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Protein phosphatase PP1-NIPP1 activates mesenchymal genes in HeLa cells

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ABSTRACT

The deletion of the protein phosphatase-1 (PP1) regulator known as Nuclear Inhibitor of PP1 (NIPP1) is embryonic lethal during gastrulation, hinting at a key role of PP1-NIPP1 in lineage specification. Consistent with this notion we show here that a mild, stable overexpression of NIPP1 in HeLa cells caused a massive induction of genes of the mesenchymal lineage, in particular smooth/cardiac-muscle and matrix markers. This reprogramming was associated with the formation of actin-based stress fibers and retracting filopodia, and a reduced proliferation potential. The NIPP1-induced mesenchymal transition required functional substrate and PP1-binding domains, suggesting that it involves the selective dephosphorylation of substrates of PP1-NIPP1.
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1. Introduction

PP1 is one of seven members of the PPP-type superfamily of protein Ser/Thr phosphatases, which together catalyze the large majority of all protein dephosphorylation events in eukaryotes [1,2]. It forms oligomeric complexes with dozens of PP1-interacting proteins (PIPs) that determine the subcellular localization, substrate selectivity and specific activity of the phosphatase. One of the major nuclear PIPs in multicellular eukaryotes is NIPP1, for nuclear inhibitor of PP1. NIPP1 forms a heterodimer with a considerable fraction of the nuclear pool of PP1 [3]. It contains an essential central PP1-anchoring domain [4,5] and a C-terminal PP1-inhibitory domain [6]. Mutation of the central PP1-anchoring motif ^{200}RVTF^{203} → ^{200}RATA^{203} (residue numbers in superscript) virtually abolishes the binding of PP1, whereas deletion of the C-terminal residues 330–351 enables associated PP1 to be constitutively active [6,7]. The recruitment of substrates by the PP1-NIPP1 holoenzyme is mediated by the ForkHead-Associated (FHA) domain of NIPP1, which specifically binds to phosphorylated threonines that are followed by a proline [8–12]. The established FHA-ligands of NIPP1 include the pre-mRNA splicing factors SAP155 and CDC5L, the chromatin modifier EZH2 and protein kinase MELK [8–10,12]. Additional investigations revealed that NIPP1 functions in spliceosome assembly [13] and transcriptional silencing by the histone methyltransferase EZH2 [12,14,15] in a PP1-dependent manner. NIPP1 also promotes the Cdc42-dependent migration of HeLa cancer cells, but the involved FHA-ligand is not known [16].

The deletion of NIPP1 in mice is embryonic lethal at E6.5–7.5, suggesting that NIPP1 is essential for lineage specification [17]. Here, we show that a mild (<10%) overexpression of NIPP1 in human epithelial HeLa cancer cells resulted in the massive expression of mesenchymal genes. This effect was critically dependent on the PP1 and substrate-binding domains of NIPP1, demonstrating a key role for dephosphorylation of PP1-NIPP1 substrates in the differentiation process.

2. Materials and methods

2.1. Antibodies

Immunoblots were incubated with anti-α-tubulin (clone B-5-1-2) and anti-Flag (M2, 200472-21; Sigma–Aldrich, St. Louis, MO),

Abbreviations: Dox, doxycycline; NIPP1, nuclear inhibitor of PP1; PP1, protein phosphatase-1

Author contributions: NVD, SB, BL and JG designed and performed the experiments, and analyzed the data. CW generated the Flp-In T-Rex cell lines. AVE and MB supervised the project and wrote the manuscript.

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anti-GFP (SC-8334; Santa Cruz, California), anti-ACTA2 (clone 1A4; Dakocytomation, Gostrup, Denmark), anti-CNN1 (464794) and anti-TAGLN (ab14106; Abcam, Cambridge, UK). Anti-NIPP1 (mAb 15B8C11) was homemade [15].

2.2. Cell culture

HeLa Tet-Off (HTO) cell lines expressing Flag-tagged fusions of NIPP1 variants in the absence of Doxycycline (Dox) were obtained as previously described [7]. HeLa Flp-In T-Rex cells (Invitrogen) that stably expressed EGFP or EGFP-tagged NIPP1 fusions in the presence of Dox were generated according to the manufacturer's instructions. Briefly, the cDNAs were cloned into the pcDNA5/FRT/TO plasmid and co-transfected into HeLa Flp-In T-Rex host cells (generous gift of Dr. Jonathon Pines, The Gurdon Institute, Cambridge, UK) with the pOG44 plasmid that encoded the Flp recombinase. Following hygromycin selection, single cell clones were picked and expanded to obtain clonal cell lines. Proper integration was verified by lack of β-galactosidase activity. Stable cells were cultured in DMEM-Low glucose medium (Sigma–Aldrich), supplemented with 10% tetracycline-reduced fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma–Aldrich), 200 μg/ml Hygromycin-B (Calbiochem) and 5 μg/ml Blasticidin (InvivoGen). Proliferation assays were performed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). HTO cells were cultured for 48 h with and without doxycycline. MTT (0.5 mg/ml) was added for 3 h. The crystals were dissolved in DMSO and the absorbance was measured at 595 nm.

2.3. Protein extracts and immunoblotting

HTO and HeLa Flp-In cells were harvested and lysed for 20 min at 4 °C in a buffer containing 50 mM Tris–HCl at pH 7.5, 0.3 M NaCl, 0.3% Triton X-100, 0.1% NP-40, 0.5 mM phenylmethanesulfonyl fluoride, 0.5 mM benzamidine, 5 μM leupeptin, 20 mM sodium fluoride and 1 μM vanadate. The lysates were clarified by centrifugation (10 min at 500×g) and the supernatant was processed for SDS–PAGE and immunoblotting. Immunoblots were visualized using eCL reagent (Perkin Elmer, Life Sciences) in the ImageQuant LAS4000 imaging system (GE Healthcare).

2.4. Confocal microscopy and immunocytochemistry

HTO cells were washed twice with PBS and fixed for 10 min with 4% formaldehyde. Cell permeabilization was performed by an incubation for 5 min in PBS, supplemented with 0.5% Triton X-100. The permeabilized cells were washed 3 times for 10 min with PBS, pre-incubated for 20 min with PBS containing 3% bovine serum albumin, and then incubated overnight with ACTA2 antibody. After three washes of 10 min each with PBS, the cells were incubated for 1 h with Alexa Fluor 546 labeled phalloidin (Life technologies) and mouse secondary antibody (Life Technologies) that was labeled with Alexa fluor 488. Finally, the cells were washed 3 times for 10 min in PBS. Confocal images were obtained with a Zeiss LSM-510(META) laser-scanning confocal microscope (Jena, Germany), equipped with the Zeiss Axiovert 200 M (Plan-Neofluar 40×/1.3 Oil DIC objective), at the Cell-Imaging-Core of KU Leuven.

2.5. Contractility assays

96 h after doxycycline removal, the HTO cells were incubated with 40 mM KCl and observed for 20 min with a bright field exposure on a motorized inverted IX-81 microscope, controlled by Cell-M software and equipped with a temperature, humidity, and CO_2-controlled incubation chamber (Olympus, Aartselaar, Belgium). Photographs were taken every 20 s.

2.6. Microarray analysis

RNA from four independent cultures of the parental and Flag-NIPP1-Pa expressing HTO cell line was labeled with a single fluorescent dye and hybridized onto Whole Human Genome Oligo microarrays from Agilent (Santa Clara, CA, USA), as previously described for the NIPP1-Wt and NIPP1-Pm cell lines [15]. Expression levels were analyzed using Significance Analysis of Microarrays (SAM) software [18]. The data from the parental cell line were used as a baseline expression for comparison with the Flag-NIPP1 cell lines. For the calculation of 95% confidence intervals on correlation coefficients, the correlation coefficient was subjected to the Fisher's z transformation, followed by back-transformation of the Student's t 95% confidence interval on the resulting z score. Data were analyzed by IPA (Ingenuity® Systems, www.ingenuity.com). The differentially regulated genes ($P < 0.01$) in Flag-NIPP1-Wt and Flag-NIPP1-Pa were uploaded into the application. The IPA analysis identified the biological functions that were most significant for the data set. Right-tailed Fisher's exact test was used to calculate a P-value determining the probability that each biological function assigned to that data set is due to chance alone. All gene expression data are available at GEO under accession numbers GSE19642 and GSE67558.

2.7. Quantitative RT-PCR

Total RNA was isolated using the Genelute Mammalian Total RNA Miniprep kit (Sigma). RNA (2 μg) was reverse-transcribed with oligo dT primer (Sigma) using the RevertAid Premium Reverse Transcriptase and RiboLock RNase inhibitor enzymes (Fermentas, GmBH, St Leon-Rot, Germany). About 1.2% of the cDNA was PCR-amplified in duplicate, using SYBR Green qPCR Mix (Invitrogen, Paisley, UK) and a Rotorgene detection system (Corbett Research, Cambridge, UK), as described by Nuytten et al. [14]. Quantitative reverse transcriptase PCR was performed to check the transcript levels of ACTA2 (5′-ACTGGGACGACATGGAAAAG-3′ and 5′-TACATGGCTGGGACATTGAA-3′), and TAGLN (5′-AGAATGGCGTGATTCTGAGC-3′ and 5′-GCTCCATCTGCTTGAAGACC-3′). Data were normalized against the housekeeping gene HPRT (5′-TGACACTGGCAAAACAATGCA-3′ and 5′-GGTCCTTTCACCAGCAAGCT-3′).

3. Results

3.1. NIPP1 induces mesenchymal-lineage specific genes in a PP1-dependent manner

We started our analysis using previously described HeLa Tet-Off (HTO) cell lines that stably express Flag-NIPP1 fusions in the absence of doxycycline [7]. The cell lines expressed a Flag-fusion with wild type NIPP1 (NIPP1-Wt), a PP1-binding mutant (NIPP1-Pm) or a mutant that is associated with constitutively active PP1 (NIPP1-Pa) (Fig. 1A). NIPP1-Pm was generated by mutation of residues 201 and 203 in the RVxF-type PP1 binding site, which largely abolishes the binding of NIPP1 to PP1 [5,6]. NIPP1-Pa lacks the C-terminal residues 330–351, comprising a PP1-inhibitory region [6]. The Flag-tagged fusions were expressed at levels that were 2–3-fold higher than that of endogenous NIPP1 (Fig. 1B). A comparative gene expression analysis using previously published data for NIPP1-Wt and NIPP1-Pm [15], and newly analyzed whole genome oligo-microarray profiling data for NIPP1-Pa revealed striking differences between the cell lines (Fig. 1C). A paired SAM analysis

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