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.

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 (RVT) 200 RVTF 203 → (RATA) 330–351 (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),...
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. Jonathan 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 phenylmethylsulfonyl 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 5000×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 fluoro 488. Finally, the cells were washed 3 times for 10 min in PBS. Confocal images were obtained with a Zeiss LSM-510META 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 CO2-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 GeneHelne 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'-ACTGGGACGACATGGAAAA G'-3' and 5'-TACATGCGTCTGGAATGAA-3'), and TAGLN (5'-AGA ATGGCCGTATCTGACG-3' and 5'-GCTCCATGTGGATGAAGC AG-3'). Data were normalized against the housekeeping gene HPRT (5'-TGACACTGGCAGAAATCTGA-3' and 5'-GGTCCCTTTCAACCAGC A-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
identified 1365 differentially expressed genes \((P < 0.01)\) in the NIPP1-Wt cells, as compared to the parental control cell line. In contrast, 2230 \((P < 0.01)\) and 185 genes \((P < 0.01)\) were differentially expressed in the NIPP1-Pa and NIPP1-Pm expressing cells, respectively (Supplemental Table 1). Thus, the number of affected genes was reduced by 90% for the PP1-binding mutant of NIPP1 and increased by 67% for the mutant that is associated with constitutively active PP1. Despite these differences, there was a large overlap between the genes that were affected in the three cell lines (Fig. 1C). For example, 67% of the genes that showed a differential expression in the NIPP1-Wt cells were also affected in the NIPP1-Pa cells. Furthermore, scatter plot analysis revealed that the genes that were significantly altered by NIPP1-Wt were also affected by NIPP1-Pa \((r = 0.95 \pm 0.0042)\), and vice versa \((r = 0.92 \pm 0.0046)\) (Fig. 1D). In general, the effects of an expression of NIPP1-Pa were more pronounced than those of NIPP1-Wt, as indicated by the different slope values (0.96 versus 0.80) of both plots (Fig. 1D).

An Ingenuity Pathway Analysis (IPA) of the genes that were significantly affected by the expression of NIPP1-Wt or NIPP1-Pa showed that 739 out of 918 commonly altered genes could be classified by their biological function (Fig. 2A). Interestingly, on average 79% of the genes in each class were upregulated by the expression of NIPP1-Wt/Pa and highly enriched for the IPA categories ‘cardiovascular system development and function’ and ‘tissue morphology’. The enrichment for genes of the mesenchymal lineage was striking given that HeLa cells have an epithelial origin. Fig. 2B illustrates the upregulation of the transcripts of some key markers of smooth-muscle, cardiac and mesenchymal-matrix specific genes by the expression of NIPP1-Wt or NIPP1-Pa, but not by the expression of NIPP1-Pm. Collectively, these data show that NIPP1 activates mesenchymal transcriptional programs in a PP1-dependent manner.

3.2. NIPP1 induces the transdifferentiation of HeLa cells

Next, we investigated the functional consequence of the altered transcriptome in Flag-NIPP1 expressing HTO cells. Consistent with the microarray data, immunoblotting revealed that ACTA2 (α-smooth muscle actin), TAGLN (smooth muscle protein 22 alpha) and CNN1 (basic smooth muscle calponin 1) were not detectable in the parental (Co) and NIPP1-Pm cell lines, but were highly expressed in the NIPP1-Wt and NIPP1-Pa cell lines (Fig. 3A). For ACTA2 and TAGLN the overexpression was more pronounced in the NIPP1-Pa cell line, in accordance with their more elevated transcript levels (Fig. 2B). However, immunoblotting did not reveal a differential expression of the cardiac-specific protein CDH13 (H-cadherin; not illustrated), which contrasts with the huge differences in the CDH13 transcript level in the HTO cell lines (Fig. 2B). Therefore, we have selected the smooth-muscle network for further analysis.

At the transcript level, the smooth-muscle genes ACTA2, TAGLN and CNN1 were among the 20 most upregulated genes in the NIPP1-Wt and NIPP1-Pa expressing HTO cells (Fig. 2B). We also detected an upregulation of numerous other transcripts encoding proteins linked to smooth-muscle contraction, including the calmodulin and actin-binding protein caldesmon (CALD1), tropomyosin (TPM1), and the calcium-activated potassium channel subunit beta-1 (KCNMB1) (Fig. 3B and Supplemental Fig. 1). The latter is predominantly expressed in vascular smooth-muscle cells and was the second highest upregulated gene in NIPP1-Pa expressing
In addition, transcripts for kinases involved in smooth-muscle contraction, including myosin light-chain kinase (MYLK) and ROCK2 kinase (ROCK2), were significantly upregulated in the NIPP1-Wt/Pa HTO cells. Also, several genes involved in actin stress-fiber formation were upregulated following the expression of NIPP1-Wt/Pa, including non-muscle myosin (MYH9) and regulatory light-chain myosin (MYL9). Consistent with this altered gene expression pattern, phalloidin stainings showed a clear increase in the number of stress fibers in the NIPP1-Wt/Pa expressing HTO cells, but not in NIPP1-Pm cells (Fig. 3C). Moreover, ACTA2 partially co-localized with the stress fibers.

The functionality of the stress fibers was determined by comparing the ability of the HTO cells to contract in the presence of a depolarizing KCl concentration [19]. In this condition, we detected retracting filopodia in 16% of the parental cells, which increased to 40% for the NIPP1-Pa expressing cells (Fig. 3D). Hence, the expression of NIPP1-Wt/Pa in HTO cells causes their conversion into smooth-muscle like cells.

As differentiation is generally associated with a reduced proliferation, we also compared the proliferation potential of the HTO cells. The expression of Flag-NIPP1-Wt reduced the proliferation rate of the HTO cells, as measured by MTT assays (Fig. 3E). NIPP1-Pa was an even stronger inhibitor of cell proliferation, whereas NIPP1-Pm had no effect.

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Since NIPP1 overexpression induced the expression of mesenchymal genes, we have also examined whether the reverse is true too. However, the knockdown of NIPP1 in differentiated C2C12 myoblasts cells did not affect the transcript levels of ACTA2 and TAGLN (not shown).

### 3.3. The induction of mesenchymal genes only requires a modest overexpression of NIPP1

Strikingly, cell proliferation was already significantly inhibited by the leaky expression of the uninduced NIPP1-Wt/Pa transgenes (Fig. 3E, +Dox). This prompted us to explore in more detail the dependency of the transdifferentiation phenotype on the level of the expressed NIPP1 fusions. TAGLN, ACTA2 and CNN1 were induced to a similar extent in the absence or presence of Dox, despite the hugely different expression levels of the Flag-NIPP1-Wt/Pa fusions (Supplemental Fig. 2). Yet, these effects appeared specific as the smooth-muscle genes were not detectably expressed in the parental and NIPP1-Pm expressing cell lines. These data therefore indicate that even a small increase (8 ± 5%) in the level of NIPP1 is sufficient to induce the expression of mesenchymal genes in HeLa cells.

To further validate our data, we generated another set of stable HeLa cell lines, using the Flp-In T-Rex system [20]. This strategy allows the insertion of transgenes in a well-defined locus, thereby eliminating artefacts due to random integration. Also, we used a different tag (EGFP) and a reversed Dox-dependency (induction by Dox). Fig. 4A illustrates the expression of EGFP or EGFP-NIPP1-Wt in a Dox-dependent manner. We again noted that the overexpression of NIPP1-Wt, but not of EGFP, resulted in the induction of smooth-muscle genes, as detected by qRT-PCR (Fig. 4B). Also, the maximal effect was already obtained in the absence of Dox, when only trace amounts of EGFP-NIPP1 were expressed due to promoter leakiness. These data confirm that the expression of smooth-muscle genes is already induced by small increments in the level of NIPP1.
Fig. 3. NIPP1-Wt/Pa endows HeLa cells with contractile properties. (A) Immunoblotting of the indicated proteins in HT3 cell lysates. The expression of the fusion proteins was confirmed with monoclonal anti-Flag and anti-NIPP1 antibodies. Tubulin served as a loading control. (B) Bar charts of microarray data of the indicated genes, after induction of NIPP1 fusion proteins. Data are expressed as a fold change ± S.E.M. (n = 4), as compared to the control cell line (Co). *P < 0.01. (C) Confocal (immuno)fluorescence images of the indicated markers in HeLa cells after the expression of the indicated NIPP1 fusions. ACTA2, α-smooth muscle actin; Phalloidin, fluorescent probe for filamentous actin. Scale bars represent 10 μm. (D) Contractility assays were performed in response to the depolarizing agent KCl. Photographs of the control and HT3 Flag-NIPP1-Pa cells were taken at 0, 10 and 20 min after the addition of 40 mM KCl. Arrows indicate a representative contracted cell. Bar diagrams show the percentage of HT3 parental (n = 64) and HT3 Flag-NIPP1-Pa (n = 48) cells that contracted a filopodium after KCl stimulation. (E) MTT proliferation assays of indicated HT3 cell lines before and after the induction with Dox.
Fig. 4. The expression of smooth-muscle genes in HeLa only requires a minimal overexpression of NIPP1. (A) HeLa Flp-In T-Rex cell lines with inducible and stably integrated EGFP or EGFP-NIPP1-Wt were cultured without (-) and with increasing amounts of Dox from 62.5 to 1000 pg/ml. The ectopic expression of EGFP and EGFP-NIPP1-Wt was analyzed by immunoblotting with anti-EGFP and anti-NIPP1 antibodies. Tubulin was used as a loading control. (B) The relative mRNA levels of ACTA2 and TAGLN were measured by qRT-PCR in the same cell lines. HPRT was used for normalization and data were presented as a percentage of control ± S.E.M. (n = 3). Data were compared to the control cell line treated with the same dose of Dox. *P < 0.05; **P < 0.01; ***P < 0.001 with the paired Student's t-test.

Fig. 5. The expression of smooth-muscle genes requires PP1 and a functional FHA domain. (A) Diagram of the EGFP-tagged NIPP1 fusions. (B) The expression of the EGFP-fusions was analyzed by immunoblotting with anti-NIPP1 (NIPP1) and anti-EGFP antibodies. Tubulin was used as a loading control. (C) The relative mRNA levels of ACTA2 and TAGLN were measured by qRT-PCR in the indicated cell lines. Data were normalized against HPRT and presented as a percentage of the EGFP control cell lines ± S.E.M. (n = 3). Stars indicate a significant difference in relative mRNA expression compared to EGFP-NIPP1-Wt. *P < 0.05; **P < 0.01; ***P < 0.001 with the paired Student's t-test.
3.4. The induction of mesenchymal genes depends on the FHA-domain of NIPP1

Using the Flip-In T-Rex system, we have also generated cell lines that expressed EGFP-NIPP1-Fm (Fig. 5A and B). Consistent with our data in HTO cells, this fusion did not induce the expression of smooth-muscle genes. In addition, we have generated cell lines with a mutated phosphate-binding loop (alanine mutation of residues 68–71) in the substrate recruiting FHA domain of NIPP1 (NIPP1-Fm). The expression of this substrate-binding mutant did not induce the expression of smooth-muscle genes (Fig. 5A–C). Collectively, our data demonstrate that the NIPP1-induced transdifferentiation of HeLa cells depends on associated PP1 and a functional substrate-recruiting domain.

4. Discussion

We have previously demonstrated that the deletion of NIPP1 in mice hampers gastrulation, resulting in an early embryonic lethality [17]. Conversely, here we show that NIPP1 induces a mesenchymal-lineage differentiation program in a PP1 and FHA-dependent manner. Our data are consistent with the notion that NIPP1 functions through regulation of the dephosphorylation of FHA-ligands by associated PP1. An excellent candidate to mediate this NIPP1-induced differentiation program is the histone methyltransferase EZH2, which has key functions in stem-cell maintenance and differentiation, including muscle differentiation [21]. Moreover, we have demonstrated that NIPP1 affects the phosphorylation status of EZH2 in a PP1 and FHA-dependent manner [12,14,15], and thereby controls its binding to a subset of target genes. However, our preliminary data (not shown) indicate that the siRNA-induced knockdown of EZH2 phenocopies some but not all of the observed phenotypes of NIPP1 overexpression, indicating that additional FHA ligands are implicated. Another good candidate to mediate differentiation effects of NIPP1 is protein kinase MLK, which has also been implicated in mesenchymal differentiation and cell proliferation [22,23].

The number of NIPP1 molecules is strictly controlled. Indeed, Nipp1+/− and wild type mice express identical NIPP1 levels [17]. Also, there is a huge discrepancy between transcript and protein levels of ectopically expressed NIPP1, which is explained by a translation control mechanism [24]. In the present study, we report that the NIPP1-induced differentiation phenotype only requires a slight (<10%) overexpression of NIPP1, explaining why the NIPP1 levels need to be so tightly controlled. We speculate that the large effects of this very moderate overexpression of NIPP1 are due to the low abundance of key FHA-ligands. For example, 10% overexpression of NIPP1 corresponds to 12,000 extra NIPP1 molecules, which is still 25 times more than the number of EZH2 molecules in HeLa cells [25].

In conclusion, we have shown here that NIPP1 induces the expression of genes of the mesenchymal lineage in HeLa cells in a PP1 and FHA-dependent manner. This leads to the enticing hypothesis that key dephosphorylation event(s) underly the mesenchymal differentiation program.

Conflict of interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.04.017.

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