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Rho-kinase signaling controls nucleocytoplasmic shuttling of class IIa Histone Deacetylase (HDAC7) and transcriptional activation of orphan nuclear receptor NR4A1

Claudia Compagnucci [a], Sabina Barresi [a], Stefania Petrini [b], Enrico Bertini [a], Ginevra Zanni [a, *]

[a] Unit of Molecular Medicine for Neuromuscular and Neurodegenerative Disorders, Department of Neurosciences, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy
[b] Research Laboratories, Confocal Microscopy Core Facility, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy

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ABSTRACT

Rho-kinase (ROCK) has been well documented to play a key role in RhoA-induced actin remodeling. ROCK activation results in myosin light chain (MLC) phosphorylation either by direct action on MLC kinase (MLCK) or by inhibition of MLC phosphatase (MLCP), modulating actin–myosin contraction. We found that inhibition of the ROCK pathway in induced pluripotent stem cells, leads to nuclear export of HDAC7 and transcriptional activation of the orphan nuclear receptor NR4A1 while in cells with constitutive ROCK hyperactivity due to loss of function of the RhoGTPase activating protein Oligophrenin-1 (*OPHN1*), the orphan nuclear receptor *NR4A1* is downregulated. Our study identify a new target of ROCK signaling via myosin phosphatase subunit (MYPT1) and Histone Deacetylase (HDAC7) at the nuclear level and provide new insights in the cellular functions of ROCK.

1. Introduction

Rho GTPase proteins (Rho, Rac, Cdc42) have relevant functions in regulating various aspects of cell development such as differentiation, migration and synaptogenesis. Rho GTPase activity is modulated through positive (GTPases activating proteins GAPs) and negative regulators (guanine nucleotide exchange factors, GEFs or guanine nucleotide dissociation inhibitors, GDIs) [1]. Loss of function of the RhoGTPase activating protein *OPHN1*, the first Rho-linked gene associated with X-linked intellectual disability and cerebellar hypoplasia [2,3], leads to hyperactivation of the downstream RhoA/Rho-kinase (ROCK) pathway and dendritic spine immaturity [4,5]. ROCK acts through phosphorylation of the Myosin Phosphatase targeting subunit 1 (MYPT1) leading to inhibition of myosin light chain phosphatase (MLCP) which triggers actin–myosin contractility [6]. Interestingly, MYPT1 and the catalytic subunit of MLCP (PP1β) were found to interact specifically with Histone Deacetylase 7 (HDAC7) dephosphorylating it. HDAC7 belongs to class IIa HDACs (including also HDAC4/5/9), and is part of a transcriptional repressor complex involved in regulation of gene expression. Nucleocytoplasmic shuttling depending on HDACs phosphorylation status, has been demonstrated as a mechanism controlling their function [7]. Nuclear import of HDAC7 leads to repression of *NR4A1* transcription in developing thymocytes [8]. *NR4A1* (also known as *NUR77*, *TR3* or nerve growth factor induced *NGFI-B*) belongs to the family of nuclear orphan receptors which act as immediate early response genes, and is important for neuronal differentiation, T cell tolerance induction and apoptosis [9–11]. In this study we demonstrate that ROCK signaling controls nucleocytoplasmic shuttling of HDAC7 and modulates the expression of its target gene *NR4A1*.

Abbreviations: ROCK, Rho kinase; OPHN1, Oligophrenin-1; HDAC7, Histone Deacetylase 7; MYPT1, myosin phosphatase subunit 1; MLCP, myosin light chain phosphatase; MLCK, myosin light chain kinase; NR4A1, nuclear receptor subfamily 4, group A, member 1; iPSCs, induced pluripotent stem cells; PKA, protein kinase A; CREB, cAMP responsive element binding; MAP2, microtubule-associated protein 2; STAT3, signal transducer and activator of transcription 3.

* Corresponding author. Bambino Gesù Children's Hospital, IRCCS, Viale San Paolo 15, 00146 Rome, Italy.
E-mail address: ginevra.zanni@opbg.net (G. Zanni).

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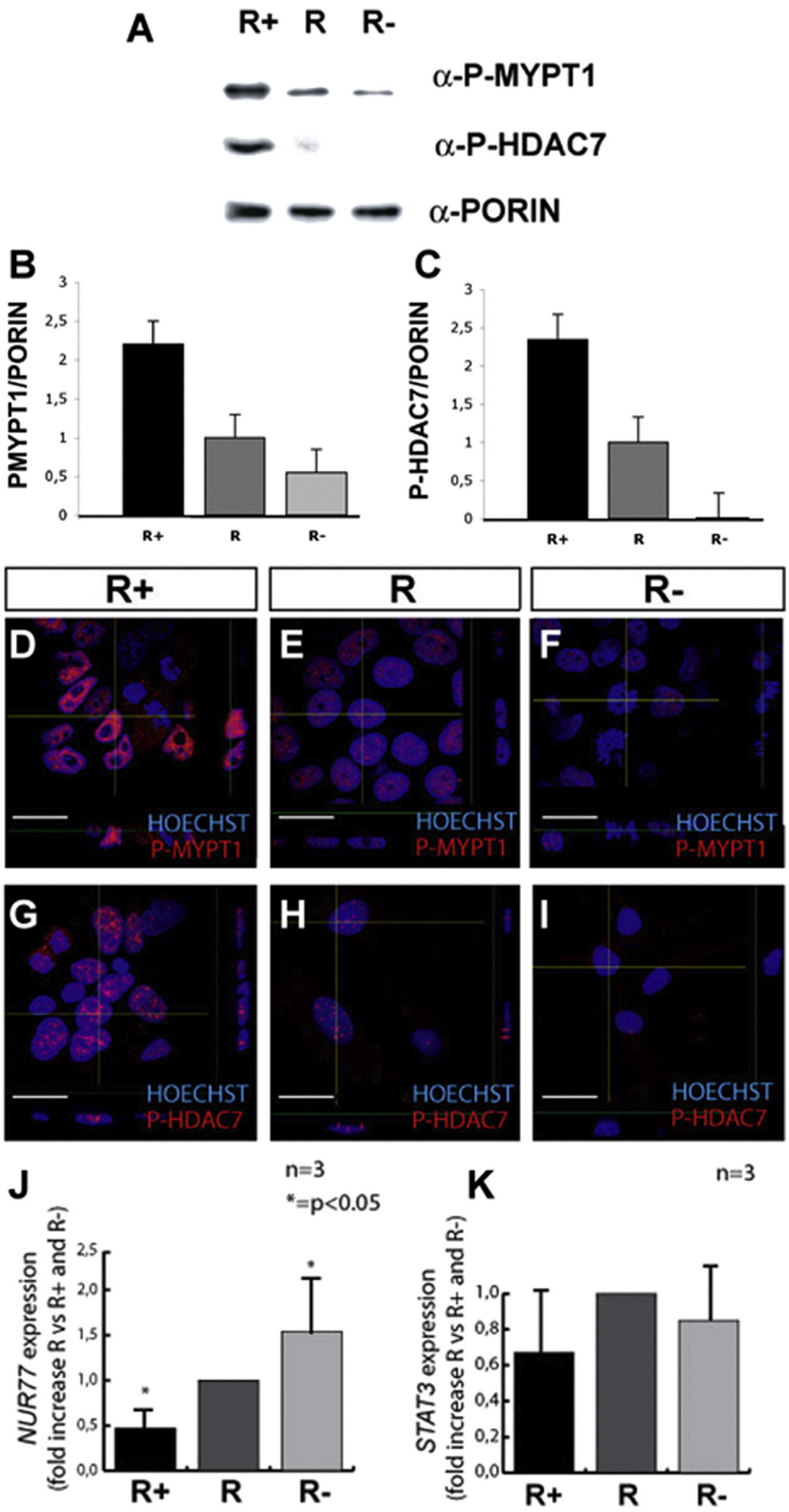

Fig. 1. Analysis of MYPT1 and HDAC7 phosphorylation status (upper panel), and *NR4A1* expression (lower panel) in iPSCs with different levels of ROCK activity. (A) Western blots of protein extracts obtained from iPS cells with *OPHN1* loss of function (R+), control iPS cells (R) and iPS cells treated with the ROCK inhibitor Y27632 (10 μM), (R−) showing phosphorylation of Thr853 in MYPT1 (P-MYPT1), phosphorylation of S318 in HDAC7 (P-HDAC7), and PORIN (used as internal standard). The data show different phosphorylation status of MYPT1 and HDAC7 at different levels of ROCK activity. (B–C) Densitometric analyses of Western blot where P-MYPT1 and P-HDAC7 values were normalized with respect to PORIN values showing decreased P-MYPT1 and P-HDAC7, respectively, in parallel with decreasing levels of ROCK activity. (D–I) Immunofluorescence analysis of iPSC colonies

2. Materials and methods

2.1. Induced pluripotent stem cells (iPSCs)

Control iPSCs were purchased from System Biosciences (USA). iPSCs from two *OPHN1* mutated patients and one non affected individual of the family, were obtained from human fibroblasts and reprogrammed using the non-integrating episomal technology (Minicircle DNA and mc-iPS Cells, Euroclone). Informed consent was obtained from all the subjects (patients and parents) involved in the study.

2.2. Cell culture conditions

Following thawing, iPSCs were grown on MEFs (Life Technologies) for the first 4–5 weeks and then in feeder free condition using Matrigel (BD Biosciences, San Diego) in mTeSR1 (Stemcell Technologies). When the iPSCs are 70–80% confluent, they were passaged (using EDTA treatment) 1:4 and transferred to new wells in feeder-free condition and incubated at 37 °C, 5% CO_2, 20% O_2, the medium were changed every day and the cells split every 3 days.

2.3. Drug treatment

Y-27632 (molecular weight 320.3) was purchased from Sigma Aldrich (Cod. Y0503, USA), dissolved in deionized water (stock solution of 10 mM) and cells treated for 24 h with a final concentration of 10 μM.

2.4. Immunofluorescence analyses

For immunocytochemistry, cells were fixed with 4% paraformaldehyde for 20 min at RT, washed with PBS, and blocked with 10% bovine serum (Vector Laboratories) and 0.1% Triton X-100 (Sigma). Primary antibodies included P(Thr853)-MYPT1 (Cell Signaling Cod. 4563), MYPT1 (Cell Signaling Cod. 2634), and P(S318)-HDAC7 (Abcam Cod ab72172). Secondary antibodies were conjugated with Alexa 488, Alexa 555 or AlexaCy5 (Life Technologies). Coverslips were mounted using PBS/Glycerol (1:1), visualized using a confocal microscope Fluoview FV1000 (Olympus) and acquired with the software FV10-ASW Version2.0.

2.5. Western blotting

For Western analysis, cells were lysed in RIPA buffer (50 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% NP-40) supplemented with complete protease inhibitor cocktail (Roche). Proteins were separated by SDS–PAGE and transferred to nitrocellulose membrane. Membranes blocked in 5% milk for 1 h at RT. Primary antibodies were blotted overnight at 4 °C. Secondary antibody-HRP conjugates were blotted for 1 h at RT and membranes stained with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnologies). The following primary antibody were used: P(Thr853)-MYPT1 (Cell Signaling Cod. 4563), P(S318)-HDAC7 (Abcam Cod ab72172), and PORIN (Abcam Cod ab15895).

2.6. RNA isolation, reverse transcription (RT-PCR) analysis

Total RNA was extracted from iPSCs with the single-step acid phenol method using TRIzol (Invitrogen, Carlsbad, CA pn: 15596018) according to the manufacturer's instructions. Each RNA sample was DNase treated (Recombinant DNase I, AM2235 – Ambion) and quantified by NanoDrop 2000 (Thermo Scientific). The reverse transcription reaction was performed in 20 μl starting from 1 μg of total RNA and cDNA was generated by ImProm-II Reverse Transcription System (A3800 – Promega, Madison, WI, USA) or Superscript II reverse transcriptase (18064 – Life Technologies) using random hexamers. Three independent RT-PCRs (reverse transcriptase-polymerase chain reactions) were performed for each sample.

2.7. Quantitative real-time polymerase chain reaction

Gene-specific exon-exon boundary PCR products (TaqMan gene expression assays, Applied Biosystems) were measured by means of a PE Applied Biosystems PRISM 7700 sequence detection system during 40 cycles. *GAPDH* mRNA was used for normalization and relative quantification of gene expression was performed according to the ΔΔCt method. Expression levels were represented in arbitrary units calculated as a relative-fold increase compared to the control sample arbitrarily set to 1. Quantitative RT-PCRs were repeated in triplicates from at least two independent experiments. The primers were supplied by Integrated DNA Technologies: *GAPDH*, Hs.PT39a.22214836; *NR4A1*, Hs.PT.56a.20808305; *MAP2*, Hs.PT.56a.20103440.g; *STAT3*, Hs.PT.56a.20367494.

2.8. Statistical analysis

Data are expressed as mean and standard deviation. Comparisons between groups were performed by two-tailed unpaired student's *t*-test and *p* values of <0.05 were considered statistically significant. Data were analyzed using Windows XP Excel.

3. Results and discussion

To study the consequences of ROCK signaling on *NR4A1* expression, we used patient derived *OPHN1*-mutated induced pluripotent stem cells (iPSCs) in which we confirmed the absence of OPHN1 protein (Supplemental Fig. 1) and the hyperactivity of ROCK through Western blot and immunofluorescence analysis of the phosphorylation status of its substrate MYPT1 [12]. We then compared ROCK activity of *OPHN1*-defective iPSCs (R+) with control iPSCs (R) and with cells treated with the ROCK inhibitor Y-27632 (R–). As it is expected, phosphorylation levels of MYPT1 increase in parallel with ROCK activity. Levels of HDAC7 phosphorylation also increases as a consequence ROCK-dependent reduction of MLCP activity (Fig. 1A–C). To determine whether the changes in phosphorylation affected the subcellular localization of HDAC7, we performed immunolocalization studies using confocal microscopy. As shown in Fig 1, in parallel with the rising of ROCK activity, there is nuclear accumulation of phosphorylated HDAC7 and MYPT1 (Fig. 1 D-I). In *OPHN1*–defective cells which displays higher levels of ROCK activity, levels of phosphorylated HDAC7 increase in the nucleus. At lower levels of ROCK activity i.e. control cells treated with the ROCK inhibitor Y-27632, HDAC7 exit the nucleus and accumulates in the cytoplasm, suggesting that MYPT1/MLCP-dependent dephosphorylation of HDAC7 promote its nuclear export (Fig. 2). At basal levels of ROCK activity, HDAC7 localizes in both nuclear and cytoplasmic cellular compartments. To determine whether the accumulation of HDAC7 in the nucleus had repressive

stained with anti P-MYPT1 (D–F) and P-HDAC7 (G–I) antibodies and nuclei counterstained with Hoechst. In R+ cells, P-MYPT1 and P-HDAC7 concentrate in the nucleus, whereas in R and R– cells both proteins progressively exit the nucleus (scale bar = 30 μm). (J) qPCR analysis of *NR4A1* transcripts levels showing that they inversely correlate with ROCK activity (K) qPCR analysis of *STAT3*. Data (mean + SD of 3 independent experiments) are expressed as fold increase of R+ and R– versus R cells, using *GAPDH* as standard control. These data show that there are no differences in *STAT3* expression in the three cell lines.

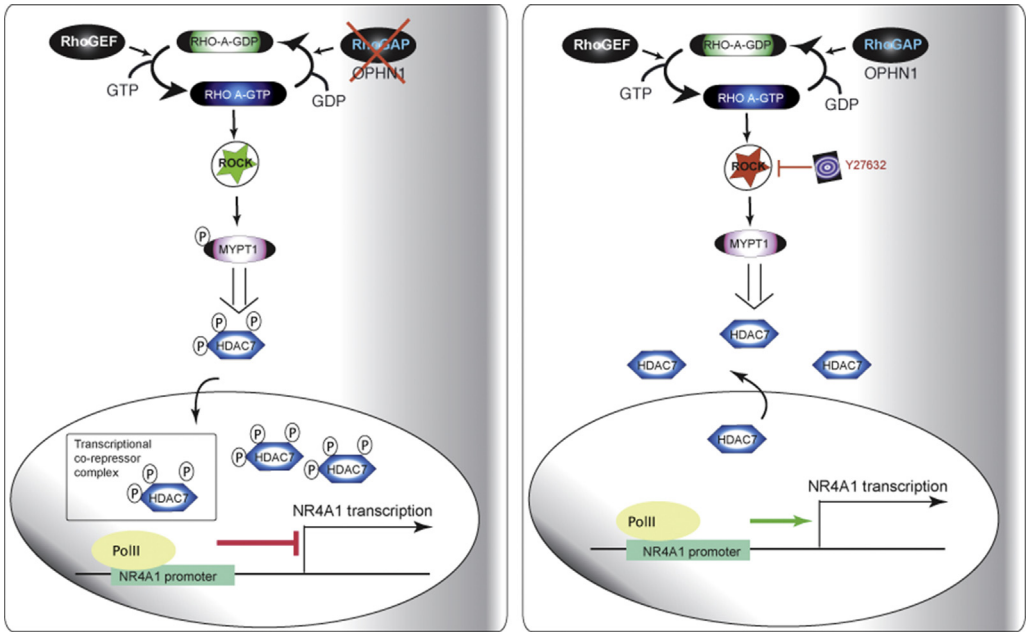

Fig. 2. Graphical overview of ROCK-dependent regulation of *NR4A1* transcription. Model showing a cell with hyperactive ROCK signaling due to *OPHN1* loss of function, leading to transcriptional repression of *NR4A1* (left panel). In a cell with decreased ROCK signaling due to ROCK inhibition, *NR4A1* transcription is activated due to nuclear export of HDAC7 (right panel).

effects of *NR4A1* transcription, we performed qPCR analysis at different levels of ROCK activation and found that expression of *NR4A1* but not of other genes such as *STAT3* or *MAP2*, is inversely correlated with ROCK activity and MYPT1-HDAC7 phosphorylation (Fig. 1J–K and Supplemental Fig. 2). ROCK inhibition thus seems to favor the expression of *NR4A1*, known to be crucial for neuronal differentiation and cell survival [13]. Another class IIa HDAC, i.e. HDAC5 is specifically phosphorylated by protein kinase A (PKA) which prevents its nuclear export and leads to the inhibition of fetal cardiac gene expression [14]. Interestingly, the cAMP/PKA pathway, which is altered in *OPHN1*-defective neuronal cells in the mouse [15], was also found to regulate *NR4A1* expression through CREB phosphorylation and activation [16]. Although further studies are necessary to determine the impact of ROCK signaling on gene transcription and to establish the contribution of *NR4A1* in the pathogenesis of *OPHN1* and other disorders related to ROCK dysfunction, the finding of a new function of the ROCK pathway in regulating gene expression through MYPT1-mediated phosphorylation and nucleocytoplasmic shuttling of HDAC7 and the identification of a target gene essential for neuronal cell development and survival such as *NR4A1*, opens new perspectives on the function of RhoGTPases signaling at the nuclear level not only as regulators of cytoskeletal remodeling.

Author contributions

G.Z., C.C. designed the study. C.C. conducted the experiments related to iPSC differentiation, immunofluorescence and western blotting. S.B. performed the gene expression experiments. C.C., E.B., G.Z. analyzed the data. S.P. performed the confocal microscopy analyses. G.Z., C.C. prepared the manuscript. G.Z. formulated the hypotheses and supervised the project.

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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.bbrc.2014.12.033.

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