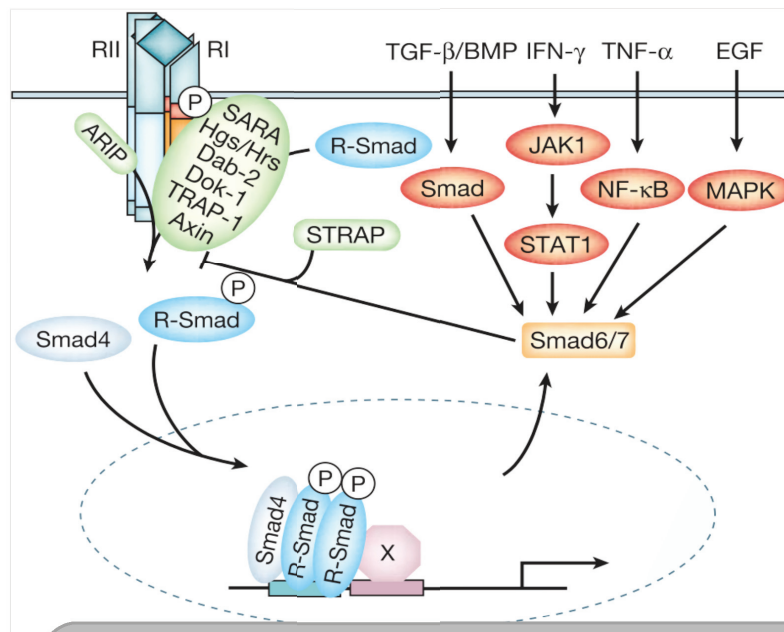


Figure 4. Schematic representation of the TGF β signaling. Smad-dependent and Smad-independent pathways are represented and annotated. (Derynck & Ying E. Zhang, *NATURE* 2003, reproduced with permission)

1.2.2 TGF β superfamily ligands

TGF β superfamily consists of more than 20 ligands (Bilezikjian, Blount, Donaldson, & Vale, 2006). They are divided on the TGF β , bone morphogenetic proteins (BMP) and activins. The largest numbers of ligands are represented by the BMP group, which contains potent regulators of development and differentiation. Activins are also primarily involved in the developmental processes.

TGF β is presented by 5 isoforms, with three isoforms found in human cells. TGF β 1, TGF β 2 and TGF β 3 all function through the same receptor signaling systems, i.e. TGF β receptors of type I and type II.

1.2.3 TGF β receptors

There are 7 type I receptors in TGF β superfamily, and 5 type II receptors. Among those, type I TGF β receptor (other name ALK5) and type II TGF β receptor (T β R-II) are known to transduce classical TGF β signaling. It has been reported that TGF β can involve ALK1 type I receptor in signaling in endothelial cell.

An important feature of activation of TGF β receptors is formation of a heterotetrameric complex of two type II and two type I receptors. The heterotetrameric complex has been found to be essential for transducing TGF β signaling. In this complex, kinases of all receptors are active. T β R-I is the receptor that phosphorylates down-stream R-Smad2 and R-Smad3. Homodimeric complexes of TGF β receptors have also been described, but their signaling impact remains to be explored (Derynck & Zhang, 2003).

The complex of activated type I and type II TGF β receptors phosphorylates a number of substrates, and initiates intracellular signaling pathways regulating transcription, protein synthesis, degradation and localization.

1.2.4 Smad-dependent signaling

The Smad family contains receptor-activated -R-Smads, Co-Smad4 and inhibitory Smads. R-Smads are receptor-activated Smads, and include Smad1, Smad5, Smad8 downstream of the BMP, and Smad2 and Smad3 downstream the TGF β and activin. Smads are recognized and phosphorylated on the C-terminus by type I receptor (T β R-I). This phosphorylation promotes formation of complexes with a co-Smad4 and other proteins. The inhibitory Smads (I-Smad) Smad6 and Smad7 are known to have an

opposite effect on TGF β signaling, by various mechanisms, with blocking phosphorylation of the R-Smads and translocation to the nucleus R-Smad/Co-Smad complex being considered as the main inhibitory mechanism.

1.2.5 Smad-independent signaling

Smad-independent signaling is a number of pathways that often were found in pathways of other regulators. TGF β can act by activating Erk1/2, p38, JNK and TAK1 (J Massagué & Wotton, 2000). A number of interacting proteins provided links to regulation of protein ubiquitylation. Another T β R-I interacting proteins indicate links to the negative regulation of TGF β signaling (Conrotto, I. Yakymovych, M. Yakymovych, & Souchelnytskyi, 2007). Thus, TGF β involved in a number of pathways that can influence practically all vital functions in a cell.

1.2.6 TGF β in cancer

TGF β was found to have a double role in tumorigenesis. At the early stage of cancer, TGF β inhibits tumor growth. However, at late stages of cancer, TGF β promotes tumorigenesis, and has a stimulatory effect on formation of metastasis. TGF β inhibits early breast cancer development by inhibition of the proliferation in mammary epithelial cells, inducing apoptosis, and suppressing motility. However, during carcinogenesis, and accumulation of various mutations, TGF β may promote epithelial to mesenchymal transition (EMT), and may induce tumor migration to the other organs (Yin, Chunyou Wang, T. Liu, Zhao, & F. Zhou, 2006).

TGF β affects nearly all cellular functions, often having both stimulatory and inhibitory effects, e.g. proliferation, apoptosis, differentiation and migration. TGF β affected phosphorylation of proteins involved in primary cellular metabolic processes, cell organization, development, differentiation, signal transduction, cell proliferation, cell cycle, cell death, transport and motility.

Systemic network analysis showed that TGF β -dependent phosphorylation might affect the cell cycle, cell death, metabolic processes, DNA damage repair, transcription, protein synthesis, degradation and metastasis. It has been shown, that Smad4 and Smad3 may stimulate metastasis into bones (Lamouille & Derynck, 2011). TGF β -dependent cell migration, invasion and metastasis are empowered by mutant p53.

Upregulation of TGF β 1 may be an early event that promotes further progression of breast tumors (Kang et al., 2005).

1.2.7 14-3-3 proteins

The family of 14-3-3 proteins have ability to bind large numbers of different proteins (Sassen et al., 2008). That makes 14-3-3 proteins to convergence points in multiple regulatory pathways, such as cell cycle, proliferation, differentiation, mitogenic activity, senescence and apoptosis (Hong et al., 2010), (Rajagopalan, Jaulent, Wells, Veprintsev, & Fersht, 2008). There are 7 isoforms of 14-3-3 proteins: beta, gamma, zeta, sigma, eta, theta and epsilon (Aitken, 2006). All of them, except sigma isoform, have an oncogenic potential (Zurita et al., 2010).

1.2.8 14-3-3 σ in cancer

14-3-3 σ - also known as a stratifin (SFN), is a scaffold protein that is known as an oncogene suppressor (Lodygin & Hermeking, 2005). SFN protein expression has been found to be suppressed in many types of the tumors (Hermeking, 2003; Li et al., 2010; Macha, Matta, Chauhan, Siu, & Ralhan, 2010; Wilker & Yaffe, 2004). SFN gene has also been found inactivated by methylation (Lodygin & Hermeking, 2005), (Toyota et al., 2009). SFN are involved in many signaling pathways that are crucial for the control of cell death, proliferation and metabolism (Wilker & Yaffe, 2004).

SFN can bind proteins that involved in signal transduction of p53, TGF β (Hong et al., 2010) and other pathways (Yang, Wen, C.-H. Chen, Lozano, & M.-H. Lee, 2003). Study of the humans' neoplasias, suggested, that underexpression of SFN protein were observed in most types of the neoplasias, however, in some tumors SFN is upregulated (Wilker & Yaffe, 2004) and (Li et al., 2010). Have been shown, that in many breast neoplasias, the low level of SFN protein correlate with poor prognosis (Dillon, Brown, Ling, Shioda, & Muller, 2007).

1.3 PROTEOMICS APPROACHES IN CANCER STUDIES

In the human genome about 30.000 genes are presented that expressed about hundred thousand proteins (Sawicki, Samara, Hurwitz, & Passaro, 1993), if to consider

more than 300 posttranslational modifications observed in proteins, a proteome may consist of about one million of the proteins. Proteomics is the study of the protein component of the genome. Proteomics has to handle very complex mixtures of proteins, by separating, identifying and characterizing hundreds of proteins.

The three main steps in a proteomics analysis are: separation of proteins, visualization of separated proteins and their identification of proteins. This may be followed by an exploration of post-translational modifications (PTMs), and by systemic analyses for the identified proteins (R. C. Stein & Zvelebil, 2002).

Several methods are used to extract and separate proteins from the complex mixture. To minimize complexity, pre-fractionations or separation by liquid chromatography (HPLC) are used. Samples may also require desalting, concentrations and depletion from the highly abundant proteins such as albumins and globulins.

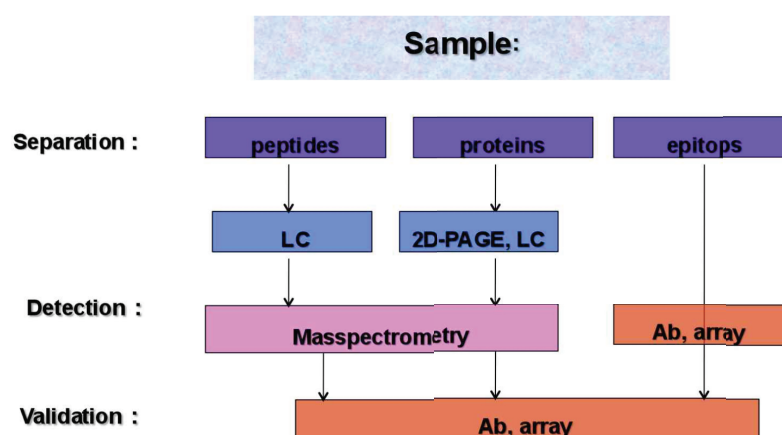


Figure 5. Proteomics workflows. Approaches to study proteins by analysis of proteins, peptides and epitops are shown.

Other method that is widely in use is 2D-PAGE based proteomics. In 2D-GE, proteins will be separated in an acrylamide gels according to their charge and molecular mass, and visualised by staining with silver or Coomassie brilliant Blue staining, and with fluorescent dyes.

After proteins were analysed and identified by mass spectrometry, all data will be processed by the systemic analysis software. That is a robust and complex analysis that will generate enormous amount of secondary data. Applied statistical, functional and other filters, will allow to extract most relevant to tumorigenesis data.

The major advantage of proteomics is the possibility of monitoring dynamic changes in protein expression and combine genetic and epigenetic influences. The contest in proteomics is the high level of complexity and large dynamic range of protein expression in cells, tissues or body fluids (Molloy & Witzmann, 2002). This makes it difficult to measure and analyze proteins in a comprehensive way. Additionally the concentrations of some biologically active proteins are low, and a large amount of biological sample-material is required (Figure 5).

1.3.1 Clinical proteomics

There are no two individuals that have an exact DNA signature and therefore there are no two people with the same proteomes. That is why a very crucial point is to define proteins that are related to the etiology of the disease and may affect treatment.

Clinical proteomics methodology includes sample selection, choice of technology for protein separation and identification, and quality control by validation studies (Apweiler et al., 2009) and (Monteoliva & Albar, 2004). This requires appropriate study design, with thorough sample collection, description, and analysis. For each experiment it is mandatory to establish protocols for the sample preparation and extraction of molecules of interest (such as DNA, proteins, metabolites, etc.) No doubt, it is important to extract maximum information from each sample, because samples are unique and not repeatable, as compared to cell lines. General clinical question need to be addressed to improve both the diagnostics and therapy of diseases. The first step is to improve the diagnostics but also the discovery of more specific biomarkers for disease (Figure 6).

The work starts with assessment of samples (tissues or cells), and includes protein extraction, separation by (2D-GE), gel image analysis and identification of cancer-related proteins, systemic analysis of identified proteins, and validation studies including tissue microarrays.

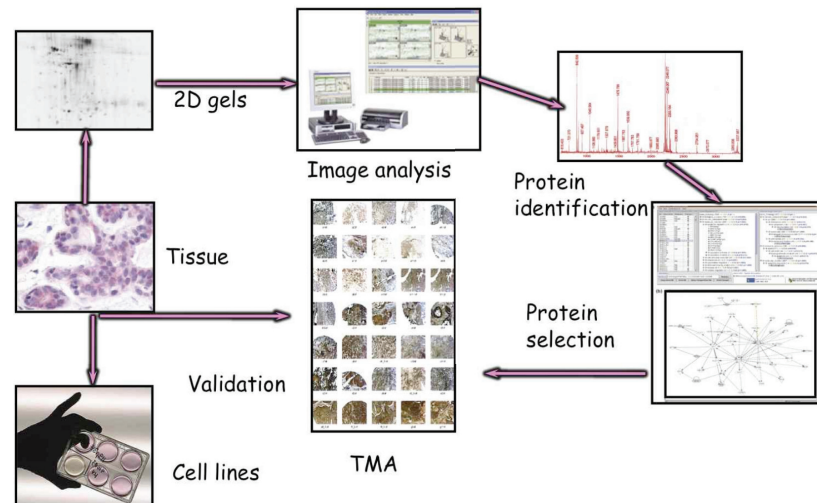


Figure 6. The workflow that were used in the clinical proteomics project. The main steps of the project are shown: sample collection, generation of the information by studying proteome, and analysis of the data and selection of the target for validation.

Significant amount of research carried out in the field of breast cancer focuses on the use of cell models. However, cell lines cannot fully reflect all changes, which appear in a tumor in the human body. Cells in culture will not be exposed to the same environment that is present in the organism, as we cannot observe all the proteins involved in carcinogenesis and the interactions between them. However, cell lines are still essential as a model for cell-signaling studies of breast cancer due to their reproducibility and possibility to manipulate expression of genes and proteins in cultured cells.

Human tissue models may be a more accurate way of reflecting changes that may occur during cancer initiation and development. Most studies about comparison between cancers versus normal have been carried out on cohorts of patients. In this way, individual differences have been ignored. However, these differences could be responsible for how some patients may respond to treatment, and account for differences in individual responses to an anti-cancer treatment.

1.3.2 Personalized medicine

The heterogeneity between the individuals is still not well understood. The anti-cancer drugs that are used today have severe side effects. The rate and the level of response may also vary significantly between patients (Figure 7). This may be due to the heterogeneity in tumors between individuals. Therefore, it's very important to decide what kind of treatment would be optimal for each patient. That strategy will require that optimization of the treatment is focused on each patient individually – a personalized medicine approach (Nevins et al., 2003; Tranin, 2005).

To introduce optimal treatment, therapeutic options need to be based on how well a patient may respond to a certain type of treatment. A little known about these factors, therefore, highly specific and sensitive markers are needed.

Successful prediction of the tumor recurrence risk in breast cancer patients is not available yet, as such predictions are based on averaged values and results of statistical correlations observed in large cohorts of patients. It is commonly accepted that the different clinical course of tumors with identical histology and stage may be the result of differences in regulatory processes at the molecular level (Foekens, Y. Wang, Martens, Berns, & Klijn, 2008; Koomen et al., 2008; Olopade, Grushko, Nanda, & Huo, 2008).

For successful treatments of breast cancer, it is important to know not only the type and grade of neoplasia, but also individual factors that may have an impact on the diagnostics and prognostics. Proteins markers can be useful tool for verification of diagnose and optimization of the treatment. Combination of tissue-proteomics and case-study approach has a great potential for identification of new markers for diagnostics of the breast cancer and prediction of a treatment outcome, which will leads to the higher rate of survival of patients (Burstein, 2009).

During the last 10 years a number of publications about personalized medicine has increased for more than 20 times (Jiang & M. Wang, 2010; Jordan, 2009; Jørgensen, 2009; Langreth & Waldholz, 1999). That confirms rapidly increasing interest for development of the new models and tools related to the diagnostics and treatments. Personalized medicine approach will leads to the improvements of the diagnostics and treatment, will help to minimize side-effects and overtreatment, and will aloud to make a more accurate prognosis for patients.

2 PRESENT STUDY

2.1 AIMS

General aims of the present here study are to unveil complexity of the cancer progression, focusing on the study of signaling implicated in the breast cancer development. Our main focus was on exploration and evaluation of the individual features of breast cancer.

The specific aims of the projects were:

- I. To optimize a procedure for sample preparation from the clinical samples (breast tissue), and to establish protein extraction protocol compatible with 2D-GE.
- II. To perform proteome profiling of human breast cancer and to determine cancer-related changes on the level of proteins. To study individual features of samples from the breast cancer patients, and their involvement in the regulatory pathways. To predict treatment outcomes for patients, based on the proteome profiling.
- III. To identify changes on the proteome level in a response to TGF β treatment in carcinogenic (MCF7) and non-carcinogenic (184A1) human breast epithelial cell lines. To explore signaling pathways activated by TGF β in these cells lines, that is crucial for regulation of cell proliferation.
- IV. To analyze the role of SFN (14-3-3 σ) in TGF β signal transduction and complex formation with Smad3, Cdk2 and p53 proteins. To identify phosphorylation sites in SFN, that are crucial for the complex formation and regulation of cell proliferation. This SFN phosphorylation may also have a role in cancer development. To explore potential role of SFN phosphorylation in tumorigenesis.

2.2 MATERIALS AND METHODS

2.2.1 Materials

In presented here studies, we used cultured mammalian cells and biopsies of human breast cancer. Clinical samples for the proteomics study the human material, were collected at Broomfield Hospital (Chelmsford, UK), under Ethical Permit 04/Q0303/28, issued by the North and Mid Essex Local Research Ethics Committee (Harlow, UK). Human breast tissues were snap-frozen in the liquid nitrogen directly after surgical procedure. Prior to start working with tissues, samples were stored at the -150°C , to prevent degradation of the protein.

2.2.2 Tissue sample preparation

To extract proteins from the breast tissue the protocol described in the paper I have been used. In brief, tissue were homogenized directly in the urea-contained buffer (Urea buffer: 8 M urea, 2 % (w/v) CHAPS, 50 mM DTT, 0.8 % (v/v) ampholytes (pH range of ampholytes depends on used strips), sonicated and centrifuged at the $+4^{\circ}\text{C}$ at 13.000 rpm, to clarify sample from the hard particles and non-dissolved proteins.

2.2.3 Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed in a two steps. First, proteins extracts were loaded on the precast (immobilized pH gradient) IPG-strips pH 3-10, and run with gradient voltage increasing (10 h – rehydration of strips with a sample, 2 h– 50 V, 1 h – 500 V, 1 h – 1000 V, and 10 h – 5.000 V). The second dimension SDS-PAGE was performed in an Ettan Dalt Six electrophoresis system (Amersham Biosciences, Uppsala, Sweden), until reached 35.000Vh. All gels were stained with 0.1 % silver nitrate.

2.2.4 Image analysis

To analyze gels and to identify differentially expressed proteins (spots) Master 2D Platinum 6.0 (GE Healthcare) software was used. Statistical significance of the reproducibility of spot expression in 2D gels, and differences in expression were evaluated with embedded statistical package. The Student's t-test was used to determine the statistical significance of the observed changes ($p < 0.05$). Proteins from 2D gels that

were shown to have either a unique expression pattern or exhibited changes by more than a 50 % increase or decrease in expression between tumor and histologically normal adjacent tissue were considered for identification.

2.2.5 Mass spectrometry

Protein spots were excised from 2D gels, destained and subjected to in-gel digestion with trypsin (modified, sequence grade porcine; Promega, USA), as described previously (Hellman, 2000).

Tryptic peptides were concentrated and desalted in ZipTip's μ C18 (Millipore, Billerica, USA). Peptides were eluted with 65 % acetonitrile, containing the matrix α -cyano-4-hydroxycinnamic acid, and applied directly onto the metal target and analyzed by MALDI TOF MS on a Bruker Ultraflex instrument (Bruker Daltonics, Bremen, Germany). Embedded software (FlexAnalysis; Bruker Daltonics) was used to collect and process mass spectra. Peptide spectra were internally calibrated using autolytic peptides from the trypsin (842.51, 1045.56 and 2211.10 Da). To identify proteins, searches in the NCBI nr (2010/05/10) RefSeq sequence database (NCBI, Bethesda, MD, USA; <http://www.ncbi.nlm.nih.gov>) were performed using the ProFound search engine (<http://65.219.84.5/service/prowl/profound.html>) and 'mammalian' for species search. Significance of the identification was evaluated according to the probability score and sequence coverage.

2.2.6 Systemic analysis

Protein names were translated into the "gi numbers" using NCBI web-based source. Functional and pathway analysis was performed using Ingenuity Pathway Analysis (IPA), a tool for description of networks and signaling pathways (<http://www.ingenuity.com>). IPAs' database contained only experimental data, which have been published and evaluated by independent researchers. This ensures that only confirmed results are taken into consideration for building a network. Experimental results that have not been reported by multiple laboratories or may have controversial interpretations were not considered for analysis. Such stringent selection of experimental data was required to exclude false-positive relations. Fischer's exact test was used to calculate a p-value determining the network connectivity.

2.2.7 Immunoprecipitation

For immunoprecipitation (IP), cell lysates were incubated with antibodies against target proteins and protein A/G-plus-Sepharose beads (Santa Cruz) over night at 4C⁰ with gentle agitation. Immunocomplexes bound to protein A/G-beads were collected by centrifugation and washed 3 times in the lysis buffer before being resolved by SDS-PAGE.

2.2.8 Immunoblotting

For immunoblotting (WB), cell lysates were resolved on 10%SDS polyacrylamide mini- gels (Mini-protean Tetra Cell, Bio-Rad) and transferred onto Hybond P membranes (Amersham Biosciences, Piscataway, NJ) in Tris-Glycine buffer 20% Methanol, on ice for 2h. Membranes were blocked with 5% (v/v) fat-free milk over night and then incubated with the primary antibody over night at 4C⁰. Membranes were washed 4 times by 10 minutes in TBX-T buffer and incubated with an HRP-conjugated secondary antibody (GE Healthcare, Uppsala, Sweden). The proteins were visualized using ECL-Advanced Luminol Reagents (GE Healthcare.).

2.2.9 Immunohistochemistry

For the TMA-slides, deparaffinisation of the slides were performed in a Xylene 60C⁰ before standard deparaffinisation and rehydration procedures. Antigen retrieval was performed using DakoCytomation target retrieval solution high pH (DAKO, Carpinteria, CA, USA). Arrays were stained with primary antibodies (used at a dilution 1:50 (v/v)). The slides were stained with VECTASTAIN Elite ABC kits (Vector Laboratories Inc Burlingame, CA, USA) following the manufacturer's instruction, and mounted with Mountex (Histolab, Sweden). The stained tissues were photographed using a Leica DFC camera and images were acquired with Leica QWin Standard software (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK).

2.2.10 Cell culture

We used followed cell lines: MCF10A, MCF7, 184A1, HEK1 and COS7. All cell lines were obtained from ATCC (Manassas, VA). MCF10A and 184A1 cells were cultured in a MEGM medium (GIBKO-BRL,) supplemented with EGF, insulin, hydrocortisone, bovine penicillin and streptomycin. MCF7, HEK1 and COS7 cells were cultured in DMEM, supplemented with 10 % FBS.

Result and discussions

2.2.11 Paper I

Optimized protocol for protein extraction from the breast tissue that is compatible with two-dimensional gel electrophoresis.

Proteomics is a highly informative approach to analyze cancer-associated transformation in tissues. The main challenge to use a tissue for proteomics studies is the small sample size and difficulties to extract and preserve proteins. The choice of a buffer compatible with the subsequent proteomics applications is also a challenge.

In paper I, the goal was to design a simple protocol with minimal amount of steps and a minimal-components buffer that is compatible with two-dimensional gel electrophoresis. This protocol is for an efficient extraction of proteins that can be useful for proteomics studies using the human breast tissue. We wanted to design a protocol that also may be useful for researches with less deep knowledge of the proteomics field.

There are several important issues that must be solved before starting a clinical proteomics project. These issues are about handling small quantities of clinical samples, efficiency of protein extraction, and removal of non-protein molecules, e.g. salt and lipids, that may interfere with protein separation.

The issue of small quantity of samples has an impact on ability to evaluate histopathological features of the sample subjected to the proteomics study. This requires that proteomics study with frozen tissue have to be made with the samples used also by pathologist for histopathological evaluation. The total quantity of proteins extracted from small size samples is low. That limits abilities of quantification of extracted proteins, as too much of the extracted proteins would be used for quantification. The solution to the quantification issues is to perform a small size SDS-PAGE and evaluate protein quantities, or use of protein quantification kits designed for work with small volumes of samples.

First of all, due to small amount of the material and no possibility of the repeats, there must be right choice of the extraction methods and buffer. The best efficiency of the extraction was observed when we used mechanical disruption of the tissue.

Extraction of proteins from tissues is the second issue to be considered. Most of the proteins in a tissue are in complexes with the others molecules. For an efficient extraction

and separation of proteins, protein complexes must be disrupted. This requires use of detergents and/or solubilization agents to break the bonds that molecules form in a complex.

The strongly charged detergents cannot be used because they may disturb isoelectrofocusing of proteins. The commonly used strong detergent for the protein solubilisation is SDS. However, SDS is not compatible with isoelectrofocusing. Therefore, zwitterionic molecules have a positive and a negative electrical charge on different sides of the molecule. The commonly used zwitterionic detergent is CHAPS. Also, urea is efficient in solubilization of proteins. DTT is often used to break disulphide bridges.

The third important issue is related to controlling non-protein components extracted from tissues. Extracts from tissues may contain such components as lipids, salts, nucleic acids, carbohydrates and other metabolites. These components may disturb separation during isoelectrofocusing, and some of them even during the second dimension SDS-PAGE.

We observed that the most problems with non-protein contaminants were caused by excessive presence of lipids in the breast tissue extracts. To remove lipids, an extensive centrifugation was applied. That allowed separation of a lipid layer from proteins in a water-layer extract. Salts could be removed by dialysis, and genomic DNA could be fragmented by ultrasound.

We explored also whether extraction with strong detergents, e.g. SDS and Triton X100, followed by precipitation of proteins and then their solubilization in 2D-compatible buffer would be an option. However, we found that the protein precipitation step significantly decreased efficacy of solubilization of proteins in the urea-containing buffer. Therefore, the most optimal protocol included careful histological evaluation of the samples by pathologists, and direct extraction in the urea-containing buffer with help of mechanical disruption of tissues. Prolonged centrifugation was found of importance to remove lipids. Other non-protein contaminants could be removed, if it would be required.

We considered also a need to remove highly abundant and/or plasma proteins. By using available on the market depletions kits, it is possible to remove proteins like globulins and albumin. We observed that the removal of highly abundant proteins was not essential, as these proteins did not interfere with 2D-GE. The depletion procedure may lead to losing of other proteins, and in additional steps in the protocol that may affect reproducibility. Therefore, depletion procedure is possible, but is not recommended.

Thus, we developed a protocol that is based on mechanical homogenization of tissues directly in the buffer that is compatible with two-dimensional separation of the proteins. Our data showed that this method is simple, robust and easy to apply in clinical proteomic experiments. Easiness of the protocol allows its use in clinical laboratories even at the places of primary health care, without the need of highly instrumental laboratory. We achieved a high quality for the proteins separations, without significant and detectable losses and degradation of the proteins. We believe that this protocol will be of help for clinicians designing a proteomics project

2.2.12 Paper II

Meta-data Analysis as a Strategy to Evaluate Individual and Common Features of Proteome Changes in Breast Cancer.

Variability of breast cancer is manifested on various levels, from a histological appearance to molecular mechanisms. Even smallest changes in a molecular composition of the proteome may lead to dramatic changes in functions of cells and in cancer development. To get insights into individual differences among human breast tumors is the most significant challenge for the treatment of breast cancer.

The goal of this paper was to develop a proteomics approach to understanding of individual features of human breast tumors. This individualization is dictated by the need of tools for personalized medicine. The cohort-based studies have shown limited success, due to the focus on features common to all studied clinical cases. We chose a two-step strategy to find individual differences and the common regulatory mechanisms that may underlie breast tumorigenesis. The first step is a full-scale proteomics analysis of individual cases on the case-by-case basis. The second step is analysis of the individual proteome-centered networks (meta-data analysis) (Figure 7).

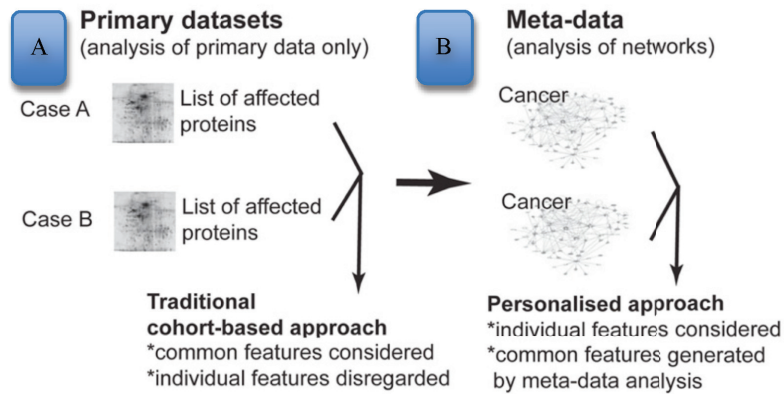


Figure 7. Representation of the common workflow for cohort-based approach (A) and personalised approach developed by us (B).

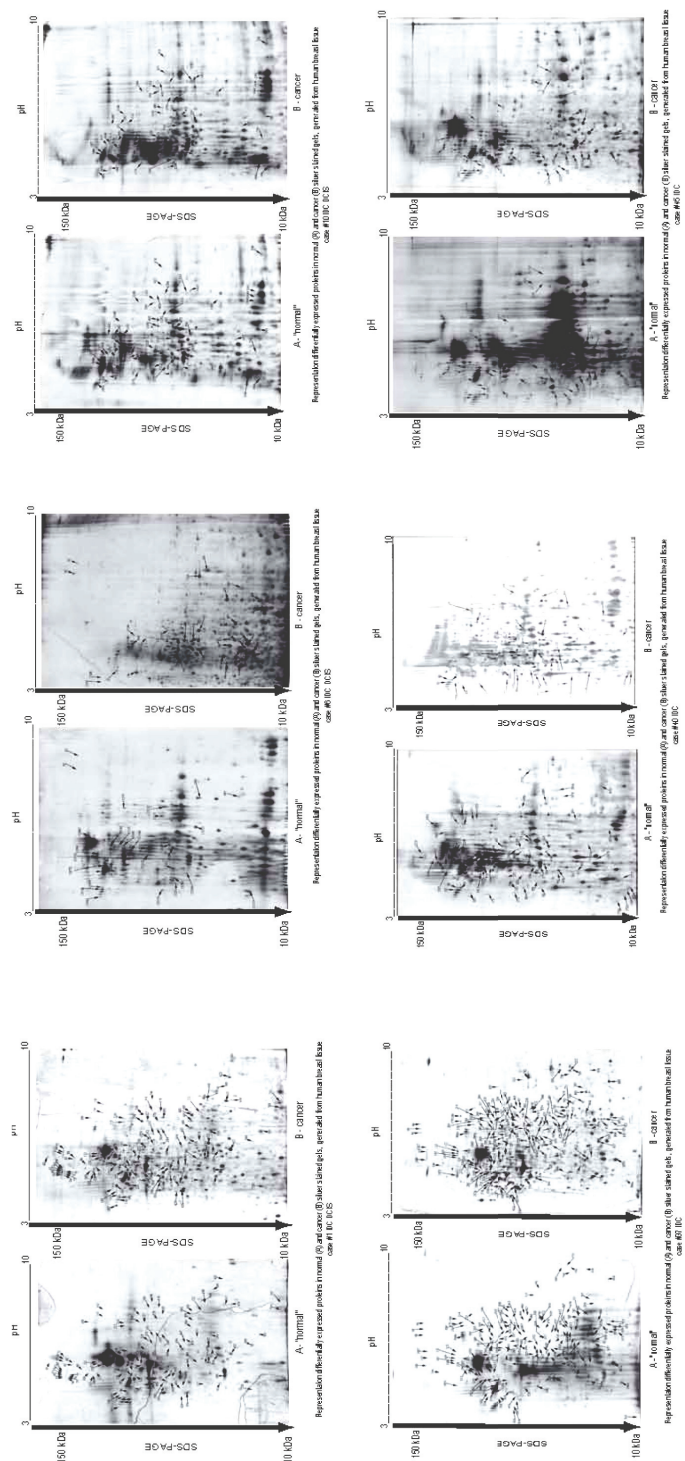


Figure 8. Heterogeneity of proteome profiles of different clinical samples. Images of 2D gels show examples of differences in the proteome patterns of the breast tissue, obtained from the differed patients. Images of 2D gels show silver stained proteins from tumors (B) and adjacent normal tissue (A). Images are clustered for each patient.

Proteome profiling of human invasive ductal carcinoma tumors was performed and each case was analyzed individually. We selected to compare tumor vs normal tissue for each case separately, and not to combine all tumor and all normal proteins. The overall pattern of protein migration in 2D gels was similar for all cases, with the majority of proteins having a tendency to shift into the area of gels corresponding to pI below 7.5. Some similarity of proteome patterns of all cases was observed. However, variability in the expression of tumour-related proteins was significant. This variability was much higher, as compared to proteome profiles of different human breast epithelial cells (Figure 8).

When we compared pools of proteins in combined tumor and combined normal categories, we found that differentially expressed proteins were mostly keratins (Figure 9A). This finding cannot be very useful to look into the molecular process of the neoplasia development. This approach leads to losing data related to the individual-specific features. That can discard regulatory mechanisms, which may be affected in a majority of samples via different components in different individuals. Proteome profiling of breast tumors, performed by others, has delivered lists of mostly high-abundance and structural proteins, e.g. keratins. This is mainly due to the averaging of primary datasets and the inability to interpret individual features. Therefore, we decided to develop a novel approach that allows exploration of individual features.

Molecular characterization of the types of breast tumors, which is different from the tumor grade system based on clinical data, was a strong confirmation of the variability of breast tumors at the molecular level. mRNA expression studies have provided signatures to discriminate patients with a worse prognosis and/or development of an aggressive tumor type, e.g. MammaPrint and Oncotype. However, their areas of application are limited, since the transcriptomics data could predict what may happen, but mRNA expression does not necessarily translate in an impact on functions.

Studies of breast tumorigenesis showed that there is a number of signaling regulating cancer. These signaling mechanisms are not fully considered in assessment breast cancer patients, because different molecules may control them in different tumors. Therefore it is challenging to obtain a list of the same signaling proteins in a large cohort, and the signaling pathways may be missed due to the variability of involved signaling components.

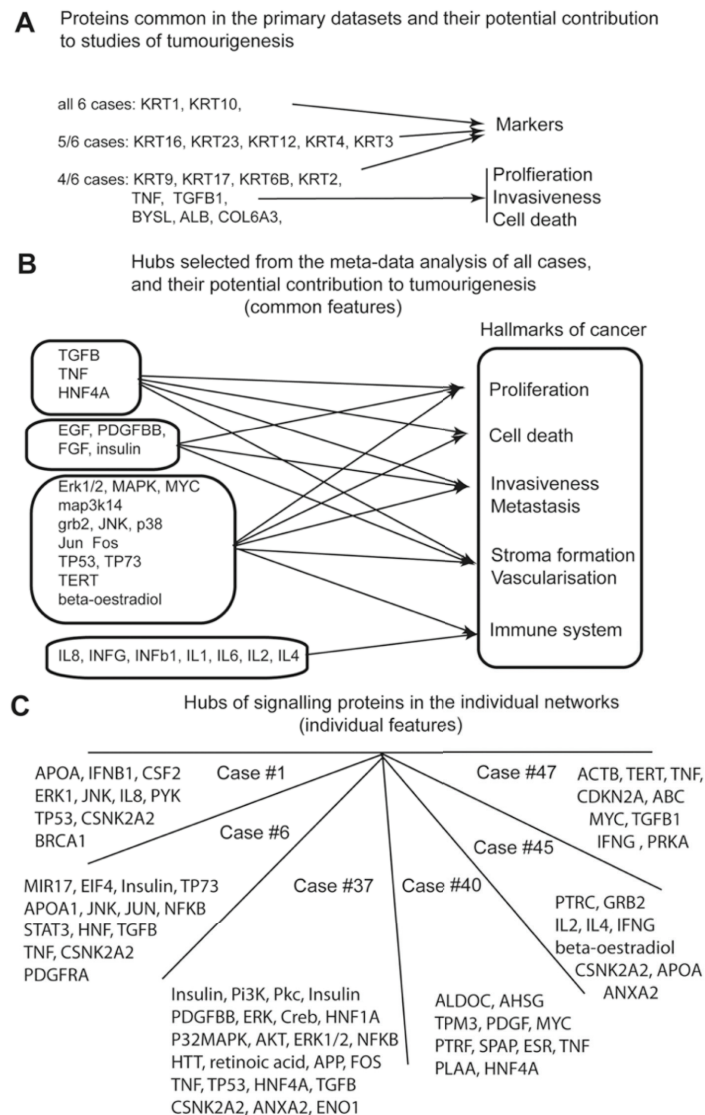


Figure 9. Prediction of common and case-specific proteins. Proteins identified as common for all cases in primary datasets (A), hubs of networks as frequently affected in many cases (B) and as case-representative (C), are shown. (A) Following a cohort-based approach, common proteins in the primary datasets were determined. The frequency of identification of proteins in each case is indicated. An impact on tumourigenesis was predicted by IPA and by the review of published reports (A, B). (B) Hubs selected upon analysis of meta-data (network-based information) are shown in 4 groups. The main impacts of each group are indicated by arrows. It should be noted that all crucial for tumourigenesis regulatory mechanisms are represented, e.g. cell proliferation, cell death, metastasis, regulation of stroma and immune system. (C) Selected hubs representing regulatory mechanisms in each studied case are shown. The annotation of proteins and hubs is in GO terms.

Our study showed that the meta-data analysis unveiled involvement of many well-known regulators of breast tumorigenesis. Notably, we observed effects on regulation of cell proliferation, death, migration, invasiveness, stroma formation and immune system. All these functions of cells are involved in tumorigenesis, and they were detected only because of meta-data analysis (Figure 9B).

Analysis of individual networks built with identified proteins predicted features and regulatory mechanisms involved in each individual case (Figure 9C). Validation of these findings by immunohistochemistry confirmed the predicted deregulation of expression of CK2 α , PDGFR α , PYK and p53 proteins. For example, CK2 α was found in a data analysis for the cases 1 and 6, and the IHC confirmed higher level of expression in these cases.

Our meta-data analysis approach may discriminate aggressive and non-aggressive tumors, despite the similar histopathological classification.

The findings from the analysis of the proteomic profiling and validation experiments reinforced the value of such a two-step approach for the development of more personalized approaches for diagnostics and a treatment. Notably, our results allow more efficient selection and application of drugs, and prediction of cancer development.

Further studies with a large cohort of patients are required to enable the introduction of this approach into the clinical practice. In future, we consider also comparing reported by us tumor profiling results with plasma profiling. That would be very useful for development of non-invasive diagnostics.

2.2.13 Paper III

Phosphoproteomic analysis of TGF β 1 signaling revealed importance of 14-3-3 σ phosphorylation for TGF β 1/Smad3-regulated transcription and CDK2/pRb-dependent cell proliferation.

Phosphoproteomics analysis of TGF β 1 signaling revealed importance of 14-3-3 σ phosphorylation for TGF β 1/Smad3-regulated transcription and CDK2/pRb-dependent cell proliferation.

Transforming growth factor- β (TGF β) is a potent regulator of tumorigenesis, although mechanisms defining its tumor suppressing and tumor promoting activities that are not fully understood. The goal of this study was to describe phosphoproteome profiling of TGF β signaling and explore one of the TGF β targets, 14-3-3 σ protein in TGF β signaling during cancer development.

We identified 60 TGF β -regulated phosphoproteins, using Fe-IMAC enrichment of phosphorylated proteins, 2D-GE and MALDI TOF mass spectrometry.

Phosphorylation of the enriched proteins was confirmed by staining of 2D gels with Pro-Q Diamond phosphor-stain, and by comparing of Fe-IMAC 2D gels with 2D gels of separated 32 P-labeled proteins. The used by us approach allows to study full-length phosphorylated proteins.

As a potent regulator of cell physiology, the TGF β would be expected to affect many signaling mechanisms. We performed a systemic study of the identified proteins that are included in a regulatory network. We observed that the identified proteins form a network with scale-free characteristics, without obvious hierarchic structure. However, presence of highly connected nodes at frequency higher than expected by a power law specified signaling sub-networks targeted by TGF β 1.

The network highlighted interactions that may distribute signaling inputs to regulation of cell proliferation, metabolism, differentiation and cell organization. Novel convergence species for TGF β and EGF, TNF, IGF and IL8 signaling were identified by the network analysis (Figure 10; see also the paper III text).

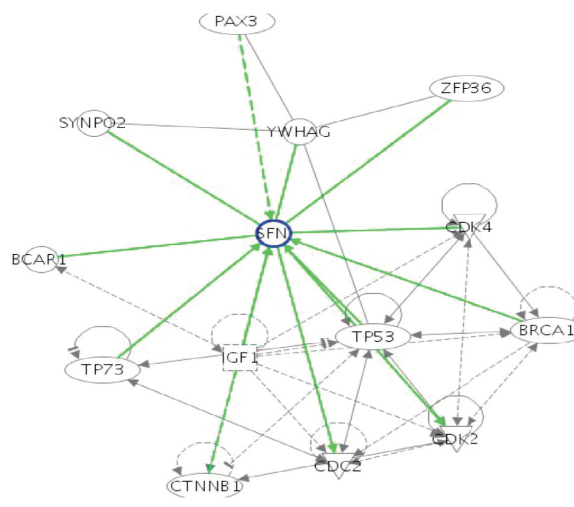


Figure 10. Signalling network centred on SFN protein. Green lines indicate direct interactions of the proteins. Proteins are annotated in GO term. Arrows indicated direction of interaction.

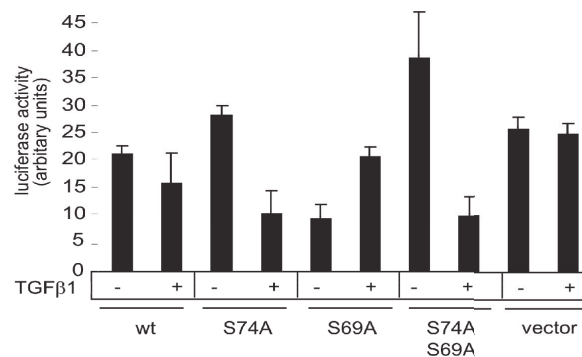


Figure 11. Role of SFN phosphorylation at Ser 69 and Ser74 in transcriptional activation of E2F-dependent luciferase reporter. Cells were transfected with empty vector, wild-type (WT) and mutants (S69A, S74A) of SFN. The SFN-mutants S74A and S69A show an opposite impact on the reporter.

To explore in details one of the identified targets, we focused on 14-3-3 σ protein.

14-3-3 σ protein has been reported as a potential tumor suppressor in breast cancer.

We hypothesized that the observed by us phosphorylation of 14-3-3 σ may have an impact

on breast carcinogenesis. First, we identified TGF β -dependent phosphorylation sites in 14-3-3 σ as Ser69 and Ser74. Experimental study of a 14-3-3 σ -centered sub-network showed that phosphorylation of 14-3-3 σ at Ser69 and Ser74 has a dual role in TGF β signaling. The first role is a feed-forward mechanism for TGF β 1/Smad3-dependent transcription, with recruitment of tumor suppressor p53 into a Smad3-14-3-3 σ complex leading to accentuating TGF β 1-dependency, e.g. p53 inhibited spurious ligand-independent but enhanced ligand-stimulated transcription. Our results showed that 14-3-3 σ is the scaffold for attracting p53 in TGF β /Smad3-dependent transcription. To our knowledge, this is the first observation of such cooperation between Smad3, p53 and 14-3-3 σ .

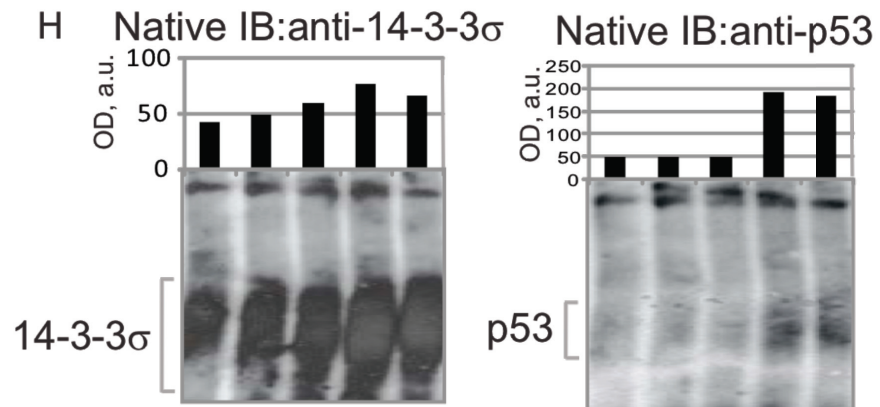


Figure 12. Blue-native gels show dynamic of the complexes formation between SFN and p53 proteins, in response of the TGF β treatment. Bars represent intensity (in arbitrary units) of a signal from the 14-3-3 σ (left panel) and p53 (right panel) proteins. Shift by molecular weigh is an indication of the complexes.

The second role of the observed TGF β 1-dependent phosphorylation of 14-3-3 σ was found to enhance complex formation between CDK2 and 14-3-3 σ .

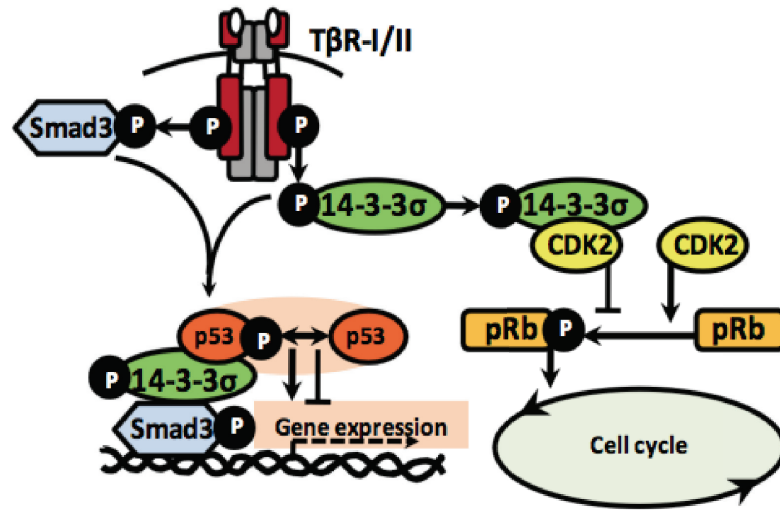


Figure 13. Model for TGF β 1-dependent phosphorylation of 14-3-3 σ and further functional interaction of TGF β /Smad3 with p53 and CDK2.

Our data suggest that TGF β 1-dependent phosphorylation of 14-3-3 σ orchestrates a functional interaction of TGF β /Smad3 with p53 and CDK2 (Figure 13), and could provide a new potential target for intervention in breast cancer. Moreover, recently it was found that 14-3-3 σ could contribute to drug resistance in human breast cancer cells by CDK-dependent mechanism. This finding together with the results of this study, suggests that TGF β 1-dependent phosphorylation of 14-3-3 σ may be considered for prediction of response to anticancer therapy in the clinic, and underscores the utility of profiling individual tumors.

Proteomics-based network signaling by TGF β 1 in 184A1 non-tumorigenic human breast epithelial cells, and its role in phosphorylation of p53 at Ser392 and regulation of cell proliferation.

It has been shown that TGF β has a double role in tumorigenesis, and that the responsiveness to TGF β may vary in cells on different stages of carcinogenic transformation. This indicates that TGF β signaling may differ in immortalized cells as compared to metastatic. As a contribution to exploration of differences in TGF β signaling in breast tumorigenesis, we studied proteome expression profile in 184A1 cells treated with TGF β 1. 184A1 cells are immortalized human breast epithelial cells that do not form tumors in mice.

Proliferation of 184A1 cells is transiently inhibited by TGF β 1 to the 50 %, at maximum. We performed proteome profiling using 2D gels, image analysis and MALDI TOF mass spectrometry. We identified 94 and 51 proteins which changed their expression and/or 35S-incorporation, respectively, upon treatment with TGF β 1 for 2 h, 8 h or 24 h. Cell proliferation, death, migration and metabolism were among main cellular functions affected by the identified proteins. The variety of affected cellular activities is expected, as other proteomics works showed that TGF β initiates a number of signaling mechanisms.

To understand whether TGF β -affected functions in 184A1 cells differ from functions affected in more transformed cells, we performed systemic analysis of the identified proteins. As we expected, analysis of networks formed by the identified proteins highlighted potential differences in TGF β 1 regulatory mechanisms in non-tumorigenic breast epithelial cells, as compared to tumorigenic cells. The network analysis suggested involvement of SSRP1, PC4, CK2 α and p53 in regulation of cell proliferation (Figure 14).

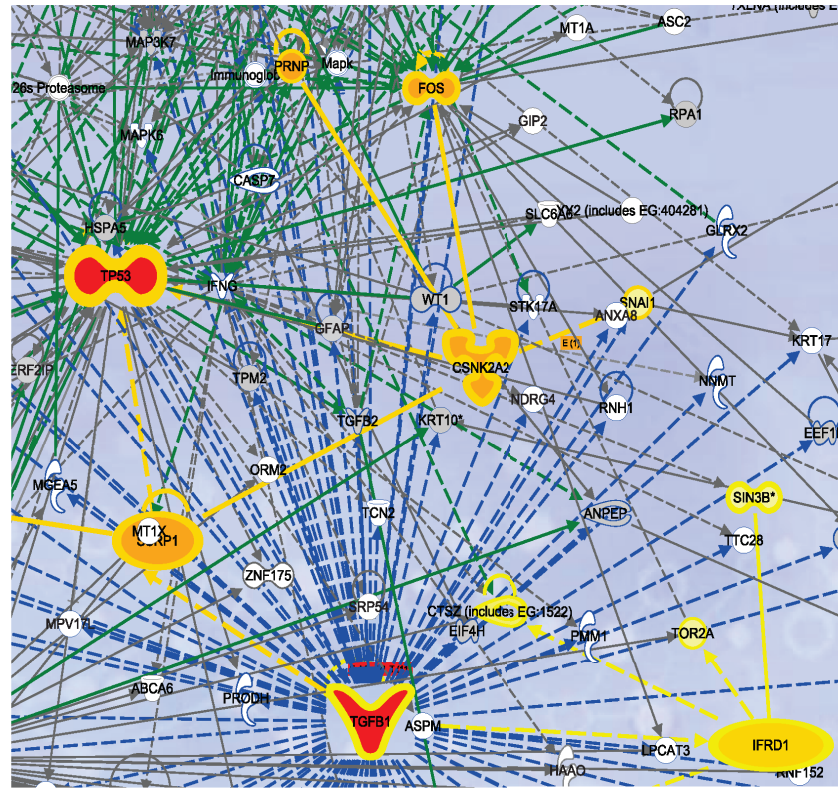


Figure 14. Fragment of the network formed by the identified proteins that were regulated by TGFβ1 in 184A1 cells. Yellow lines indicate interactions related to p53, SSRP1, CK2α and PC4. For detailed description, see the paper IV text and figures.

To explore impact of CK2α, SSRP1 and PC4 on cell proliferation, we interrogated a proliferation sub-network by manipulating expression of CK2α, SSRP1 and PC4. We confirmed predicted impact on p53 phosphorylation, and effects on cell proliferation and apoptosis.

Our results showed that TGFβ signaling might vary in different types of cells, and we described a proteome profile of TGFβ-dependent changes in 184A1 cells. These variations may be of importance for evaluation of TGFβ role in tumorigenesis. Our results open also up for further explorations of TGFβ signaling in cancer, by providing a list of novel TGFβ targets. Our results may be of help in development of anti-cancer treatment methods with inhibitors of TGFβ type I receptor kinase that currently enter clinical trials

2.3 CONCLUSIONS

Described by us results showed feasibility of application of proteomics to individualization of treatment of breast cancer patients. We expect that the combination of tissue-proteomics and case-study approach has a great potential for identification of novel markers for the diagnostics of breast cancer.

The case-by-case approach for proteome profiling of IDC and ILC will help to identify individual changes in protein expression and/or modifications. This will lead to discoveries of novel proteins involved in the processes of malignant transformation in human breast cancer, and also to understanding of new functions of proteins already found to be involved in cancer development.

We believe that our research will lead to a better understanding of the mechanisms involved in cancer development, through the use of human tissues and cell lines. We also expect, that our result will lead to better diagnostic and treatment options for patients.

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I

Optimized Protocol for Protein Extraction from the Breast Tissue that is Compatible with Two-Dimensional Gel Electrophoresis

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Abstract: Proteomics is a highly informative approach to analyze cancer-associated transformation in tissues. The main challenge to use a tissue for proteomics studies is the small sample size and difficulties to extract and preserve proteins. The choice of a buffer compatible with proteomics applications is also a challenge. Here we describe a protocol optimized for the most efficient extraction of proteins from the human breast tissue in a buffer compatible with two-dimensional gel electrophoresis (2D-GE). This protocol is based on mechanically assisted disintegration of tissues directly in the 2D-GE buffer. Our method is simple, robust and easy to apply in clinical practice. We demonstrate high quality of separation of proteins prepared according to the reported here protocol.

Keywords: 2D-GE, breast tissue, sample preparation, SBO4 buffer

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Introduction

Proteins are main components of the organisms. They control many different and important functions related to the cell proliferation, differentiation and death. Changes in the protein expression and structure affect protein's functions.¹ A number of reports have shown that even small changes in protein functions may lead to a disease development.² The ultimate role of proteins in tumorigenesis has also been reported.³ Proteins are primary targets of anti-cancer drugs and the source of cancer markers.⁴

Tissue is a perfect source for monitoring changes and modifications of proteins that are related to carcinogenesis. However, there are pitfalls in using tissues for proteome analysis. For the first, the breast tissue is not a homogenous substance, but consists of a number of various cells and extracellular matrix, such as stromal and blood cells, adipocytes, collagen, etc. For the second, non-protein elements are present in tissues. In the case of the breast tissue, the high content of lipids may have a strong impact on protein extraction and separation.

Therefore, several issues must be solved for an efficient preparation of proteins from tissues: 1) maximal efficiency of extractions and 2) removal of non-protein components must be achieved, and 3) extracted proteins have to be under controlled conditions to ensure their suitability for a proteomics study in terms of concentration and stability upon storage. Table 1 presents these 3 challenges, and suggests how they may be solved.

For proteomics applications, proteins have to be solubilized in buffers compatible with separation techniques. For two-dimensional electrophoresis, it has to be a low-conductivity buffer, e.g. urea containing buffer. For shotgun applications, proteins are digested directly by a protease, and therefore have to be solubilized in a digestion-compatible buffer, e.g. ammonium bicarbonate buffer. It is known that the best solubilization of proteins requires strong detergents, e.g. SDS, in a buffer containing salts, such as Tris-HCl buffers and NaCl. Such solubilization conditions are not compatible with a direct analysis of extracted proteins by proteomics techniques. Protein purification procedures often lead to losses of proteins. Especially significant losses can be upon protein precipitation followed by a solubilization from a pellet, or upon an extensive dialysis. Therefore, there is a need for a robust and easy protocol

for extraction of proteins, which would be compatible with protein separation by 2D-GE. Here we report a protocol which is easy to use and allows protein solubilization directly in a buffer compatible with 2D-GE.

Materials and Methods

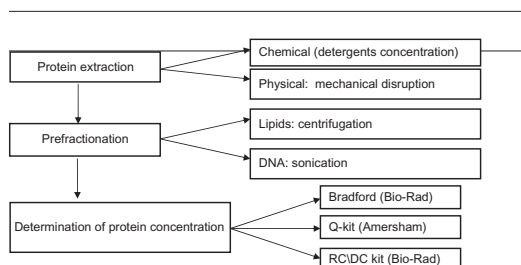
Studied samples

Cell line K562 (human myeloid leukemia) was obtained from ATCC (Manassas, USA). As a control tissue, we used a commercially available fresh frozen chicken liver. Breast tissue was collected at Broomfield Hospital (Chelmsford, UK), under Ethical Permit 04/Q0303/28, issued by the North and Mid Essex Local Research Ethics Committee (Harlow, UK). Clinical samples were collected immediately upon surgery and stored on wet ice before being dissected by a pathologist. Samples of breast epithelial tissue were immediately frozen in liquid nitrogen. K562 cells were used as a control to determine an efficiency of proteins extraction and a quality of proteins separation, as compared to the tissue. Frozen chicken liver tissue was used as a model to optimize the protocol for tissue extraction in terms of exploring an impact of interfering detergents, salts and any other chemicals in used buffers and solutions. As frozen chicken liver is available commercially, it allowed performing a significant part of trials without using the human breast tissue. The final optimization and validation of the protocol was performed with an aliquot of the human breast tissue.

Composition of buffers

1% Triton X-100 buffer contained 1% Triton X-100, 20 mM Tris-HCl pH. 8.0, 150 mM NaCl and Protease Inhibitor Cocktail "Complete" (Roche) as recommended by the supplier. RIPA buffer contained phosphate buffer

Table 1. Sample preparation from the tissue for 2D-GE experiments.





saline (PBS), 0.1%, SDS, 1% NP-40, and 0.5% sodium deoxycholate. Urea buffer contained 8 M urea, 2% (w/v) CHAPS, 50 mM DTT, and 0.8% (v/v) ampholytes.

Protein concentration measurement

Bradford reagent (Bio-Rad), 2D-Quant Kit (Amersham/GE Healthcare) and RC/DC Kit (Bio-Rad) were used as recommended by the suppliers.

Two-dimensional gel electrophoresis

2D-GE was performed as described earlier.⁵ In brief, total amount of proteins loaded was from 50 µg to

100 µg per an IPGstrip. Proteins were solubilized in the rehydration buffer containing 8 M urea, 2% (w/v) CHAPS, 50 mM DTT, 0.8% (v/v) ampholytes (pH 3–10), and were loaded on the 18 cm NL-IPG strips pH 3–10, (GE Healthcare, Uppsala, Sweden). Strips were rehydrated in the urea-containing buffer for 12 hours. Isoelectrofocusing (IEF) was performed with stepwise increasing voltage as follows: 50 v for 10 min, 100 v for 30 min, 500 v for 1 h, 1000 v for 1 h and 5000 v for the time needed to reach 35,000 Vh. After isoelectrofocusing was completed, strips were equilibrated for 15 min as described earlier.⁶ 12% PAGE was performed

Table 2. Detailed description of the protocol and procedure.

Step by step protocol (overview)	<ol style="list-style-type: none">1. A piece of tissue (about 1 mm³) is added to the extraction urea-containing buffer and glass beads. Buffer must cover the sample completely.2. Tissue is disrupted first by vortexing.3. To disintegrate the tissue further, perform sonication for 30 min, tube with sample must be kept on ice during procedure to avoid heating of the sample.4. Centrifugation at 13,000 g for 30 min on +4 °C is performed to clarify sample and to provide better compression of the pellet and better concentration of lipids in the top layer of the sample.5. After collection of the supernatant, concentration of the protein was determined with the use of a RC/DC kit.6. 80–100 µg of proteins is loaded on the IPG strip for 2D-GE.		
Steps	Description	Reagents and tools	Troubleshoots
Evaluation of a sample	Clinical samples have to be evaluated by a pathologist. Quantity and quality of cellular, stromal and other histological features have to be evaluated.	Light microscope.	Selection of a non-appropriate sample will affect results of the experiment.
Extraction of proteins from the tissue	Tissue must be homogenized, proteins extracted and separated from the pellet. Sonication can be performed in steps, eg, 3 times of 10 min each. It is important that the sample is not heated upon extraction. Centrifugation can be repeated to improve separation from lipids and the pellet.	Urea buffer: 8 M urea, 2% (w/v) CHAPS, 50 mM DTT, 0.8% (v/v) ampholytes (pH range of ampholytes depends on used strips). Glass beads can be added in a proportion of 1/3 (beads/sample; v/v). Water-bath, sonicator, a centrifuge with cooling.	Particles, lipids and impurities in the sample lead to the distortions of protein separation during 2D-GE. During extraction procedure, it is important to control temperature. Especially during sonication and centrifugation. Urea may crystallize at low temperature (eg, +4 °C), while temperature higher than +20 °C may induce degradation processes in a sample. Shorter than 30 min centrifugation time may not be sufficient for efficient separation.
Evaluation of protein concentration	Optimal concentration of the protein required for achieving good quality of 2D gels. Usually, 80–100 µg of protein is enough to prepare one 2D maxi-gel (20 cm × 20 cm).	RC/DC kit or a similar kit.	Overload with proteins will make analysis of the separated proteins difficult due to very intense staining. If concentration is too low only few protein will be visualized in the 2D gels.



at 70 W/6 gels for 7 hours. Proteins were visualized by staining with 0.2% silver nitrate as described.⁷

Results and Discussion

Table 2 presents the optimized protocol for extraction of proteins from breast tissue in a solution compatible for 2D-GE. Briefly, the protocol consists of 3 following steps: 1) evaluation of a sample, 2) extraction of proteins, and 3) evaluation of a quantity of extracted proteins.

The first step in this protocol is an evaluation of the sample by a pathologist. This is required for an estimation of the presence of the cellular and stromal components. Specifically, relative volume of malignant cells, tumor fibroblasts, adipocytes, cells of the immune system and vessels have to be evaluated. The sections have to be taken from the same sample that is prepared for 2D-GE. As a rule, the part of the malignant tumor cells has to be not less than 50%. It also has to be noted whether there are any areas of necrosis or macrophage infiltration, which should be excluded from proteomics analysis. The necrosis is often manifested as areas with destroyed tumor cells, fragments of cells and infiltration of macrophages. Macrophages can be identified by their specific staining pattern and morphology, eg, strong staining with Hematoxylin-Eosin and appearance as multiple dots in sections. Such evaluation has to be performed by a trained pathologist. The sections of the biopsies have to be stored for a validation study, when expression of proteins of interest identified by proteomics would be monitored by immunohistochemistry with specific antibodies in the sections of the same sample.

The first step in protein extraction from the tissue is selection of an optimal extraction buffer. We prepared experiments with buffers that contained different detergents and concentration (Fig. 1), because different detergents solubilize cells membranes with different efficiencies.^{8,9} Extraction conditions (time, treatments) were similar for all tested conditions. For composition of tested buffers, see the materials and methods section. The output of experiments was monitored by intensity of prepared and separated in 1D SDS-PAGE gels proteins, stained with coomassie blue R-250 (Sigma). To evaluate the maximal level of protein extraction, a similar sample was extracted using SDS. SDS provides maximal extraction, but is

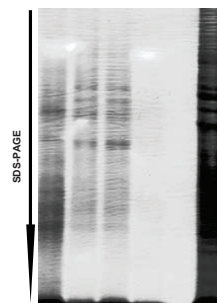


Figure 1. Optimization of protein solubilization and recovery in urea-containing solution. 1D SDS-PAGE of proteins prepared by direct extraction (a, e), or by extraction, precipitation and re-solubilization (b–d). Proteins were prepared by a) direct extraction in the urea-containing solution. For other extraction/precipitation/re-solubilization, proteins were b) extracted in 1% Triton X100-containing buffer, c) in RIPA buffer, d) in 1% SDS, followed by precipitation with 20% acetic acid and 40% methanol, and solubilization in the urea-containing solution. e) As a control of maximal extraction, proteins were extracted directly with 1% SDS. Separated proteins were stained with Coomassie Brilliant Blue R-250.

not compatible with 2D separation due to SDS interfering with isoelectrofocusing of proteins.¹⁰ We estimated the yield of proteins by comparing quantities of proteins extracted using our protocol and a direct boiling in 2.5% SDS-containing electrophoresis sample buffer, followed by 1D SDS-PAGE. An efficiency of protein extraction was evaluated as a quantity of proteins detected in 1D SDS-PAGE. We observed that the highest efficiency of protein recovery with 2D-GE-compatible solution was in the case of direct extraction with urea buffer (Fig. 1).

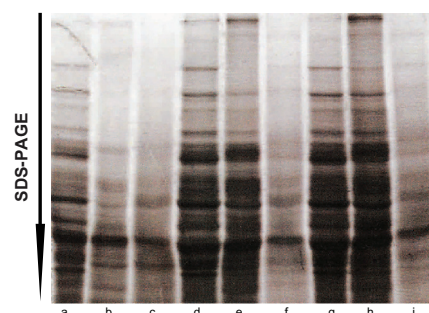


Figure 2. Mechanical disintegration enhanced extraction of proteins. Extraction of proteins from fresh frozen chicken liver was monitored after extraction of proteins after sonication only (a–c), after treatment with glass beads only (d–f; vortex only) and after sonication with glass beads (g–i). Lanes a, d and g show proteins extracted directly with SDS. Lanes b, e and h show separated proteins extracted with the urea-containing buffer, and lanes c, f and g show proteins extracted with SDS from the pellet after urea-buffer extraction.

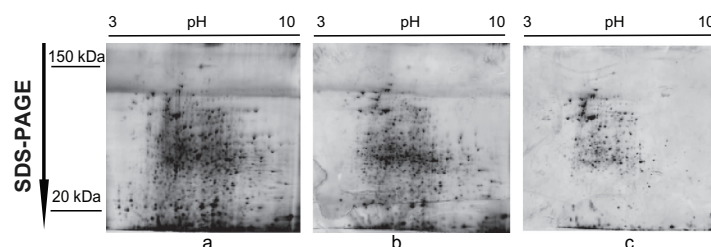


Figure 3. No disturbance of protein separation in 2D gel electrophoresis of clinical samples prepared according to the described protocol. Various quantities of proteins prepared from human breast tumors separated by 2D-GE. Note lack of distortions in protein migration. Protein quantities were 100 µg (a), 85 µg (b) and 70 µg (c). Gradient of pH and migration of molecular mass markers are shown. Gels were stained with silver.

We performed tests to find optimal conditions for extraction, separation and clarification of the proteins extracted from the tissue (Fig. 2). We optimized further condition of the extraction using a tissue sample (frozen chicken liver). This tissue was available commercially, and allowed extensive optimization experiments. The best results were achieved by combining disruption of the tissue with glass beads, sonication on ice and prolonged time of the centrifugation (Table 2). The use of glass beads significantly improved disintegration of tissue, as compared to vortexing or extraction by end-to-end mixing (data not shown). Sonication up to 30 min was found to be important for an efficient separation of the lipid fraction that was observed as a layer on the top of the protein extract. Shorter sonication times were less efficient (data not shown). Prolonged centrifugation for 30 min, as compared to often used 10–15 min, was found to be more efficient for separation of lipids and formation of a pellet (data not shown).

To confirm that the presented protocol is suitable for human clinical samples, we used breast tumors and histologically normal adjacent tissues. Proteins from tumors samples were separated in 10% acrylamide gels prepared according to the described above protocol, and stained with silver nitrate to detect separated proteins. The 2D gel images showed no distortions in separation of proteins (distribution of high or low molecular mass proteins, or preferences in pI of proteins) (Fig. 3). Repeats of sample preparation confirmed reproducibility and good quality of protein extraction and separation in 2D-GE. We estimated that the yield of proteins was in the range of 80% to 100%. It is also important to mention that the extracted proteins were mostly soluble proteins not associated with

cellular structures, eg, cytoskeleton (pellet). Thus, validation experiments with human breast tumors and histologically normal tissue confirmed efficiency of the described here protocol for protein extraction.

Conclusion

Here we report a protocol suitable for an efficient extraction of proteins from breast tissue. The high extraction and preservation of proteins was achieved by using glass beads for disruption and extraction in the urea-containing buffer. Sonication and prolonged time of centrifugation allowed removing contaminants and small particles that can distort protein separation. This is especially valid for removal of lipids. The proposed protocol may also be used for other tissues with minimal optimization.

Disclosures

This manuscript has been read and approved by all authors. This paper is unique and not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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II

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Meta-data Analysis as a Strategy to Evaluate Individual and Common Features of Proteome Changes in Breast Cancer

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Abstract. *Background:* Individual differences among breast tumours in patients is a significant challenge for the treatment of breast cancer. This study reports a strategy to assess these individual differences and the common regulatory mechanisms that may underlie breast tumourigenesis. *Materials and Methods:* The two-step strategy was based firstly on a full-scale proteomics analysis of individual cases, and secondly on the analysis of common features of the individual proteome-centred networks (meta-data). *Results:* Proteome profiling of human invasive ductal carcinoma tumours was performed and each case was analysed individually. Analysis of primary datasets for common cancer-related proteins identified keratins. Analysis of individual networks built with identified proteins predicted features and regulatory mechanisms involved in each individual case. Validation of these findings by immunohistochemistry confirmed the predicted deregulation of expression of CK2 α , PDGFR α , PYK and p53 proteins. *Conclusion:* Meta-data analysis allowed efficient evaluation of both individual and common features of the breast cancer proteome.

Variability of breast cancer is manifested on various levels, from histological appearance to molecular mechanisms (1-3). This variability calls for individual assessment of each patient so that the best treatment is provided. Currently, the selection of treatment for breast cancer is based on clinical data, histopathological examinations and some molecular markers. Size and location of a tumour, lymph node status

and presence of distal metastases are at the core of clinical evaluation (2, 3). Histopathological examination of a biopsy or a resected tumour provides important information about types and differentiation status of cells in a tumour. Expression of HER2/neu, oestrogen and progesterone receptors is often used in determining appropriate treatment. In some clinics, expression of p53 and VEGF receptor, vascularisation level, inflammation areas and structure of the tumour stroma are considered (2-4). Molecular diagnostics of breast cancer commenced with the introduction of mRNA expression arrays (5). Molecular characterisation of the types of breast tumours, which is different from the tumour grade system based on clinical data, was a strong confirmation of the variability of breast tumours at the molecular level (6). mRNA expression studies have since provided signatures to discriminate patients with a worse prognosis and/or development of an aggressive tumour type, e.g. MammaPrint and OncoPrint (3-7). However, their areas of application are limited, and the array-based tools still have to prove their clinical value.

Molecular diagnostics is of high importance, as it has a potential to detect novel drug targets. However, practically all reports of molecular markers have been focused on the identification of common features in the studied cohorts (2-7). Such an approach tends to disregard any individual-specific features. It also tends to minimise the insight into regulatory mechanisms which may be affected in a majority of samples via different components in different individuals. As a consequence, markers of differentiation of human breast epithelial cells are mostly keratins, however there are a number of signalling mechanisms that have been shown to regulate the differentiation of cells (4, 8). It is believed that these signalling mechanisms are not considered because different molecules may control them in different tumours, and therefore it is challenging to obtain a list of the same signalling proteins in a large cohort. Therefore, although the

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Key Words: Proteomics, breast cancer, signalling.

same regulatory processes may be affected, in many cases they may be missed due to the variability of involved signalling components.

Proteins offer a rich source of markers for diagnostics, prediction and monitoring of cancer treatment (9, 10). The importance of such proteins is emphasised by the fact that all anti-cancer drugs act on or via proteins (11). Therefore, proteome profiling of breast tumours has been approached extensively. Proteins extracted from tumours, microdissected cells or tumour interstitial fluids have all been studied (12-19). The main methodological approaches used in such studies are two-dimensional gel electrophoresis (2D-GE), peptide-based shotgun mass spectrometry techniques and various arrays (10-20). 2D-GE is currently the only technology which allows the separation of hundreds of full-length proteins (21). As practically all proteins *in vivo* have post-translational modifications, the use of full-length proteins, as analytes, is essential for high quality proteome studies.

Attempts to develop a general 'one-fit-for-all' proteome profile of breast tumourigenesis have delivered lists of mostly high-abundance and structural proteins, *e.g.* keratins (9, 10, 22). This is mainly due to the averaging of primary datasets and the inability to interpret individual differences. However, the combination of proteomics technologies, systems biology tools and modern molecular and cell biology in the field of cancer studies provides a platform to achieve a new depth in tumour profiling. This study shows that a complete analysis of individual cases, followed by comparison of identified protein-dependent networks, is informative in delivering insight into the molecular mechanisms that may be present in either all cases or only in an individual patient.

Materials and Methods

Clinical samples and their preparation. Clinical samples were collected at Broomfield Hospital (Chelmsford, UK), under Ethical Permit 04/Q0303/28, issued by the North and Mid Essex Local Research Ethics Committee (Harlow, UK). Clinical samples were collected immediately upon surgery and stored on wet ice before being dissected by a pathologist. Samples of breast epithelial tissue were snap-frozen immediately in liquid nitrogen for use in the proteomics analysis. Samples for immunohistopathological diagnostics were collected and embedded in paraffin blocks in the Department of Histopathology, Broomfield Hospital (UK) before being sectioned onto glass slides. For the proteomics study, tissue was extracted directly in a buffer for isoelectrofocusing (8 M Urea, 2.5% CHAPS, 50 mM DTT, IPGbuffer with pH 3-10, traces of bromophenol blue), with mechanical disintegration with glass beads at room temperature. Extracts were centrifuged at 13,000 rpm (15,000 g) for 15 min, and supernatants were used for 2D-GE. Proteome profiling. Proteome profiling, 2D-GE, gel image analysis and MALDI TOF mass spectrometry were used, as described previously (23). In brief, first dimension isoelectrofocusing (IEF) was

performed using IPG Dry strips (linear, pH 3-10, 18-cm long) in an IPGPhor instrument (Amersham Biosciences, Uppsala, Sweden), using the following protocol: 10 h – rehydration of strips with a sample, 2 h – 50 V, 1 h – 500 V, 1 h – 1000 V, and 10 h – 5,000 V. The second dimension SDS-PAGE was performed in an Ettan Dalt Six electrophoresis system (Amersham Biosciences, Uppsala, Sweden), using the following protocol: 0.5 W/gel – 15 min, 1 W/gel – 30 min, 10 W/gel – to completion of the run (45,000 Vhrs). Three to four 10% SDS-PAGE gels were generated for each sample, depending on the quantity of extracted proteins. Generated gels were stained with 0.1% silver nitrate. Protein spots were analysed using Image Master Platinum version 6.0 software (GE Healthcare, Uppsala, Sweden). Statistical significance of the reproducibility of spot expression in 2D gels and differences in expression were evaluated by using the Image Master 2D Platinum Version 6.0 statistical package (GE Healthcare). Proteins from 2D gels which were shown to have either a unique expression pattern or exhibited changes by more than a 50% increase or decrease in expression between tumour and histologically healthy adjacent tissue were considered for identification. The Student's *t*-test was used to determine the statistical significance of the observed changes ($p < 0.05$).

Protein identification. Protein spots were excised from the gels, destained and subjected to in-gel digestion with trypsin (modified, sequence grade porcine; Promega, USA), as described previously (23). Tryptic peptides were concentrated and desalted in ZipTip's μ C18 (Millipore, Billerica, USA). Peptides were eluted with 65% acetonitrile, containing the matrix α -cyano-4-hydroxycinnamic acid, and applied directly onto the metal target and analyzed by MALDI TOF MS on a Bruker Ultraflex instrument (Bruker Daltonics, Bremen, Germany). Embedded software (FlexAnalysis; Bruker Daltonics) was used to collect and process mass spectra. Peptide spectra were internally calibrated using autolytic peptides from the trypsin (842.51, 1045.56 and 2211.10 Da). To identify proteins, searches in the NCBI nr (2010/05/10) RefSeq sequence database (NCBI, Bethesda, MD, USA; <http://www.ncbi.nlm.nih.gov>) were performed using the ProFound search engine (<http://65.219.84.5/service/prowl/profound.html>). One miscut, alkylation and partial oxidation of methionine were allowed. Search parameters were set to 'no limitations of pI', 'Mr', 'tolerance less than 0.1 Da', and 'mammalian' for species search. Significance of the identification was evaluated according to the probability value ('Z') and sequence coverage.

Systemic analysis. Protein names were translated into gene ontology (GO) terms (<http://www.geneontology.org>). Functional and pathway analysis was performed using Ingenuity Pathway Analysis (IPA), a tool for description of networks and signalling pathways (<http://www.ingenuity.com>). IPA operates with a proprietary database and considers only those experimental data which have been evaluated by independent researchers. This ensures that only confirmed results are taken into consideration for building a network. Experimental results which have not been reported by multiple laboratories or may have controversial interpretations were not considered for analysis. Such stringent selection of experimental data was required to exclude false-positive relations. Fischer's exact test was used to calculate a *p*-value determining the network connectivity.

Immunohistochemical study. BRC961 USBiomax breast cancer arrays (US Biomax Inc., Rockville, MD, USA) were used to

Table 1. Clinical and pathological description of cases subjected to proteome profiling in the present study.

Case number ^a	Histopathological diagnostics ^b	Grade ^c	ER ^d	PR ^d	HER2/neu ^d	Size total (max diam; mm) ^e	Lymph node positive ^e	Lymph node examined total ^e	Lymphovascular invasion ^e
#1	IDC DCIS	2	+	–	+	25	0	0	Yes
#6	IDC DCIS	3	–	–	–	20	0	6	No
#37	IDC	3	+	–	+	28	0	0	Yes
#40	IDC	2	+	+	n/a	14	0	9	Yes
#45	IDC	3	–	–	+	27	0	4	n/a
#47	IDC DCIS	2	+	–	–	42	0	5	n/a

^aIdentification number of the cases; ^bIDC – invasive ductal carcinoma; DCIS – ductal carcinoma *in situ*. IDC DCIS – IDC with inclusions of DCIS; ^cGrade of tumours; ^dER, PgR and HER2/neu status was evaluated by immunohistochemistry; ^eSize of tumours and invasive areas were measured by a pathologist upon pathological examination. The number of positive lymph nodes indicates the number of lymph nodes with detected metastasis. Lymphovascular invasion in tumours was evaluated upon histo-pathological analysis; n/a, not available.

evaluate the expression of CK2 α , PDGFR α , PYK and p53. Each array slide contained 35 cases of malignant tumours, three cases of hyperplasia, five cases of benign tumours and three cases of non-neoplastic tissues (Supplementary Figure S17). Arrays were stained with anti-CK2 α (H-286; sc-9030), anti-PDGFR α (C-20; sc-338), anti-PYK (H-102; sc-9019) and anti p53 (DO-1; sc-126) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). All antibodies were used at a dilution 1:50 (v/v), according to the supplier's recommendations. Anti-Smad2 C-terminal phosphorylation (pS2) antibodies were described previously (23). pS2 antibodies were used at dilution 1:25 (v/v). Antigen retrieval was performed using DakoCytomation target retrieval solution high pH (DAKO, Carpinteria, CA, USA). The slides were stained with VECTASTAIN Elite ABC kits (Vector Laboratories Inc., Burlingame, CA, USA) following the manufacturer's instruction, and mounted with Fluoromount G (Southern Biotechnology, Birmingham, AL). The stained tissues were photographed using a Leica DFC camera and images were acquired with Leica QWin Standard software (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK). Intensity of staining was evaluated as absent (–); no stained cells), weak ((+); <10% of stained cells), moderate ((++); 10% to 50% of cells stained) and strong ((+++); >50% of stained cells). The staining was evaluated in malignant (epithelial) cells of tumours and epithelial cells of healthy tissues.

Results

Generation of individual proteome profiles. Previous studies which highlighted the significant variability between clinical samples (1-19) prompted the present study to develop a new strategy, based on a proteomics study of each clinical breast cancer case separately before attempting to find changes common for all cases in a studied cohort of patients. Every breast tumour tissue sample (case) was subjected to a complete proteome analysis which included proteome profiling, identification of proteins from the individual tumours and functional clustering and network building. The aim of using this method was to identify and predict regulatory mechanisms affected by identified proteins specific to each breast tumour case. Consequently, sets of

proteins and predicted regulatory mechanisms affected in individual cases were compared against all studied cases (Figure 1A).

All the studied cases were described as invasive ductal carcinomas (IDCs); cases #1, #6 and #47 were described as IDCs with elements of ductal carcinoma *in situ* (DCIS). Tumour grades were 2 (3 cases) or 3 (3 cases) and no lymph metastases were observed in the examined cases (Table I). The aim of this selection of cases was to focus on IDCs. As expected, the histopathological evaluation of sections of tumours showed some variability in the histology of the samples, although more than 50% of the cellular component was composed of malignant cells (Figure 1B). Differences in the presence of epithelial cells and stromal elements were observed. These histological differences may reflect molecular variability between the cases. It was hypothesised that a proteome analysis of each case separately would expose individual features of the cases. Therefore, proteome analysis was performed for each case separately, *i.e.* each tumour was compared to the corresponding adjacent histologically healthy tissue.

2D gels were generated for each of the studied cases, as a set of a tumourous and a corresponding histologically healthy adjacent tissue. The overall pattern of protein migration in 2D gels was similar for all cases, with the majority of proteins having a tendency to shift into the area of gels corresponding to pI below 7.5 (Figure 2, Supplementary Figures S1 to S6). A similar distribution of proteins in 2D gels was observed previously in studies of breast tumours (12, 15, 16, 18). Despite the similarity in the overall protein patterns of all cases, there was variability in the expression of tumour-related proteins. For example, 46 protein spots were detected for the case #47, while for the case #37 there were 180 tumour-related protein spots. The numbers of identified proteins which changed their expression are indicated in Table II and the lists of the identified proteins for each of the cases are given in

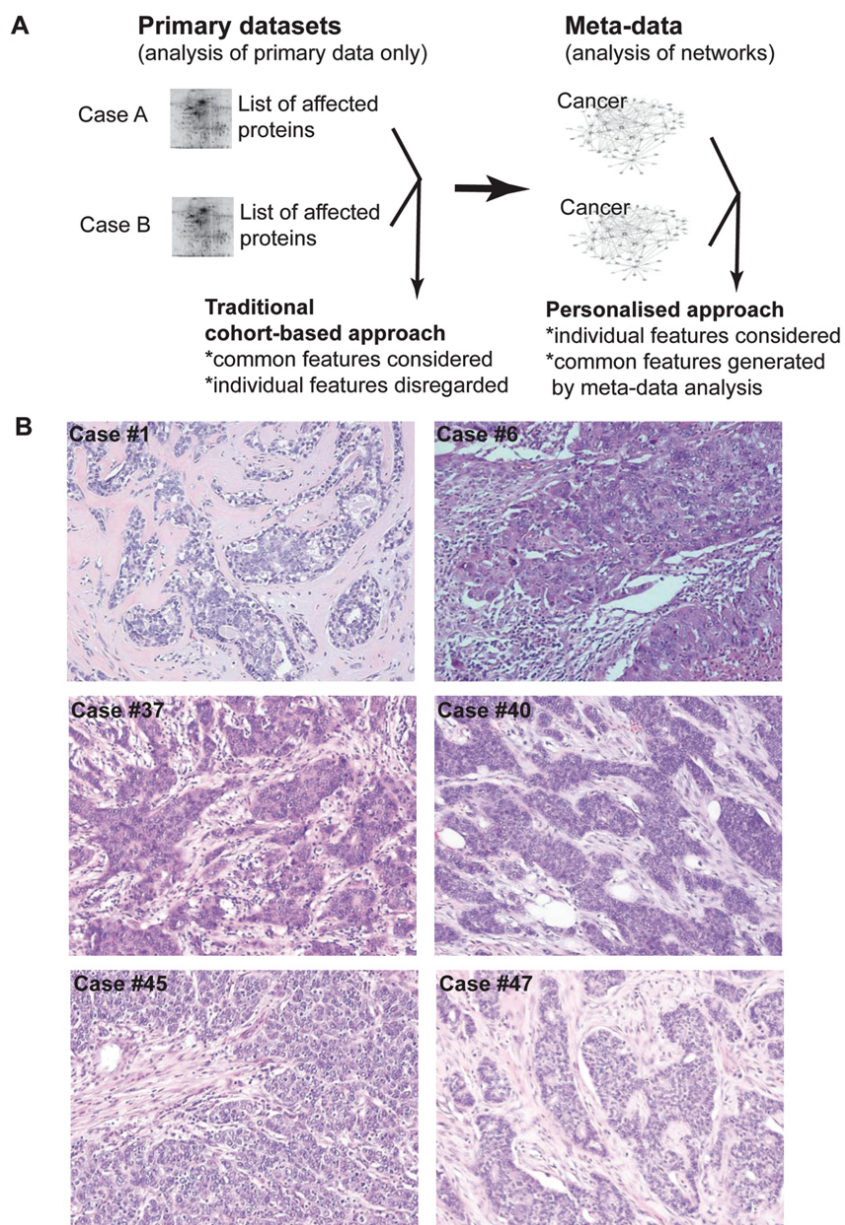


Figure 1. Presentation of studied cases. (A) The workflow of a traditional cohort-based analysis and the proposed approach. Only two cases are shown as examples. The workflow can be applied to unlimited number of patients. (B) Haematoxylin-eosin stained images of tumour sections representing cases used in this proteomics study (magnification: $\times 100$). The cases shown are described in the Results section.

Table II. Summary of detection of protein spots and identified proteins.

Case number ^a	Total number of affected protein spots ^b	Number of affected spots with identified proteins ^c	Identified proteins, as up-regulated in tumours ^c	Identified proteins, as up-regulated in normal tissues ^c	Total number of uniquely identified proteins ^c
#1	114	81	79	3	44
#6	141	100	86	24	54
#37	180	131	122	9	69
#40	116	52	29	23	31
#45	44	26	18	8	19
#47	46	46	38	8	34

^aCase number is annotated in the text (Table I); ^bNumbers of proteins spots were obtained following gel image analysis; ^cNumbers of uniquely identified proteins. These numbers are lower than the numbers of spots with identified proteins due to identification of some of the proteins in multiple spots.

Supplementary Tables S1 to S6. It should be noted that a number of proteins were identified in multiple spots. In these cases, there were between 5 to 11 proteins identified in multiple spots. The number of spots for a unique protein varied from 2 to 15. This confirmed the importance of studying full-length proteins without prior digestion to peptides.

The key aspect of the strategy presented here is a full-scale proteomics study of each case separately, before making an analysis of the common and individual features. The results of individual proteome profiling are briefly described below and detailed information is presented in the Supplementary figures and tables.

Case #1. One hundred and fourteen protein spots were detected as showing changes in expression levels in the tumour tissue compared to the histologically healthy adjacent tissue. (Table II, Supplementary Figure S1). Forty-four unique proteins in 81 spots were identified (Supplementary Table S1). Among them, CK2 α , BRCA1, vimentin and annexin A2 were identified. Systemic analysis of the identified proteins suggested changes in the regulatory processes involving interferon β 1, IL8, Erk1/2, Jnk, p53, ApoA1, CSF2 and BRCA1. The network formed by the tumour-related identified proteins for this case included 51 components (Supplementary Figure S7).

Case #6. One hundred and forty-one protein spots were detected as showing changes in the expression levels in the tumour tissue compared to the histologically healthy adjacent tissue (Table II, Supplementary Figure S2). Fifty-four unique proteins in 100 protein spots were identified (Supplementary Table S2). Among them, CK2 α , PDGFR α , phospholipase C and protein tyrosine phosphatase 14 were identified. Systemic analysis of the identified proteins suggested changes in the regulatory processes involving TGF β , TNF, insulin, TP73, JNK, Jun and HNF. The network formed by the tumour-related identified proteins included 147 components (Supplementary Figure S8).

Case #37. One hundred and eighty protein spots were detected as showing changes in the expression levels in the tumour tissue compared to the histologically healthy adjacent tissue (Table II, Supplementary Figure S3). Sixty-nine unique proteins were identified in 131 protein spots (Supplementary Table S3). Among them, CK2 α , GDF2, RB binding protein 7, vimentin and annexin A2 were identified. Systemic analysis of the identified proteins suggested changes in the regulatory processes involving TP53, Fos, NFkB, ERK1/2, PDGF, TGF β , TNF, insulin, PKC, HNF and AKT. The network formed by the tumour-related identified proteins included 144 components (Supplementary Figure S9).

Case #40. One hundred and sixteen protein spots were detected as showing changes in expression levels in the tumour tissue compared to the histologically healthy adjacent tissue (Table II, Supplementary Figure S4). Thirty-one unique proteins were identified in 52 protein spots (Supplementary Table S4). Among them, annexin A2 and phospholipase A2 activating protein were identified. Systemic analysis of the identified proteins suggested changes in the regulatory processes involving PDGF, MYC, TNF and HNF. The network formed by the tumour-related identified proteins included 122 components (Supplementary Figure S10).

Case #45. Forty-four protein spots were detected as showing changes in expression levels in the tumour tissue compared to the histologically healthy adjacent tissue (Table II, Supplementary Figure S5). Nineteen unique proteins were identified in 26 protein spots (Supplementary Table S5). Among them, CK2 α , steroid 21-monooxygenase, annexin A2 and apolipoprotein A-IV precursor were identified. Systemic analysis of the identified proteins suggested changes in the regulatory processes involving β -estradiol, IL2, IL4, GRB2 and interferon γ . The network formed by the tumour-related identified proteins included 69 components (Supplementary Figure S11).

Case #47. Forty-six protein spots were detected as showing changes in expression levels in the tumour tissue

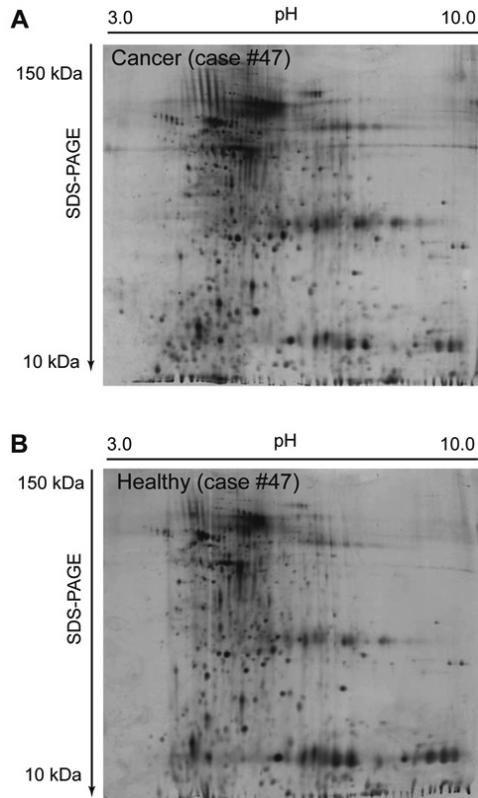


Figure 2. Representative images of 2D gels. The images show separation of proteins extracted from tumour (A) and from histologically healthy adjacent tissue (B). The images represent gels generated with the samples of case #47. For each image, pH gradient, direction of SDS-PAGE and migration positions of molecular mass markers are shown. Images of annotated gels of all cases are shown in Supplementary Figures S1 to S6 and lists of identified proteins are given in Supplementary Tables S1 to S6.

compared to the histologically healthy adjacent tissue (Table II, Supplementary Figure S6). Thirty-three unique proteins were identified in 46 spots (Supplementary Table S6). Among them, ribosomal protein S6 kinase, protein kinase A anchor protein 2 and obscurin were identified. Systemic analysis of the identified proteins suggested changes in the regulatory processes involving TGF β , TNF, Myc, interferon γ and CDK inhibitor p16. The network formed by the tumour-related identified proteins included 40 components (Supplementary Figure S12).

Table III. Summary of immunohistochemical detection of CK2 α , PDGFR α , PYK and p53 in human breast cancer tissue microarray (healthy tissues and malignant tumours).

CK2 α staining	(-)	(+)	(++)	(+++)
Healthy (3)	2	1		
Malignant tumours (34)	2	5	9	18
PDGFR α staining	(-)	(+)	(++)	(+++)
Healthy (3)				3
Malignant tumours (35)			10	25
PYK staining	(-)	(+)	(++)	(+++)
Healthy (3)		2	1	
Malignant tumours (35)		4	22	9
p53 staining	(-)	(+)	(++)	(+++)
Healthy (3)		1	2	
Malignant tumours (35)	6	8	11	10

Immunohistochemical analysis and the grading for staining are described in the Materials and Methods section.

Thus, proteome profiling showed individual differences between cases, as primary datasets and as a prediction of interacting networks.

Generation of a common profile of deregulated signalling mechanisms. Analysis of primary proteomics datasets showed that different keratins were the only proteins common for all cases (keratins were common in 5 of 6 and 6 of 6 cases; Figure 3A). When the cut-off frequency of protein detection was decreased to 4 cases out of 6, TNF and TGF β signalling were represented (Figure 3A; Supplementary Figures S13 and S14). This was in contrast to a number of proteins with proven roles in intracellular signalling and tumourigenesis that were identified as cancer-related in each individual case (Supplementary Tables S1 to S6).

The differences between the lists of identified proteins with altered expression levels in tumours, as compared to adjacent histologically healthy tissues, may be interpreted as a representation of the high variability between the cases. However, many of the regulatory mechanisms in a cell may employ different proteins to achieve the same impact on cellular functions, such as proliferation or death. Therefore, the regulatory mechanisms which may be deregulated in the tumour samples of the present study were investigated through the building of networks based on the identified proteins from each case. To predict which pathways may be involved, highly connected hubs were analysed in the individual networks. This analysis showed that TGF β , TNF,

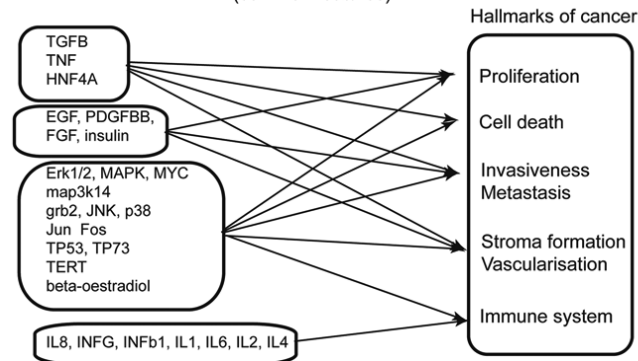
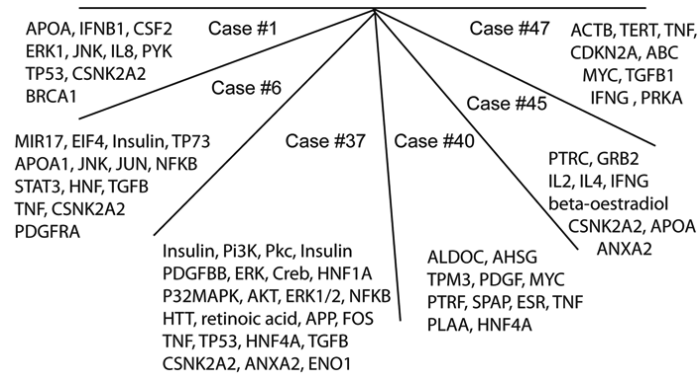
A Proteins common in the primary datasets and their potential contribution to studies of tumourigenesis**B** Hubs selected from the meta-data analysis of all cases, and their potential contribution to tumourigenesis (common features)**C** Hubs of signalling proteins in the individual networks (individual features)

Figure 3. Prediction of common and case-specific proteins. Proteins identified as common for all cases in primary datasets (A), hubs of networks as frequently affected in many cases (B) and as case-representative (C), are shown. (A) Following a cohort-based approach, common proteins in the primary datasets were determined. The frequency of identification of proteins in each case is indicated. An impact on tumourigenesis was predicted by IPA and by the review of published reports (A, B). (B) Hubs selected upon analysis of meta-data (network-based information) are shown in 4 groups. The main impacts of each group are indicated by arrows. It should be noted that all crucial for tumourigenesis regulatory mechanisms are represented, e.g. cell proliferation, cell death, metastasis, regulation of stroma and immune system. (C) Selected hubs representing regulatory mechanisms in each studied case are shown. The annotation of proteins and hubs is in GO terms. The networks of each case and the networks of common primary dataset- and network-selected molecules are given in Supplementary Figures S7 to S16.

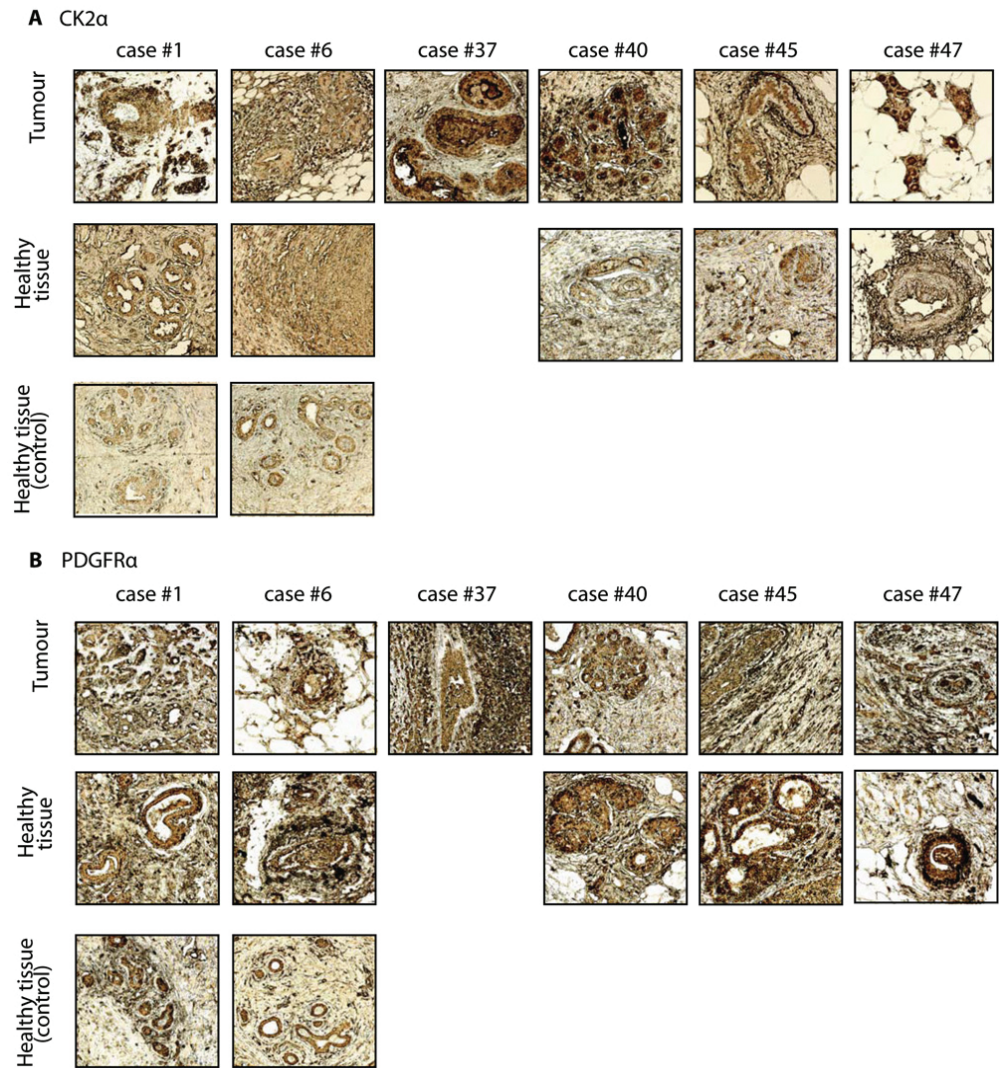


Figure 4 Continued

mitogenic (EGF, PDGF, FGF) and interleukin (IL1, IL2, IL4, IL6 or IL8) related signalling responses are overrepresented (Figure 3B; Supplementary Figures S15 and S16). Various components of the generic MAP kinase cascade were also represented. This finding is in line with reports showing the

involvement of known predicted mechanisms in tumourigenesis, such as proliferation, death, invasiveness, angiogenesis, stroma development and corruption of the immune surveillance (24). Therefore, despite differences in the primary datasets, the approach described here showed

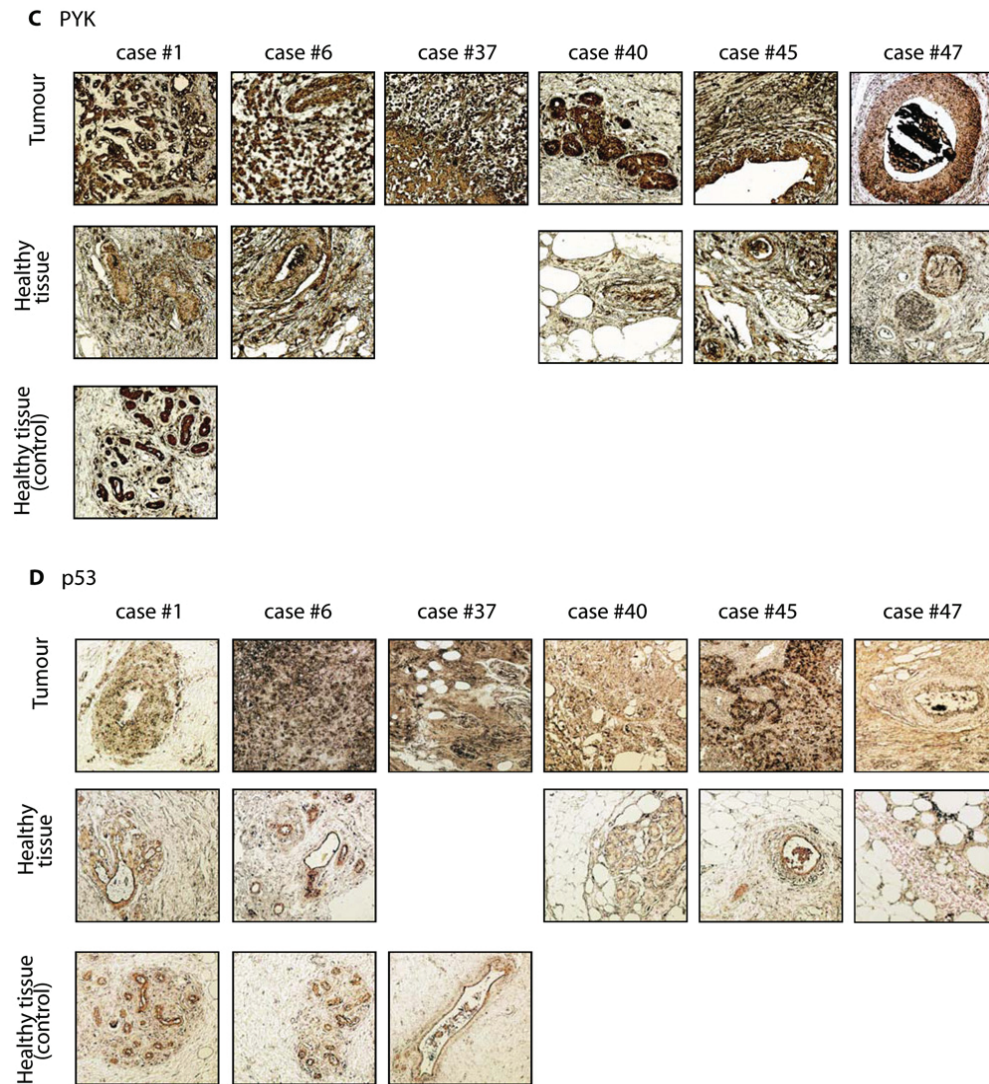


Figure 4 Continued

that there are significant similarities in the predicted signalling mechanisms deregulated in individual tumours.

Another important conclusion from this type of analysis was that the employed strategy allows the prediction of mechanisms which may have a more significant impact on

tumourigenesis in each specific case (Figure 3C). As an example, the deregulation of BRCA1-dependent signalling was suggested in the tumour of case #1. For the case #6, the status of TP73 may have a role in the growth of this tumour. For the case #47, areas of DCIS were observed in addition

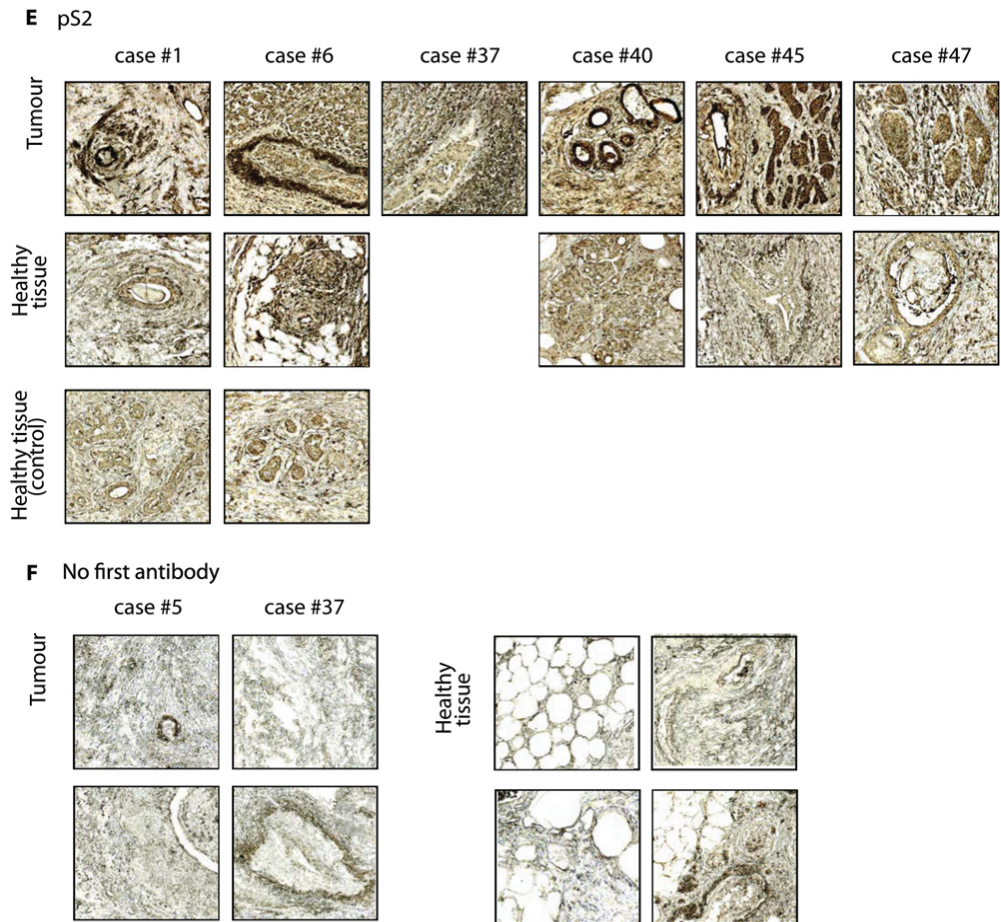


Figure 4. Expression of CK2 α and PDGFR α in the studied cases. Expression of CK2 α (A) and PDGFR α (B), PYK (C), p53 (D) and pS2 (E) in tumour and healthy adjacent tissues (healthy tissue) was monitored by IHC. Healthy breast tissue from non-cancerous patients (healthy tissue control) was also stained. Control of the staining, without primary antibodies, is shown in (F). Brown colour indicates positive staining.. Case #37 did not have available for IHC adjacent histologically healthy tissue. Representative images are shown (magnification: $\times 50$).

to IDC and for this case, telomerase reverse transcriptase (TERT) was predicted as a highly connected hub, indicating changes relevant to early stages of tumourigenesis. Other examples of proteins with predicted impact on tumourigenesis in individual cases were CK2 α , pyruvate kinase M1/M2 (PYK), p53 and TGF β and PDGF signalling (Figure 3C; Supplementary Figures S15 and S16). The methodology described here allows the generation of predictions based on targets that are deregulated in individual

tumours. This approach is crucial for gaining a greater understanding of the underlying mechanisms in individual tumours. Furthermore, this information may be essential in developing a more personalised regime of treatment options for patients.

Validation of common and individual features of tumours by immunohistochemistry. To validate the 2D-GE based findings immunohistochemistry (IHC) was performed on the samples

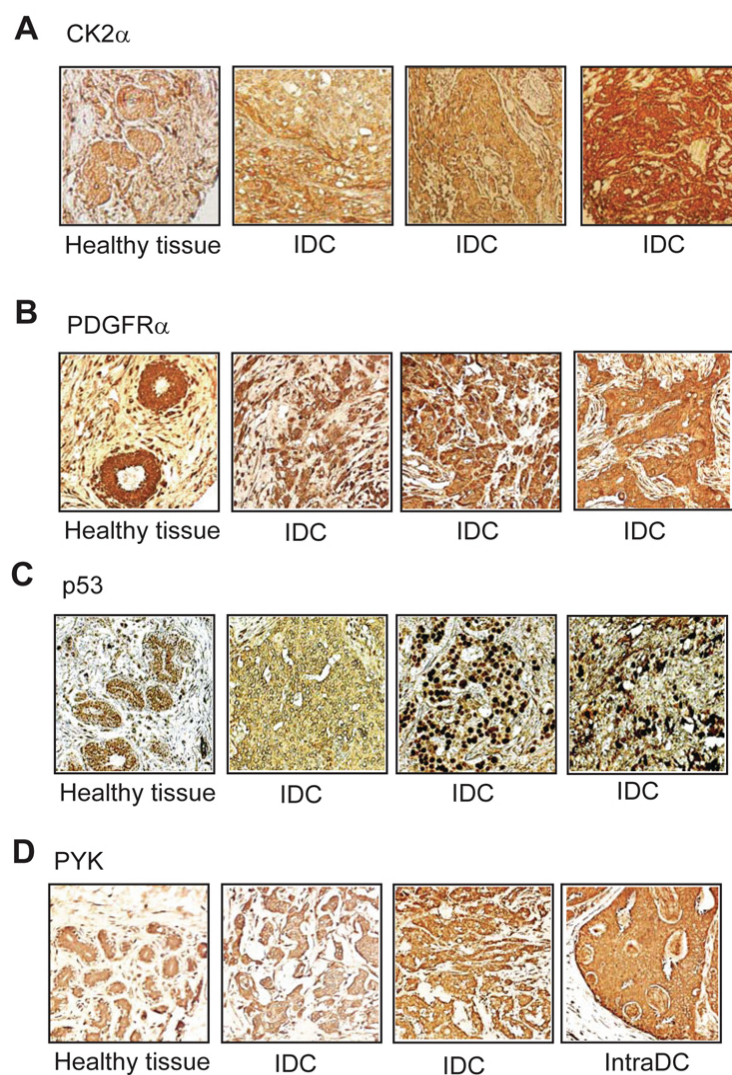


Figure 5. Expression of CK2 α , PDGFR α , PYK and p53 in human breast tumours. The expression of CK2 α (A), PDGFR α (B), PYK (C) and p53 (D) in IDC and healthy tissues is shown. TMA of human breast malignant and benign tumours, and healthy tissues were stained with respective antibodies. Representative images are shown, where brown colour indicates positive staining (magnification: $\times 50$). Case #37 did not have enough histologically healthy tissue for IHC.

of cases subjected to the initial proteome profiling (Figure 4) and through the use of a tissue microarray (TMA) (Figure 5). The TMA contained 35 cases of malignant tumours, 3 cases of fibroadenomas, 6 cases of non-malignant conditions

(e.g. hyperplasia) and 3 cases of healthy breast tissues (Supplementary Figure S17). In contrast to immunoblotting of extracts from whole tumour or tissue, IHC allows the evaluation of the expression of proteins in different cell-

types. Therefore, IHC is a good methodological approach to demonstrate whether the findings observed from the proteome profiling are specific to the malignant cells of tumours.

For validation, the levels of expression of CK2 α , PDGFR α , PYK, p53 and TGF β receptor-induced phosphorylation of Smad2 protein were assessed (Figure 4). These proteins were selected due to their identification in the proteome profiling primary datasets, by network analysis and their potential involvement in breast tumorigenesis (Figure 3; Supplementary data) (25-30). These proteins are known to regulate tumorigenesis-related processes, but they are not accepted in clinic as markers. One of the reasons may be that their correlation to tumorigenesis may not be high in a large cohort study, but may be highly relevant for the individual cases. Therefore, the expression of these proteins is expected to alter in cancer, but with significant variability between individual cases.

IHC staining of sections of the studied cases with anti-CK2 α , anti-PDGFR α , anti-PYK, anti-p53 and anti-phosphorylated Smad2 antibodies confirmed the proteomics and network analysis results. Notably, the expression of CK2 α was enhanced in all tumours; however it showed varying levels in staining between the individual cases, with an increased expression in tumour cells (Figure 4A). PDGFR α expression also showed variable staining among the cases, with a significant staining of epithelial cells in histologically healthy adjacent tissues. Compared to histologically healthy tissues, PDGFR α staining was similar or less pronounced in tumour cells, although the total PDGFR α signal was enhanced in tumour sections (Figure 4B). PYK staining was increased in tumours, as compared to adjacent histologically healthy tissues (Figure 4C). p53 staining also showed tumour-related changes, with moderate (cases #1, #40 and #47) to strong (cases #6 and #45; Figure 4D) signal increase. TGF β signalling was identified by the two-step strategy as deregulated in the studied cases. IHC showed that the activated C-terminal phosphorylation of Smad2 is enhanced in tumour cells, as compared to histologically healthy adjacent tissue (Figure 4E) Therefore the, IHC staining of the individual cases confirmed the deregulation of the identified and predicted proteins, and showed them to be relevant to breast tumour tissues.

To explore whether the observed deregulation of specific proteins would be observed similarly in new cases of breast cancer, IHC staining was performed on a TMA set of human breast cancers with focus on healthy tissues and malignant tumours, *e.g.* IDC (Figure 5). Results of IHC staining of non-malignant cases in TMA are mentioned in Supplementary Figure S18. IHC staining of the TMAs showed that CK2 α expression is increased in almost half of IDC cases, as compared to weak or no expression in benign neoplasias and healthy tissues (Table III; Figure 5A). Thus, the deregulation

of expression of CK2 α may be characteristic for part of tumours. Case-to-case variability in staining for PDGFR α and PYK was also observed (Figure 5B, C; Table III). An evaluation of IHC staining for PDGFR α and PYK based on staining intensities showed tumour-related changes in less than 30% of cases (Table III). IHC staining for p53 showed that in IDC cases the expression level of p53 is also deregulated. Notably, a moderate expression of p53 was observed in healthy tissues, while in IDC there were cases with no detectable p53 (6 cases) and cases with strong expression (10 cases) In a cohort-based study the levels of changes observed for CK2 α , PDGFR α , PYK and p53 would not be considered as representative for the whole cohort, despite the fact that these changes may be relevant for individual patients. The relevance to individual patients is even more pronounced as the studied proteins are potent regulators of cellular functions and are known to affect tumorigenesis. Therefore, the results suggest that many of the changes in regulatory processes may not be random events but characteristic for the development of breast tumours in individual patients. Identification of such individual traits in tumour development would be beneficial for the individualisation of anti-cancer treatment.

Discussion

Studies of genome, transcriptome and proteome changes in human breast cancer have delivered a number of markers for detection, selection of treatment and prognosis (2-6, 31). The main trait of previously reported studies is a search for 'common for all cases' markers, which would have acceptable sensitivity and specificity. The drawback of this approach is that individual differences in primary data would be lost, and only common features would be considered. This cohort-based approach does not take into consideration systemic properties of cellular functions. Multiplicity of ways to control cellular functions is the basic principle of cell physiology, and it is ensured by a similar impact of different proteins on a same signalling mechanism (32). In its application to cancer, this means that even if different sets of proteins would be identified as cancer-specific in different tumours, they may reflect de-regulations of the same cellular functions. This similarity will be visible only if a systemic analysis is performed with primary datasets, and then meta-data compared. In addition, systemic analysis of individual cases allows identification of proteins and signalling pathways specific for that patient. This was a pilot study which used a two-step approach to identify breast cancer markers; firstly using an individual proteome profiling and systemic analysis, followed by a case-specific meta-data analysis for all cases. This is the first report of such two-step approach in the search of breast cancer related markers for their potential use in the management of breast cancer.

Proteome profiling of breast tumours and cultured cells established from human breast epithelial cells have delivered lists of potentially cancer-specific proteins (2-8, 10). However, comparison of these lists showed that common proteins were predominantly of high abundance, *e.g.* keratins. At the top of the list of common cancer-related proteins were also keratins (Figure 3A). This is in contradiction to results of molecular studies of breast carcinogenesis, when a number of involved signalling pathways have been described (4, 9, 10, 24, 30). Proteins directly involved in these pathways have been seldom proposed as markers, with the exception of HER2/neu, oestrogen and progesterone receptors, p53, BRCA1 and BRCA2 (1-6). Recent reports indicated that even these molecular markers are not always efficient predictors, probably due to their mutations and intracellular compensatory mechanisms (1-4). Studies of signalling pathways involved in breast tumourigenesis indicated that the possible reason for such a disproportion in output between signalling and marker studies may be in the multiplicity of cellular regulatory mechanisms. When the potential functional impact of components identified by systemic analysis of individual cases was analysed, it was found that practically all cancer hallmarks were represented (Figure 3B). This confirms that the described approach allowed gaining a more comprehensive overview of molecular changes in tumour proteomes, as compared to conclusions based on primary datasets only.

Furthermore, the developed approach allowed for the identification of regulatory mechanisms specific for individual patients (Figure 3C). Meta-data analysis predicted changes in regulatory processes which otherwise would not be detectable by a direct analysis of only identified proteins. Knowledge of these mechanisms is important for the selection of patient treatment, as it provides information about the status of potential drug targets. The IHC validation study confirmed that the observed changes in the proteome profiles are not random events, but may be specific for a subset of tumours. The size of such subsets would not be large, with up to 10% or 50% of all cases. However, as these changes may be relevant to an individual patient, to know these unique specifics would be of great importance when designing anti-cancer treatment regimes. The developed two-step methodology with the analysis of meta-data was a pilot study established to evaluate the feasibility of this approach. Further studies with a large cohort of patients are required to enable the introduction of this approach into the clinical practice.

This pilot study proposed that a two-step strategy in the analysis of proteome profiles of human breast tumours is more informative in providing insight into affected molecular mechanisms than an analysis of only primary datasets. The first step was a full-scale proteome profiling of each case

separately. The second step was a comparison of meta-data from all cases. The findings from the analysis of the proteomic profiling and validation experiments reinforced the value of such a two-step approach for the development of more personalised medicinal regimes

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III

Phosphoproteomic analysis of TGFβ1 signaling revealed importance of 14-3-3σ phosphorylation for TGFβ1/Smad3-regulated transcription and CDK2/pRb-dependent cell proliferation.

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Abstract

Transforming growth factor- β (TGF β) is a potent regulator of tumorigenesis, although mechanisms defining its tumor suppressing and tumor promoting activities are not understood. Here we describe phosphoproteome profiling of TGF β signaling and show that 60 identified TGF β -regulated phosphoproteins form a network with scale-free characteristics. Presence of highly connected nodes at frequency higher than expected by a power law specified signaling sub-networks targeted by TGF β 1. The network highlighted interactions which may distribute signaling inputs to regulation of cell proliferation, metabolism, differentiation and cell organization. Novel convergence species for TGF β and EGF, TNF, IGF and IL8 signaling are identified by the network analysis. Study of a 14-3-3 σ -centered sub-network showed that phosphorylation of 14-3-3 σ at Ser69 and Ser74 has a dual role in TGF β signaling. The first role is a feed-forward mechanism for TGF β 1/Smad3-dependent transcription, with recruitment of tumor suppressor p53 into a Smad3-14-3-3 σ complex leading to accentuating TGF β 1-dependency, e.g. p53 inhibited spurious ligand-independent but enhanced ligand-stimulated transcription. The second, TGF β 1-dependent phosphorylation of 14-3-3 σ enhanced complex formation between CDK2 and 14-3-3 σ , and correlated with decreased phosphorylation of pRb and TGF β 1-dependent inhibition of cell proliferation. Thus, our data showed that a) TGF β 1 regulates phosphorylation of a plethora of proteins which form a scale-free network for coordinated regulation of various cellular functions and b) TGF β 1-dependent phosphorylation of 14-3-3 σ is important for functional and physical interactions of TGF β /Smad3 with p53, CDK2 and pRb in regulation of transcription and cell proliferation.

Introduction

TGF β is a potent regulator of cell proliferation, death, migration and differentiation (Derynck *et al*, 2003; Shi. *et al*, 2003). TGF β binds to serine/threonine kinase receptors on the cell surface. The complex of activated type I and type II TGF β receptors phosphorylates a number of substrates, and initiates intracellular signaling pathways regulating transcription, protein synthesis, degradation and localization. The output of TGF β treatment of cells is dependent on a type of cells and their status. The importance of Smad proteins has been shown, as well as a number of so-called Smad-independent pathways (Shi. *et al*, 2003; Attisano *et al*, 2002). In other words, the result of challenging of cells with TGF β depends on functional interactions between a number of components in cells, e.g. proteins.

Protein phosphorylation is one of the most crucial post-translational modifications in regulations of cellular functions. Phosphorylation at serine, threonine and tyrosine residues initiate conformational changes leading to changes in activity of proteins, and affect protein-protein and protein-nucleic acids interactions (Johnson *et al*, 2005).

Proteomics has proven to be the only technology which is capable to provide a large-scale unbiased analysis of protein phosphorylation. Phosphopeptide- and phosphoprotein-based approaches have been employed with various degree of successfulness (Morandell *et al*, 2006; Mukherji *et al*, 2005). We reported previously modification of IMAC technique for enrichment of phosphorylated proteins (Dubrovskaya *et al*, 2005). The advantage of this phosphoprotein Fe-IMAC over a phosphopeptide studies is in providing information about full-length proteins and not selected sites/peptides. This is especially important for studies of proteins with many phosphorylation sites with different dynamics of phosphorylation, as each combination of phosphorylated sites will be well distinguishable for full-length proteins, but will be difficult to deduct from phosphopeptides.

Changing a cellular status, e.g. proliferation or inhibition of cell growth, requires coordinated changes of hundreds of proteins (Sandhu *et al*, 2005; Desrivieres *et al*, 2003). Proteomics provides an overview of such alterations in protein expression and selected post-translational modifications. However, unveiling of key components in large datasets requires use of tools of systems biology. This includes various clustering methods, network building and modeling of relations (Bosl *et al*, 2007; Hu *et al*, 2007). The principals underlining mechanisms of interaction between proteins have been extensively studied. The structure of protein-based networks is important for distribution of triggering signals to various cell function-controlling units, e.g. distribution of signals triggered by TGF β to mechanisms regulating the cell cycle, differentiation, migration and apoptosis. Scale-free characteristics have been claimed for a number of networks, although scale-rich features have also been described (Deeds *et al*, 2006). Understanding of network features is of ultimate importance for unveiling of how an extracellular stimulus may trigger such different outputs, as inhibition of cell growth and stimulation of apoptosis.

Here we report a comprehensive phosphoproteomics screen of TGF β 1 signaling in MCF10A human breast epithelial cells. Systemic analysis showed that TGF β 1-regulated phosphoproteins form a scale-free network which regulates cell metabolism, development, various signaling pathways and cell organization. The phosphoproteome analysis showed an importance of TGF β 1-dependent phosphorylation of 14-3-3 σ for a network of 14-3-3 σ , p53, Smad3, CDK2 and pRb, which contributed to regulation of transcription and cell proliferation.

Results

Phosphoproteome profiling of TGF β 1 signaling

We generated phosphoprotein expression maps using immobilized metal-affinity chromatography technique developed by us (Dubrovskaya *et al*, 2005). MCF10A cells were treated with TGF β 1 for 15, 30, 60 and 120 minutes and phosphorylated proteins were enriched by Fe-

IMAC (Fig. 1; Supplementary Fig. S1). We detected in average 393 phosphoprotein spots in non-treated cells, 370 after 15 min, 371 after 30 min, 474 after 60 min and 436 after 120 min of treatment of cells. Although most of the protein spots migrated in the region of 2D gels corresponding to pI lower than 7.0, this shift was not dominant. Presence of proteins of various molecular masses in 2D gels indicated that upon Fe-IMAC enrichment was no selection related to the size of proteins. An increase in the number of phosphorylated proteins upon TGF β treatment was expected, as TGF β activates serine/threonine kinase receptors. However, a slight decrease in the number of phosphoproteins during the first 30 min indicated that de-phosphorylation of proteins had occurred. This is an important observation, as it showed that TGF β 1 initiated both phosphorylation and de-phosphorylation events, in contrary to the previous suggestion of predominantly phosphorylation-inducing signaling (Derynk *et al*, 2003; Shi *et al*, 2003; Attisano *et al*, 2002).

We confirmed that Fe-IMAC-enriched proteins are phosphoproteins by performing [32 P]orthophosphate labeling followed by Fe-IMAC and 2-D gel electrophoresis, and by staining of Fe-IMAC 2D gels with a phospho-specific dye Pro-Q Diamond (Kang *et al*, 2007). After exposure in a phosphorimager, comigration of silver-stained and 32 P-labeled spots indicated that Fe-IMAC-enriched proteins were phosphorylated. Staining with Pro-Q Diamond also confirmed that enriched proteins were phosphoproteins (data not shown). Our control mass spectrometry analysis of protein phosphorylation showed significant variability in phosphopeptide detection. This was expected due to the well known phenomenon of variability in peptide ionization. The mass spectrometry-based phosphopeptide analysis was found inferior to the phosphoprotein approach in detection and coverage of phosphorylated proteins (data not shown). Thus, 3 different phosphorylation-specific techniques provide strong confirmation of phosphorylation of detected proteins, i.e. directed detection of presence of phosphoryl groups (32 P) in proteins, detection of

phosphoproteins by a phosphor-specific dye, and previously confirmed high specificity of used by us Fe-IMAC protocol (Dubrovskaya *et al*, 2005).

Gel image analysis identified 85 protein spots which changed their appearance upon treatment of cells with TGFβ1. The spots were selected for identification of proteins if TGFβ1 induced changes of more than 50% of their level of phosphorylation in at least one of the time-points of treatment, as compared to any of the other time-points. The level of phosphorylation was defined as a volume of a protein spot in a Fe-IMAC 2D gel. For identification of proteins we used peptide mass fingerprinting by MALDI TOF mass spectrometry, and each protein was identified in at least two different preparations of respective phosphoprotein spots. Thus, we identified 60 unique proteins in 85 protein spots (Table 1).

Thirteen proteins were identified in multiple spots, with heterogenous nuclear ribonucleoprotein A2/B1 identified in 7 protein spots, enolase-1 in 5 spots, HSP-70 in 4 spots, MLAA-34 antigen and fructose 1,6-biphosphate aldolase in 3 spots each, eukaryotic translation initiation factor 3, keratin 10, keratin 9, zink finger protein 62, vasodilator-stimulated phosphoprotein, stress-induced phosphoprotein 1, ribosomal protein P0 and 14-3-3σ in 2 spots each (Table 1). An identification of the same protein in different spots is a strong indication of phosphorylation at multiple sites and may indicate combinations of phosphorylated sites. As an example, identification of 7 phosphoprotein spots for hnRNP A2/B1 indicated that this protein may have 7 predominant combinations of phosphorylated sites.

Phosphorylation may affect apparent molecular mass of a protein upon migration in SDS-PAGE, which may result in deviation of observed molecular mass from theoretical one. We observed such deviations for a number of identified proteins (Table 1). However, we also observed that TGFβ1 affected appearance of phosphorylated fragments of proteins, e.g. HSP-70 and cytokeratin 9. This corroborates importance of studying full-length proteins, as performed in this work. Phosphorylation of selected identified proteins was validated by immunoblotting of

MCF10A cell extracts with anti-phosphoSer/phosphoThr/ phosphoTyr antibodies (FKBP12, Actin, Enolase1, 14-3-3 σ ; Supplementary Fig. S2). Thus, we identified 60 unique proteins phosphorylation of which is regulated by TGF β 1.

Systemic analysis of TGF β 1 targets

TGF β affects practically all cellular functions, often having both stimulatory and inhibitory effects, e.g. proliferation, apoptosis, differentiation and migration (Derynk *et al*, 2003; Shi *et al*, 2003; Attisano *et al*, 2002). To gain insights into mechanisms of TGF β action, we performed a systemic analysis of our phosphoproteomics data. This included functional and dynamics clustering, building of a network of dependencies between identified TGF β -regulated proteins and analysis of systemic properties of the network.

Functional clustering showed that TGF β 1 affected phosphorylation of proteins involved in primary cellular metabolic processes, cell organization, development, differentiation, signal transduction, cell proliferation, cell cycle, cell death, transport and motility (Supplementary Fig. S3A). Dynamics of protein phosphorylations were variable, without predominant up- or down-regulation (Supplementary Fig. S3B, C). Dynamics of protein phosphorylation in selected functional clusters was also variable; as an example, dynamics of cell proliferation- or apoptosis-regulating proteins is shown (Supplementary Fig. S3B, C). It has to be noted that the most of the identified proteins and their phosphorylation have not been earlier described as components of TGF β signaling, which makes predictions of functional input of these phosphorylations uncertain and requires separate detailed study of each protein. However, our observation showed that TGF β -dependent phosphorylation had a similar high dynamics of phosphorylation observed in other regulatory systems, e.g. EGF signaling (Olsen JV *et al*, 2006; Lim *et al*, 2003).

Large-scale analysis of identified phosphoproteins showed that they form a network with scale-free characteristics (Supplementary Fig. S4). The network consists of 102 species (proteins

or their genes), with 58 species identified as functional or physical interactors with TGF β 1-regulated proteins, e.g. “guilt by association”, in addition to identified by us proteins (Table 1). Two clusters including elongation initiation factors and chaperonins were detected (Supplementary Fig. 4). The average number of connections (or strings) for a single species in the whole network is 9, and for the identified proteins the average number of connections is 3. This indicates that by generation of the network we detected highly connected hubs which otherwise would not be identified. The average number of intermediate connections between two TGF β 1-regulated proteins is 2.4, suggesting that all TGF β -dependent phosphoprotein-inputs are closely connected.

Distribution of node connections showed that the network contains highly connected hubs which appeared in the network with a frequency higher than it would be expected by a power law relationship (Fig. 2A). The network analysis pointed to TGF β as one of the main hubs, although TGF β ligand itself was not in the experimental dataset. This strongly indicated that we detected functional dependencies previously assigned to TGF β and provides confidence that we were able to identify previously reported TGF β -specific activities.

Among other highly connected growth factor species in the network were found EGF, TNF, IGF receptor and IL8. This suggests that these growth factors and TGF β may converge on the same components of the network. For example, RPSA, RPS6KA3, BRCA1, cdk2, RET and HNRPK are novel predicted convergence points for TGF β and EGF, in addition to those species which have been described earlier, e.g. H-Ras, AKT1, Src and NF- κ B. For TNF and TGF β signaling predicted convergence points are CDKN2A, P4HB, SMC1B, 14-3-3s, PRDX6, CAST, RPSA, PCDS, CREBP, Src, hnRNP, NF κ B and CLEC11A. For IGF and TGF β signaling they are ANXA2, NDY, Src, ALDOA and CCNA1. And for IL8 and TGF β , they are TPT1, NOS2A, NKRF, RLDZ, NFB, AKT1 and Src.

Thus, systemic network analysis predicted that TGFβ1-dependent phosphorylation might affect in a coordinated way the cell cycle, cell death, metabolic processes, DNA damage repair, transcription, protein synthesis and degradation. Our results showed also that the TGFβ1 initiated a network signaling with predominantly scale-free characteristics, although the number of key hubs is higher than would be expected by a power law relationship characteristic for ideal scale-free networks. Our results pointed also to intersection components between TGFβ and EGF, TNF, IGF and IL8 signaling.

TGFβ1 induced phosphorylation of 14-3-3σ at Ser69 and Ser74

Network analysis indicated a potential role of 14-3-3σ in regulation of cell proliferation with involvement of p53 (Fig. 2B). We selected 14-3-3σ for further analysis, as it has been most directly linked to cancer of all the 14-3-3 genes. The high frequency of 14-3-3σ inactivation by epigenetic silencing or p53 mutations indicates that it has a critical role in tumor formation (Hermeking *et al*, 2003; Kastan *et al*, 2007). First, we confirmed TGFβ1-dependent phosphorylation of endogenous 14-3-3σ protein in MCF10A cells (Fig. 3). In 2-D gels, 14-3-3σ was identified in two protein spots in 2-D gel. Notably, p72 migrated at a position corresponding to molecular mass of 32 kDa and pI 6.5, while p74 spot migrated at 24 kDa and pI 4.2 position. These two forms of 14-3-3σ are believed to be due to post-translational modifications, e.g. phosphorylation (Fig. 3A). In 1D SDS-PAGE, these two forms would migrate in a single band, and phosphorylation status would be a sum of these two forms. We observed increased phosphorylation of 14-3-3σ after TGFβ1 treatment for 1 h using two types of assay. First, we immunoprecipitated phosphorylated cellular proteins with anti-phosphoserine, anti-phosphothreonine and anti-phosphotyrosine antibodies and immunoblotted with antibodies to endogenous 14-3-3σ (Fig. 3B). In the second assay, cells were metabolically labeled with ³²P, 14-

3-3 σ was precipitated with specific antibodies and detected after exposure in a phosphorimager (Fig. 3B). Similar TGF β 1-dependent induction of 14-3-3 σ phosphorylation was observed for an ectopically expressed 14-3-3 σ (Fig. 3C). Phosphorylation of transfected 14-3-3 σ was evaluated in assays of the same types as phosphorylation of endogenous protein. Thus, the phosphorylation pattern of 14-3-3 σ was confirmed for both endogenous and ectopically expressed protein.

As 14-3-3 σ may be phosphorylated on multiple sites, we performed two-dimensional phosphopeptide mapping which allows monitoring of all phosphopeptides and the level of ^{32}P incorporation in these peptides (Fig. 4A). We found that TGF β 1 induced phosphorylation of two phosphopeptides of ectopically expressed 14-3-3 σ , indicated as phosphopeptides #1 and #2 (Fig. 4A). The same phosphopeptides were observed in endogenous 14-3-3 σ in TGF β 1-treated MCF10A cells (Fig. 4B). To identify sites of phosphorylation, TGF β 1-regulated phosphopeptides were subjected to radiochemical sequencing and to phosphoamino acid analysis. We found that the phosphopeptide #1 was strongly phosphorylated at the position 6, and the phosphopeptides #2 showed two sites of phosphorylation at positions 1 and 6 (Fig. 4C). Alignment of possible tryptic peptides showed that the peptide Ser69-Lys77 has serine residues at positions 1 and 6. Ser69 and Ser74 were mutated to alanine residues to abrogate phosphorylation at these sites. 14-3-3 σ with mutated Ser69 and Ser74 did not show TGF β 1-dependent induction of phosphorylation, as compared to the wild-type construct (Fig. 4D). Two-dimensional phosphopeptides maps of mutated 14-3-3 σ showed disappearance of phosphopeptides #1 for Ser74Ala mutant, phosphopeptides #2 for Ser69Ala mutant, and both phosphopeptides for the double Ser69/74Ala mutant (Supplementary Fig. 5). The peptide sequence around Ser69 and Ser74 residues showed similarity to the Casein Kinase-2 (CK2) consensus (Meggio *et al*, 2003). Co-expression of CK2 α with the wild-type 14-3-3 σ led to enhancement of 14-3-3 σ phosphorylation, as quantified by ^{32}P incorporation in peptides #1 and #2 (data not shown). Thus, we have identified Ser69 and Ser74

as the sites of TGF β 1-dependent phosphorylation. Ser69 and Ser74 residues are located within amino-terminal domain of 14-3-3 σ protein in close proximity to CDK binding sequence WRVL (residues 59-63) (Laronga *et al*, 2000) (Fig. 4E). Crystal structure and mutational studies revealed that the 14-3-3 σ target binding is mediated by residues from both the amino- and carboxy-terminal part of the 14-3-3 σ protein and demonstrated that 14-3-3 σ dimerization is mediated by the amino-terminal part of the protein (Tzivion *et al*, 2001; Wilker *et al*, 2005). Moreover, recent study demonstrated an important role of 14-3-3 dimerization in the phosphorylation-dependent target binding (Shen *et al*, 2003). These findings point to possibility of involvement of Ser69 and Ser74 residues in the binding of 14-3-3 target proteins.

Phosphorylation of 14-3-3 σ is a feed-forward mechanism in Smad3-dependent transcription which is controlled by recruitment of p53.

14-3-3 σ is known to act as a scaffold by interacting with over 200 target proteins in phosphoserine-dependent and phosphoserine-independent manners (Hermeking *et al*, 2006; Benzinger *et al*, 2005). We observed that the wild-type 14-3-3 σ interacted with the full-length and the MH1 domain of Smad3 in GST pull-down assay (Fig. 5A). We observed that the interaction between Smad3 and wild-type 14-3-3 σ was induced by treatment of cells with TGF β 1 and co-transfection with constitutively active T β R-I (Fig. 5B). Abrogation of 14-3-3 σ phosphorylation at Ser69 and Ser74 completely blocked the interaction, while single Ser69Ala and Ser74Ala mutants were able to form a complex with Smad3. We showed that treatment of cells with TGF β 1 modulates interaction between endogenous Smad3 and 14-3-3 σ in time dependent manner. Moreover, this interaction correlates with profile of 14-3-3 σ phosphorylation at Ser69 and Ser74 residues (Fig. 5C). We also observed co-localization of Smad3 and wild-type 14-3-3 σ in cells using immunofluorescence staining (Supplementary Fig. S6). Thus, the interaction of Smad3 and 14-3-3 σ requires phosphorylation on Ser69 and Ser74 in 14-3-3 σ .

Smad3 is a transcription factor which binds directly to a specific promoter element CAGA (Dennler *et al*, 1998). Thus we explored whether phosphorylation and interaction of 14-3-3 σ with Smad3 regulates TGF β /Smad3-dependent transcriptional activation of CAGA(12)-luciferase reporter (Fig. 6A, B). Our data suggest that overexpression of the wild type or a single Ser74 mutant 14-3-3 σ proteins could significantly increase TGF β and Smad3-dependent transactivation activity of the reporter (up to 2 folds). However, the abrogation of TGF β dependent phosphorylation at Ser69 and Ser74 abolished the ability of 14-3-3 σ to co-activate TGF β and Smad3-dependent transcription (Fig. 6A).

Previous reports indicated that wild-type p53 may associate with SMAD2 and SMAD3 in a TGF- β -dependent manner, although the exact molecular mechanism of such association has not been reported (Atfi *et al*, 2008; Cordenonsi *et al*, 2003). Our study demonstrated that co-expression of p53 correlated with a restrictive effect of p53 on Smad3-dependent transcriptional activity in cells not treated with TGF β 1, while upon treatment with TGF β 1 the level of transcription activation was more than 10 fold increased in the cells overexpressing Smad3, p53 and 14-3-3 σ proteins (wild-type, Ser74Ala, Ser69Ala and double mutant Ser69/74Ala), as compared to TGF β 1 non-treated cells (Fig. 6A). Notably, we observed significantly lower activation of TGF β -induced transcription in the cells with overexpressed Smad3 and 14-3-3 σ proteins (a 1.3-2.1 fold increase). Smad3 co-transfected with 14-3-3 σ double mutant Ser69/74Ala and p53 was two-fold more efficient in transcriptional activation, comparable to Smad3 only (Fig. 6A). The transcription assays showed that 14-3-3 σ proteins stimulated TGF β 1 transcriptional responses in phosphorylation-dependent manner and p53 had an accentuating role in Smad3-dependent transcription by inhibiting ligand-independent and promoting TGF β -stimulated transcription. Analysis of proteins bound to CAGA element (DNA precipitation assay) showed that the presence of Smad3 and its upper migrating form correlated with stimulatory effect on transcriptional activity (Fig. 6B). No detection of the double mutant of 14-3-

3 σ also correlated with decreased transcriptional activation. Moreover, TGF β dependent transactivation of Smad3 responsive genes PAI-1 and COL7A1, possibly through CAGA box elements in their promoters, correlated with involvement of 14-3-3 σ protein in a transcriptional complex with endogenous Smad3 (Fig. 6C, D).

Post-translational modifications play an essential role in driving p53 transcriptional activation. CK2 is a serine/threonine kinase phosphorylating p53 at serine 392 in response to stress, and therefore activating p53 transcriptional activity (Blaydes *et al*, 1998; Keller *et al*, 2002). We observed that p53 phosphorylated at Ser392 can be recruited to the CAGA element. DNA precipitation experiments showed that Ser392-phosphorylated p53 was recruited to the CAGA element in presence of the wild-type or single mutants of 14-3-3 σ and Smad3. Double mutant Ser69/74Ala of 14-3-3 σ abrogated recruitment of p53 (Fig. 6E). Notably involvement of phospho-p53 into the Smad3/14-3-3 σ /CAGA complex positively correlated with expression of Smad3-responsive genes PAI and COL7A1 suggesting that 14-3-3 phosphorylation could be one of the mechanism regulating cooperation between p53 and TGF β signals (Fig. 6C, D).

Moreover, involvement of p53 in a transcriptional complex with Smad3 and 14-3-3 σ proteins correlated with transactivation of genes p21 and MDM2, suggesting that Smad3/14-3-3 σ /p-p53 complex could contribute to transactivation of p53 responsive genes not having CAGA box elements in their promoter (Supplementary Fig. S7). p53 Ser392 phosphorylation is important for damage-induced p53 activation and tumor suppression (Blaydes *et al*, 1998; Keller *et al*, 2002; Brook *et al*, 2003). Activated p53 then up-regulates a number of target genes involved in the DNA damage response including MDM2 and p21. Our data suggest that Smad3/14-3-3 σ /phospho – p53 complex could regulate MDM2 and p21 transcription.

Co-immunoprecipitation assay showed that 14-3-3 σ contributes to enhancement of Smad3 and p53 interaction (Fig. 6F). p53 was found to co-precipitate with 14-3-3 σ ; this interaction is slightly decreased upon treatment of cells with TGF β 1 and upon expression of Smad3, and is

dependent on phosphorylation on both serine residues of 14-3-3 σ (Fig. 6F). Additionally, we showed that the treatment of cells with TGF β 1 modulated interaction between endogenous p53 and 14-3-3 σ in time dependent manner. This interaction correlated with the profile of 14-3-3 σ phosphorylation at Ser69 and Ser74 residues (Fig. 6G). To identify physiological 14-3-3 σ -p52 interactions we performed the blue native PAGE. Consistent with immunoprecipitation results, we observed 14-3-3 σ -p53 protein complex formation in time dependent manner (Fig. 6H).

Thus, TGF β -dependent phosphorylation of 14-3-3 σ is a feed-forward mechanism for TGF β /Smad3 transcriptional regulation. The feed-forward tuning included a 14-3-3 σ -dependent recruitment of p53 to a Smad3-initiated transcriptional complex, which led to restriction of ligand-independent transcription and to enhancement of the ligand-induced effect. This indicates that p53 is an enhancer of bi-stability for Smad3-dependent transcriptional activation, e.g. on-off accentuation.

TGF β 1-dependent phosphorylation of 14-3-3 σ modulates cdk2 and pRb role in regulation of cell proliferation.

TGF β is claimed to be a growth inhibitor, although the potency of its action may vary. 14-3-3 σ is also known to inhibit the G2/M cell cycle progression (Laronga *et al*, 2000; Tzivion *et al*, 2001). Thus, we explored an impact of 14-3-3 σ phosphorylation on TGF β effect on the cell cycle. The 14-3-3 σ -centered network suggested involvement of CDK2, a kinase regulating diverse aspects of the mammalian cell cycle. 14-3-3 σ shares cyclin-CDK2 binding motifs with different cell cycle regulators, including p107, p130, p21^{CIP1}, p27^{KIP1}, and p57^{KIP2}. Previous studies demonstrated that overexpression of 14-3-3 σ impedes cell cycle transition by inhibiting cyclin-CDK activity in many cell lines (Laronga *et al*, 2000; Tzivion *et al*, 2001). We found that the wild-type 14-3-3 σ formed a complex with CDK2 in agreement with previous observations (Fig.

7A). Abrogation of 14-3-3 σ phosphorylation at Ser69 or Ser74 led to inhibition of interaction between 14-3-3 σ and CDK2. Treatment of cells with TGF β 1 and overexpression of CK2 stimulated this interaction. Notably, deficient interaction between 14-3-3 σ Ser74Ala mutant and CDK2 could be restored to wild type 14-3-3 σ level in the presence of ectopically expressed CK2 and treatment of cells with TGF β 1, suggesting that TGF β 1 dependent phosphorylation of 14-3-3 σ at Ser 69 plays more significant role for the interaction with CDK2 than phosphorylation at Ser74 (Fig. 7A).

Our data is consistent with previous reports demonstrated that a consensus cyclin – CDK binding sequence (4 amino acid residues WRVL at positions 59-63) is located at the N-terminal region of 14-3-3 σ in close proximity to Ser 69 (Laronga *et al*, 2000). Analysis of cell proliferation in response to TGF β 1 treatment showed that 14-3-3 σ Ser74Ala and Ser 69Ala mutations enhanced growth inhibitory effect of TGF β 1 (Fig. 7B). However, overexpression of the 14-3-3 σ Ser69/74Ala double mutant did not decrease cell proliferation as compared to wild type 14-3-3 σ indicating that that phosphorylation at Ser 69 and Ser 74 could play unique role for cell cycle regulation. The inhibitory effect of the 14-3-3 σ Ser69Ala mutant on cell proliferation could be abolished by knockdown of CDK2 suggesting that phosphorylation of 14-3-3 σ at Ser 69 plays an important role in CDK2-dependent regulation of cell cycle progression. In contrast, knockdown of CDK2 could not attenuate the inhibitory effect of 14-3-3 σ Ser74Ala mutant on cell proliferation indicating that phosphorylation at Ser74 may regulate other cell-cycle related targets in addition to CDK2 (Fig. 7B).

Activity of cycling - CDK complexes is dependent not only on post translational modifications of CDKs, but *CDK2* gene expression. High CDK2 expression level is associated with poor survival in breast cancer and aggressive tumor behavior (Bonin *et al*, 2006). We found that expression of the wild-type 14-3-3 σ markedly enhanced expression of CDK2 mRNA and protein in TGF β 1-dependent manner (2-fold). In contract, CDK2 protein expression was

decreased by 1.8 fold in the presence of 14-3-3 σ Ser74Ala mutant and treated with TGF β 1 (Fig. 7E, F). Remarkably, the results of co-immunoprecipitation analysis of ectopically expressed 14-3-3 and endogenous CDK2 can be distorted by changes in the expression level of endogenous CDK2 protein (Supplementary Fig. S8). Thus, phosphorylation of 14-3-3 σ modulates its interaction with CDK2 and contributes to regulation of CDK2 expression (Fig. 7A-F, Supplementary Fig. S8).

CDK2 is known to phosphorylate pRb at Ser612 which contributes to disruption of pRb interaction with E2F transcription factors (Zarkovska *et al*, 1997; Knudsen *et al*, 1997). We observed that expression of the wild-type 14-3-3 σ inhibited pRb phosphorylation at Ser612 in TGF β 1 non-treated cells, while Ser74Ala mutant did not. Ser74Ala mutant showed TGF β 1-dependent de-phosphorylation of pRb at Ser612, which was stronger than pRb de-phosphorylation observed in vector-transfected cells (Fig. 7G). Ser69Ala mutant had a similar effect as the wild-type 14-3-3 σ , although no significant TGF β 1-dependent decrease in pRb phosphorylation was observed. Ser69/74Ala double mutant led to the similar level of pRb phosphorylation in non-treated cells, as in vector-transfected cells. However, for the Ser69/74Ala mutant TGF β 1-dependent inhibition of pRb phosphorylation was less evident, as compared to the vector-transfected cells. Thus, phosphorylation of 14-3-3 σ at Ser74 (Ser69Ala mutant) may impede TGF β 1-dependent inhibition of pRb phosphorylation, while phosphorylation at Ser69 (Ser74Ala mutant) promotes TGF β 1-dependency (Fig. 7G).

CDK-dependent phosphorylation of pRb triggers the cell cycle by activating E2F transcription factors (Zarkovska *et al*, 1997; Knudsen *et al*, 1997). Luciferase reporter assay which monitors activity of E2F2 confirmed results of pRb phosphorylation study (Fig. 7H). We observed that the wild-type 14-3-3 σ which can be phosphorylated by TGF β , inhibited slightly luciferase activity, as compared to the vector-transfected cells. Mutations of Ser69 and Ser74 modulated basal reporter activity, with mutation of Ser74 leading to enhancement and mutation of

Ser69 to inhibition of the reporter. Mutations of the serine residues unmasked TGF β -responsiveness; mutation of Ser74 lead to a TGF β -dependent inhibition of the reporter, while mutation of Ser69 had an opposite effect. The bi-directional way of 14-3-3 σ -dependent E2F2 activation was reflected in TGF β 1-stimulated inhibition of cell proliferation. Ser74Ala mutation enhanced growth inhibitory effect of TGF β 1, while inhibitory effect of Ser69Ala mutation is less significant (Fig. 7B). Remarkably, the inhibitory effect of the 14-3-3 σ Ser69Ala mutant on cell proliferation could be abolished by knockdown of CDK2 suggesting that phosphorylation at Ser74 is mainly playing a role for CDK2-14-3-3 σ assembly (Fig. 7B). These results are in agreement with our previous data demonstrating the importance of phosphorylation at Ser 69 for TGF β 1 dependent 14-3-3 σ -CDK2 interaction and *CDK2* gene expression. The bi-directional Ser74 and Ser69 dependent way of 14-3-3 σ -mediated E2F2 activation may contribute to TGF β 1-stimulated inhibition of cell proliferation (Fig. 8).

Thus, phosphorylation at Ser69 (Ser74Ala mutant) is primarily required for 14-3-3 σ interaction with CDK2, TGF β 1-dependent inhibition of pRb phosphorylation, E2F2 reporter inhibition and TGF β 1 inhibitory action on the cell proliferation. Phosphorylation at Ser74 (Ser69Ala mutant) in contrary may hinder TGF β 1-dependency. Together with unidirectional role of Ser69 and Ser74 phosphorylations in transcription (Fig. 6), phosphorylations at these sites may have a bi-directional impact on cell proliferation (Fig. 7).

Discussion

Signaling by a network, as compared to the model of unidirectional signaling pathways (Souhelnytskyi *et al*, 2005), is required for coordination of various functions in cells undergoing significant changes, e.g. proliferation or carcinogenic transformation. Our data identified proteins which may mediate coordinated regulation of cell metabolism, proliferation, death and migration

of human breast epithelial cells (Supplementary Fig. S3). The network built with TGF β -regulated phosphoproteins showed characteristics of a scale-free network (Supplementary Fig. S4). However, the frequency of highly-connected hubs is higher than would be expected according to a power law distribution of connections in an ideal scale-free network. The presence of such hubs indicates the key points of convergence for various signals. In the TGF β phosphoprotein-network (Fig. 3), these hubs represent signaling activities initiated and/or mediated by EGF, TNF, IGF, AKT, Src, H-Ras, CDK2 and NF- κ B. Therefore the status of these hubs may dictate the output of TGF β action on cells. For many of the above mentioned hubs, functional cross-talks with TGF β have been reported in model systems different for each of the hubs (Derynk *et al*, 2003; Shi *et al*, 2003; Attisano *et al*, 2002). Phosphoproteome profiling of TGF β 1 in MCF7 cells using in vivo metabolic labeling with 32 P was the first step in an exploration of TGF β phosphoproteome. Despite use of different cell lines, we identified in MCF10A cells some of the proteins which were regulated by TGF β 1 in MCF7 cells, e.g. keratin 10, enolase-1 and HSP70 (Stasyk *et al*, 2005). IMAC-enrichment of phosphoproteins showed capability to enrich for low abundance proteins, which explains high representation of regulatory proteins (Table 1). Thus, our approach provided the most comprehensive description of phosphorylation events initiated by TGF β 1.

To confirm that our network-based approach unveils novel crucial regulatory mechanisms, we explored the role of TGF β 1-dependent phosphorylation of 14-3-3 σ ; 14-3-3 σ -centered sub-network showed possible involvement of CDC2, CDK2, CDK4, p53 and BRCA1 (Fig. 2B). 14-3-3 σ and p53 have been reported to cooperate in suppression of tumorigenesis (Laronga *et al*, 2000), although the role of 14-3-3 σ expression in breast tumorigenesis has been disputed (Moreira *et al*, 2005; Hondermarck *et al*, 2001). Three phosphorylation sites in 14-3-3 σ important for its function have been identified, e.g. Thr198, Ser216, Thr291, Ser428, Ser642 (Hondermarck *et al*, 2000; Fu *et al*, 2000). In this report, we identified two novel and TGF β -dependent phosphorylation sites, i.e. Ser69 and Ser74. Identified by us network (Fig. 2B) suggested that

phosphorylation of 14-3-3 σ at Ser69 and Ser74 may play crucial role in TGF β signaling. Indeed, we observed that 14-3-3 σ phosphorylation is a feed-forward mechanism in TGF β /Smad3-dependent transcription. 14-3-3 σ through interactions with Smad3 and p53 may provide a scaffold for a complex which includes both Smad3 and p53 at the Smad3-specific CAGA element (Fig. 6, 8). Our results show that the recently reported p53 phosphorylation-dependent complex with Smad3, which lead to inhibition of cell proliferation (Cordenonsi *et al*, 2007), may be mediated by 14-3-3 σ (Fig. 6). At the same time, Ser69 and Ser74 phosphorylation of 14-3-3 σ regulates a complex of CDK2 and 14-3-3 σ , which results in inhibition of pRb phosphorylation and cell proliferation (Fig. 7, 8). Our data suggest that TGF β 1-dependent phosphorylation of 14-3-3 σ orchestrates a functional interaction of TGF β /Smad3 with p53, CDK2 and pRb (Fig. 8) and could provide a new potential target for intervention in breast cancer. Moreover, recently it was also found that 14-3-3 σ could contribute to drug resistance in human breast cancer cells by CDK-dependent mechanism (Maxwell *et al*, 2009; Liu *et al*, 2006). This finding together with the results of this study, suggest that TGF β 1-dependent phosphorylation of 14-3-3 σ may be considered for prediction of response to anticancer therapy in the clinic and underscores the utility of profiling individual tumors.

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Material and Methods

Cell cultures

293T and MCF10A cells were obtained from American Type Culture Collection (Manassas, VA). 293T cells were cultured in DMEM with 10% of foetal bovine serum, penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO). MCF10A cells were cultured in a MEGM medium (Clonetics, Inc., San Diego, CA) supplemented with EGF, insulin, hydrocortisone, bovine pituitary extract, and 5% horse serum.

Luciferase reporter assay

Reporter assays with CAGA(12)-luc and E2F2-luc reporters were performed as described previously (Stasyk *et al*, 2005). 293T cells were used, because they are responsive to TGF β and allow efficient expression of proteins.

Cell transfection

Day before transfection 293T cells were subcultured to reach 50% confluent the next day for transfection. 293T cells were transfected in 12 wells plates using calcium phosphate-based transfection procedure. MCF10A cells were transfected in 12 wells plate by LipofectAMINE 2000 reagent. Medium was changed 6 hours after transfection and then cells were incubated in serum-free MEBM medium for 72 hours prior to addition of TGF β 1.

Cell proliferation assay

MCF10A proliferation in response to TGF β 1 treatment was measured by using CellTiter-Glo $\text{\textcircled{R}}$ Reagent (Promega) according to the manufacturer's recommendations. Cells were grown in DMEM/F12 medium supplemented with 15 mM Hepes buffer, 10 μ g/ml insulin, 20 ng/ml EGF and 0.5 μ g/ml hydrocortisone, with and without TGF β 1 treatment at concentration 5 ng/ml. Alternatively, proliferation of MCF10A cells in response to TGF β 1 treatment was analyzed by using CellTiter 96 $\text{\textcircled{R}}$ Non-Radioactive Cell Proliferation Assay (MTT) (Promega) according to the manufacturer's recommendations. MCF10A cells were cultured in a MEGM medium (Clonetics,

Inc., San Diego, CA) supplemented with EGF, insulin, hydrocortisone, bovine pituitary extract, and 5% horse serum, with and without TGF β 1 treatment at concentration 5 ng/ml.

GST-pull down assay

For GST-pull down assay 293T cells were transfected with GST, GST-Smad3, GST-Smad3MH1, GST-Smad3MH2 expressing pGEX vectors and with pcDNA3.1 vector expressing 14-3-3 σ -Flag protein. The proteins were extracted with a lysis buffer containing 1% NP-40, 50mM Tris-HCl pH 8.0, 150mM NaCl, 10 mkg/ml aprotinin and 1 mM PMSF. Equal amounts of control GST and fusion proteins GST-Smad3, GST-Smad3MH1 and GST-Smad3MH2 bound to glutathione Sepharose beads were added to the cell lysate (lysate from 6x10⁶ cells overexpressing 14-3-3 σ protein per pull down) and incubated overnight at 4 °C. After 3 washes with ice-cold lysis buffer, the samples were re-suspended in a sample buffer for SDS-PAGE.

Immunoblotting and immunoprecipitation

For immunoblotting, cell lysates were resolved on SDS polyacrylamide gels and transferred onto Hybond P membranes (Amersham Biosciences, Piscataway, NJ). Membranes were blocked with 5% (v/v) BSA for one hour and then incubated with the primary antibody against target proteins with dilution as recommended by manufacturer followed by an HRP-conjugated secondary antibody (GE Healthcare, Uppsala, Sweden). The proteins were visualized using Western Blotting Luminol Reagents (Santa Cruz Biotechnology Inc.). For immunoprecipitation, cell lysates were incubated with antibodies against target proteins and protein A-Sepharose beads (Sigma-Aldrich) for 6 hours at 4 °C with gentle agitation. Immunocomplexes bound to protein A-Sepharose beads were collected by centrifugation and washed 3 times in lysis buffer before being resolved by SDS-PAGE.

Two-dimensional gel electrophoresis

Samples for two-dimensional gel electrophoresis were prepared according to the protocol described for Fe-IMAC (Dubrovskaya *et al*, 2005). Two-dimensional gel electrophoresis was

performed as described earlier (Dubrovskaya *et al*, 2005; Stasyk *et al*, 2005). Briefly, prepared samples were subjected to isoelectric focusing using IPG Dry strips with immobilized pH gradient, pH range 3–10, 18 cm, linear (GE Healthcare, Uppsala, Sweden). 2D-GE was performed according to the protocol described earlier (Dubrovskaya *et al*, 2005; Stasyk *et al*, 2005), SDS-PAGE was performed in 12% polyacrylamide gels. After the electrophoresis, gels were fixed in 10% acetic acid and 20% methanol for 10–12 h. Proteins were detected by silver staining, as described earlier (Dubrovskaya *et al*, 2005; Stasyk *et al*, 2005). Totally, 6 gels with samples from three experiments were prepared and subjected to analysis.

Gel analysis

Silver stained gels were scanned and analyzed by the ImageMaster 2D Platinum Version 6.0 (GE Healthcare, Uppsala, Sweden). Gels that did not show deviations in pattern of protein migration were used to generate master gels of the phosphoproteome of cells treated or not with TGFβ1. Cellular proteins changing their phosphorylation after treatment with TGFβ1 were considered for identification. Statistical significance of changes was evaluated using the ImageMaster 2D Platinum Version 6.0 software.

Protein identification

Protein spots were excised from the gels, destained and subjected to in-gel digestion with trypsin (modified, sequence grade porcine, Promega, USA), as described earlier (Dubrovskaya *et al*, 2005; Stasyk *et al*, 2005). Tryptic peptides were concentrated and desalted on a “nano-column”, i.e. ZipTip. Peptides were eluted with 65% acetonitrile, containing the matrix α-cyano-4-hydroxycinnamic acid, and applied directly onto the metal target and analyzed by MALDI TOF MS on a Bruker Biflex (Bruker Daltonics, Bremen, Germany). Peptide spectra were internally calibrated using autolytic peptides from the trypsin. To identify proteins, we performed searches in the NCBI nr sequence database using the ProFound (<http://65.219.84.5/service/prowl/profound.html>) search. One miscut, alkylation, and partial

oxidation of methionine were allowed. Significance of the identification was evaluated according to the probability value, “Z” value, and sequence coverage.

Pathway analysis

Functional and pathway analysis was performed using Ingenuity Pathway Analysis, a proprietary tool for description of networks and signaling pathways (www.ingenuity.com). A data set containing identified proteins was uploaded into the Ingenuity Pathway Analysis application and TGF β 1-dependent networks regulating cell proliferation, death, migration and differentiation were generated. Fischer’s exact test was used to calculate a p-value determining the network connectivity.

Blue native polyacrylamide gel electrophoresis

Samples were prepared as described (Wittig *et al*, 2006). Briefly, MCF10A cells were treated with TGF β at concentration 5ng/ml for 24 hours. Cells were subsequently rinsed twice with ice-cold PBS and incubated with solubilization buffer A (50 mM NaCl, 2 mM 6-aminohexanoic acid, 1 mM EDTA, 50 mM imidazole/HCl, pH 7.0) for 15 min on ice. Cells lysates were clarified by centrifugation and incubated with Triton X-100 at a final concentration 3 % for 15 min on ice for solubilization of protein complexes. Glycerol at a final concentration 5.4% and the anionic dye coomassie brilliant blue were added to the sample prior to loading. The protein complexes were separated by SDS-PAGE using 3.5%-13% % linear gradient gels. Electrophoresis was performed using anode buffer (25 mM imidazole/HCl, pH 7.0) and cathode buffer B (50 mM tricine, 7.5mM imidazole, 0.02% coomassie brilliant blue dye, pH 7.0). When coomassie dye migrated about one third of the gel length, cathode buffer B was replaced with cathode buffer B/10 (50 mM tricine, 7.5 mM imidazole, 0.002% coomassie brilliant blue dye, pH 7.0).

Two-dimensional Phosphopeptide Mapping

MCF-10A or 293T cells were treated as indicated in the text. Metabolic labeling of cells with [³²P] orthophosphate (GE Healthcare) was performed as described previously³¹. Briefly, radioactively labeled 14-3-3σ proteins were subjected to digestion with trypsin (modified, sequence grade porcine; Promega), and the tryptic digest was separated on thin-layer cellulose plates by electrophoresis and chromatography. Plates were exposed in a FujiX2000 PhosphorImager (Fuji). Phosphopeptides of interest were subjected to phosphoamino acid analysis and to Edman degradation.

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Figure legends

Figure 1. Representative 2D gel of Fe-IMAC enriched phosphoproteins of MCF10A cells treated with TGF β 1. Migration positions of proteins regulated by TGF β 1 are indicated by lines, with annotation of proteins as in Table 1. Direction of isoelectrofocusing is indicated on the top of the gel image. Migration positions of molecular mass markers upon SDS-PAGE are indicated on the side of the image.

Figure 2. Network of TGF β 1-regulated phosphoproteins (A) Graphs show distribution of connections for species of the network. Distribution for proteins identified by phosphoproteomics (rhombs; experimental data) and as would be expected by a power law distribution of connections of species in an ideal scale-free network (squares; predicted distribution) are shown. (B) A sub-network of 14-3-3 σ (SFN). Proteins which are in proximal dependencies to 14-3-3 σ were extracted from the complete network (Supplementary Fig. S4) into the presented sub-network.

Figure 3. Validation of 14-3-3 σ phosphorylation upon treatment of cells with TGF β 1. (A) Images of areas of 2D Fe-IMAC gels with annotation of phosphoprotein spots p72 and p74 in which 14-3-3 σ was identified are shown. Values of volumes of protein spots are shown below images of gels for both protein spots. (B) Phosphorylation of endogenous 14-3-3 σ in MCF10A cells was evaluated by immunoprecipitation with anti-phosphoserine, threonine and tyrosine antibodies (upper panel) or by incorporation of ^{32}P (middle panel). Control immunoprecipitation of 14-3-3 σ is shown in lower panel. Densitometry analysis of the protein immunoblots or ^{32}P incorporation is shown in accompanying graphs. (C) Phosphorylation of Flag-14-3-3 σ expressed in 293T cells was evaluated in the same way as for endogenous protein. The upper panel shows detection of phosphorylation by immunoprecipitation and the middle panel shows incorporation of ^{32}P . The lower panel shows expression of 14-3-3 σ . Migration positions of 14-3-3 σ are shown

by arrows, and treatments with TGFβ1 are indicated. Densitometry analysis of the protein immunoblots is shown in accompanying graphs. Representative experiments out of 3 performed are shown (B, C).

Figure 4. 14-3-3 σ is phosphorylated at Ser69 and Ser74. (A) Two-dimensional phosphopeptides mapping showed appearance of 2 phosphopeptides upon TGFβ1 treatment. Migration positions of these phosphopeptides are shown by arrows, as #1 and #2 respectively. (B) Phosphopeptide map of endogenous 14-3-3 σ precipitated from MCF10A cells. Treatment with TGFβ1, and directions of electrophoresis and chromatography are indicated (A, B). (C) Elution positions of ³²P-labeled amino acids upon Edman degradation are shown for phosphopeptides #1 and #2, respectively. Corresponding tryptic peptide is aligned below the panels. (D) Phosphopeptide maps of the wild-type and Ser74Ala(S74A), Ser69Ala (S69A) and Ser69, 74Ala (S69,74A) mutants of 14-3-3 σ are shown. Abrogation of appearance of phosphopeptides #1 and #2 are indicated by arrows. (E) Ser69 and Ser74 residues are located within amino-terminal dimerization domain in close proximity to CDK binding sequence WRVL (residues 59-63). The image shows the structure of the p53 C-terminus bound to 14-3-3 σ (Schumacher *et al.*, 2010), PDB entry 3LW1.

Figure 5. 14-3-3 σ forms a complex with Smad3. (A) 14-3-3 σ interacts with Smad3 in vitro. The interaction is mediated by the MH1 domain. GST constructs are indicated on the top of panels. The left panel shows co-precipitated 14-3-3 σ , the right panels show inputs of 14-3-3 σ (upper part) and GST constructs (lower part). (B) Complex formation between Smad3 and 14-3-3 σ is dependent on phosphorylation at Ser69 and Ser74. 293T cells were transfected, as indicated. Migration position of co-precipitated 14-3-3 σ is shown by an arrow in upper panel, and expression of 14-3-3 σ constructs and Smad3 are shown in lower panels. (C) Treatment of cells

with TGF β 1 modulates interaction between endogenous Smad3 and 14-3-3 σ in time dependent manner. MCF7 cells were treated with TGF β at concentration 5ng/ml for the indicated times. Cell lysates were immunoprecipitated with anti-14-3-3 σ antibody. Anti-Smad3 antibody was used in Western blot analysis. Densitometry analysis of Smad3 immunoblot is shown in accompanying graphs.

Figure 6. Smad3-dependent transcriptional activation is dependent on phosphorylation of 14-3-3 σ at Ser69 and Ser74 which regulate recruitment of p53 to the promoter. (A) CAGA(12)-luc reporter activation upon expression of 14-3-3 σ constructs, Smad3 and p53. Combinations of various transfections, treatment of cells with TGF β 1 and control of expression of Smad3, 14-3-3 σ and p53 are indicated, * - p value < 0.05 (B) DNA precipitation assay shows that wild-type 14-3-3 σ enhances interaction of Smad3 with the promoter (upper panel). An abrogation of 14-3-3 σ phosphorylation at both Ser69 and Ser74 inhibited 14-3-3 σ recruitment to the CAGA element (second from the top panel). Expression controls for Smad3 and 14-3-3 σ constructs are shown in two lower panels. Densitometry analysis of the protein precipitation is shown in accompanying graphs. (C, D) TGF β dependent transcriptional activation of PAI-1 and COL7A1 genes through CAGA box elements in their promoter correlates with involvement of 14-3-3 σ proteins in a transcriptional complex with endogenous Smad3. For gene expression analysis, MCF10A cells stably transfected with wild type or mutated 14-3-3 σ were treated with TGF β at concentration 5ng/ml for 12 hours. (E) p53 phosphorylated at Ser392 is recruited to the CAGA element in presence of 14-3-3 σ and Smad3 (top panel). Expression controls are shown in lower panels, as indicated. (F) Smad3 is recruited in a complex with p53 via 14-3-3 σ . Smad3, p53 and 14-3-3 σ constructs were expressed in 293T cells, as indicated, and their expression was monitored by immunoblotting of whole cell extracts (IB-WCE). Upper panel shows co-precipitation of p53 and 14-3-3 σ . Densitometry analysis of the protein immunoprecipitation

is shown in accompanying graphs. (G) Treatment of cells with TGF β 1 modulates interaction between endogenous p53 and 14-3-3 σ in time dependent manner. MCF7 cells were treated with TGF β at concentration 5ng/ml for the indicated times. Cell lysates were immunoprecipitated with anti-14-3-3 σ antibody. Anti-p53 antibody was used in Western blot analysis. Densitometry analysis of p53 immunoblot is shown in accompanying graphs.

Figure 7. TGF β 1-dependent phosphorylation of 14-3-3 σ affects interaction of 14-3-3 σ with cdk2, pRb phosphorylation and cell proliferation. (A) Complex formation between 14-3-3 σ and cdk2 is dependent on phosphorylation at Ser69 and Ser74. 293T cells were transfected, as indicated. Migration positions of 14-3-3 σ proteins co-precipitated with cdk2 are shown by arrows in upper panels, and expression of 14-3-3 σ and CDK2 are shown in middle and lower panels. Densitometry analysis of the immunoblots is shown in accompanying graphs. (B) Analysis of cell proliferation in response to TGF β 1 treatment. Rate of TGF β 1-dependent inhibition of MCF10A cells proliferation is enhanced by abrogation of phosphorylation at Ser74 and to a lesser extent by abrogation of phosphorylation at Ser69. Knockdown of CDK2 could abolish the antiproliferative activity of the 14-3-3 σ Ser69Ala mutant. MCF10A cells stably transfected with wild type or mutated 14-3-3 σ and stably transfected with shRNA for CDK2 or with scrambled shRNA were treated with TGF β 1 at concentration 5ng/ml replenished daily for 7 days. * - p value < 0.05; ** - p value < 0.01. (C) Control RT-PCR analyses showed that CDK2 mRNA expression was knocked down by around 80% compared to the scrambled shRNA control. (D) Control immunoblot analyses of 14-3-3 σ showed equal protein loading. (E, F) Effect of 14-3-3 σ phosphorylation on expression of CDK2 mRNA (E) and protein (F). For RT-PCR and Western blot analysis, MCF10A cells were treated with TGF β 1 at concentration 5ng/ml for 12 hours. CDK2 mRNA was monitored by RT-PCR; GAPDH is used as the internal control (E). Expression of CDK2 protein was monitored by immunoblotting. Migration positions of CDK2 and actin

control are indicated by arrows (F). Densitometry analysis of the immunoblot and DNA electrophoresis is shown in accompanying graphs. (G) CDK2-dependent phosphorylation of pRb at Ser612 is dependent on TGFβ1-induced phosphorylation of 14-3-3σ. Migration positions of phosphorylated pRb, total pRb and actin (loading control) are shown. Densitometry analysis of the immunoblot is shown in accompanying graph. (H) TGFβ1-dependent inhibition of E2F2-dependent luciferase reporter correlated with the level of TGFβ1 inhibition of pRb phosphorylation. Transfection of cells and treatment with TGFβ1 are indicated.

Figure 8. Schematic presentation of the role of 14-3-3σ phosphorylation in TGFβ1/Smad3-dependent transcription and CDK2/pRb regulation of cell proliferation. The model shows that phosphorylated 14-3-3σ interacts with Smad3 and mediates recruitment of p53 into transcription complex. Phosphorylated 14-3-3σ sequesters also CDK2, leading to decrease of pRb phosphorylation. The model suggests that phosphorylation of 14-3-3σ coordinated transcription and the cell cycle.

Supplementary Information

Supplementary Figure S1

Representative 2D gel of Fe-IMAC enriched phosphoproteins of MCF-7 cells treated with TGFβ1 for indicated time periods are shown. Directions of isoelectrofocusing are indicated on the top of the gel image. Migration positions of molecular mass markers upon SDS-PAGE are indicated on the side of the image. Migration positions of proteins regulated by TGFβ1 are indicated by lines in Figure 1, with annotation of proteins as in Table 1.

Supplementary Figure S2

Validation of phosphorylation of identified proteins. Protein spots in which (A) FKBP12, (B) Actin and (C) Enolase1 were identified are shown with quantification of protein expression. (D) Phosphorylation of these proteins was monitored by immunoprecipitation with anti-phosphoSer/phosphoThr/phosphoTyr and immunoblotting with specific antibodies, as indicated. Loading control is shown in lowest panel.

Supplementary Figure S3. Functional and dynamic clustering of TGFβ1-regulated phosphoproteins. (A) Functional clusters and number of proteins assigned to the clusters are indicated. (B) Heatmap of TGFβ1-regulated phosphoproteins clustered according to changes in their expression. (C) Dynamics of phosphoproteins involved in regulation of cell proliferation and cell death. Proteins are annotated in Gene Ontology (GO) terms.

Supplementary Figure S4. Network of TGFβ1-regulated phosphoproteins. TGFβ1-regulated proteins are presented in a network with their known targets and regulators. Strings between proteins/species represent dependencies which describe physical and/or functional interactions between these species.

Supplementary Figure S5. Mutation of Ser69 and Ser74 resulted in abrogation of TGF β 1-dependent phosphorylation of 14-3-3 σ . Phosphorylation of the wild-type (wt), Ser74Ala (S74A) and Ser69Ala (S69A) mutants are shown in upper (wt), middle (S74A) and lower (S69A) double panels. In each pair, the upper panels show phosphorylation, and the lower panels show expression of protein. Migration positions of 14-3-3 σ proteins are shown by arrows, and treatment of cells with TGF β 1 was as indicated.

Supplementary Figure S6. 14-3-3 σ and Smad3 co-localize in cells. Images of immunofluorescent staining of cells transfected with 14-3-3 σ and Smad3 constructs, as indicated, are shown.

Supplementary Figure S7. p53 driven transcriptional activation of MDM2 and p21 genes is modulated by TGF β dependent phosphorylation of 14-3-3 σ . For gene expression analysis, MCF10A cells were treated with TGF β at concentration 5ng/ml for 12 hours.

Supplementary Figure S8. Co-immunoprecipitation analysis of ectopically expressed 14-3-3 and endogenous CDK2. MCF10A cells stably transfected with wild type or mutated 14-3-3 σ were treated with TGF β at concentration 5ng/ml for 18 hour. Migration positions of 14-3-3 σ proteins co-precipitated with cdk2 are shown by arrows in upper panels, and expression of 14-3-3 σ and CDK2 are shown in middle and lower panels. Complex formation of ectopically expressed 14-3-3 and endogenous CDK2 depend on changes in the expression level of endogenous CDK2 protein. Densitometry analysis of the protein immunoprecipitation and immunoblotting is shown in accompanying graphs.

Table 1

Table 1. Proteins identified as changing their phosphorylation upon TGFβ1 treatment

No a)	Protein Identity b)	Accession No b)	Probability b)	Est'd Z b)	Sequence coverage, %	Theoretical value		Experimental value		Matched peptides
						pI	Mr (kDa)	pI	Mr (kDa)	
1	Heat shock 70 kDa protein 8 isoform 2	NP_69488.1	1.0e+000	2.16	36	5.6	53.61	4.8	60	15
2	Heat shock 70 kDa protein 9B (mortalin -2)	AAH30634.1	1.0e+000	2.28	34	6.0	74.12	5.0	60	21
3	Mitosis -specific chromosome segregation protein SMC1 homolog	AAB34405.1	1.0e+000	2.28	18	7.6	143.81	6.5	47.5	17
4	Keratin 14	AAP35850.1	1.0e+000	2.39	38	5.1	51.92	4.8	47	27
5	ENO-1 protein	AAH50642.1	1.0e+000	1.98	35	7.0	47.49	7.5	45	13
6	ENO-1 protein	AAH50642.1	1.0e+000	2.15	42	7.0	47.49	7.4	45	18
7	Eukaryotic translation initiation factor 3, subunit 5 epsilon (EIF3S5)	AAP35540.1	1.0e+000	1.97	27	5.2	37.13	4.7	37	10
8	ENO-1 protein	AAH50642.1	1.0e+000	2.08	49	7.0	47.49	7.3	45	19
9	Keratin 5	AAH71906.1	9.6e-001	1.45	24	7.7	62.59	7.8	40	15
10	A+U -rich element RNA binding factor	BAA22860.1	1.0e+000	2.43	29	8.9	30.34	7.0	32	10
11	Annexin A2 , isoform 2	AAH23990.1	1.0e+000	2.30	35	7.7	38.79	7.2	32	15
12	CNN2 (calponin 2)	CAG46609.1	1.0e+000	1.04	24	7.0	34.09	7.0	32	10
13	Keratin 10	AAH59199.1	1.0e+000	1.70	18	4.7	39.84	8.0	26	9
14	Peroxiredoxin 6	NP_004896.1	1.0e+000	1.44	37	6.0	25.13	6.5	25	8
15	Peroxiredoxin 1	CAI13096.1	1.0e+000	2.26	44	6.4	19.13	9.0	19	10
16	Keratin 14	NP_000517.2	1.0e+000	2.35	40	5.1	51.89	4.6	45	26
17	Keratin 17	NP_000413.1	1.0e+000	2.32	39	5.0	49.37	4.3	40	23
18	Laminin -binding protein	CAA43469.1	9.7e-001	0.69	20	4.8	31.89	4.0	33	6
19	RAB6 -interacting protein 2 (RAB6IP2)	AAH68006.1	9.4e-001	0.61	19	6.8	71.88	4.8	33	14
20	Eukaryotic translation initiation factor 3, subunit 2 beta (EIF3S2)	NP_003748.1	1.0e+000	2.22	25	5.4	36.88	5.2	33	14
21	RSAFD1 (radical S -adenosyl methionine and flavodoxin domains 1)	AAH51888.1	9.9e-001	0.70	17	5.8	44.08	5.0	32	8
22	Eukaryotic translation initiation factor 3, subunit 2 beta (EIF3S2)	NP_003748.1	1.0e+000	1.68	39	5.4	36.88	4.2	32	13
23	Keratin 10, type I, cytoskeletal	KRHU0	1.0e+000	1.87	26	5.2	59.74	5.2	26	17
24	Zinc finger protein 62 homolog	NP_689496.1	1.0e+000	0.86	16	9.9	58.78	8.3	20	9
25	RAB6-interacting protein 2 isoform alpha	NP_055879.1	6.3e-001	0.74	15	6.2	108.89	8.3	20	14
26	Antigen MLLA -34	AAQ93064.1	9.0e-001	0.93	26	8.7	39.24	8.0	18	8

a - Selected phosphoprotein spots from 2-D gels.

b - NCBI sequence identification numbers. Probability, Z-value, coverage and theoretical pI and M_r were obtained from the ProFound search. The calculation of experimental pI and M_r was based on migration of proteins on 2-D gels.

27	Eukaryotic translation initiation factor 4H, isoform 2	AAH10021.1	1.0e+000	2.19	44	7.8	25.24	8.0	25	9
28	Keratin 10, type I, cytoskeletal	KRHU0	1.0e+000	1.78	22	5.2	59.74	9.5	20	12
29	Heterogeneous nuclear ribonucleoprotein A2/B1 isoform A2	NP_002128.1	1.0e+000	1.84	20	8.7	36.05	9.2	30	8
30	Heterogeneous nuclear ribonucleoprotein A2/B1 isoform A2	NP_002128.1	1.0e+000	2.36	48	8.7	36.05	9.3	30	13
31	Heterogeneous nuclear ribonucleoprotein A2/B1 i soform A2	NP_002128.1	1.0e+000	2.18	40	8.7	36.05	9.0	32	15
32	Heterogeneous nuclear ribonucleoprotein A2/B1 isoform A2	NP_002128.1	1.0e+000	2.31	29	8.7	36.05	9.2	29	11
33	Heterogeneous nuclear ribonucleoprotein A2/B1 isoform A2	NP_002128.1	1.0e+000	2.38	42	8.7	36.05	8.7	28	12
34	Heterogeneous nuclear ribonucleoprotein A2/B1 isoform A2	NP_002128.1	1.0e+000	1.09	15	8.7	36.05	8.7	25	7
35	Human Muscle Fructose 1,6-Biphosphate Aldolase Complexed With Fructose 1,6-Biphosphate	4ALD	1.0e+000	2.09	28	8.8	39.73	8.8	35	10
36	Human Muscle Fructose 1,6-Biphosphate Aldolase Complexed With Fructose 1,6-Biphosphate	4ALD	1.0e+000	2.25	24	8.8	39.73	9.1	35	9
37	Human Muscle Fructose 1,6-Biphosphate Aldolase Complexed With Fructose 1,6-Biphosphate	4ALD	1.0e+000	1.28	22	8.8	39.73	9.0	35	8
38	Vasodilator-stimulated phosphoprotein	CCA67147.2	1.0e+000	2.15	26	9.2	39.76	9.5	45	6
39	Vasodilator-stimulated phosphoprotein	CCA67147.2	1.0e+000	1.77	21	9.2	39.76	9.3	45	11
40	Keratin 10, type I, cytoskeletal	KRHU0	8.6e-001	0.60	13	5.2	59.74	7.2	46	8
41	ENO1 protein	AAH50642.1	1.0e+000	2.35	43	7.0	47.49	6.8	38	19
42	ENO1 protein	AAH50642.1	1.0e+000	2.40	35	7.0	47.49	7.0	38	14
43	IMP (inosine monophosphate) dehydrogenase 2	AAH15567.1	9.7e-001	0.69	12	6.4	56.24	6.5	47	8
44	DEAD (Asp - Glu -Ala -Asp) box polypeptide 48	NP_055555.1	9.9e-001	0.79	20	6.3	47.25	6.3	47	7
45	Stress-induced phosphoprotein 1 (Hsp70/Hsp90-organizing protein)	AAV38814.1	1.0e+000	1.98	31	6.4	63.25	6.5	50	20
46	Stress-induced phosphoprotein 1 (Hsp70/Hsp90-organizing protein)	AAV38814.1	1.0e+000	1.98	33	6.4	63.25	6.7	50	11
47	Fumarate hydratase	AA P88841.1	1.0e+000	1.32	17	9.0	54.79	7.5	40	9
48	Keratin 10, type I, cytoskeletal 10	K1CJ	1.0e+000	1.14	13	5.1	59.73	7.2	40	9
49	Zinc finger protein 62 homolog	NP_689496.1	9.7e-001	0.63	19	9.9	58.78	7.8	35	9
50	Antigen MLAA -34	AAQ93064.1	9.7e-001	0.61	18	8.7	39.24	6.8	30	6

51	Cytokeratin 9	AAC60619.1	1.0e+000	1.83	23	5.1	62.20	6.5	33	8
52	RNA binding motif protein 4	AAH_32735.1	1.0e+000	1.94	23	6.6	40.70	6.6	33	9
53	Keratin 9	NP_000217.2	1.0e+000	2.18	26	5.1	62.27	6.9	33	14
54	Antigen MLAA -34	AAQ93064.1	9.8e-001	0.68	23	8.7	39.24	7.3	27	6
55	Zinc finger protein 62 homolog	NP_689496.1	1.0e+000	0.99	18	9.9	58.78	7.2	26	8
56	Guanyl nucleotide releasing protein 4	AAL87858.1	9.4e-001	0.51	9	9.1	76.22	7.5	27	9
57	Transcription factor NRF	AAH47878.1	9.6e-001	0.57	11	9.2	78.34	7.9	28	7
58	Heterogeneous nuclear ribonucleoprotein A2/B1 isoform A2	NP_002128.1	1.0e+000	2.31	34	8.7	36.05	7.9	30	11
59	Heat shock 70 kDa protein 9B (mortalin -2)	AAH30634.1	1.0e+000	2.26	41	6.0	74.12	5.0	48	25
60	Heat shock 70 kDa protein 9B (mortalin -2)	AAH30634.1	1.0e+000	2.26	33	6.0	74.12	4.5	60	19
61	MTHSP75 (heat shock 70kDa protein 9B precursor)	AAA67526.1	1.0e+000	2.37	18	6.0	74.05	5.0	50	14
62	Heat shock 70 kDa protein 9B (mortalin -2)	AAH30634.1	1.0e+000	1.71	17	6.0	74.12	5.3	50	11
63	Unknown (chaperonin containing TCP1)	AAH02971.1	1.0e+000	1.99	23	5.4	59.91	5.2	47	12
64	Prolyl 4 -hydroxylase, beta subunit	NP_000909.2	1.0e+000	2.28	32	4.8	57.50	4.0	47	17
65	Chaperonin containin TCP1, subunit 2 (beta)	NP_689496.1	1.0e+000	0.86	16	9.9	58.78	8.3	20	10
66	Tubulin, beta polypeptide	NP_821133.1	1.0e+000	2.39	38	4.8	50.11	4.3	46	34
67	Keratin 7	NP_005547.2	1.0e+000	2.39	35	5.4	51.46	5.0	46	17
68	Heterogeneous nuclear ribonucleoprotein K isoform a	NP_112553.1	1.0e+000	2.32	28	5.2	51.30	7.5	46	13
69	Gamma -adin	JC5818	1.0e+000	1.80	35	5.3	41.99	5.0	35	10
70	Ribosomal protein P0	AAH01127.1	1.0e+000	1.98	34	5.4	34.43	5.3	31	10
71	Ribosomal protein P0	AAH01127.1	1.0e+000	1.98	34	5.4	34.43	5.5	31	9
72	SFN (14-3-3sigma, stratifin)	AAH01550.1	1.0e+000	2.18	37	4.8	24.38	6.5	32	10
73	Eukaryotic translation elongation factor 1 beta 2	AAP35742.1	1.0e+000	1.54	29	4.5	24.92	3.7	25	8
74	SFN (14-3-3sigma, stratifin)	AAH01550.1	1.0e+000	2.18	45	4.8	24.38	4.2	24	21
75	Mitochondrial short-chain enoyl - coenzyme A hydratase 1 precursor	NP_004083.2	1.0e+000	2.29	50	8.9	31.81	6.0	24	16
76	Proteasome alpha 5 subunit	NP_002781.2	1.0e+000	2.43	18	4.7	26.56	4.0	23	8
77	Ribosomal protein S6 kinase, 90kDa, polypeptide 3	BAC 81131.1	1.0e+000	1.05	16	6.4	82.48	6.3	10	10

78	Keratin 9	NP_000217.2	1.0e+000	1.14	18	5.1	62.27	9.0	10	14
79	Chain B, Human Mitochondrial Single Strand Dna Binding Protein (Hmssb)	1S3O B	1.0e+000	1.98	49	8.2	15.18	9.2	8	7
80	RAS guanyl releasing protein 4	AAK85_701.1	9.7e-001	0.63	11	9.0	76.19	9.2	6	8
81	Fk506 Binding Protein Fkbp Mutant R42KH87V Complex With Immunosuppressant Fk506	1BKF	1.0e+000	2.24	46	8.1	11.79	9.0	7	10
82	Heat shock 70kDa protein 8 isoform 1	NP_006588.1	1.0e+000	2.37	32	5.4	71.11	4.1	63	19
83	Eukaryotic translation elongation factor 2	NP_001952.1	8.2e-001	0.64	13	6.4	96.29	3.9	25	11
84	Endoplasmic reticulum protein 29 precursor	NP_006808.1	1.0e+000	2.26	34	6.8	29.03	6.2	25	10
85	Lamin A/C isoform 2	NP_005563.1	1.0e+000	2.31	35	6.4	65.17	6.6	49	18

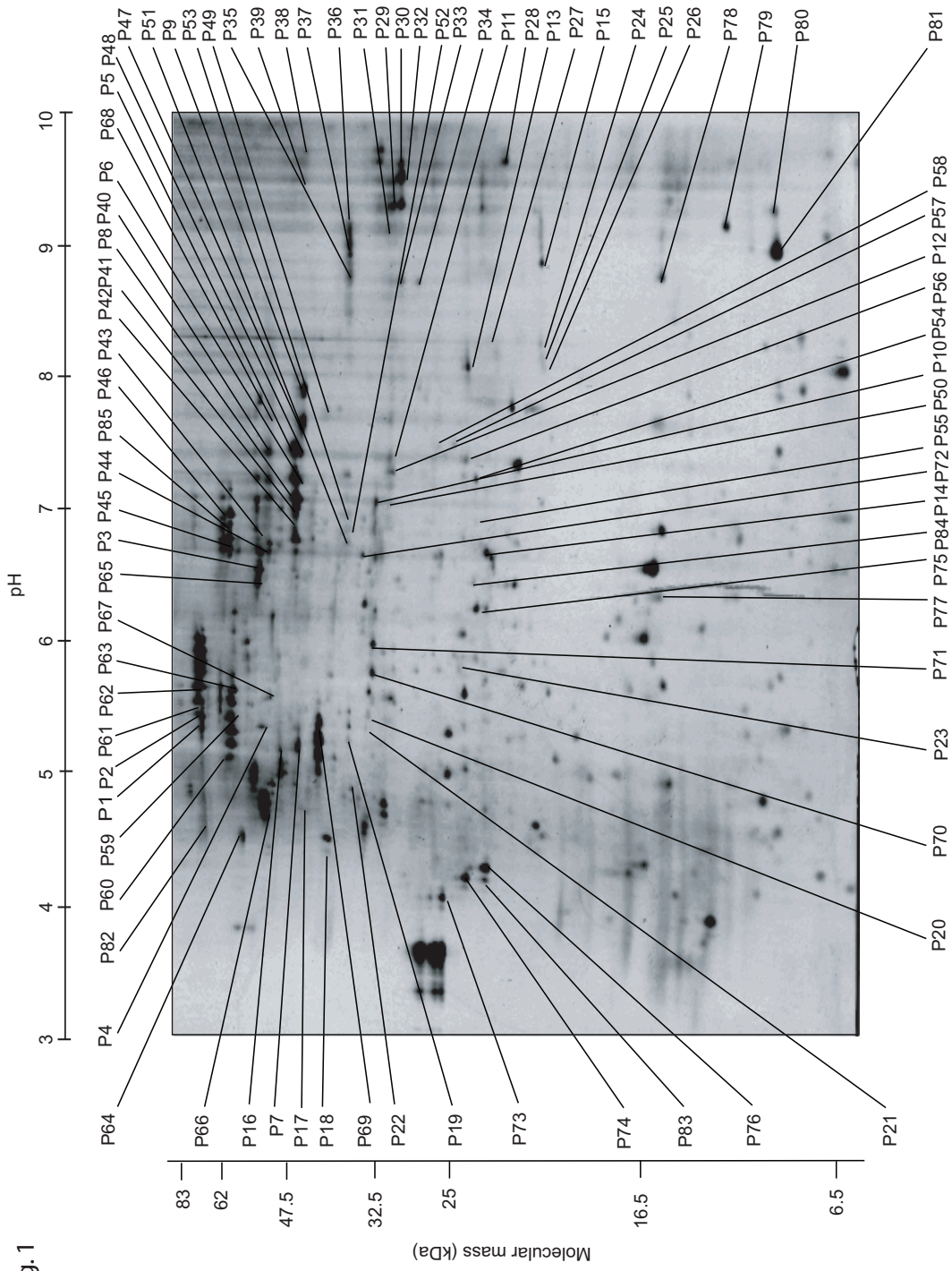
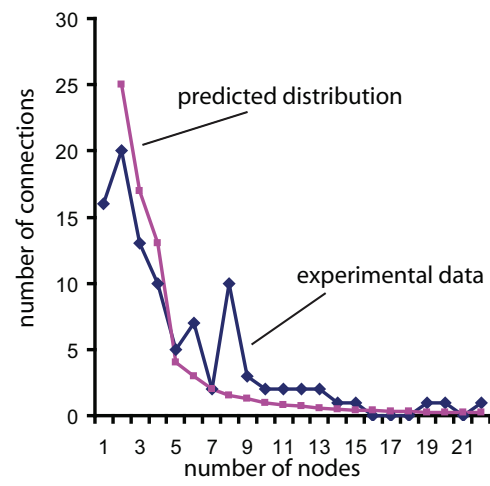


Fig. 1

Fig. 2

A



B

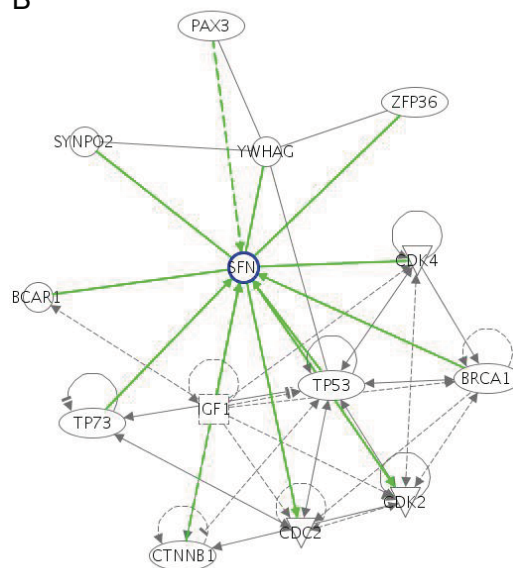


Fig. 3 A

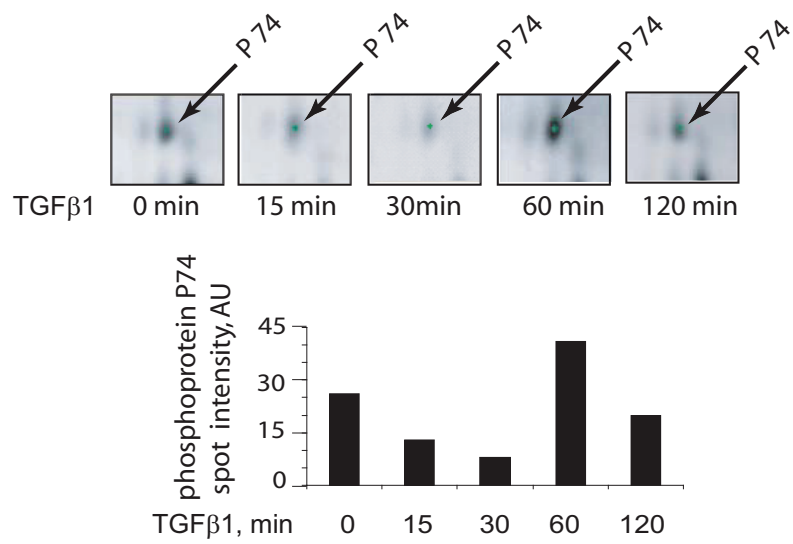
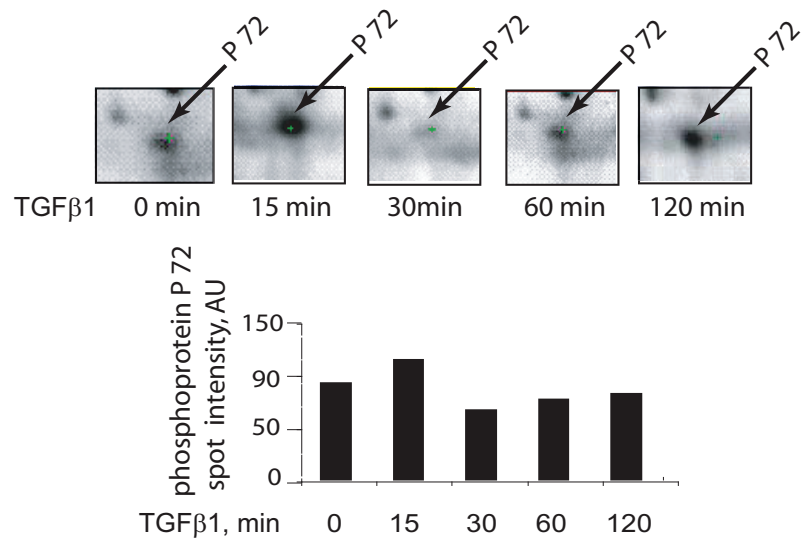
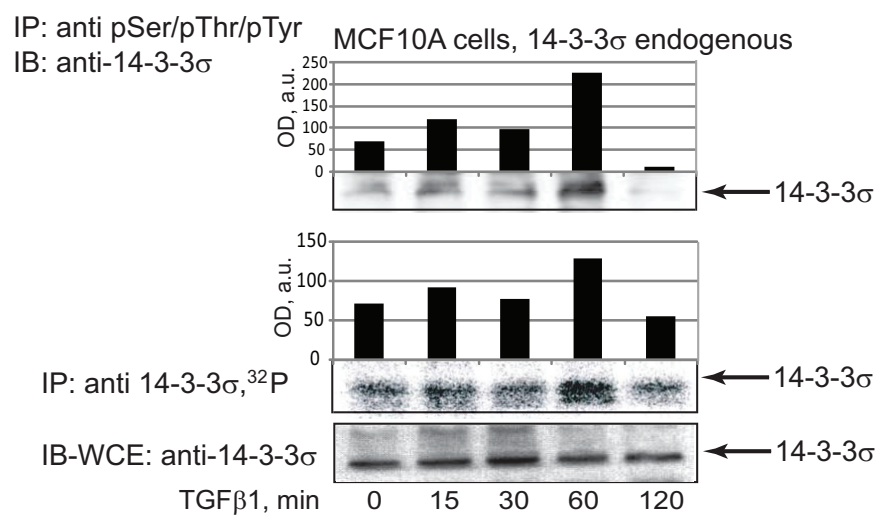


Fig. 3 B, C

B



C

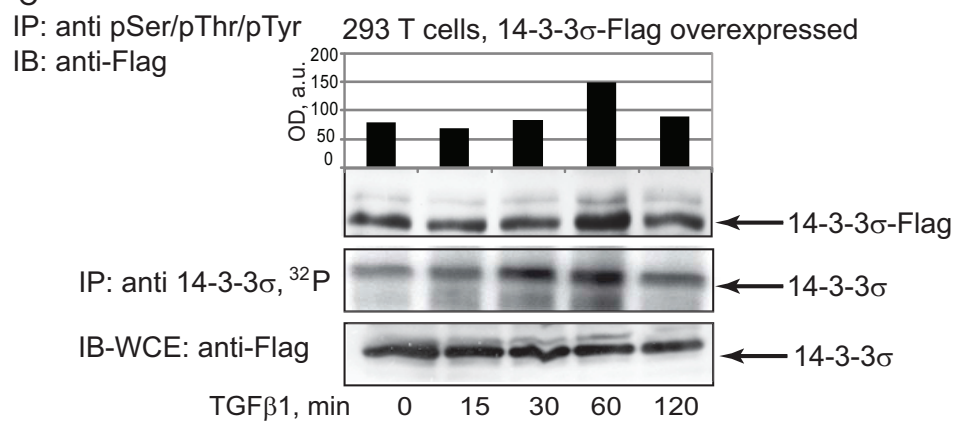


Fig. 4 A

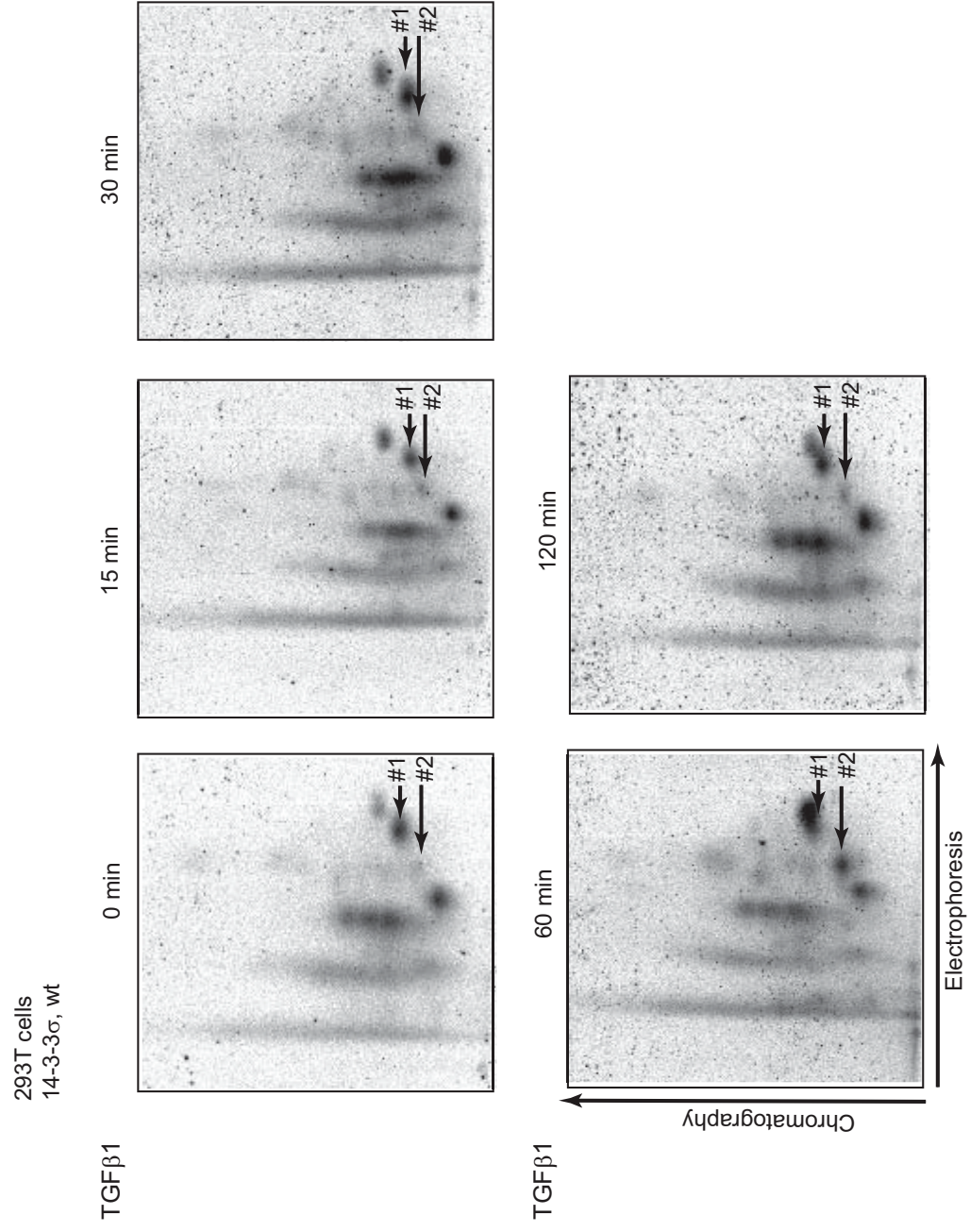
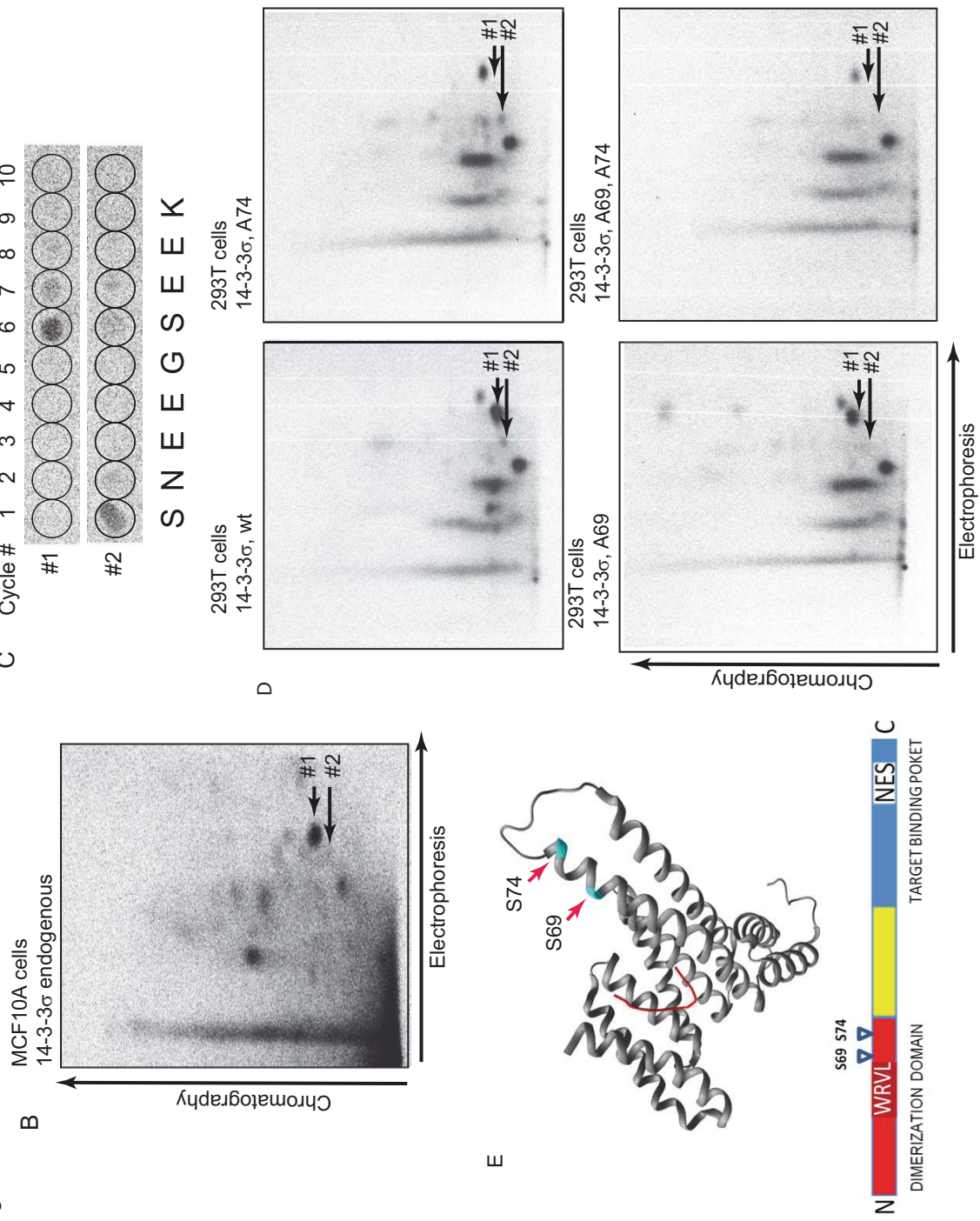


Fig. 4 B, C, D, E



Fig, 5

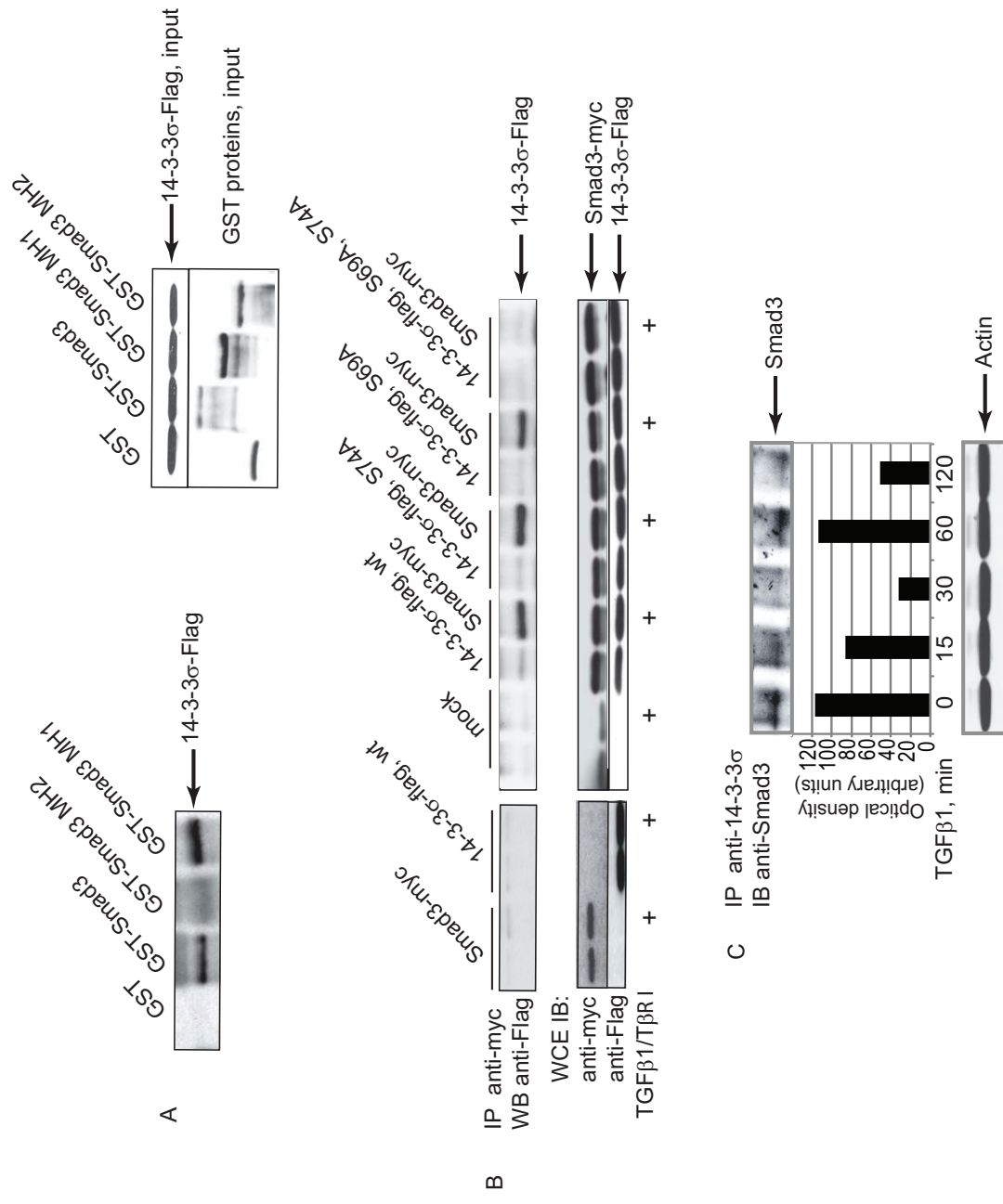
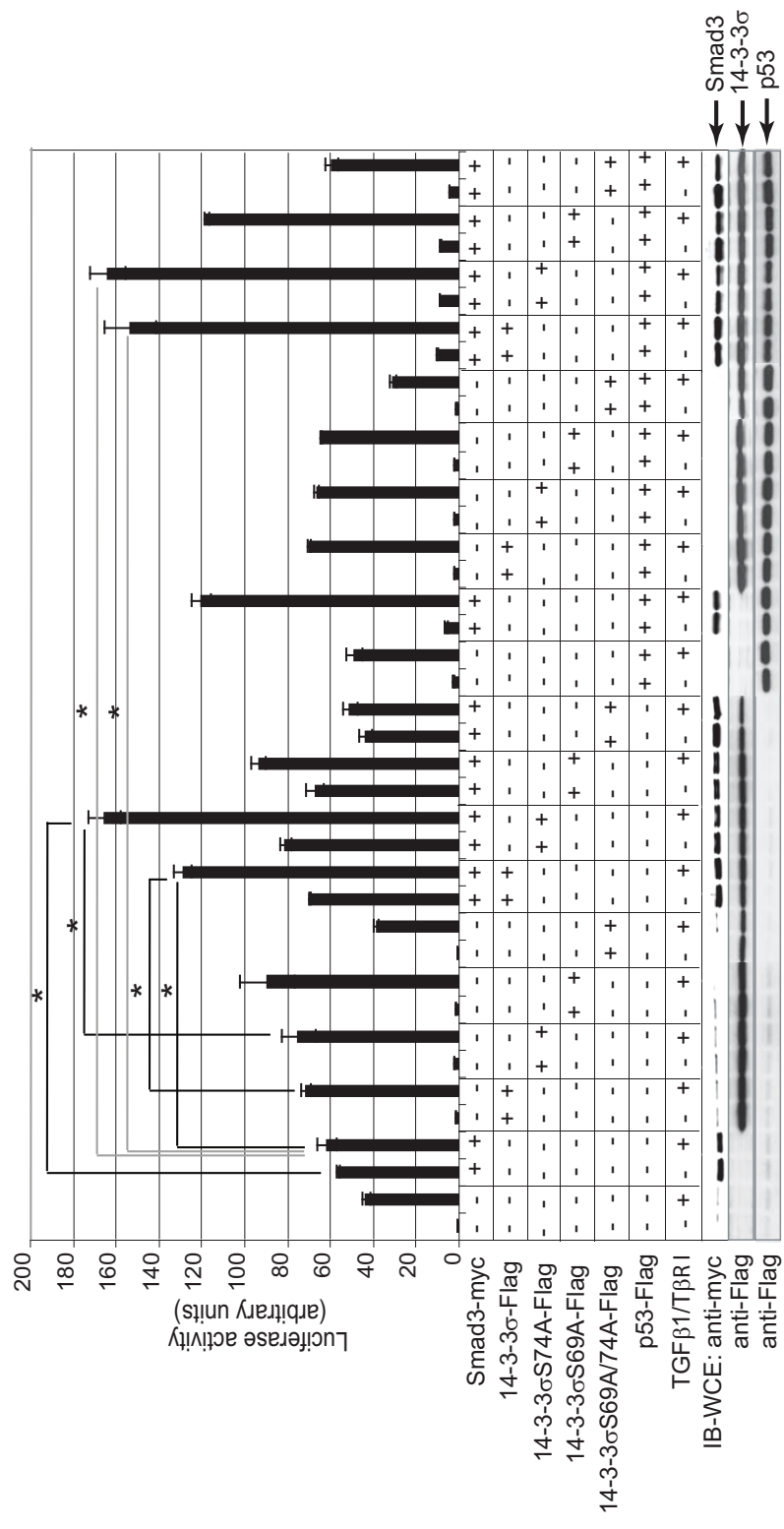


Fig. 6 A



B DNAP: CAGA-probe

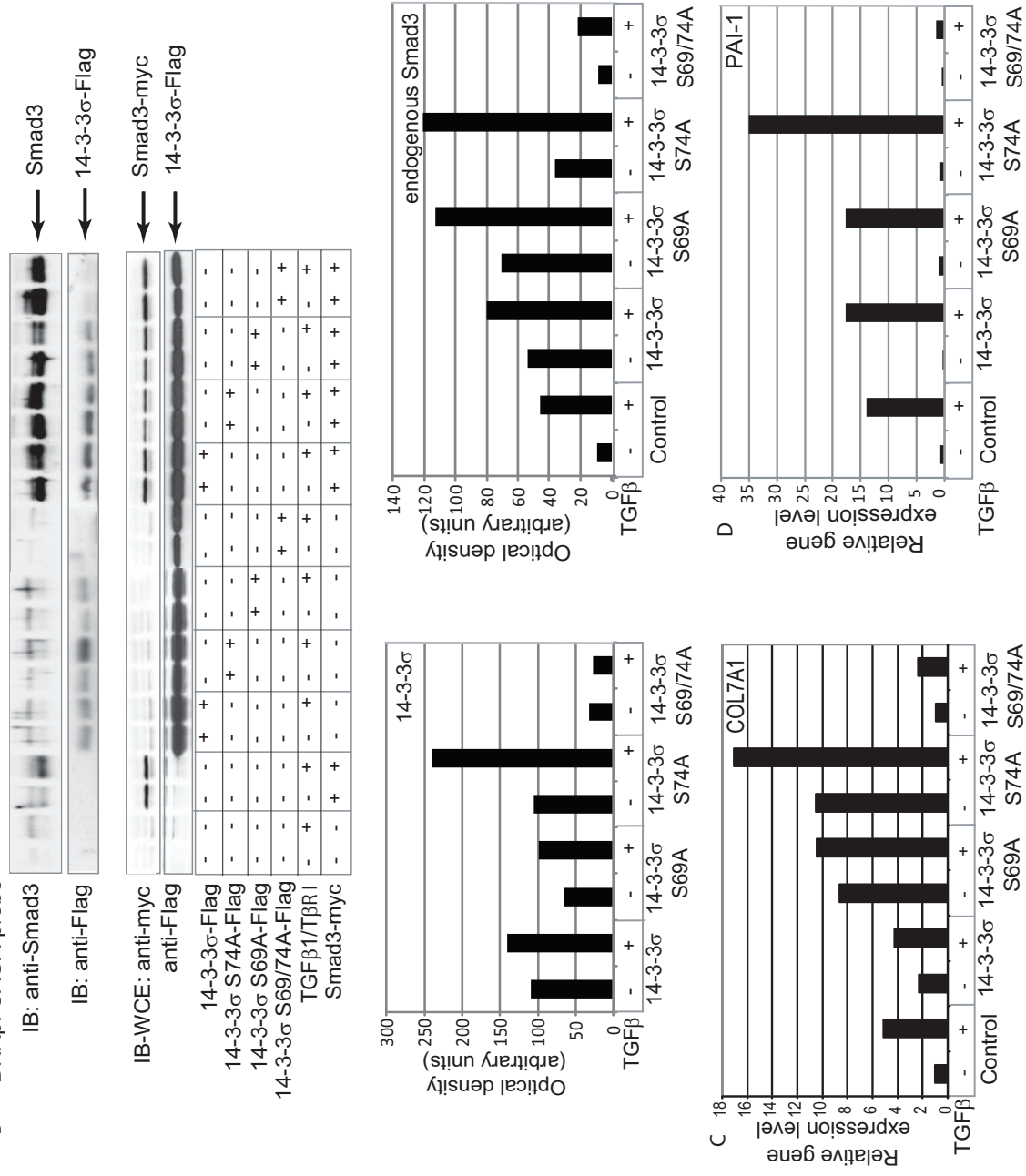
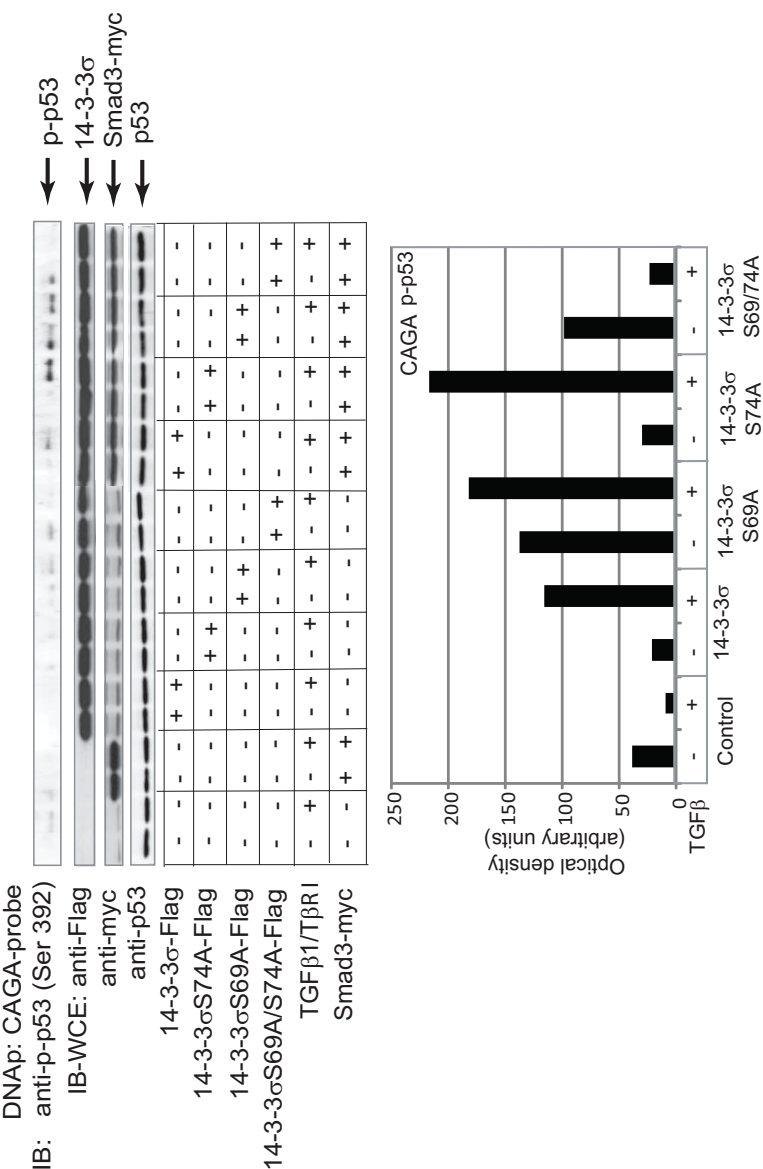


Fig. 6 E



F IP: anti-p53
IB: anti-Flag

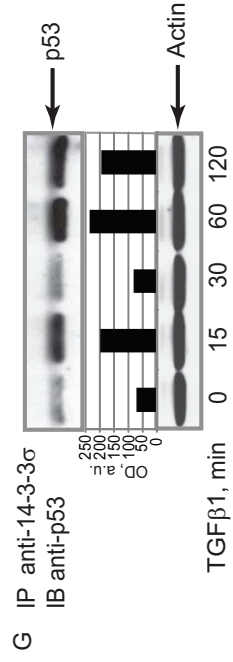


Fig. 7 A, B, C, D

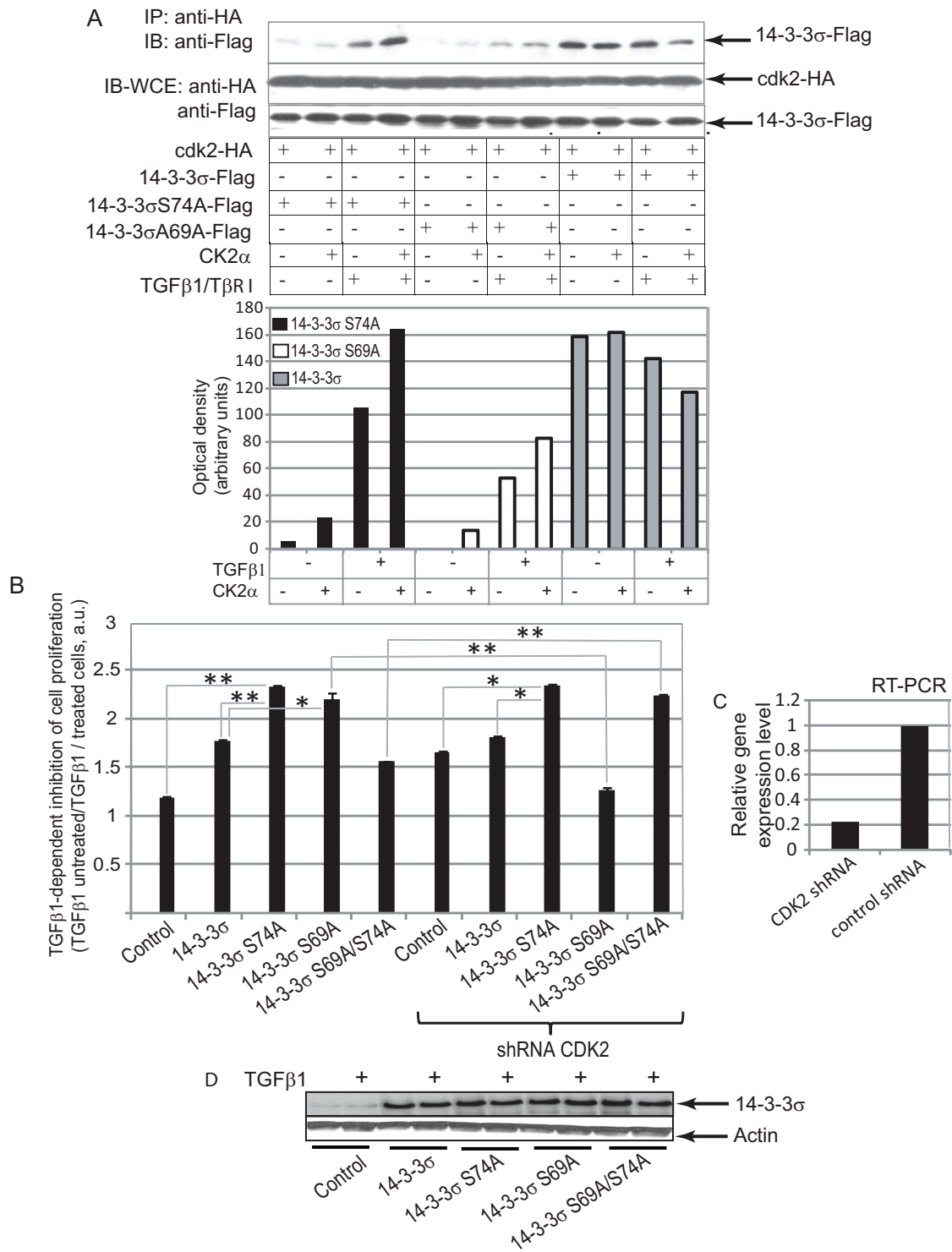


Fig. 7 E, F

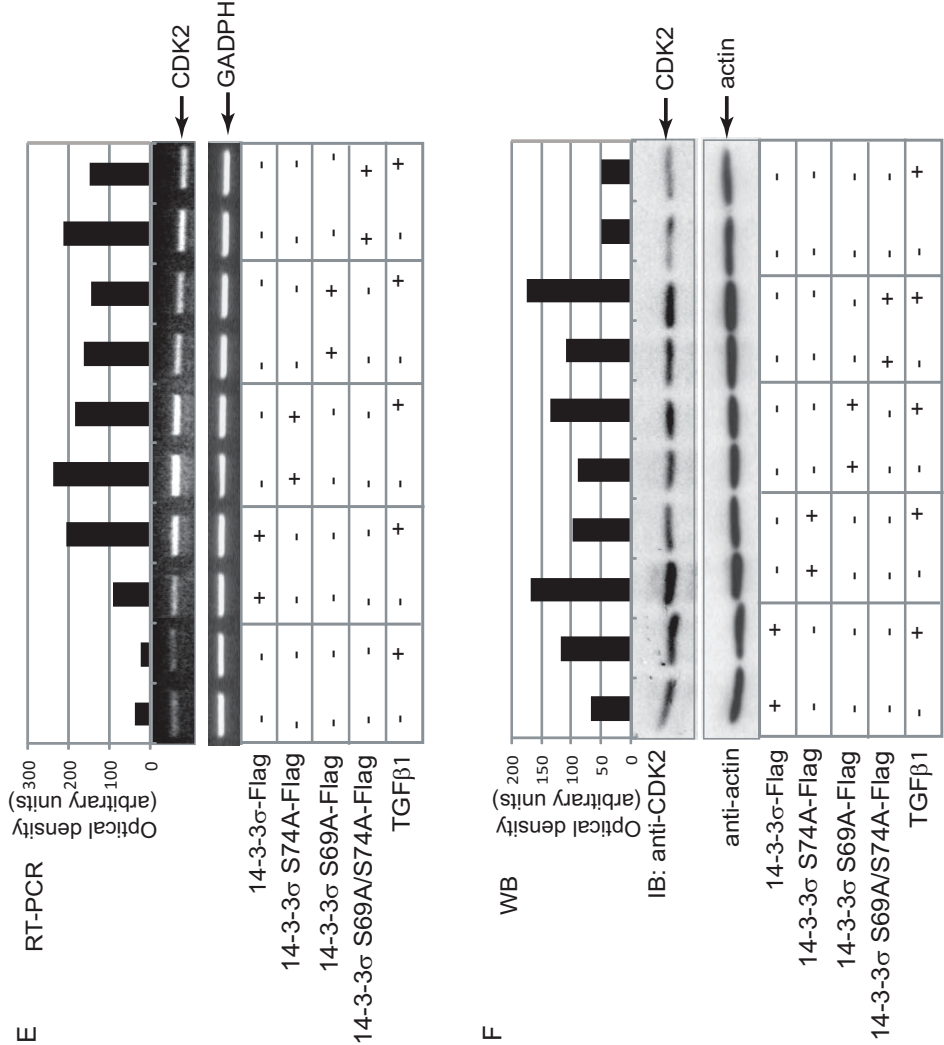


Fig. 7 G, H

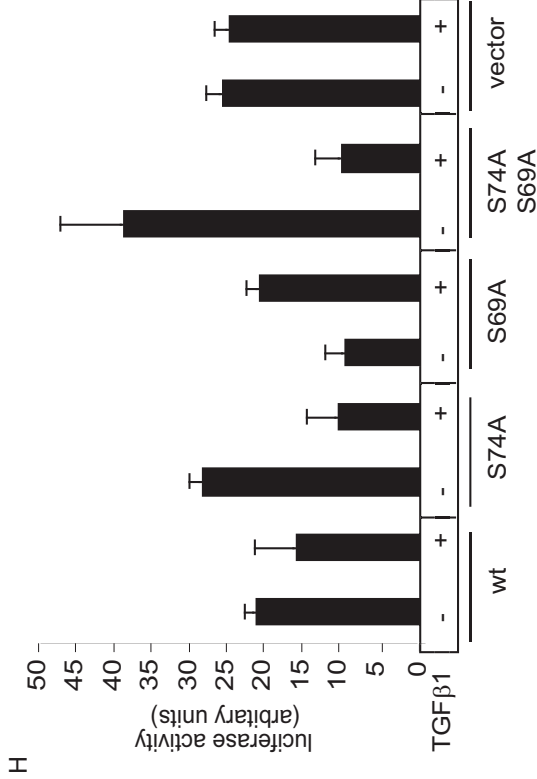
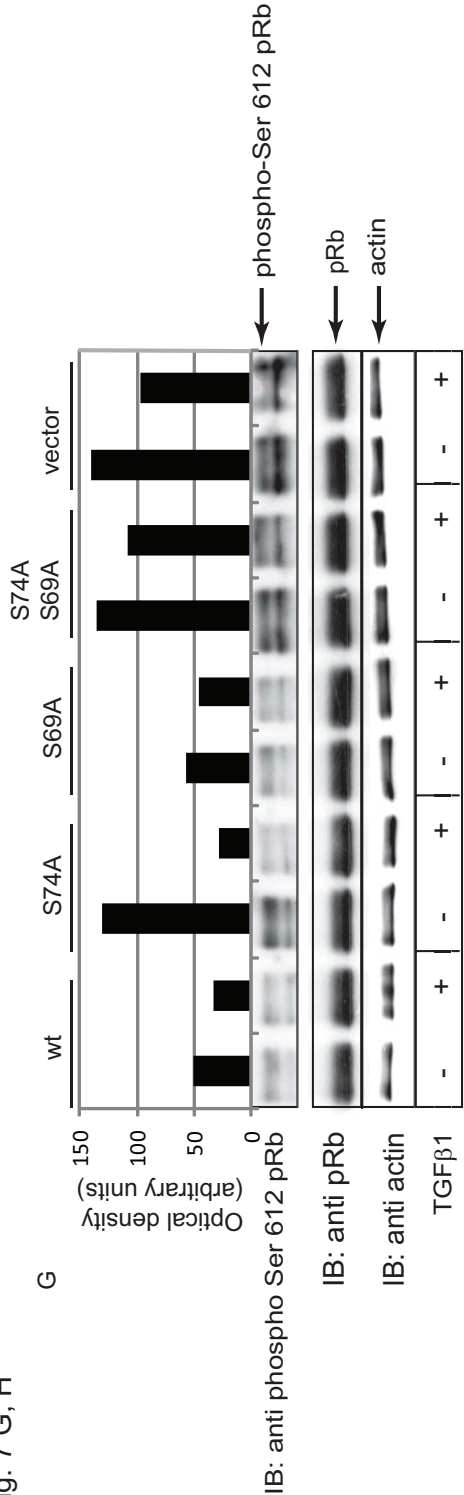
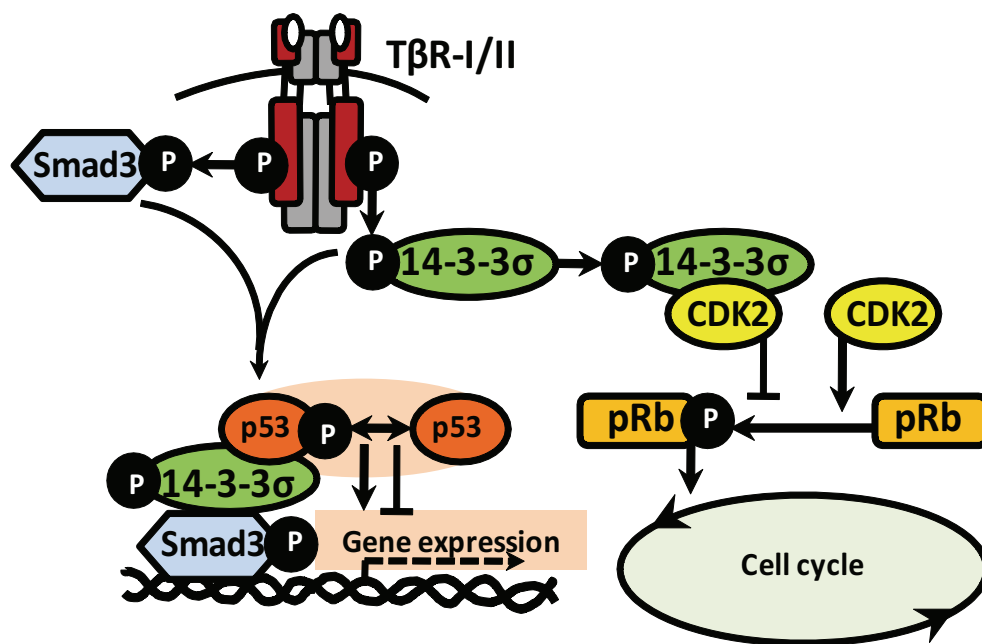
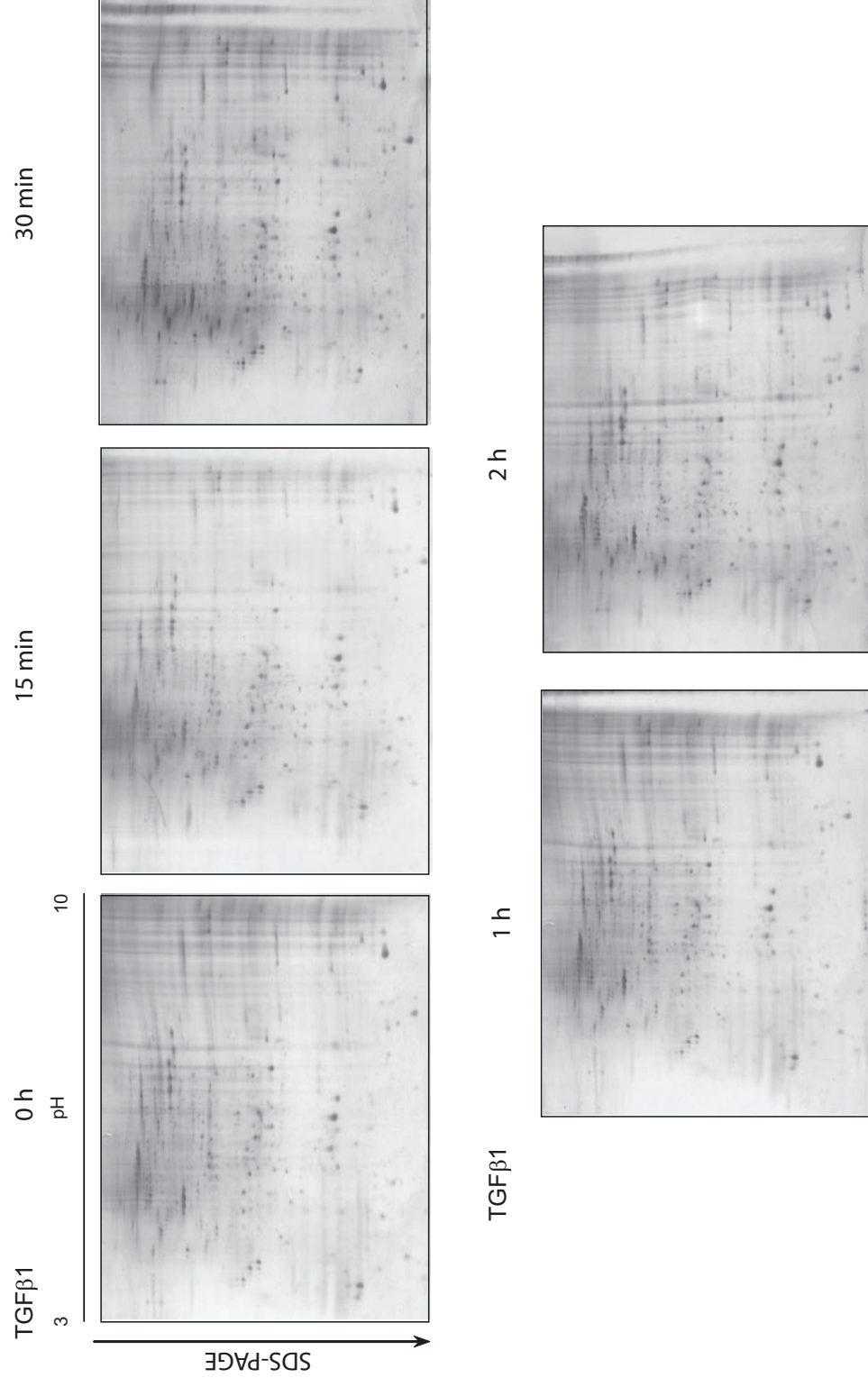


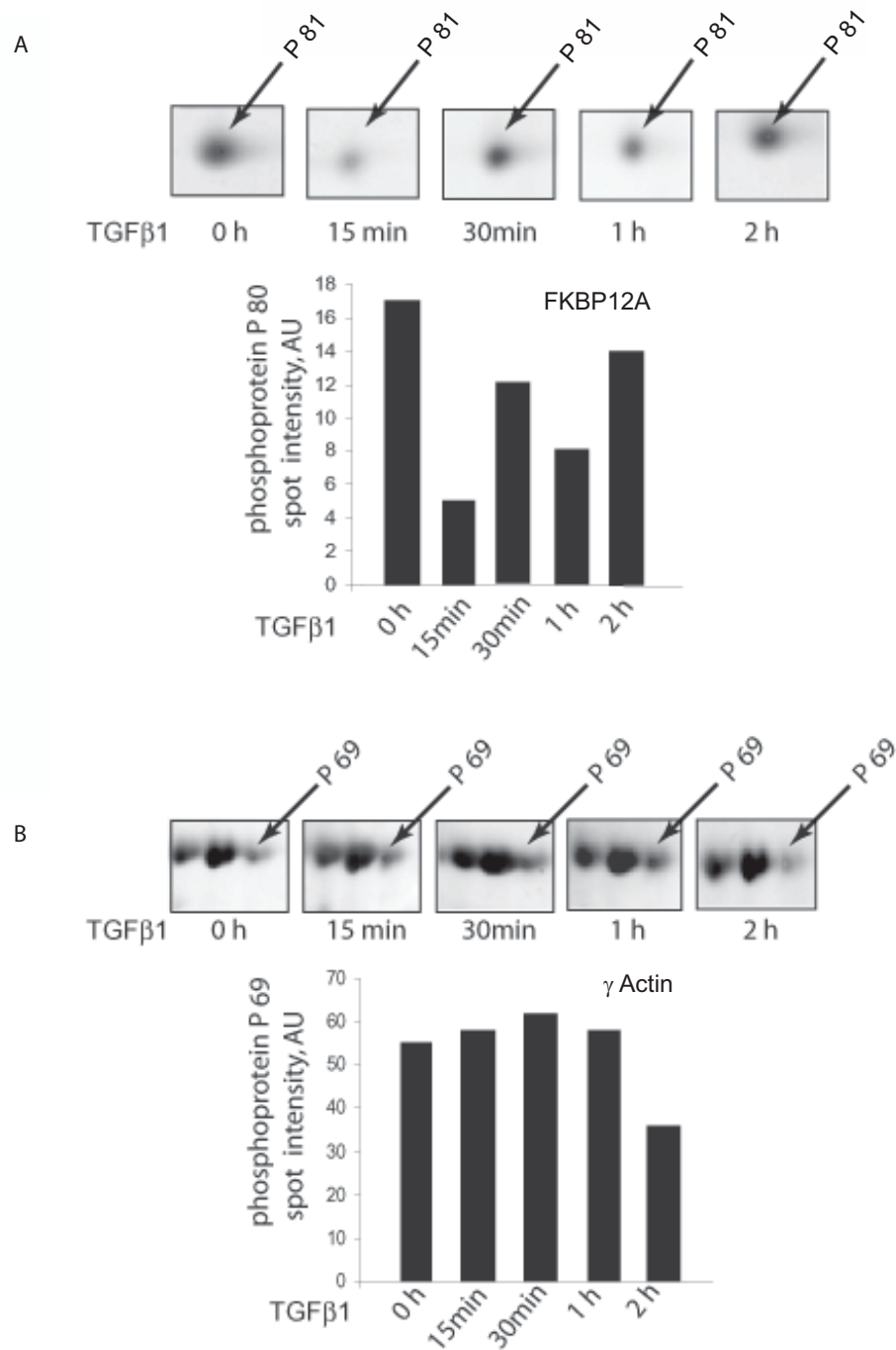
Fig. 8



Supplementary Figure S1

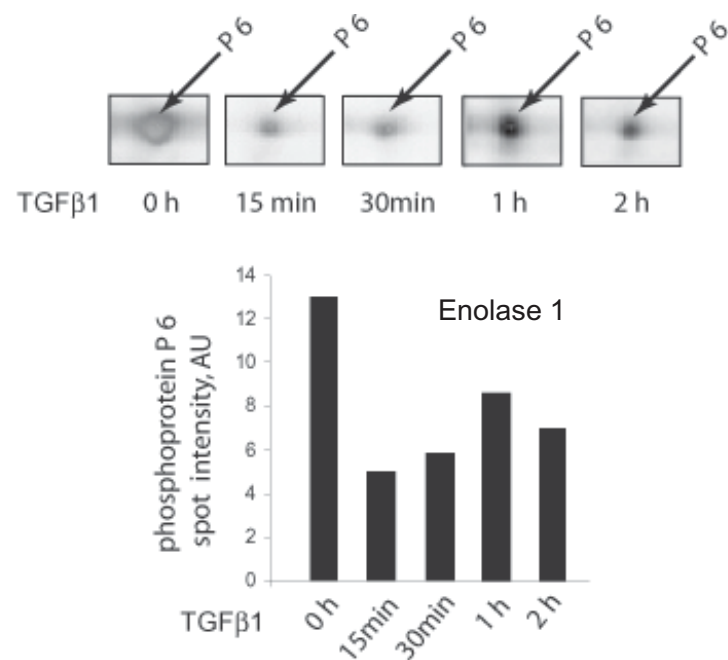


Supplementary Figure S 2 A, B

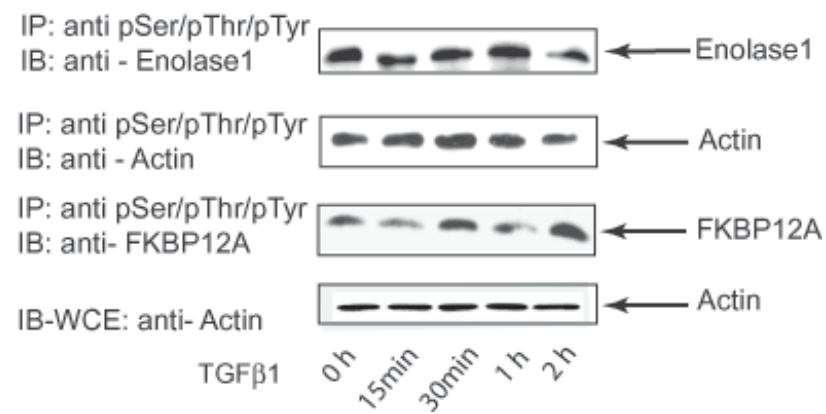


Supplementary Fig. S2 C, D

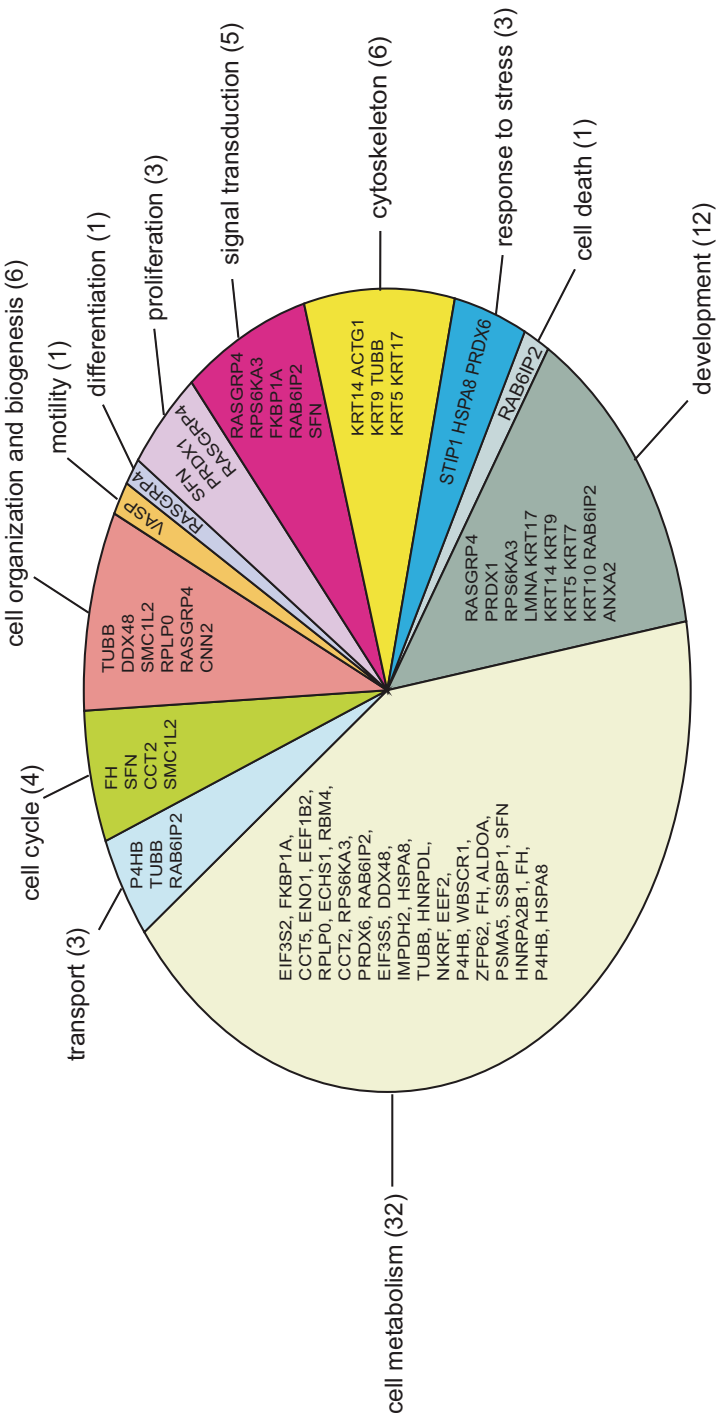
C



D

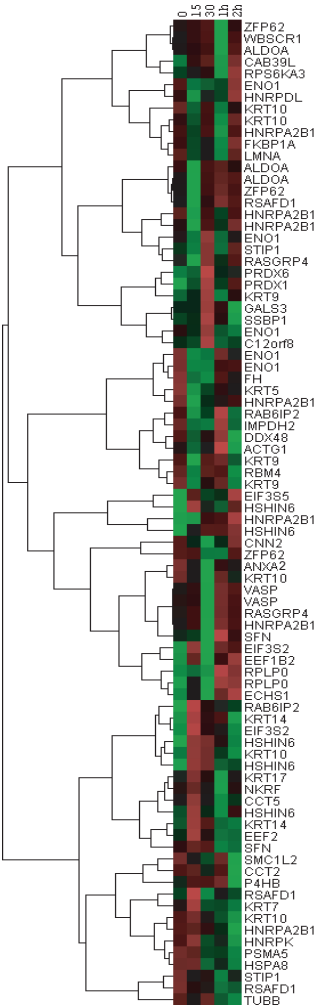


Supplementary Fig.S3 a

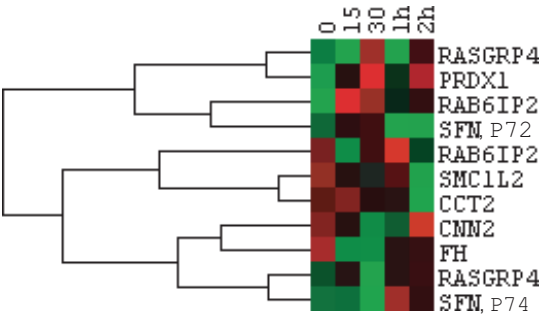


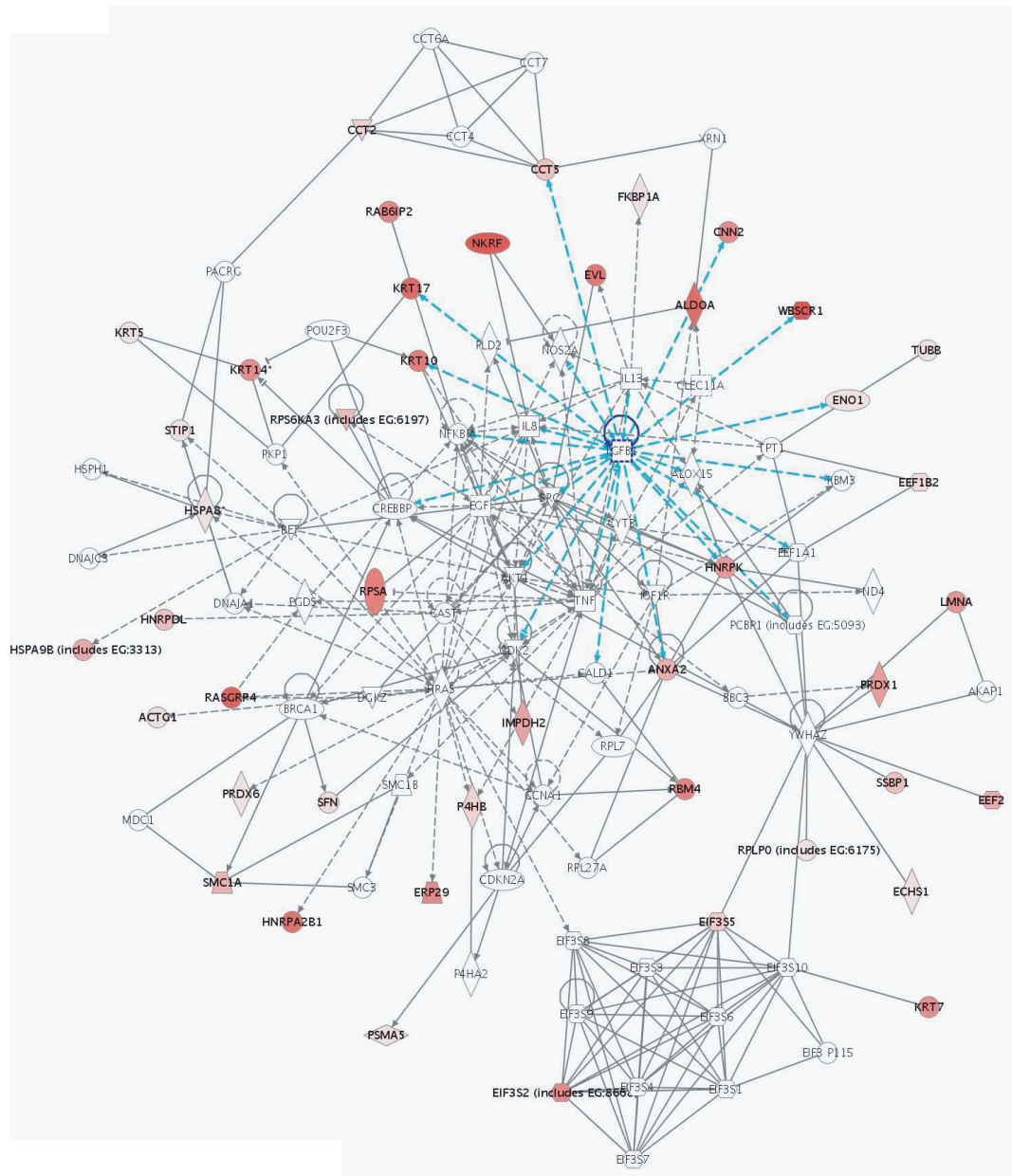
Supplementary Fig. S3 B, C

B

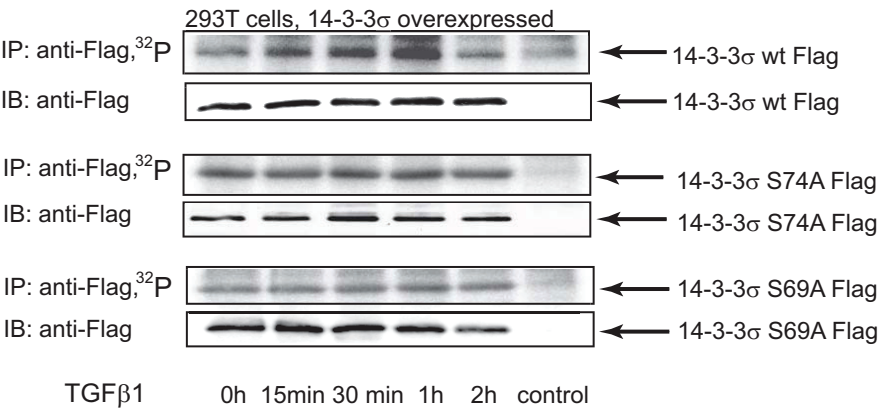


C

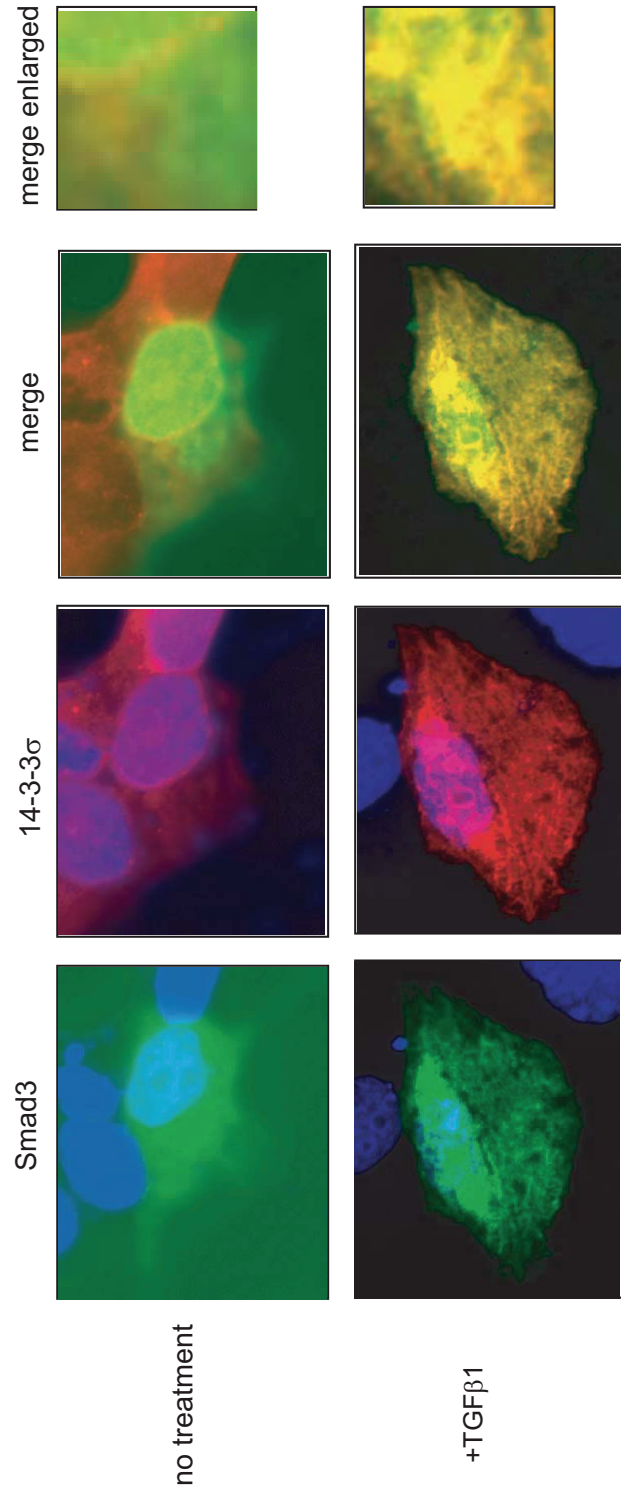




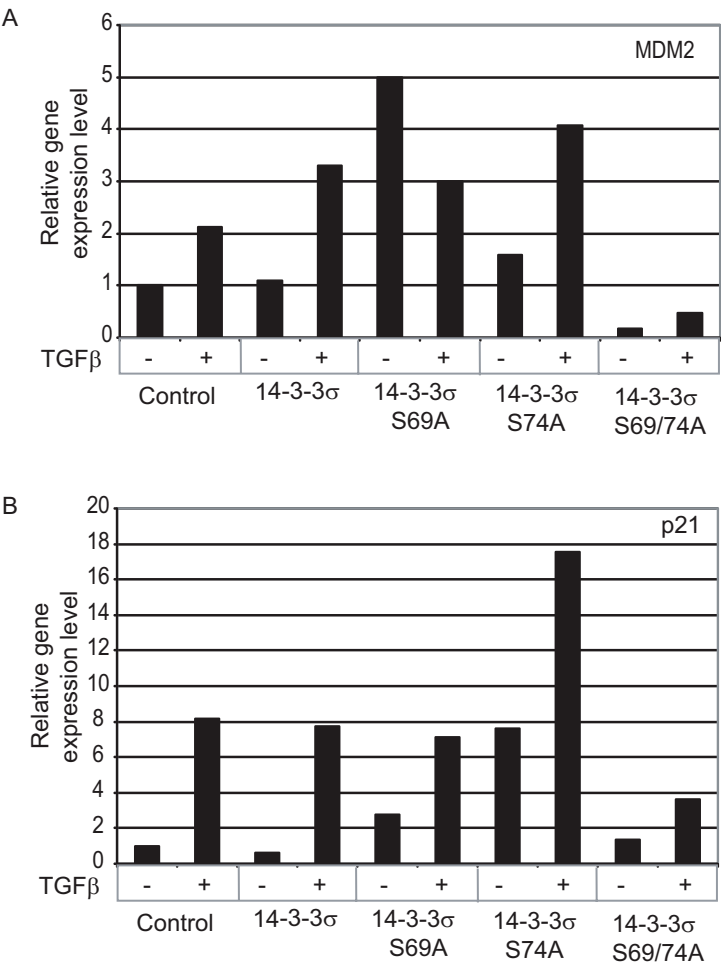
Supplementary Fig. S5



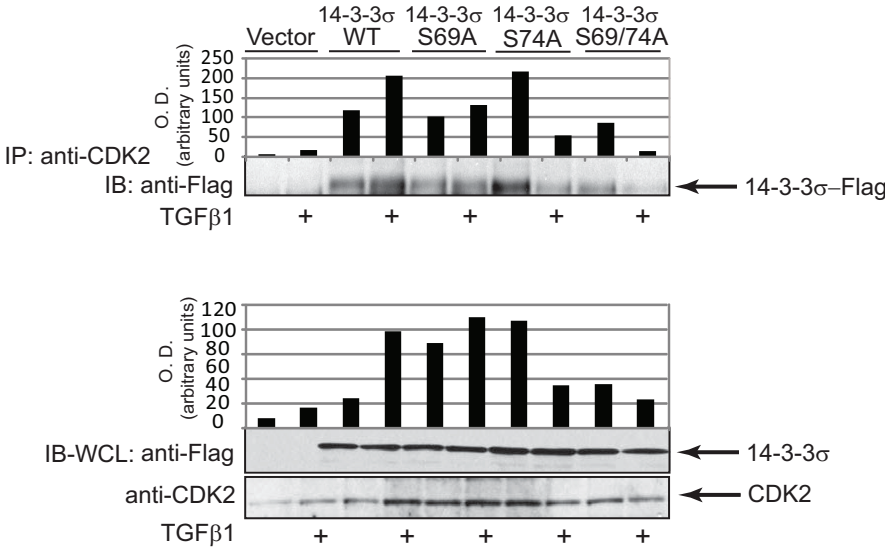
Supplementary Fig. S6



Supplementary Fig. S7



Supplementary Fig. S8



IV

Proteomics-based network signaling by TGF β 1 in 184A1 non-tumorigenic human breast epithelial cells, and its role in phosphorylation of p53 at Ser392 and regulation of cell proliferation

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Key words: transforming growth factor- β , proteomics, CK2, p53

Abbreviations used: TGF β , transforming growth factor- β ; CK2 α , casein kinase-2 α ; SSRP1,

Structure-Specific Recognition Protein-1; PC4, proprotein convertase-4.

Abstract

Transforming growth factor- β (TGF β) is known as an inhibitor of proliferation of epithelial cells, but the strength of its inhibitory action varies depending on type of cells. It implies that TGF β may employ different regulatory mechanisms in different cell types. Here we report proteome profiling of TGF β 1 action on non-tumorigenic human breast epithelial cells 184A1 that show phenotype of normal non-cancerous cells. Proliferation of these cells is transiently inhibited by TGF β 1 to the 50 %, at maximum. We identified 94 and 51 proteins which changed their expression and/or ^{35}S -incorporation, respectively, upon treatment with TGF β 1 for 2 h, 8 h or 24 h. Cell proliferation, death, migration and metabolism were among main cellular functions affected by the identified proteins. Analysis of networks formed by the identified proteins highlighted potential differences in TGF β 1 regulatory mechanisms in non-tumorigenic breast epithelial cells, as compared to tumorigenic cells. The network analysis suggested involvement of SSRP1, PC4, CK2 α and p53 in regulation of proliferation of 184A1 cells. Interrogation of the proliferation sub-network by manipulating expression of CK2 α , SSRP1 and PC4 confirmed predicted impact on p53 phosphorylation, and effects on cell proliferation and apoptosis. Thus, we report here identification of TGF β 1-regulated proteins in non-tumorigenic human breast epithelial cells, and explored involvement of the network-signaling in regulation of p53 phosphorylation and cell proliferation.

Introduction

TGF β is a key regulator of cell proliferation, apoptosis, migration and differentiation, and is involved in practically all aspects of normal human physiology. Changes in responsiveness to TGF β have been associated with tumorigenesis, suggesting that TGF β may be a tumor suppressor as well as a promoter of metastasis (Tan et al., 2009; Wharton and Derynck, 2009; Massagué, 2008). Such so different impact on tumorigenesis of the same growth factor has been explained by the variability in employed signaling mechanisms in different cells. As an example, TGF β -dependent inhibition of cell proliferation was found to vary from pronounced to almost negligible for breast epithelial cells (Tan et al., 2009; Souchelnytskyi, 2005). This has been explained by differences in engagement by TGF β signaling different sets of intracellular regulatory molecules.

TGF β consists of a family of 3 isoforms in mammals, TGF β 1, TGF β 2 and TGF β 3, which all can act via type II and type I TGF β receptors (Massagué, 2008, Tan et al., 2009; Wharton and Derynck, 2009). TGF β binds first to a dimer of type II receptors, which then recruits two type I receptors. Activated heterotetrameric TGF β receptor complex phosphorylates Smad2 and Smad3 proteins, which form complexes with other proteins, including common Smad4 and various transcriptional regulators. A number of important so-called non-Smad mechanisms can also be initiated by the activated receptors. These pathways include regulation of Erk1/2, p38, JNK, acetylation (HDACs) and ubiquitylation (E3-ligases) of proteins (Massagué, 2008, Tan et al., 2009; Wharton and Derynck, 2009; Souchelnytskyi, 2005a). An important component of TGF β signaling is a direct impact on protein synthesis via TGF β -dependent phosphorylation of eEF1A1 (Lin et al., 2010)

TGF β inhibition of cell proliferation has been attributed to effects of Smads, with modulation by non-Smad pathways, such as MAP kinases and protein synthesis. Cyclin-dependend kinases, their inhibitors, cyclins and cdc25a have been proposed as ultimate targets in the regulation of the

cell cycle (Hahn and Weinberg, 2002). However, regulatory mechanisms from the TGF β receptors to these targets have been shown to be complex and of a network-signaling feature rather than a set of straight pathways (Souchelnytskyi, 2005b). Recent proteomics studies of TGF β signaling confirmed complexity of signaling mechanisms initiated by TGF β (Souchelnytskyi, 2005a, Kanamoto et al., 2002; Stasyk et al., 2005; Bhaskaran and Souchelnytskyi, 2008; Friedman et al., 2007). These studies showed some similarities in functional domains affected by TGF β in different types of cells. However, these studies showed also significant differences in sets of targets affected by TGF β . These differences indicated that TGF β may employ different mechanisms in different types of cells. Therefore proteome profiling of cells representing various stages of tumorigenesis may provide insights into specifics of TGF β action during tumorigenesis.

Here we report proteome profiling of TGF β action on human breast epithelial cells 184A1. These cells are non-tumorigenic and have phenotype similar to normal breast epithelial cells. We identified 104 unique proteins regulated by TGF β , and showed that Casein Kinase 2 α (CK2 α), Structure-Specific Recognition Protein-1 (SSRP1) and proprotein convertase-4 (PC4) may be involved in TGF β -dependent inhibition of cell proliferation by modulating p53 phosphorylation.

Materials and methods

Cells and reagents

184A1 human breast epithelial cells (Stampfer and Yaswen, 2000) were obtained from ATCC, and were cultured in recommended by the ATCC medium (Mammary Epithelial Growth Medium (MEGM), complemented with penicillin/streptomycin, 5 % horse serum, hydrocortisone, insulin,

bovine pituitary extract, Epidermal Growth Factor (EGF), Gentamicin sulfate, Amphotericin B (GA) and transferrin.

Proteome profiling

For analysis of TGF β 1-regulated proteins, 184A1 cells were treated with human TGF β 1 at 10 ng/ml for 2 h, 8 h and 24 h (see Supplementary Figure S1 for the treatment scheme). Specifically, cells were seeded in 10 cm dishes at 70 % confluence, and the next day 10 % FBS containing medium was changed to the medium with 3 % FBS. TGF β 1 was added to cells to be treated for 24 h. For 8 h or 2 h incubation, TGF β 1 was added 8 h or 2 h before harvesting the cells, respectively. Control non-treated cells were cultured all the time period in 3 % FBS-containing medium. For ^{35}S -labeling, [^{35}S]methionine and [^{35}S]cysteine isotopes (Promega) were added to the medium during last 2 h of incubation of cells, before harvesting. Final concentration of ^{35}S -label in culture medium was 10 $\mu\text{Ci/ml}$. Upon collection of proteins, cells were extensively washed with PBS and with 250 mM sucrose in 10 mM Tris-HCL, pH 7.2. Protein solubilization buffer was added directly to cells (8 M urea, 2.5 % CHAPS, IPGPhor buffer (3.4 $\mu\text{L/ml}$), pH 3-10, and DTT (100 mM)), and proteins were extracted for 30 min at room temperature (18 $^{\circ}\text{C}$ – 20 $^{\circ}\text{C}$). Extract was centrifuged (13.000 rpm, 15 min), protein concentration was measured, and aliquots of the extracts were frozen at -70 $^{\circ}\text{C}$ until use.

Extracted proteins (70 $\mu\text{g/gel}$) were subjected to isoelectrofocusing in an IPGPhor instrument (Amersham Biosciences/GE Healthcare, Uppsala, Sweden), in 18 cm IPG Drystrips, pH3-10, linear. Isoelectrofocusing was performed as follows: 10 h passive rehydration, 3 h 50 V, active rehydration, 1 h 1,000 V, and 10 h 5,000 V, or until 50.000 Vh. Strips after isoelectrofocusing were equilibrated in SDS-containing buffer, with DTT (100 mM) and then the same buffer with iodoacetamide (200 mM), and were transferred onto 10 % SDS gels. Second dimension SDS PAGE was performed in DaltSix, as follows: 1 W/gel, 20 min, 5 W/gel, 1 h, and 10 W/gel for 5 –

8 h. After electrophoresis, gels were fixed, stained with silver and dried, as described earlier (Stasyk et al., 2005). To detect incorporation of ^{35}S -label, gels were exposed and scanned in a phosphorimager FujiX-3000 to generate images of ^{35}S -labeled proteins. 2D gels were also scanned in a light scanner to generate images of silver-stained proteins. Images from visual scanning and from ^{35}S -exposure scanning were up-loaded in Image Master Platinum (AmershamBiosciences/GE Healthcare, Uppsala, Sweden) for detection of differentially expressed spots. Statistical tools embedded in the software were used to ensure statistical significance of differential expression of spots, as normalized volumes, with probability threshold set at $p < 0.05$. Spots which showed changes of expression more than 50 % between at least two experimental conditions were considered for identification.

Protein identification

Selected protein spots were cut from gels, and subjected to in-gel digestion, as described earlier (Stasyk et al., 2005). In brief, dried gel-spot was rehydrated, de-stained, extensively washed in 0.1 M ammonium bicarbonate, then in 100 % acetonitrile, and dried. Aliquot of activated trypsin (Promega) was added to the gel, and upon rehydration with trypsin solution, protein digestion was initiated. After 15-18 h incubation at 37°C , generated peptides were extracted, de-salted using ZipTips C18 μ and loaded with matrix (α -cyano-4-hydroxycinnamic acid) on a metal target for mass spectrometry. Mass spectra were collected on Ultraflex MALDI TOF/TOF instrument (Bruker Daltonics) using FlexControl and FlexAnalysis software (Bruker Daltonics). Spectra were internally calibrated with tryptic peptides (842.51, 1045.56 and 2211.10 Da). Peptide mass fingerprinting was performed by searching NCBI nr database (RefSeq) with ProFound engine. One miscut, partial oxidation of methionine, alkylation of cysteine residues, tolerance less than 0.5 Da, and “mammalian” were set for searches. No restrictions for pI, and (+) and (–) 30 kDa to a migration position in 2D gels were set for molecular mass definition. Probability and Z-value,

which calculate significance of identification, were considered, and only significant identifications were considered in our analysis.

Systemic analysis of TGF β -regulated proteins

Functional and pathway analysis was performed using Ingenuity Pathway Analysis (IPA), a tool for description of networks and signalling pathways. See www.ingenuity.com for detailed description of IPA. IPA operates with a proprietary database which is based on a thorough analysis of reported experimental data. IPA considers only those experimental data which have been evaluated by independent researchers. This ensures that only confirmed results are taken into consideration for building a network. Experimental results which have not been reported by multiple laboratories or may have controversial interpretations are not considered by IPA. Such stringent selection of experimental data is required to exclude building of false-positive dependencies. Settings for the network analysis were taken as recommended by IPA, e.g. the number of connections and components between two dataset-defined components. A dataset containing identified proteins was uploaded into the Ingenuity Pathway Analysis application, and networks were generated. Fischer's exact test was used to calculate a p-value determining the network connectivity.

Transfections and immunoblotting

Cells were transfected in 6-well plates by LipofectAMINE 2000 reagent, as recommended by the supplier (Invitrogen, Carlsbad, USA). siRNA to CK2 α , PC4 and SSRP1 and control scrambled siRNA (sc-37007) were obtained from SantaCruz Biotech (Santa Cruz, USA). The control siRNA is designed and extensively tested by the supplier, in order to ensure that it would not interfere with expression of any known genes. siRNAs to CK2 α , PC4 and SSRP1 were tested by the supplier for specificity and off target effects. Medium was changed 6 hours after transfection. For immunoblotting, cell lysates were resolved on SDS polyacrylamide gels and transferred onto Hybond P membranes (GE Healthcare, Piscataway, NJ). Membranes were blocked with 5 % (w/v)

BSA and then incubated with a primary antibody against target proteins with dilutions, as recommended by the manufacturer, and followed by an HRP-conjugated secondary antibody (GE Healthcare, Uppsala, Sweden). The following antibodies were used: casein kinase II α (CK2 α ; sc-9030, H-286, Santa Cruz, USA), and actin (sc-1615, C-11, broad range of actin isoforms, Santa Cruz, USA). The proteins were visualized using Luminol Reagents (Santa Cruz Biotechnology Inc.). For transfection with siRNA, cells were seeded in 24-well plates, and transfection procedure was performed the next day, as recommended by the siRNA suppliers. After transfection, cells were cultured in a medium supplemented with 10 % serum, and used in assays within 24 hours of transfection.

Cell proliferation and cell death assays

Cell proliferation was measured by using [3 H]thymidine incorporation assay and CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT assay) (Promega, Promega Biotech AB, Stockholm, Sweden). 184A1 and MCF10A cells were seeded in plates for proliferation assays. Cells were incubated with 0.1 μ Ci/ml of [3 H]thymidine for the last 24 h of the indicated time periods. Radioactivity incorporated into DNA was measured, as described earlier (Stasyk et al., 2005). MTT assay was performed in parallel with [3 H]thymidine-incorporation test, except that no radioactivity was added. Cells were grown for the time periods indicated in the text, and MTT assay was performed according to the manufacturer's recommendations. Statistical significance of observed differences was evaluated with Student's t-test.

Cell death assay was performed using Cell Death Detection ELISA plus, as recommended by the supplier (Roche, Stockholm, Sweden). Cells were treated in the same way as for the proliferation assay. Statistical significance of observed differences was evaluated with Student's t-test.

Results

Proteome profiling of TGF β 1 action on 184A1 cells

To explore TGF β signaling in non-tumorigenic human breast epithelial cells, we performed proteome profiling of 184A1 cells. TGF β 1 inhibited cell proliferation and induced C-terminal phosphorylation of Smad2 protein, indicating that TGF β signaling was intact in these cells (Figure 1A, B). We observed that the effect of TGF β 1 was rather transient. TGF β 1 inhibited to 50 % cell proliferation after 24 h of treatment, but after 48 h the cell proliferation was restored to up to 80 % of its original level (Figure 1A). To monitor initiation of the intracellular signaling, phosphorylation of the substrate of type I TGF β receptor, Smad2, was analyzed. We observed that Smad2 was phosphorylated after 2 h of treatment, but then its level of phosphorylation decreased on the 8th h and the 24th h (Figure 1B). Thus, 184A1 cells were responsive to TGF β 1, although the inhibition of cell proliferation was less pronounced, as compared to tumorigenic breast epithelial cells, e.g. MCF7 or MCF10A cells (Stasyk et al., 2005; Dubrovska et al., 2010; Bhaskaran et al., 2009).

We generated two-dimensional gels of proteins extracted from 184A1 cells treated with 10 ng/ml of TGF β 1 for 2 h, 8 h and 24 h (Figure 2). To evaluate protein expression, we stained proteins in 2D gels with silver. To evaluate protein synthesis, we labeled 184A1 cells with [³⁵S]methionine and [³⁵S]cysteine for the last 2 hours of incubation with TGF β 1. In average, we observed 1600 protein spots in silver-stained and in ³⁵S-labeled gels. Changes in the total number of protein spots in gels representing all experimental conditions were less than 10 %. Using gel image analysis, we identified protein spots, which changed their expression levels for more than 50 % between at least 2 experimental conditions. Only spots with statistically significant differences in expression were considered for identification (p<0.05, Student's t-test, embedded in the image analysis software). MALDI TOF mass spectrometry was used to identify proteins in

these spots. Ninety-four unique proteins regulated on the level of expression were identified in 128 protein spots, and 51 unique proteins were identified in 65 ³⁵S-labeled spots (Supplementary Tables S1 to S4). The overlap between the expressed and ³⁵S-labeled proteins was 41 proteins, which would be expected. The total number of TGFβ1-regulated unique proteins was 104.

To validate proteomics results, we performed immunoblotting (for expression, silver-stained proteins) and immunoprecipitation (for ³⁵S-labeled proteins) of selected proteins. Validation of changes in expression of DNA polymerase κ, cullin5, replication protein-1 (RPA1) and RAP1 confirmed proteomics results (Figure 3).

Systemic analysis of proteins regulated by TGFβ

Functional clustering of identified proteins showed that regulation of the cell cycle, cell movement, morphology, antigen presentation and metabolic processes were among the most affected functional domains (Figure 4). The number and functional roles of identified proteins indicated that the depth of this study was on the level of signaling and low abundance proteins.

For an overview of signaling pathways and various cellular processes affected by TGFβ1, we explored relations between identified proteins by generating two networks. The first network was based on proteins changing expression (silver stained), and the second was based on proteins changing their ³⁵S-incorporation levels (Supplementary Figure S2). General topology of the networks showed scale-free features, i.e. no strictly defined hierarchy of species, and distribution of connectivity of nodes approximate to the power law distribution (Supplementary Figures S2). Such topology has been claimed for many regulatory processes (Bhaskaran and Souhelnytskyi, 2008; Kitano, 2004; Wiley et al., 2003), and our findings confirm that TGFβ1 signaling in 184A1 cells has the similar features.

To extract information suitable for experimental interrogation, we extracted sub-networks related to regulation of specific functions. As TGF β 1 regulates cell proliferation, the proliferation-related network was analyzed (Figure 5). Among other components, p53, CK2 α , PC4 and SSRP1 proteins were found to be connected, with p53 being predicted as regulated by CK2 α , PC4 and SSRP1. p53 was found mutated or inactivated in approximately 50 % of cancers, and is known to regulate cell proliferation and apoptosis. Therefore, we focused further experimental interrogation on CK2 α , PC4 and SSRP1, as potential regulators of p53 activation.

CK2 α , PC4 and SSRP1 are involved in TGF β 1-dependent phosphorylation of p53 at Ser392, and regulation of cell proliferation and apoptosis

For interrogation of the sub-network (Figure 5), we down-regulated CK2 α , PC4 and SSRP1 or overexpressed CK2 α , alone or in combinations. Transfection of specific siRNAs was used for down-regulation, and CK2 α expression vector for enhanced expression. Phosphorylation of p53 at serine residue 392, expression of p53, activating phosphorylation of Erk1/2, cell proliferation and apoptosis were used to monitor responsiveness of cells to TGF β 1 (Figures 6, 7 and 8). As expected from proteomics data, TGF β 1 enhanced expression of CK2 α (Figure 6). We also observed enhanced expression of PC4 and stimulation of p53 phosphorylation at Ser392. The expression level of p53 did not change significantly (data not shown). An unexpected observation was TGF β 1-dependent inhibition of phosphorylation of Erk1/2 (Figure 6). Thus, we confirmed that TGF β 1 may affect not only CK2 α , as was observed in proteomics, but also CK2 α -dependent components indicated by the network analysis.

Observed changes in expression of CK2 α , PC4 and phosphorylation of p53 may be coordinated but also may be independent. The network analysis suggested that these changes may be dependent. Therefore, we performed an interrogation of the suggested network by down-or up-

regulation of selected components. CK2 α was up-regulated by overexpression, and SSRP1, PC4 and CK2 α were down-regulated by using specific siRNAs (Figure 7). Down-regulation of SSRP1 or PC4 alone led to enhanced phosphorylation of p53. However, combined siRNAs were less efficient, with preservation of TGF β 1-dependent induction of p53 phosphorylation. Manipulations of CK2 α alone showed that CK2 α is a potent stimulator of p53 phosphorylation. However, this potent effect is dependent on SSRP1 and PC4, as their combined down-regulation prevented CK2 α -dependent induction of p53 phosphorylation. Thus, SSRP1 and PC4 are modulators of CK2 α -dependent phosphorylation of p53.

We further investigate whether observed changes in p53 phosphorylation upon interrogation of cells with modulated expression of SSRP1, PC4 and CK2 α would have an impact on cell proliferation and death (Figure 8). We observed that the down-regulation of CK2 α had an inhibitory effect on cell proliferation, probably due to the enhanced rate of cell death.

Discussion

Multiplicity of TGF β effects on cells is a strong indication of multiple regulatory mechanisms that have to be engaged by TGF β (Wharton and Derynck, 2009; Massagué, 2008; Souchelnytskyi, 2005a). Reported here proteome profiling contributed to understanding of the regulatory mechanisms initiated by TGF β in human breast epithelial cells with a phenotype corresponding to the normal cells. This is the first report of such proteome profiling, and it shows that TGF β may employ different mechanisms in cells with different degree of carcinogenic transformation.

Systemic analysis of proteomics data significantly increased possibilities to decipher the complexity of TGF β signaling. We reported here use of the network building to unveil dependencies between identified proteins. The first conclusion from analysis of the generated network is that the topology of the network is similar to scale-free networks. Scale-free features

have been observed in signaling by TGF β and other growth factors, e.g. EGF (Wiley et al., 2003; Oda and Kitano, 2006; Bhaskaran and Souchelnytskyi, 2008). Moreover, carcinogenic transformation of human breast epithelial cells showed also scale-free features (Jia et al., 2010; Bhaskaran and Souchelnytskyi, 2008; Zakharchenko et al., 2010). This is an important observation that allows to conclude that different regulatory mechanisms follow in principle a similar signal wiring model. Scale-free features provide robustness and resistance to perturbations, but also provide efficient adaptation to changed environment (Kitano, 2004). Reported here results suggested which components are of the key importance for TGF β signaling in normal breast epithelial cells.

Systemic analysis of TGF β signaling in 184A1 cells indicated that p53 may play an important role in regulation of proliferation of 184A1 cells (Figure 5; Supplementary Figure S2). p53 is one of the key regulators of cell proliferation and apoptosis (Farnebo et al., 2010; Hahn and Weinberg, 2002). The network analysis indicated also that the effect on p53 may be mediated by CK2 and may be modulated by SSRP1 and PC4. SSRP1 was found in a complex with CK2 (Keller et al., 2001; Keller et al., 2002). However, an interaction between PC4, SSRP1 and CK2 in relation to regulation of p53 has not been reported earlier. Our interrogation data showed that regulation of p53 phosphorylation is the subject of a tight control by at least SSRP1 and PC4 (Figure 7). p53 is subjected to a number of regulatory mechanisms including post-translational modifications, e.g. phosphorylation, ubiquitylation, acetylation, protein-protein and protein-nucleic acid interactions (Meek and Andreson, 2009; Farnebo et al., 2010). Our results showed additional mechanism that involves CK2 α , SSRP1 and PC4. Further studies would be needed to explore details of molecular mechanisms of reported here observations. Knowledge of these mechanisms may be used in development of novel anti-cancer therapeutics.

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H.W., O.Z. and A.G. performed experiments, N.Z. participated in protein identification by MS, U.H. helped with mass spectrometry, S.S. designed and managed the project, wrote the manuscript.

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Figure legends

Figure 1. Proliferation of 184A1 cells is inhibited by TGFβ1.

A) 184A1 cells were treated with 10 ng/ml TGFβ1 for 6 h, 24 h and 48 h, as indicated. [³H]thymidine incorporation was measured during the last 2 hours of incubation. Lower panel shows scheme of treatment. As 100 % is taken incorporation in cells not treated with TGFβ1. B) Smad2 is phosphorylated at the C-terminal serine residues upon treatment of 184A1 with TGFβ1. The whole cell extracts from cells treated as indicated, were subjected to immunoblotting with pS2 antibodies. Migration position of phosphorylated Smad2 is indicated by the arrow. Representative experiments out of 4 (A) and 3 (B) performed are shown.

Figure 2. Representative 2D gels of proteins from cells treated or not with TGFβ1.

A-D) Representative 2D gels stained with silver are shown. Treatment of cells is indicated on the images of gels. Annotation of proteins is as in Supplementary Table S1. E-F) Images of representative ³⁵S-labeled gels obtained after exposure in a phosphorimager of gels shown in panels A-D. ³⁵S-labeled proteins identified as regulated by TGFβ1, are annotated as in Supplementary Table S3. Migration position of identified proteins are shown by lines in all panels. Directions of isoelectrofocusing and SDS-PAGE are indicated.

Figure 3. Validation of protein expression

Expression of DNA polymerase κ, RPA1, Cullin5 and RAP1 was validated by immunoblotting of cell extracts with corresponding specific antibodies, as indicated. Migration positions of the proteins are indicated by arrows. Upper parts of panels show changes observed in 2D gels, and lower panels show immunoblotting images.

Figure 4. Functional clustering of the identified proteins

Main functional domains affected by TGFβ1-regulated proteins on the level of expression (A) and ³⁵S incorporation (B) are shown. Relative representation of the functional domains is annotated as pie-charts, domains and numbers of assigned proteins are indicated between the pie-charts.

Figure 5. Sub-network formed by proteins potentially involved in regulation of cell proliferation.

Cell proliferation and growth sub-networks extracted from the full networks formed by proteins regulated by TGFβ1 as expression (A) and as ³⁵S incorporation (B), are shown. The full networks are shown in Supplementary Figure S2.

Figure 6. Expression of PC4, CK2α, p53 phosphorylated at Ser 392 and phosphorylated Erk1/2 in 184A1 cells upon treatment with TGFβ1.

184A1 cells were treated with TGFβ1 (10 ng/ml) for indicated period of time. Expression of PC4, CK2α and phosphorylation of p53 and Erk1/2 were monitored by immunoblotting of total cell extracts. Specific bands in immunoblotting experiments are shown, and proteins of interest are indicated. Representative experiments out of 4 performed are shown.

Figure 7. Modulation of expression of CK2α, PC4 and SSRP1 showed coordinated impact on phosphorylation of p53.

184A1 cells were transfected with siRNA to SSRP1, PC4 and CK2α, expression vector of CK2α, and control scrambled siRNA, as indicated. Phosphorylated p53 was detected by immunoblotting

of total cell extracts. Expression of p53 and actin in cells subjected to manipulations as in the lower panel of phosphor-p53 immunoblotting. This immunoblotting was used to control the loading of samples. Representative experiments out of 4 performed are shown.

Figure 8. Modulation of expression of CK2 α , PC4 and SSRP1 showed coordinated impact on proliferation of cells.

Proliferation (**A**) and cell death (**B**) were measured in cells subjected to modulation of expression of CK2 α , SSRP1 and PC4, as indicated. Transfections were performed with siRNA constructs (siCK2 α , siSSRP1, siPC4), or with expression vector for CK2 α (CK2 α), alone or in combinations, as indicated in panels. Representative experiments out of 3 performed (A, B) are shown.

Supplementary materials

Supplementary Table S1. Complete list of TGFβ1-regulated proteins, identified by changes in expression (silver-stained gels)

Supplementary Table S2. List of TGFβ1-regulated proteins with indication of changes in their expression. Folds of changes were calculated with normalized volumes of spots in 2D gels, in which proteins were identified.

Supplementary Table S3. Complete list of TGFβ1-regulated proteins, identified by changes in ³⁵S incorporation.

Supplementary Table S4. List of TGFβ1-regulated proteins, identified by changes in ³⁵S incorporation, with indication of changes in their expression. Folds of changes were calculated with normalized volumes of spots in 2D gels, in which proteins were identified.

Supplementary Figure S1. Scheme of treatment of cells used in this study.

Time points when cells were seeded for the experiments, treated with human TGFβ1, labeled with [³⁵S]methionine and [³⁵S]cysteine, and harvested for extraction, are shown.

Supplementary Figure S2. Complete networks formed by proteins which changed expression (A; silver stained proteins) and ³⁵S-label incorporation (B) are shown.

Figure 1, Woksepp, Zakharchenko, et al

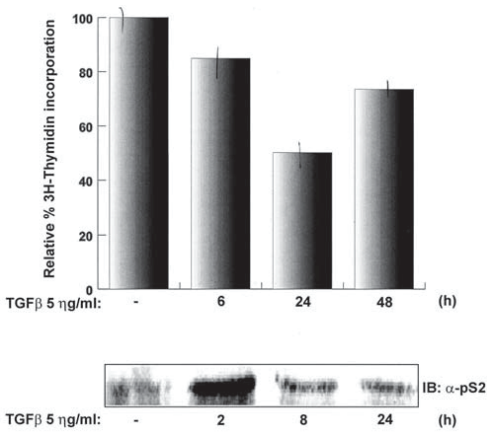


Figure 2 A, B, Woksepp, Zakharchenko, et al

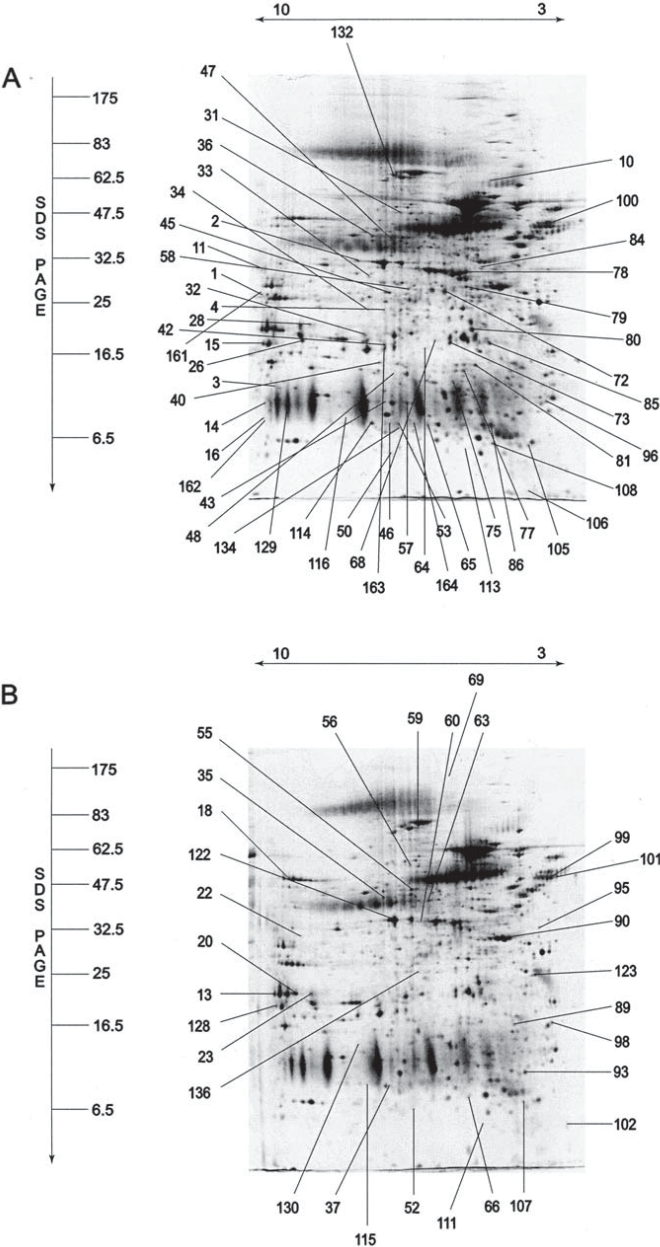


Figure 2 C, D, Woksepp, Zakharchenko, et al

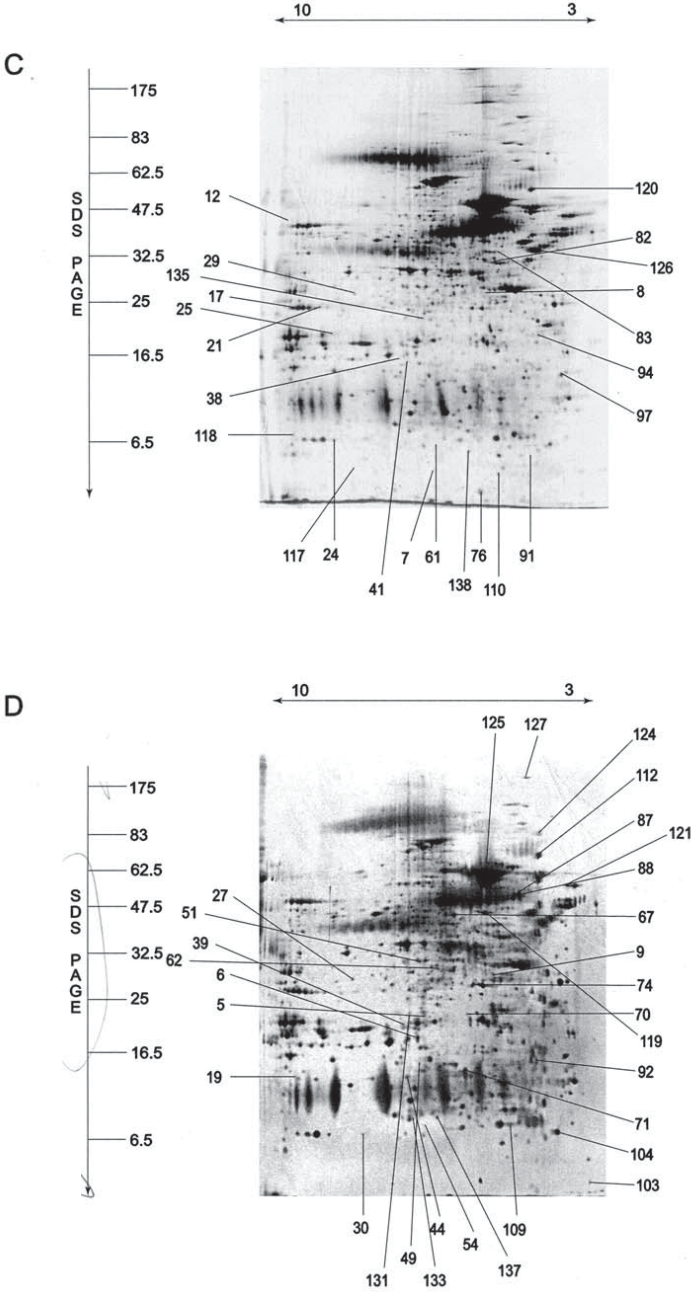


Figure 2 E, F, Woksepp, Zakharchenko, et al

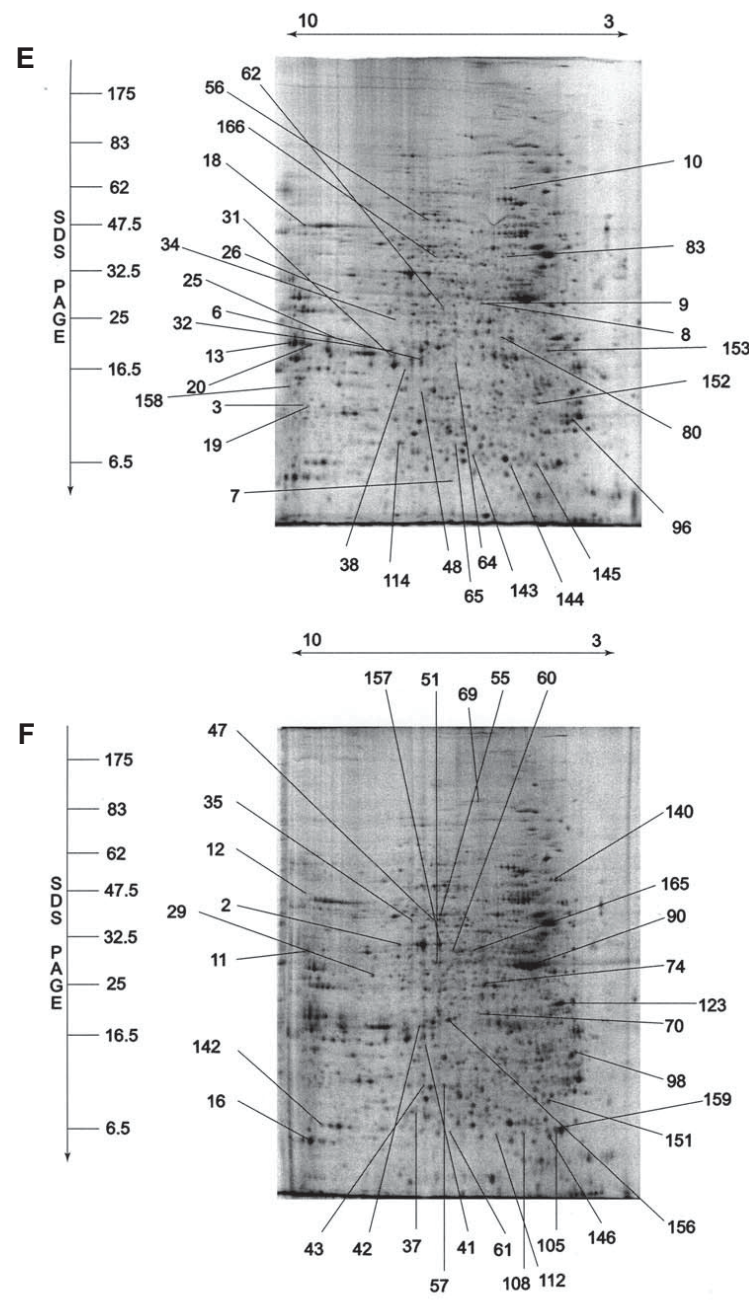


Figure 3, Woksepp, Zakharchenko, et al

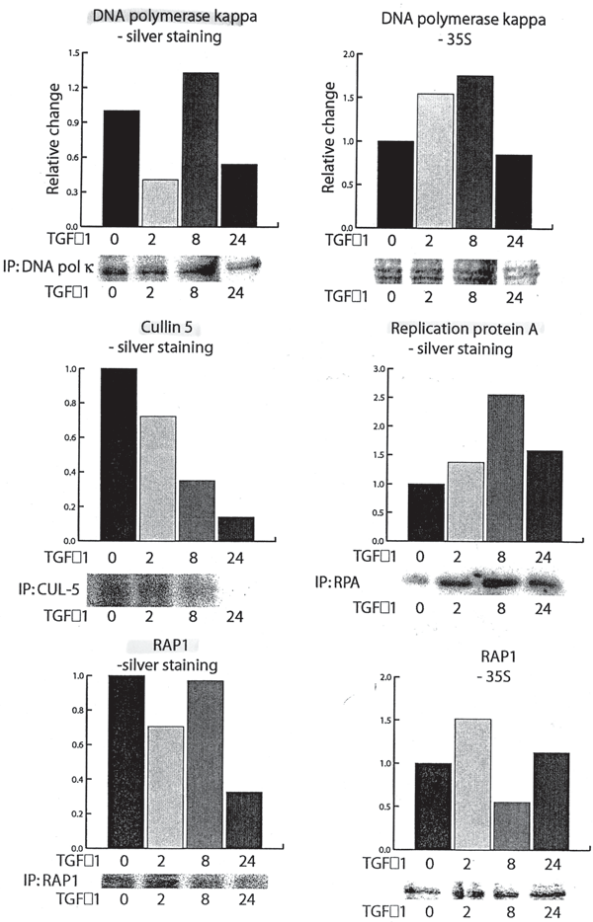
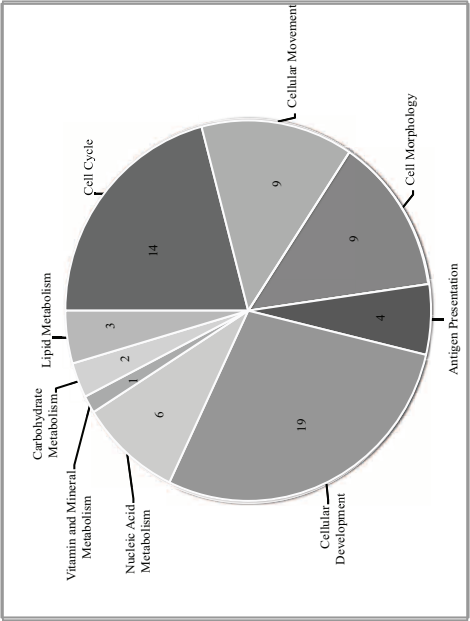


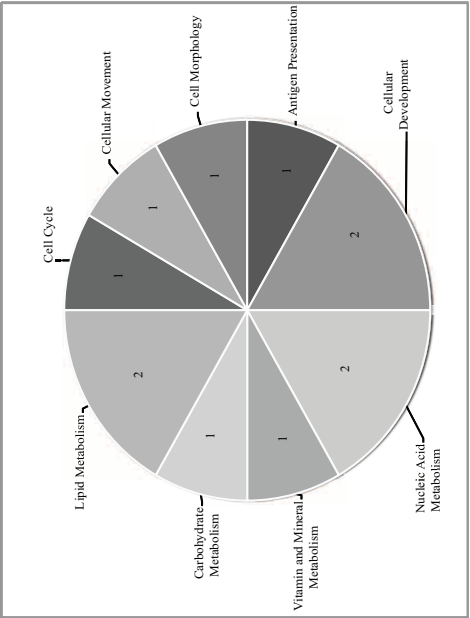
Figure 4.

Functional and dynamic clustering of the identified proteins

A)



B)



Sub-network formed by proteins potentially involved in regulation of cell proliferation

B) ^{35}S -labeled

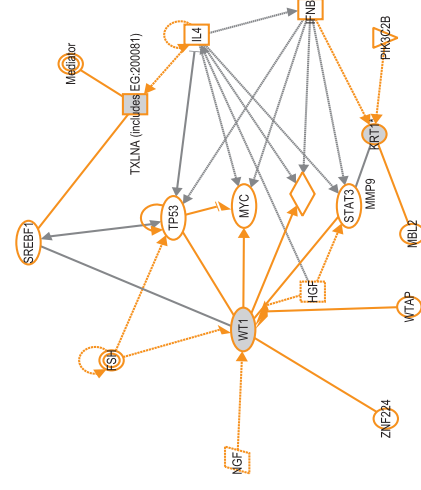


Figure 6, Woksepp, Zakharchenko, et al

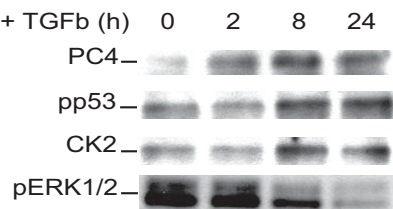
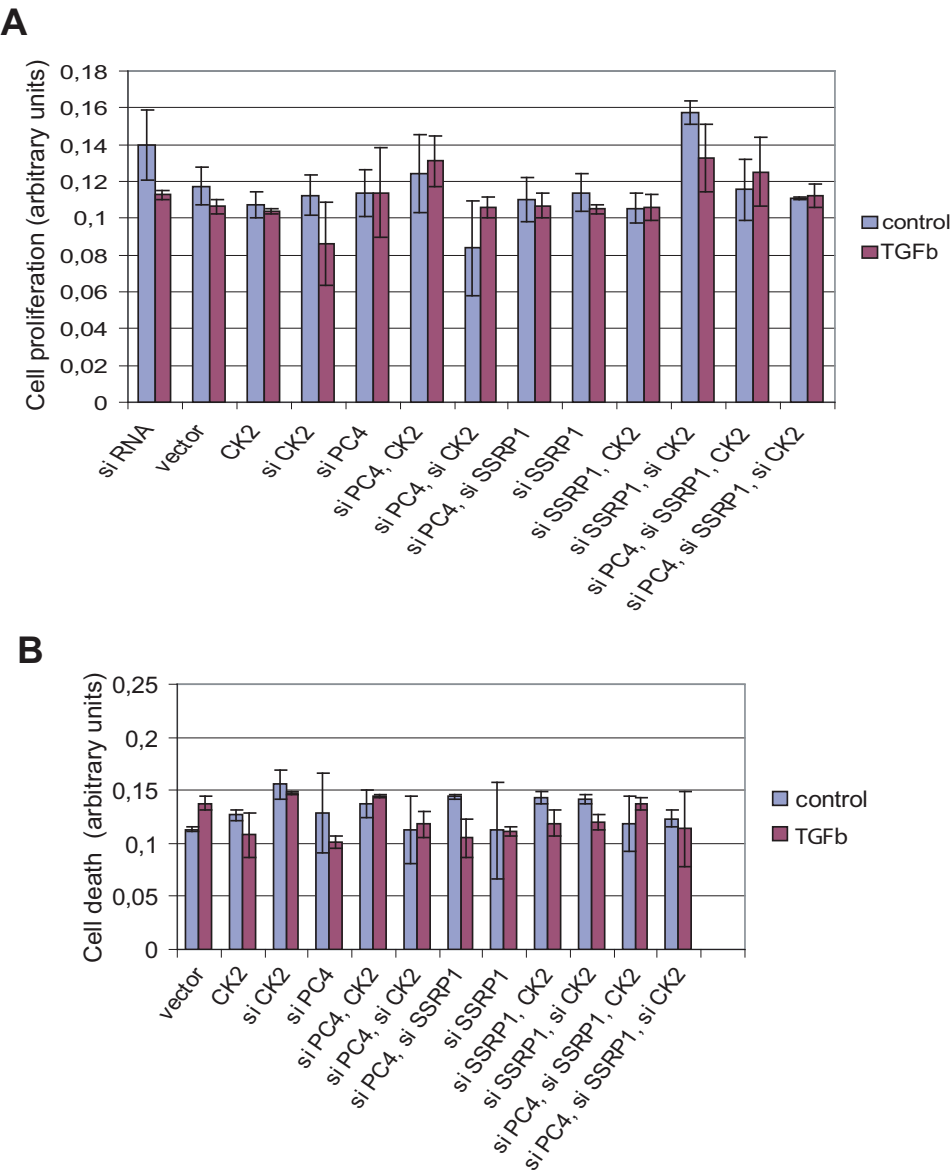


Figure 7, Woksepp, Zakharchenko, et al



Figure 8, Woksepp, Zakharchenko, et al





**Karolinska
Institutet**

DEPARTMENT OF ONCOLOGY AND PATHOLOGY

PROTEOME PROFILING OF HUMAN BREAST CANCER

AKADEMISK AVHANDLING

som för avläggande av medicine doktorsexamen vid Karolinska
Institutet offentligens försvaras i Lecture Hall, CCK R8:00.

Friday 29 April, 2011, kl 09.00

av

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PhD degree

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intervention och teknik

Stockholm 2011

ABSTRACT

Breast cancer (BC) takes thousands of woman's lives yearly. Several factors have been found to influence initiation and development of breast cancer, and to affect prognosis and treatment of this disease. This thesis is focuses on opening-out this complexity and search for approaches that may lead to individualized treatment of breast cancer patients.

We studied clinical samples of breast tumors and adjacent normal tissues using protein-based proteomics. By studying each patient individually, we identified proteins that changed expression during carcinogenesis (p53, Smad2, etc). We observed significant differences in the lists of cancer-related proteins between individual patients. We demonstrated that meta-data analysis of the identified proteins is the most efficient way to describe common and individual features of tumors from different patients. Our validation study by immunohistochemistry analysis of identified molecules (PYK, Smad2, CK2 α) confirmed the changed expressions between tumor and normal tissue, and thereby confirmed the conclusions obtained with proteomics analysis. Thus, we found that meta-data analysis approach is suitable for improved and individualized diagnostics and selection of treatment.

Transforming growth factor- β (TGF β) is a potent regulator of tumorigenesis. In our study of the clinical cases, we demonstrated that TGF β signaling might be influenced in breast tumorigenesis. Phosphoproteomics analysis of TGF β action on MCF10A human breast epithelial cells showed a complex regulation of cell signaling, with strong representation of functional domains such as metabolism. One of the targets of TGF β is 14-3-3 σ protein, and we found that 14-3-3 σ was of a crucial importance for the cross-talk between TGF β and p53 signaling.

We reported also proteins identified by expression proteomics, which are regulated by TGF β in human breast epithelial cells that have phenotype similar to normal breast epithelial cells. We found more than 100 proteins that were regulated by TGF β . Among them, Casein Kinase 2 α (CK2 α), Structure-Specific Recognition Protein-1 (SSRP1) and protein convertase-4 (PC4) may be involved in TGF β -dependent inhibition of cell proliferation by modulating p53 phosphorylation.

Therefore, presented here study describes development of tools for individualized treatment of patients, and provides insights in the complexity of cancer related signaling in breast epithelial cells.

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