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NR4A orphan nuclear receptors influence retinoic acid and docosahexaenoic acid signaling via up-regulation of fatty acid binding protein 5

Nikolaos Volakakis [a], Eliza Joodmardi [a], Thomas Perlmann [a,b,*]

[a] Ludwig Institute for Cancer Research Ltd., Box 240, S-17177 Stockholm, Sweden
[b] The Department of Cell and Molecular Biology, Karolinska Institute, S-17177 Stockholm, Sweden

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ABSTRACT

The orphan nuclear receptor (NR) Nurr1 is expressed in the developing and adult nervous system and is also induced as an immediate early gene in a variety of cell types. *In silico* analysis of human promoters identified *fatty acid binding protein 5* (FABP5), a protein shown to enhance retinoic acid-mediated PPARβ/δ signaling, as a potential Nurr1 target gene. Nurr1 has previously been implicated in retinoid signaling via its heterodimerization partner RXR. Since NRs are commonly involved in cross-regulatory control we decided to further investigate the regulatory relationship between Nurr1 and FABP5. FABP5 expression was up-regulated by Nurr1 and other NR4A NRs in HEK293 cells, and Nurr1 was shown to activate and bind to the *FABP5* promoter, supporting that *FABP5* is a direct downstream target of NR4A NRs. We also show that the RXR ligand docosahexaenoic acid (DHA) can induce nuclear translocation of FABP5. Moreover, via up-regulation of *FABP5* Nurr1 can enhance retinoic acid-induced signaling of PPARβ/δ and DHA-induced activation of RXR. We also found that other members of the NR4A orphan NRs can up-regulate FABP5. Thus, our findings suggest that NR4A orphan NRs can influence signaling events of other NRs via control of FABP5 expression levels.

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Introduction

NRs comprise a family of transcription factors that have critical functions during development and in adult physiology. NRs include steroid hormone receptors and receptors for other lipophilic ligands such as vitamin D3 and retinoids [1]. Several members of the NR family lack identified ligands and are referred to as orphan receptors [2]. Nurr1 is an orphan receptor, which together with NGFI-B and Nor1, constitutes the NR4A orphan NRs [3]. Nurr1 is expressed within the embryonic central nervous system where it plays a key role for the development of dopamine neurons [4] and continues to be expressed in the adult brain where it may be critical for dopamine neuron survival and other functions [5,6]. Nurr1, NGFI-B and Nor1 are unique within the NR family in being encoded by immediate early genes that are rapidly induced by various stimuli such as growth factors, ischemia and seizures [7].

The structural features of the Nurr1 and DHR38 (NR4A-homologue in *Drosophila*) ligand binding domain show that the NR4A family members lack a hydrophobic pocket for ligand binding and thus function as ligand-independent NRs [8,9]. NR4A proteins bind to DNA either as monomers or homodimers and promote constitutive activation of transcription [10,11]. In addition, Nurr1 and NGFI-B, but not Nor1, can form heterodimers with the retinoid X receptor (RXR), which have the ability to promote strong transcriptional activation after binding to RXR ligands like 9-*cis*-retinoic acid [12] or fatty acids such as docosahexaenoic acid [13].

In this study we identify *fatty acid binding protein 5* (*FABP5*) as a Nurr1-regulated gene. Fatty acid binding proteins are cytosolic proteins that bind long-chain fatty acids [14]. *FABP5* is expressed in a variety of tissues [15] and has been implicated in regulation of water permeability barrier of the skin, neurite outgrowth [16] and fatty acid transport during neuronal regeneration [17,18]. Retinoic acid binding to FABP5 leads to its translocation to the nucleus and enhancement of retinoic acid-induced activation of PPARβ/δ, which in turn can promote cellular survival [19]. DHA, a ligand for RXR, has also been shown to bind to FABP5 [20]. Since Nurr1 is indirectly linked to retinoid and DHA-signaling via its ability to form heterodimers with RXR we were interested to further investigate if Nurr1-mediated regulation of *FABP5* could influence cross-regulation between Nurr1 and other NR-mediated signaling pathways. Our experiments indicate that Nurr1 can regulate *FABP5* expression via direct binding to the *FABP5* promoter and we also provide evidence indicating that Nurr1 can modulate RXR signaling via this regulation. Thus, *FABP5* could be a regulated target of Nurr1 and the two other NR4A NRs in an immediate early context and thereby influence signaling by ligands binding to RXR.

* Corresponding author. Address: Ludwig Institute for Cancer Research Ltd., Box 240, S-17177 Stockholm, Sweden. Fax: +46 8 332812.
E-mail address: thomas.perlmann@licr.ki.se (T. Perlmann).

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Materials and methods

Chemicals. Retinoic acid and DHA (*cis*-4,7,10,13,16,19-docosahexanoic acid, C22:6) were purchased from Sigma–Aldrich. LG268 was kindly provided by Mark Leibowitz at Ligand Pharmaceuticals.

siRNA. A control siRNA and a pool of three siRNAs designed to knock down human *FABP5* expression were purchased from Santa Cruz Biotechnology. Cells were transfected in 24-well plates with 20 pM siRNA per well using Lipofectamine 2000.

Plasmids. pCMX-Nurr1, -Nor1, -NGFI-B, -Nurr1^{R334A} and -Nurr1^{dim} have been described by [21]. The luciferase reporter MH100-tk-luc contains four copies of the GAL4 binding site upstream of the herpes simplex virus thymidine kinase promoter [12]. pCMX-RXRα, -LacZ, -GAL4-Nurr1 and -GAL4-Nurr1^{dim} are described by [22]. PPREx3-tk-luc has been described by [23], while PSG5-PPARβ/δ was provided by Dr. Desvergne (University of Lausanne, Switzerland). The *FABP5* promoter fragment was amplified from genomic DNA extracted from HEK293 cells using QuickExtract DNA extraction solution (Epicentre Biotechnologies). The following primers were used: 5′-AAAAGCTAGC GTGGTCTGATTTCATAAGGT-3′ and 5′-AAAAAGATCTGCACCCGGCGCC GGCGGCTG-3′. The PCR-amplified promoter fragment was ligated into the pGL2 basic plasmid (Promega). The *FABP5* promoter construct with the mutated NBRE site was generated with a Pfu ultra polymerase (Stratagene) reaction with the wild-type promoter construct as a template and the following primers: F: 5′-GCGAGGAGCAGAAGG AAAGGGAGGCACCGTAG-3′ and R: 5′-CTACGGTGCCTCCCTTTCCTTCT GCTCCTCGC-3′. The human *FABP5* cDNA was amplified from the IRAVp968H094 cDNA clone (RZPD, Accession No. BC002008) using the following primers: 5′-AAAAAAGCTTATGGCCAGTCTTAAGGATCT-3′ and 5′-AAAAGCTAGCTCATTGCACCTTCTCATAGA-3′. The PCR-amplified fragment was cloned into the pCMX vector.

Cell culture and transfections. Human embryonic kidney (HEK) 293 cells and chorion carcinoma JEG-3 cells were maintained in DMEM and MEM (Invitrogen), respectively. Media were supplemented with 10% heat-inactivated fetal calf serum (Invitrogen) at 37 °C under 5% CO_2 humidified atmosphere. Cells were transfected with the appropriate plasmids using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. Cells were lysed in 0.65% NP40, 10 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0 and cell extracts were assayed for luciferase and β-galactosidase activity using Lucy luminometer (Anthos Labtec Instruments). The ratio of luciferase activity to β-galactosidase activity was calculated to normalize the luciferase value. For all transient transfections, values represent the mean of nine samples.

Real-time PCR. Total RNA was prepared using the RNAeasy mini kit (Qiagen) and reverse-transcribed using SuperScriptII (Invitrogen). Real-time PCR analysis was performed on the Rotor-Gene 3000A (Corbett Research) using SYBR PCR Mastermix (Applied Biosystems). The following primers were used: *FABP5* F: 5′-GGAAGAT GGCGCCTGGTGGA-3′, *FABP5* R: 5′-CCGAGTACAGGTGACATTGT-3′, GAPDH F: 5′-CATGGCCTTCCGTGTTCCTA-3′ and GAPDH R: 5′-GCG GCACGTCAGATCCA-3′. All values were normalized against the housekeeping gene GAPDH and then presented as relative increase of *FABP5* expression. Data are presented as the mean + SEM of quadruplicate determinations of a representative experiment. Similar results were obtained in at least three independent experiments.

Western blot analysis. HEK293 cells were lysed in 10 mM Hepes–KOH pH 7.9, 0.4 M NaCl, 1 mM EDTA, 5% glycerol). For the experiment described in Fig. 4C, extracts were performed using the protocol described by [24]. Equal amounts of extracts were electrophoresed on 10% SDS–polyacrylamide gels and blotted onto PVDF membranes (BioRad). After transfer, the membrane was washed with PBS pH 7.6 and saturated with 5% dry milk in PBS 0.1% Tween 20 (PBS-T) for 1 h at room temperature. The membrane was then incubated with anti-human *FABP5* (Biovendor), anti-GAPDH and anti-histone 1 (Santa Cruz Biotechnology) rabbit polyclonal antibodies in PBS-T 0.5% milk for 18 h at 4 °C. The membrane was incubated with horseradish peroxidase-coupled anti-rabbit IgG antibody (Pierce) for 60 min at room temperature. Labeling was performed as described in the ECL Plus detection kit (Amersham).

Chromatin immunoprecipitation. Chromatin immunoprecipitation was performed using the ChIP assay kit (Upstate). The protein–DNA complexes were cross-linked using 37% formaldehyde (Sigma–Aldrich), sheared by sonication and immunoprecipitated overnight at 4 °C with 2 μg rabbit anti-Nurr1 antibody (E-20, Santa Cruz biotechnology) or rabbit IgG antibody (R&D Systems). The antibodies were eluted from the DNA–protein complexes and the cross-links were reversed by overnight incubation at 65 °C with 0.2 M NaCl. Samples were purified (MinElute PCR purification kit, Qiagen)

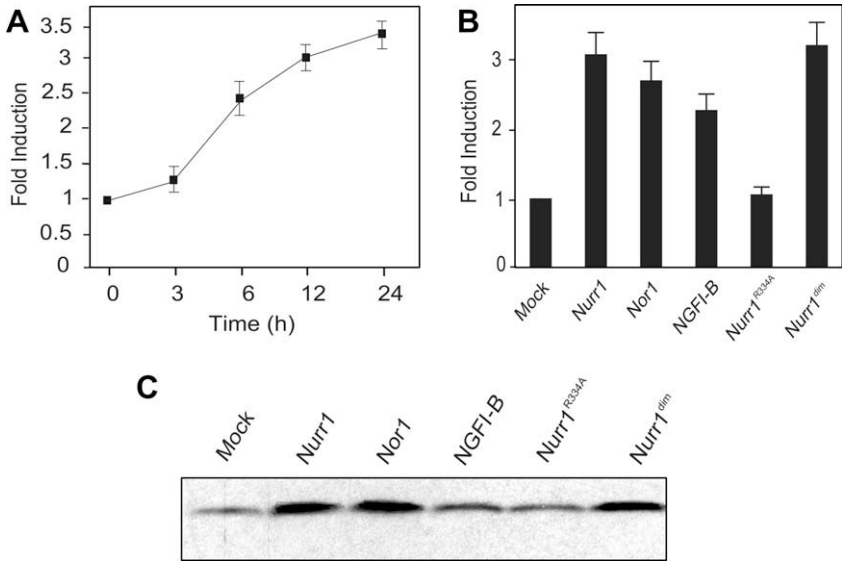

Fig. 1. Nurr1 regulates endogenous FABP5 expression in HEK293 cells. (A) Total RNA was extracted at different time points post transfection with cmx-Nurr1 and the expression levels of FABP5 mRNA were determined with real-time PCR. (B) HEK293 cells were transiently transfected with the indicated plasmids. Cells were harvested 24 h after transfection and real-time PCR analysis of FABP5 expression was performed. (C) HEK293 cells were transfected with the indicated plasmids. Whole cell extracts were prepared after 24 h. Resolved on SDS–PAGE and probed with anti-FABP5 antibody.

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