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Nucleophosmin1 is a negative regulator of the small GTPase Rac1

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Abstract
The Rac1 GTPase is a critical regulator of cytoskeletal dynamics and controls many biological processes, such as cell migration, cell-cell contacts, cellular growth and cell division. These complex functions are mediated by Rac1 signaling through effector proteins. We previously identified several target proteins of Rac1 that mutually act as Rac1 regulatory proteins, including caveolin-1 and PACSIN2. Here, we report that Rac1 interacts through its C-terminus with nucleophosmin1 (NPM1), a multifunctional nucleocytoplasmic shuttling protein with oncogenic properties. We show that Rac1 controls NPM1 subcellular localization. In cells expressing active Rac1, NPM1 translocates from the nucleolus to the cytoplasm. In addition, Rac1 regulates NPM1 phosphorylation as determined by the disappearance of the phosphorylated pool of NPM1 from the nucleus. Importantly, we found that NPM1 overexpression limits Rac1 GTP loading and cell spreading. In conclusion, this study identifies NPM1 as a novel regulator of Rac1 and describes the role of Rac1 in recruiting a nuclear oncogene, which has previously been associated with the development of leukemia.
Introduction

Cell migration is a vital process playing an essential role in many physiological activities, such as organogenesis, wound healing and immune responses. Cell motility is controlled by the actin cytoskeleton, which determines cell polarity, contributes to the formation of adhesive structures and, most importantly, drives forward movement by inducing both protrusive force at the front and contraction at the lateral sides and rear of the cell. In addition to the actin cytoskeleton, the microtubule network also contributes importantly to cell migration. For example, vesicular transport along the microtubule filaments allows specific spatio-temporal localization of important signaling proteins. This step is important for inducing and maintaining cell polarity which, in turn, is essential for persistent directional movement of the cell.

Cytoskeletal dynamics and cellular adhesion are regulated through signaling by Rho-like small GTPases, such as RhoA, which controls myosin-based contraction, and CDC42 and Rac1, that induce actin polymerization and membrane protrusions at the leading edge. These GTPases act as molecular switches, cycling between an inactive GDP-bound state and an active GTP-bound state. This cycling is regulated by guanine nucleotide exchange factors (GEFs) that promote the exchange of GDP for GTP and by GTPase-activating proteins (GAPs) that accelerate the intrinsic GTPase activity. Rho GTPase activity is also regulated by Rho guanine nucleotide dissociation inhibitor (RhoGDI), which binds to inactive RhoGTPases in the cytosol and controls the cytosol-to-membrane translocation of the activated GTPase. This is key to specific Rho GTPase functions, since most GTPases require membrane localization for proper activation and subsequent downstream signaling.

One of the most studied Rho GTPase is Rac1. Rac1 contributes to cell proliferation, participates in the signaling pathway promoting cell survival and is known for its central role in the control of cell adhesion and cell migration. Following activation, Rac1 interacts with a series of downstream targets, such as p21-activated kinase1 (Pak1) that regulates cytoskeletal dynamics and cell adhesion. Importantly, Rac1-mediated actin polymerization and consequent membrane ruffling at the leading edge is regulated through the WAVE/Arp2/3 complex, which controls actin polymerization and branching.

The members of Rho family GTPases show high sequence homology. The functional difference between the almost identical GTPases is explained by their different localization in cells and their binding to different subsets of effector proteins. Rho GTPase specificity is mainly determined by the hypervariable C-terminal domain. Our laboratory has previously identified a number of proteins, which bind to the C-terminus of Rac1 and translocate to cell adhesion sites or the plasma membrane upon Rac1 activation. For example, the adapter proteins caveolin-1 and PACSIN2 are recruited to integrin-regulated focal adhesions and specific tubular structures, respectively, upon Rac1 activation. Reciprocally, we found that these proteins negatively regulate Rac1 activity. We found that caveolin-1 mediates Rac1 poly-ubiquitylation and degradation and that PACSIN2 targets Rac1 to an endocytic pathway involving GAP proteins.

In this study, we describe the identification of nucleophosmin1 (NPM1) as a novel Rac1 binding protein, which, like caveolin-1 and PACSIN2, acts as a negative regulator of Rac1. NPM1, also known as B23, is a highly conserved, ubiquitously expressed phosphoprotein that shuttles rapidly between the nucleus and cytoplasm, although its main location is in the
nucleolus. NPM1 is a multifunctional protein taking part in various cellular processes, such as ribosome biogenesis, the maintenance of genomic stability and the inhibition of pro-apoptotic pathways\textsuperscript{18-20}. Nucleo-cytoplasmic shuttling and proper subcellular localization of NPM1 are important determinants for NPM1 functioning and cellular homeostasis. NPM1 mutations are frequent in acute myeloid leukemia (AML) and are characterized by aberrant NPM1 accumulation in the cytoplasm\textsuperscript{18,21,22}. Many phosphorylation sites have been identified in NPM1 and different phosphorylation sites have been associated with different functions\textsuperscript{19}. NPM1 is phosphorylated by several kinases, including casein kinase 2 and cyclin-dependent kinases\textsuperscript{23-25}. Here, we show that NPM1 interacts with the C-terminus of Rac1 and negatively regulates Rac1 activity and cell spreading. Importantly, we show that Rac1 activity, in turn, promotes NPM1 nuclear export and alters the NPM1 phosphorylation pattern inside the nucleus. These findings identify a new, bidirectional signaling unit involving two proto-oncogenes NPM1 and the RhoGTPase Rac1.

Materials and methods

Cell lines and cell culture
The Jurkat T-lymphocyte cell line (from the ATCC, Rockville, MO, USA) and HeLa cells were maintained at 37°C and 5% CO\textsubscript{2} in Iscove’s Modified Dulbecco’s Medium (IMDM; Lonza, Basel, Switzerland) containing 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (all purchased from PAA laboratories, Pasching, Austria) and 10% v/v FCS (Bodinco, Alkmaar, The Netherlands). Hela cells were passaged using trypsin (PAA laboratories). HeLa cells were transiently transfected using TransIt (Mirus, Madison, WI, USA) according to the manufacturer’s recommendations.

Antibodies
The following antibodies were used: anti-GFP (JL8, Clontech), anti-Rac1 (Transduction laboratories), anti-NPM1 (Cell Signaling), anti-phospho NPM1 (recognizes threonine 199, Cell Signaling), rabbit anti-myc and mouse anti-HA (both from Sigma-Aldrich), goat anti-rabbit IgG Alexa 488 or 568 and rabbit anti-mouse IgG Alexa 568 (Invitrogen), Alexa-Fluor-633-labeled Phalloidin (Invitrogen).

Biotinylated peptides and GST fusion proteins
Cell permeable peptides containing the amino acid sequence of the C-terminal region of Rac1, the Rac1 C-terminal mutants of the proline motif or polybasic region and the C-terminal region of the related GTPases RhoA and RhoG (Figure 1A) were fused to a protein transduction domain (PTD). These and a control peptide that only comprises the PTD (control) were produced by F-moc protein synthesis (Department of Peptide Synthesis, Netherlands Cancer Institute, Amsterdam, The Netherlands). GST-Rac1 WT and GST-Rac1 lacking the C-terminal tail (ΔC) proteins were produced as described previously\textsuperscript{26}.

Pull-down assays
HeLa cells transiently transfected with the indicated constructs (24 hours) or Jurkat T-cells were washed twice with ice-cold phosphate-buffered saline (PBS; Fresenius Kabi’s Hertogenbosch, The
Figure 1. Rac1 interacts through its C-terminus with NPM1. (A) Schematic representation of the Rho-like GTPase C-terminal peptides fused to a protein transduction domain as used in this study. (B) Pull-down (PD) experiments were performed using lysates from HeLa cells (upper panel) or Jurkat T-cells (lower panel) with a control peptide, wild-type and mutant Rac1 C-terminal peptides, Rac2, RhoA and RhoG C-terminal peptides. Association of NPM1 was detected by immunoblotting (IB) with an NPM1 specific monoclonal antibody (representative example out of three independent experiments is shown). (C) Pull-down (PD) experiment was performed using lysates from HeLa cells with a control peptide, wild-type and mutant Rac1 C-terminal peptides. Association of phosphorylated NPM1 (pNPM1) was detected by immunoblotting (IB) with a phospho-specific NPM1 antibody. (representative example out of two independent experiments is shown). (D) Pull-down (PD) experiment using full-length Rac1 and Rac1 lacking the C-terminus (∆C) both fused to GST or GST alone was performed with lysates from HeLa cells exogenously expressing GFP-NPM1. Association of NPM1 was detected by immunoblotting (IB) with a GFP specific monoclonal antibody. The ponceau staining shows the presence of the different GST constructs. ED: effector domain of Rac1, HV: hypervariable domain of Rac1, PTD: protein transduction domain, Rac1 PPPàAAA, Rac1 RKRàAAA: Rac1 C-terminal peptide mutants where the three prolines, or RKR sequence were replaced by alanine residues, respectively, TCL: total cell lysates, PD: pull-down, IB: immunoblotting.
Netherlands) (supplemented with 1 mM CaCl2 and 0.5 mM MgCl2) and lysed in NP-40 lysis buffer (i.e. 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM MgCl2, 10% glycerol and 1% NP-40) supplemented with protease inhibitors (Complete mini EDTA, Roche, Mannheim, Germany), centrifuged at 20,000 g for 10 minutes at 4°C. The supernatant was pre-cleared by incubation with streptavidin-coated beads (Sigma-Aldrich) for 1 hour at 4°C. The pre-cleared lysate was then incubated with the RhoGTPase C-terminal peptide (6 µg) or the control peptide (6 µg) in the presence of streptavidin-coated beads at 4°C for 1 hour with rotation. To determine Rac1 activity, the same procedure was followed and GTP-bound Rac1 was isolated with a biotinylated Pak1-CRIB peptide (20 µg). GST-fusion proteins were coupled to glutathione-coated beads and used at a concentration of 50 µg in each pull-down. GST fusion constructs were incubated with cell lysates at 4°C for 1 hour with rotation.

Cell lysates and pull-down samples were analyzed by SDS-PAGE and immunoblotting using specific monoclonal antibodies and HRP-coupled secondary antibodies.

**Electric resistance measurements**

For Electrical Cell Substrate Impedance Sensing (ECIS)-based cell spreading experiments, golden ECIS electrodes (8W10E; Applied Biophysics) were treated with 10 µM L-cysteine for 15 minutes and subsequently coated with 5 µg/ml fibronectin in 0.9% NaCl for 1 hour at 37°C. Next, HeLa cells, transfected with the indicated constructs, were seeded at a concentration of 100,000 cells per well in a volume of 400 µl IMDM with 10% FCS. Impedance was measured continuously at 45 kHz using ECIS model 9600. The increased impedance, as a measure of cell spreading, was recorded for several hours. Measurements and graphs representing the normalized resistance were calculated using the ECIS software (Applied Biophysics).

**Confocal Laser Scanning Microscopy**

HeLa cells were seeded on fibronectin-coated glass coverslips and transfected with the indicated constructs for 24 hours. Cells were then fixed with 3.7% formaldehyde (Merck) in PBS for 10 minutes. When required for antibody staining, cells were washed and permeabilized with 0.5% Triton X-100 in PBS for 5 minutes. Coverslips were then incubated with 2% BSA in PBS at room temperature (RT). Immunostaining was performed using the indicated specific antibodies and secondary labeled antibodies successively for 1 hour at RT. Fluorescent imaging was performed with a confocal laser scanning microscope (LSM510/Meta; Carl Zeiss MicroImaging Inc., Jena, Germany).

**Results**

**NPM1 interacts with the hypervariable C-terminus of Rac1**

Our laboratory previously established the association and mutual regulation of Rac1 and SET/I2PP2A (inhibitor 2 of protein phosphatase 2A)\textsuperscript{27,28}, a nuclear proto-oncogene which, as a protein-fusion with Nup214, is associated with myeloid leukemia\textsuperscript{29,30}. SET binds to Rac1 through its hypervariable C-terminus, specifically through the polybasic region within this domain. Similar to SET, NPM1 is a nuclear protein that has been implicated in the onset of leukemia\textsuperscript{7,8} and that can shuttle from the nucleus to the cytoplasm and vice versa\textsuperscript{27}. We therefore questioned whether Rac1 would also interact with NPM1.
To assess whether Rac1 and NPM1 associate, we used a biotinylated peptide encoding the C-terminus of Rac1 and included a series of similar peptides derived from related small GTPases as controls (Figure 1A). Binding to endogenous NPM1 was tested by a streptavidin-based pull-down assay using cell lysates of HeLa or Jurkat T-cells. We found that endogenous NPM1 clearly interacts with the peptide encoding the C-terminus of Rac1 (Figure 1B). In contrast, biotinylated C-terminal peptides derived from Rac2 and RhoG did not bind to NPM1, while weak binding was observed to the RhoA C-terminal peptide (Figure 1B).

The Rac1 C-terminus contains a proline-rich domain and a polybasic domain, that are both involved in the binding of specific regulatory proteins. To examine whether these domains are involved in NPM1 binding, we used the same Rac1 peptide, but now with substitutional alanine mutations of the proline-rich amino acid sequence or the polybasic amino acid sequence (Figure 1A). Binding of endogenous NPM1 was minimally affected by mutation of the proline-stretch, while mutating the polybasic region completely abolished the association of NPM1 to the Rac1 C-terminus (Figure 1B).

These data show that NPM1 specifically interacts with the C-terminus of Rac1. In addition, in Figure 1C, we show that the Rac1 C-terminal peptide strongly interacts with the phosphorylated form of NPM1. This interaction was also dependent on the polybasic domain of the Rac1 C-terminus (Figure 1C). Finally, to investigate the interaction between NPM1 and full-length Rac1, we performed a pull-down assay with bacterially purified GST or GST-Rac1 proteins or with GST-Rac1ΔC, which encodes full length Rac1 that lacks the hypervariable C-terminus, using cell lysates of HeLa cells expressing GFP-NPM1. Our results show that GFP-NPM1 interacts with full-length Rac1 protein and that this interaction is completely abolished by deletion of the C-terminus of Rac1 (Figure 1D). Together, these results show that the Rac1 C-terminus, in particular the polybasic domain, is necessary and sufficient for the interaction of Rac1 with NPM1.

Rac1 activity regulates NPM1 localization

To test if there is also a functional connection between Rac1 and NPM1, we first examined whether Rac1 controls NPM1 localization. To this end, we co-transfected HeLa cells with GFP-NPM1 and a constitutively active Rac1 mutant, Rac1Q61L. As a control for nuclear membrane integrity, we confirmed that expression of the active Rac1 mutant does not alter nuclear histone localization. In cells expressing the GFP-NPM1 construct, NPM1 was mainly detected in nucleoli, identified as small circular structures inside the nucleus (Figure 2A). Interestingly, co-expression of a constitutively active form of Rac1 drove a fraction of GFP-NPM1 out of the nucleus and promoted its accumulation in the cytoplasm (Figure 2B).

Because the polybasic region of Rac1 mediates its association to NPM1, we anticipated that a constitutively active Rac1 mutant lacking this region would not induce NPM1 relocalization outside the nucleus. Interestingly, in approximately half of the cells expressing the active Rac1 polybasic region mutant, NPM1 showed detectable extra-nuclear localization (Figure 3). This demonstrates that, to a limited extend, the polybasic region is dispensable and that Rac1 activity alone is sufficient to promote NPM1 extranuclear localization.

We then asked whether the closely related GTPase RhoA induces the same effects on NPM1 translocation as activated Rac1. Expression of a constitutively active RhoAV14 mutant did not...
Figure 2. Rac1 activity drives NPM1 out of nucleoli. HeLa cells were grown on glass cover slips and (co)-transfected with GFP-NPM1 (A) or mCherry Rac1Q61L and GFP-NPM1 (B). After 24 hours, cells were fixed and stained with the nuclear dye DAPI and the F-actin binding toxin Phalloidin fluorescently labeled with Alexa633. Samples were analyzed by confocal laser scanning microscopy. Higher magnification images of the boxed areas are included. Scale bars, 20 µm.
Figure 3. Active Rac1 poybasic mutant has decreased capacity to relocalize NPM1. HeLa cells were grown on glass cover slips and co-transfected with mCherry Rac1Q61L-RKR and GFP-NPM1. After 24 hours, cells were fixed and stained with the nuclear dye DAPI and the F-actin binding toxin Phalloidin fluorescently labeled with Alexa633. Samples were analyzed by confocal laser scanning microscopy. Higher magnification images of the boxed areas are included. Scale bars, 20 µm. Bar graph figure represents the percentage of cells expressing Rac1Q61L-RKR with either nuclear NPM1 (SD ± 0.054) or extranuclear NPM1 localization (SD ± 0.054) quantified in two independent experiments.

alter NPM1 nucleolar localization (Figure 4), suggesting that Rac1 activity specifically regulates NPM1 subcellular localization.

Because NPM1 function is regulated by phosphorylation and because the Rac1 C-terminus associates to phosphorylated NPM1 (pNPM1), we questioned whether Rac1 activity alters the distribution of pNPM1. First, we used a phospho-specific NPM1 antibody to document the localization of pNPM1. This staining revealed that the phosphorylated fraction of endogenous NPM1 localized markedly different from the total NPM1 pool. Phospho-NPM1 was diffusely dispersed throughout the nucleus and also localized to small dotted structures inside the nucleoplasm (Figure 5).
**Figure 4. Active RhoA does not affect NPM1 localization.** HeLa cells were grown on glass cover slips and co-transfected with HA-tagged active RhoA mutant, RhoAV14, and GFP-NPM1. After 24 hours, cells were fixed and stained with the nuclear dye DAPI and the F-actin binding toxin Phalloidin fluorescently labeled with Alxa633. RhoA was detected by a monoclonal anti-HA antibody followed by an anti-mouse Alexa568 antibody. Samples were analyzed by confocal laser scanning microscopy. Higher magnification images of the boxed areas are included. Scale bars, 20 µm.

**Figure 5. Phospho-NPM1 shows dispersed localization throughout the nucleus.** HeLa cells were grown on glass cover slips and transfected with GFP-NPM1. After 24 hours, cells were fixed and stained with a phospho-specific rabbit antibody against NPM1 followed by a goat anti-Rabbit IgG Alexa668, the nuclear dye DAPI and the F-actin binding toxin Phalloidin fluorescently labeled with Alexa633 and analysed by confocal laser scanning microscopy. Higher magnification images of the boxed areas are included. Scale bars, 20 µm.
Figure 6. Rac1 activity alters NPM1 phosphorylation pattern. HeLa cells were grown on glass cover slips and transfected with either mCherry Rac1Q61L (A) or mCherry Rac1V12G (B). After 24 hours, cells were fixed and stained with a phospho-specific rabbit antibody against NPM1 followed by a goat anti-Rabbit IgG Alexa488, the nuclear dye DAPI and the F-actin binding toxin Phalloidin fluorescently labeled with Alexa 633 and analysed by confocal laser scanning microscopy. Higher magnification images of the boxed areas are included. (C) HeLa cells were transfected with an empty vector, myc-tagged Rac1 WT or two different myc-tagged constitutively active Rac1 mutants; Rac1V12G and Rac1Q61L and after 24 hours lysates were made and endogenous NPM1 as well as phospho-NPM1 (pNPM1) were detected by immunoblotting (IB) with an NPM1 specific or an NPM1 phospho-specific antibody. EV: empty vector. Scale bars, 20 µm.
Interestingly, in cells expressing a constitutive active Rac1 mutant, either Rac1Q61L or Rac1V12G, staining of phospho-NPM1 completely disappeared from the nucleus and was no longer clearly detectable (Figure 6A and 6B). This could either be due to a diffuse localization of the pool of pNPM1 or to Rac1 induced de-phosphorylation of pNPM1. To determine this, we detected pNPM1 levels by Western Blot in lysates from cells expressing either of the Rac1 mutants. This showed that expression of the activated Rac1V12G or Rac1Q61L mutants did not affect neither the levels of endogenous NPM1 nor of phosphorylated NPM1 (Figure 6C). Collectively, these data show that Rac1 activity promotes NMP1 nucleo-cytoplasmic shuttling and induces a diffuse localization, most likely throughout the cytoplasm, of pNPM1.

NPM1 negatively regulates Rac1 activity

Given that NPM1 interacts with the GTPase Rac1, we set out to determine the functional consequences of this interaction. We first tested the effect of NPM1 overexpression on the GTP-loading of Rac1. We found that the basal levels of endogenous, active Rac1 were clearly decreased in cells transfected with GFP-NPM1 (Figure 7A). Importantly, expression of NPM1 did not affect the total levels of endogenous Rac1, excluding that increased degradation would account for the loss of active Rac1. In line with these data, we found that NPM1 overexpression reduced cell spreading on fibronectin, a process which is dependent on Rac1 activity (Figure 7B). These results suggest that NPM1 act as a negative regulator of Rac1. We then questioned whether blocking Rac1 activation by GEF proteins could be the mechanism by which NPM1 reduces Rac1 GTP loading. To investigate this, we tested whether the Rac1 GEF Tiam1-C1199 is still able to activate endogenous Rac1 in the presence of NPM1. Tiam1-C-1199 is a N-terminally truncated mutant Tiam1 that is widely used because of its enhanced stability and increased activity as compared to full-length Tiam1. As expected, we observed NPM1-mediated downregulation of Rac1GTP levels in the control situation (Figure 7C). In addition, expression of Tiam1-C1199 increased Rac1GTP levels. However, co-expression of NPM1 did not reduce Tiam1-C1199-induced activation of endogenous Rac1 (Figure 7C). We also found that NPM1 did not block the activation of Rac1 upon expression of another Rac1GEF, TrioD (data not shown). These data show that NPM1 does not interfere with the Rac1 activation by GEF proteins and suggest that NPM1 acts downstream of Rac1 to promote its inactivation, possibly by promoting the association of activated Rac1 to GAP proteins.

Figure 7. NPM1 is a negative regulator of Rac1. (A) Rac1 GTP loading was measured by biotinylated Pak-CRIB peptide-based pull-down (PD) with lysates of GFP control-transfected HeLa cells or HeLa cells transfected with GFP-NPM1. Rac1, Rac1 GTP and GFP-NPM1 were detected by immunoblotting with Rac1 and GFP specific monoclonal antibodies respectively. The bar graph shows the relative levels of Rac1 GTP levels compared with that in control as determined by quantification of Western blots (representative example out of three independent experiments is shown). TCL: total cell lysates, PD: pull-down. (B) Cell spreading on fibronectin coated ECIS-electrodes was determined for mock-transfected HeLa cells or HeLa cells expressing GFP-NPM1. The results are depicted as normalized mean resistance of three independent experiments. (n=3) *p< 0.05, **p< 0.01. (C) Rac1 GTP loading was measured by biotinylated Pak-CRIB peptide-based pull-down (PD) with lysates of GFP control-transfected HeLa cells or HeLa cells expressing GFP-NPM1. The results are depicted as normalized mean resistance of three independent experiments. (n=3) *p< 0.05, **p< 0.01.
Discussion

The link between Rac1 and NPM1 was previously only described in nucleophosmin-anaplastic lymphoma kinase (NPM-ALK)-positive lymphomas. NPM-ALK is an oncogenic fusion protein, which acts as a constitutive active tyrosine kinase. NPM-ALK signals in part through Rac1 thereby contributing to the pathogenesis of ALK-positive human lymphomas. Here, we present data indicating that NPM1 and Rac1 interact and reciprocally regulate each other.

Like many other regulatory proteins, NPM1 interacts with Rac1 via the hypervariable C-terminus of Rac1. Importantly, the association to NPM1 was specific for Rac1 as compared to other, highly related, small GTPases. This suggested that NPM1 could be a selective regulator of Rac1. Indeed, overexpression of NPM1 reduced GTP loading of Rac1 and cell spreading on fibronectin. We attempted to detect the opposite effect, i.e. increased basal Rac1 activity, after silencing NPM1 with two different shRNAs. However, NPM1 knock-down did not affect Rac1 GTP loading (data not shown). This may be the result of redundancy. Three NPM family members, NPM1, NPM2 and NPM3 have been identified in mammals. Moreover, two isoforms of NPM1 exist. Therefore, it is possible that another NPM homologue or isoform compensates for the loss of NPM1. How NPM1 regulates Rac1 activity still remains to be investigated. Rac1 is regulated at two important levels. The first is at the level of activation, where GEFs play an important role. A proper intracellular GEF protein localization, usually at the plasma membrane, and a cascade of signaling events, are essential determinants for efficient GEF biological activity and subsequent Rac1 activation. However, this hypothesis can be excluded, since expression of NPM1 did not interfere with GEF-induced Rac1 GTP loading. The potentially second means of regulating the Rac1 activity cycle is at the level of inactivation. Inactivation is achieved by extracting Rac1 from the plasma membrane followed by GAP-promoted GTP hydrolysis. Some proteins, such as RhoGDI, caveolin-1 and PACSIN2, have been described to facilitate Rac1 internalization from the plasma membrane. Most known Rac1-regulatory proteins are localized at the plasma membrane or throughout the cytoplasm. Therefore, it is intriguing how NPM1 regulates Rac1 GTP loading, since NPM1 is localized in nucleoli, which are specialized nuclear compartments.

NPM1 is known to rapidly shuttle between the nucleus and the cytoplasm and may limit Rac1 activity during its short stay outside the nucleus. NPM1 contains both a nuclear localization signal (NLS) as well as a nuclear export signal (NES). However, despite the NES motif, NPM1 is mainly localized in nucleoli. Thus, under physiological conditions, nuclear import of NPM1 outweighs its export. Increasing the levels of activated Rac1 in cells reversed this balance and promoted NPM1 recruitment to the cytoplasm, possibly to promote Rac1 inactivation.

The hypervariable C-terminus of Rac1 comprises a NLS motif, meaning that a subset of Rac1 resides in the nucleus. Regarding the considerable number of effector proteins, scaffolding proteins and GEFs for small GTPases that are found in the nucleus, it is thought that Rac1 is involved in nuclear signaling pathways. Therefore, it is intriguing to speculate that NPM1 regulates Rac1 signaling in the nucleus by limiting its activation. We are currently pursuing this issue by the use of NPM1 mutants, exclusively expressed either in the cytoplasm or in the nucleus to clarify the relevant location of NPM1 where it accomplishes its role as a negative regulator of Rac1.
Another key finding of the current study is that Rac1 activity alters the import/export dynamics of NPM1, thereby promoting its translocation into the cytoplasm. Aberrant NPM1 cytoplasmic accumulation is associated with AML, where it is implicated in promoting malignant cell growth\textsuperscript{16,20,21}. Similarly, our laboratory recently identified the oncogene SET as a target of Rac1\textsuperscript{27,28}. Constitutive active Rac1 induced the nuclear export of SET into the cytoplasm. The role of Rac1 in cellular transformation is established and is mainly attributed to Rac1 modulation of the Bcl-2 family that regulates apoptosis\textsuperscript{31,45-48}. We propose a model in which aberrant Rac1 signaling alters the subcellular localization of several oncogenes and triggers their oncogenic activity. Our finding that the constitutively active Rac1 with a mutated polybasic region relocalizes NPM1 in the cytoplasm in a portion of the transfected cells was unexpected. This mutant is not anticipated to bind NPM1 and also misses the NLS that targets Rac1 to the nucleus. This suggests that active Rac1 is able to initiate a signaling cascade from the cytosol to stimulate NPM1 relocalization. This makes it unlikely that the direct interaction between Rac1 and NPM1 is essential to induce NPM1 translocation from the nucleus. However, in another portion of the transfected cells, transfection with the active Rac1 polybasic region mutant did not affect NPM1 localization in the nucleoli, showing that in addition to Rac1 activity, this domain is required for optimal regulation of NPM1 subcellular localization.

We also found that expression of a constitutively active mutant of Rac1 altered NPM1 phosphorylation pattern. The fraction of phosphorylated NPM1 observed by confocal microscopy became largely undetectable in the presence of an active mutant of Rac1. Since we could not detect significant de-phosphorylation of pNPM1 in the presence of activated Rac1 mutants, this result indicates that Rac1 activity disperses pNPM1 from the nucleus into the cytoplasm, obscuring clear-cut detection by microscopy.

In conclusion, we establish NPM1 as a novel Rac1 interactor that acts as a negative regulator of activated Rac1. Like other Rac1-regulatory proteins, such as caveolin-1 and PACSIN2, NPM1 and Rac1 showed reciprocal regulation with Rac1 promoting NPM1 nuclear exit. Future experiments are directed at identifying the molecular mechanisms of Rac1 inactivation by NPM1 and to determine the biological consequences of NPM1 accumulation into the cytoplasm upon Rac1 activation.

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