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Research Paper

Decreased Usage of Specific Scrib Exons Defines a More Malignant Phenotype of Breast Cancer With Worsened Survival

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ABSTRACT

SCRIB is a polarity regulator known to be abnormally expressed in cancer at the protein level. Here we report that, in breast cancer, an additional and hidden dimension of deregulations exists: an unexpected SCRIB exon usage pattern appears to mark a more malignant tumor phenotype and significantly correlates with survival. Conserved exons encoding the leucine-rich repeats tend to be overexpressed while others are underused. Mechanistic studies revealed that the underused exons encode part of the protein necessary for interaction with Vimentin and Numa1, a protein which is required for proper positioning of the mitotic spindle. Thus, the inclusion/exclusion of specific SCRIB exons is a mechanistic hallmark of breast cancer, which could potentially be exploited to develop more efficient diagnostics and therapies.

1. Introduction

In epithelial cells planar polarity is maintained by proteins that are encoded by tumor suppressor genes such as SCRIB (Bilder et al., 2000; Bilder and Perrimon, 2000). Scribble, the protein product of SCRIB, is crucial for the proper maintenance of epithelial cell integrity and function (Zhan et al., 2008); it is required for E-cadherin-mediated cell-cell adhesion and, when its expression is down-regulated, epithelial cells acquire mesenchymal appearance and their migration is augmented (Qin et al., 2005). In epithelial cells the orientation of the mitotic spindle is restricted to the plane of the epithelium to ensure that daughter cells will remain within the layer. It was shown recently that Scribble is involved in this process and when its expression is knocked down in Drosophila the orientation of the mitotic spindle becomes random. When combined with inhibition of apoptosis, Scribble knock-down is sufficient to induce epithelial to mesenchymal transition and formation of tumor-like structures (Nakajima et al., 2013). Scribble expression and localization is frequently deregulated in human cancers, including breast cancer, where it is either lost or abnormally overexpressed and localized (Zhan et al., 2008, Feigin et al., 2014). In this study we show that, in human breast cancer, the expression of several exons, which share very little similarity with the sequence from Drosophila, is decreased relative to the exons encoding the rest of the protein. Furthermore, this pattern of exon usage correlates with long-term survival. The underused exons encode part of the C-terminal proline-rich domain of Scribble, which we found to become increasingly phosphorylated in mitosis, leading to association with Numa1, a protein known to be crucial for the proper positioning of the mitotic spindle in polarized cells.

2. Materials and Methods

2.1. Reagents

Chemicals and HPLC solvents, unless indicated otherwise in the text, were purchased from Thermo Fisher. The highest available grades were used. The following antibodies were used for Immunoprecipitation and indirect immunofluorescence: mouse monoclonal anti-GFP (B-2, Santa Cruz, CA), rabbit polyclonal anti-Numa1 (H300, Santa Cruz, CA), mouse monoclonal anti-Scribble (C6, Santa Cruz, CA), goat anti-mouse Alexa Fluor 467 (Invitrogen, USA), goat anti-rabbit Texas red-conjugated (Jackson IR laboratories). Protein A/G coated magnetic microparticles (Thermo Fisher). MS-grade trypsin was used for protein digestion (Promega).

2.2. Plasmids and Mutagenesis

The GFP-Scribble sequence cloned in pEGFP-N1 (Clonthech) used to generate Scribble mutants was a generous gift from Professor Ian Macara, Vanderbilt University School of Medicine. The following

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oligonucleotides were used to change S1448 to A and D, and to delete the C-terminus: GCTGGAGGGAAGATGGCTTAATCTCCCTGCTCCCCTAG to change codon 1293 to TAA for stop; AGGCAGAGCCCGGCGGCCCCCCCGCCCCTGGGA to change codon 1448 to GCC for alanine; AGGCAGAGCCCGGCGGACCCCCCGCCCCTGGGA to change codon 1448 to GAC for aspartate. Mutagenesis was confirmed by sequencing and mass spectrometry at the protein level.

2.3. Cell Culturing and Transfection

HEK293T cells were grown on DMEM medium supplemented with 10% FCS at 37 °C and 5% CO_2 and split every third day. For confocal imaging the cells were transfected in in Nunc™ Lab-Tek™ chamber slides or on coverslips in 24 well dishes. For co-IP experiments the cells were transfected in 10 cm dishes. In both cases the transfection was with TurboFect (Thermo Fisher) according to the manufacturer's instructions.

2.4. Immunoprecipitation

Cells were harvested, washed with PBS and lysed with buffer containing 1% Igepal 40, 50 mM Tris-HCl, pH 6.8, protease and phosphatase inhibitors. Lysates were cleared by centrifugation and incubated with 4 μg anti-GFP antibody (B-2, Santa Cruz Biotechnology) or anti-Scribble antibody (C-6, Santa Cruz Biotechnology) for 45 min at RT on a rotator. 20 μl magnetic protein A/G microbeads (Thermo Fisher) were added and incubated for 1 h at RT on the rotator. Beads were washed 3× with 1 ml PBS containing 0.1% Tween 20 and once with 1 ml PBS without detergent. The Magnarack system was used to pellet the magnetic microbeads between washes. After the removal of the final wash volume 20 μl of trypsin solution (30 mg/ml trypsin in 25 mM NH_4HCO_3 containing 1 M urea) was added to the microbeads and the digestion was carried out at 30 °C for 16 h.

2.5. Nano-scale LC-MS/MS and Analysis of MS/MS Data

The analysis of protein digests by electrospray ionization MS was performed using a hybrid LTQ/Orbitrap Velos instrument as previously described (Greenwood et al., 2012; Metodieva et al., 2013; Croner et al., 2014). MaxQuant was used to analyze MS/MS data to identify and quantify proteins (Cox and Mann, 2008).

2.6. Identification of Co-precipitated Proteins

To evaluate the statistical significance of the IP-LC/MS results we used the spectral counts for GFP, Scribble and co-precipitated proteins and computed p-values using two different tests: the G test and Chi squared test. For the Chi squared test we constructed contingency tables of observed and expected counts. The expected values for each candidate protein were derived from either the observed Scribble counts or the observed GFP counts. This analysis was performed in R.

2.7. Targeted Detection of Numa1

A SRM scan was added to the LC-MS/MS method to isolate and fragment the doubly-charged Numa1 peptide AALMESQGQQQEER at m/z of 802.87. The precursor was accumulated for 100 ms in the linear ion trap and then fragmented by collision-induced dissociation. The intensity of the y7 fragment ion ($m/z = 874.45$) was used for quantification. Label-free quantitation was performed by integrating the ion intensities as described previously (Metodieva et al., 2009).

2.8. Calculation of Relative Phosphorylation Site Occupancy Rates

We followed the approach described in Olsen et al. for SILAC-labelled peptides (Olsen et al., 2010). The occupancy rate calculation as described in Olsen et al. requires SILAC labeling. Here we modify the procedure to allow relative occupancy rate calculation from label-free experiments provided there exist co-eluting peptides derived from the same protein that can serve as internal normalizing standards. The procedure as used to analyze S1448 phosphorylation dynamics during the cell cycle is explained below.

Two co-eluting unmodified Scribble peptides were used to normalize the ion current for the phosphopeptide and its unmodified counterpart: RLPDGIGQLK for the phosphopeptide QSPAS(ph)PPPLGGGAPVR and GHAGNPRDPTDEGIFISK for QSPASPPPLGGGAPVR. E_0 is ionization efficiency of base peptide; E_1 is ionization efficiency of mod peptide; I_0 is ion intensity of base peptide; I_1 is ion intensity of mod peptide; E_2 is ionization efficiency of normalizing peptide co-eluting with base peptide; E_3 is ionization efficiency of normalizing peptide co-eluting with mod peptide; I_2 is ion intensity of normalizing peptide co-eluting with base peptide; I_3 is ion intensity of normalizing peptide co-eluting with mod peptide. To calculate molar ratio (mod/base) we assume concentration (M) $= I * E$ so mod/base $= I_1E_1/I_0E_0$.

Since ionization intensities are unknown we use control/treatment ratios to write equation and eliminate E:

We assume that the concentrations of the normalizing peptides do not change relative to the protein concentration because they do not undergo modification under cell cycle control. The ratio I_1E_1/I_3E_3 is run-invariant for a given biological replicate because the two peptides co-elute so any fluctuations in spray conditions would affect them equally and we assume that ionization efficiencies depend only on the sequence. On the other hand this ratio would be changed in response to treatment if the phosphorylation site occupancy is treatment-dependent. It follows then that:

$$\frac{I_1^t}{I_3^t} / \frac{I_1^c}{I_3^c} = \frac{\lfloor mod^t \rfloor}{\lfloor mod^c \rfloor} = X \tag{1}$$

$$\frac{I_0^t}{I_2^t} / \frac{I_0^c}{I_2^c} = \frac{\lfloor base^t \rfloor}{\lfloor base^c \rfloor} = Y \tag{2}$$

From equations 1 and 2 we now calculate the site occupancy rates in control and treatment to be related as follows:

$$\frac{\lfloor mod^t \rfloor}{\lfloor base^t \rfloor} = \frac{X}{Y}\left(\frac{\lfloor mod^c \rfloor}{\lfloor base^c \rfloor}\right) \tag{3}$$

$$\frac{a}{b} = \frac{X}{Y} \tag{4}$$

Where a is the occupancy rate $\frac{\lfloor mod^t \rfloor}{\lfloor base^t \rfloor}$ and b is the occupancy rate $\frac{\lfloor mod^c \rfloor}{\lfloor base^c \rfloor}$

The ratios from Eq. 4 are then used to graph the corresponding data points for the different phases of the cell cycle assuming G1-S as a baseline.

2.9. Confocal Microscopy

Cells were seeded on Nunc™ Lab-Tek™ chamber slides or on coverslips in 24 well plates at 30%–50% confluency. The next day they were washed with PBS and fixed with 4% formaldehyde for 15 min RT. They were washed with PBS and permeabilized with 0.5% Triton X-100 in PBS for 5 min and blocked for 1 h with 5% BSA in PBS. Primary antibody was added in 5% BSA, at 1:100 dilution for 1 h. Samples were washed 3 times with 1% BSA in PBS with 0.1% Tween 20. Secondary fluorophore – conjugated antibody was applied for 1 h and after 3× washing with PBS – 0.1% Tween 20, samples were stained with DAPI for 15 min and washed with PBS. Coverslips were mounted on glass slides and chamber slides had coverslips mounted using Vectashield (Vector Laboratories,