Supporting information for

Identification of a Binding Site for Unsaturated Fatty Acids in the Orphan Nuclear Receptor Nurr1

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SUPPLEMENTARY MATERIALS AND METHODS

Plasmids and ligands

DNA encoding the Nurr1 LBD (NR4A2; residues 353–598) and RXRα LBD (NR2B1l residues 223–462) were cloned into the expression vector pET-46 using the Ek/LIC system (EMD Chemicals/Novagen) as tobacco etch virus (TEV) protease-cleavable N-terminal 6x-polyhistidine tag fusion proteins. Full-length human Nurr1 was cloned into the expression vector pTriEx-4. The 3xNBRE-*luciferase* reporter was generated by cloning three copies of the monomeric NR4A DNA-response element upstream (5'- GAGTTTTAAAAGGTCATGCTCAATT TGTC-3') into the pGL2 luciferase reporter vector (Promega). Fatty acids were obtained from Sigma-Aldrich and dissolved in ethanol-d_6.

Protein expression and purification

Nurr1 LBD protein (unlabeled and NMR-labeled) was expressed in *Escherichia coli* BL21(DE3) cells (Life Technologies) in autoinduction media; or M9 media supplemented with D_2O, ^{13}C-glucose and/or $^{15}NH_4Cl$ (Cambridge Isotope Labs, Inc.). Proteins in wash buffer (50 mM Tris pH 7.4, 500 mM NaCl, 5 mM TCEP) were eluted against a 500 mM imidazole gradient through a Ni-NTA column, subsequently incubated with TEV protease at 4 °C overnight to cleave the 6xHis tag, and loaded anew onto the Ni-NTA column. The flow through containing purified protein was collected, buffer-exchanged into NMR buffer (20 mM KPO_4 pH 7.4, 50 mM KCl, 5 mM TCEP and 0.5 mM EDTA), and stored at –80 °C. RXRα LBD protein was expressed and purified using similar methods and as previously described.[1]

Enrichment of DHA via liquid chromatography-mass spectrometry (LC-MS)

Ni^{2+}-NTA agarose beads (Qiagen) were washed and equilibrated in an enrichment assay buffer (10 mM KH_2PO_4, 50 mM KCl, 5 mM TCEP, pH 7.4). The resin (50 µL of 50% v/v slurry) was then treated with either 200 µL of 50 µM 6xHis-Nurr1 LBD diluted in enrichment assay buffer or 200 µL enrichment assay buffer as control. The samples were incubated for 1 h at room temperature (RT) under slight agitation. Subsequently, the beads were briefly centrifuged and unbound protein was removed by 3x washing using protein buffer (40 mM KH_2PO_4, 500 mM KCl, 5 mM TCEP, 15 mM imidazole, pH 7.4), followed by washing with 800 µL enrichment assay buffer and transfer of the resin into 2 mL glass vials. A freshly prepared DHA stock solution (50 mM in ethanol) was diluted to 100 µM in enrichment assay buffer, and 400 µL of the diluted DHA solution was added to the resin that was incubated for 1 h at RT under slight agitation. Thereafter, any unbound DHA was removed by centrifugation and washing 1x with enrichment assay buffer, then 2x with 10 mM Tris pH 8.0. The elution of bound ligand was accomplished by addition of 100 µL of 100% methanol for 7 minutes. The solvent was removed by argon flushing. Any residual coeluted protein was removed by acetone precipitation. Finally, the acetone fraction was concentrated by argon flushing, followed by resuspension in 75% acetonitrile in water. Subsequently, the sample was analyzed by LC-MS on a 6230 TOF LC/MS (Agilent) mass spectrometer in the negative ion mode employing a 5% to 95% acetonitrile gradient over 25 min using a C18 column. To calculate the enrichment of DHA, a pairwise comparison of the peak area of the integrated mass ion intensity (retention time: 18.53 min) was performed. The identity of the analyzed peak was confirmed by inspection of the high resolution MS data, revealing a mass of 327.2329 Da (expected mass for DHA $(M - H)^-$: 327.2330 Da).

Tryptophan fluorescence spectroscopy

Steady-state tryptophan fluorescence emission spectra were collected using an ISS K2 Phase and Modulation Fluorometer with 2 nm band-pass filters employed for both excitation and emission. Samples were excited at 295 nm using a Xe lamp, with emission data collected in the range 320 to 420 nm in 1 nm steps after passing through a 320 nm high-pass filter. The emission spectrum of a 3.6 μM solution of Nurr1 LBD in NMR buffer at 20 °C was collected using a stirred 1 cm path length quartz cuvette. DHA was added to a final concentration of 150 μM, maintaining a Nurr1 concentration of 3.6 μM, and spectra collected 3 hours post addition.

In addition, seventeen DHA, AA and SA stock concentrations ranging from 0.23 μM to 50 mM were prepared in ethanol; 2 μL ligand was spiked to 2 wells per concentration point in a 96-well black quartz microplate (Hellma) containing 200 μL of 2.5 μM Nurr1 LBD protein or 25 μM L-tryptophan. For RXRα LBD experiments, DHA was titrated into 2.5 μM protein. The plate was read in a Tecan Safire II or Molecular Devices Spectramax M5e microplate reader at 23–25 °C with excitation and emission wavelengths set to 280 nm and 335 nm, respectively, or with an emission scan from 300–500nm at 5 nm increments at the same excitation wavelength. Fluorescence units were converted to percentage fluorescence quenching with respect to vehicle controls (i.e., Nurr1 LBD or L-tryptophan with 1% v/v ethanol). Data were fit using nonlinear regression to a one-site binding equation using GraphPad Prism (v6).

Nuclear magnetic resonance (NMR) spectroscopy

Protein NMR data were acquired on Bruker 700 MHz NMR spectrometer equipped with a QCI cryoprobe at 25 °C. For backbone and side-chain CHD_2-methyl NMR chemical shift assignments, [^{2}H,^{13}C,^{15}N]-Nurr1 LBD was used to collect 2D [^{1}H,^{15}N]-TROSY and 3D TROSY-based assignment data (HNCO, HNCA, HN(CO)CA, HN(CA)CB, and HN(COCA)CB) and 3D ^{15}N-NOESY-HSQC data using standard NMR experiments provided with Bruker Topspin 3.0; as well as 2D [^{1}H,^{13}C]-CHD_2-HSQC and 3D C-TOCSY-CHD_2 data on side-chain methyl groups [2]. For titrations, 2D [^{1}H,^{15}N]-TROSY and/or one-dimensional proton (^{1}H) spectra were acquired at 25 °C for 100 μM of ^{15}N-labeled Nurr1 LBD, mixed with 10% D_2O in NMR buffer and 1% ethanol-d_6 or ligand at 37.5 μM, 75 μM, 150 μM, 300 μM, and 500 μM. Data were analyzed with NMRViewJ (OneMoon Scientific, Inc; v9.1). Chemical shift perturbations were calculated using the minimum chemical shift perturbation procedure using the following equation: $CSP = \sqrt{\left(d_H^0 - d_H^i\right)^2 + \left(0.101 \times (d_N^0 - d_N^i)\right)^2}$.

Fluorescence polarization (FP) peptide interaction assay

The FP peptide interaction assay was carried out in black, non-binding surface, 384-well plates (Greiner) at room temperature in buffer A (20 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM TCEP, 1 mM EDTA, 0.01% w/v Tween 20). Purified recombinant Nurr1 LBD protein was diluted by serial dilution, and the corresponding protein dilution was added to the plate in dose-response format. A peptide derived from PIASγ, FITC-Ahx-MSFRVSDLQMLLGFVGRSK (Ahx=aminohexanoic acid), was diluted in buffer A supplemented with either ethanol (vehicle) or DHA. The corresponding mixture was added to the plate, resulting in a final peptide concentration of 100 nM, DHA concentration of 90 μM (or 0.45% vehicle), and a highest protein concentration of 90 μM. Controls with no protein with either 0.45% ethanol or 90 μM DHA were also made. Plates were sealed and incubated at 4 °C. Measurements were taken on a Molecular Devices SpectraMax M5e or BioTek Synergy Neo plate reader at 485 nm emission and 528 nm excitation wavelengths. Data were fit using nonlinear regression to a one-site binding equation using GraphPad Prism (v6).

Time-resolved fluorescence resonance energy transfer (TR-FRET)

The TR-FRET assay was carried out in black, non-binding surface, 384-well plates (Greiner) at room temperature using the same reagents as the FP assay above along with an Anti-His-Terbium (Tb) antibody (CisBio). DHA stocks were prepared via serial dilution in ethanol, and added to wells containing a protein/antibody/peptide mixture with final concentrations (20 µL well volume) of 8 nM 6xHis-Nurr1 LBD protein, 2 nM Anti-His-Tb antibody, and 400 nM FITC-PIASγ peptide. Plates were sealed and incubated at 4 °C. Measurements were taken on a Molecular Devices SpectraMax M5e or BioTek Synergy Neo plate reader. The Tb donor was excited at 340 nm, its emission was monitored at 492 nm, and the acceptor FITC emission was measured at 520 nm. Data were fit using nonlinear regression to a sigmoidal dose response equation using GraphPad Prism (v6).

Cellular luciferase transactivation assay

HEK293T or MN9D cells were seeded in 6-well plates at 350,000 cells per well. HEK293 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 50 units ml^{-1} of penicillin, streptomycin, and glutamine. MN9D cells were cultured in DMEM media supplemented with 15 mM sodium bicarbonate (pH 7.2), 10 % fetalclone III fetal bovine serum (Hyclone), and 50 units of penicillin and streptomycin. The following day, cells were transfected using Lipofectamine 2000 (Thermo Fisher Scientific) and Opti-MEM with full-length Nurr1 expression plasmid (0.5 µg) or empty plasmid, 3xNBRE3-luciferase reporter plasmid (1 µg), with empty vector added to a total of 2.5 µg total DNA and incubated for 18 h. Cells were transferred to white 384-well plates (Thermo Fisher Scientific) at 10,000 cells/well in 20 µL and incubated for 4 h. DHA or vehicle control was added (20 µL), cells incubated for 18 h and harvested for luciferase activity quantified using Britelite Plus (Perkin Elmer; 20 µL) on a Synergy Neo plate reader (Biotek). Data were analyzed using GraphPad Prism (v6). Experiments using similar conditions were also performed to determine the effect of DHA on cell toxicity using CellTiter-Glo Luminescent Cell Viability Assay (Promega), of which it was found that concentrations >100 µM resulted in some toxicity to the cells.

Size exclusion chromatography (SEC)

The oligomeric state of apo-Nurr1 and DHA-bound Nurr1 was examined by size exclusion chromatography using a 5 µm silica bead standard bore Wyatt SEC column MP030S5 (Wyatt Technology) equilibrated with NMR buffer. Nurr1 was loaded at 163 µM alone or supplemented with 3-fold molar excess of DHA into the column in 150 µl injections at room temperature at a flow rate of 0.3 mL min^{-1}.

SUPPLEMENTARY REFERENCES

1. Kojetin, D. J., Matta-Camacho, E., Hughes, T. S., Srinivasan, S., Nwachukwu, J. C., Cavett, V., Nowak, J., Chalmers, M. J., Marciano, D. P., Kamenecka, T. M., Shulman, A. I., Rance, M., Griffin, P. R., Bruning, J. B., and Nettles, K. W. (2015) Structural mechanism for signal transduction in RXR nuclear receptor heterodimers, *Nature communications 6*, 8013.

2. Otten, R., Chu, B., Krewulak, K. D., Vogel, H. J., and Mulder, F. A. (2010) Comprehensive and cost-effective NMR spectroscopy of methyl groups in large proteins, *J Am Chem Soc 132*, 2952-2960.

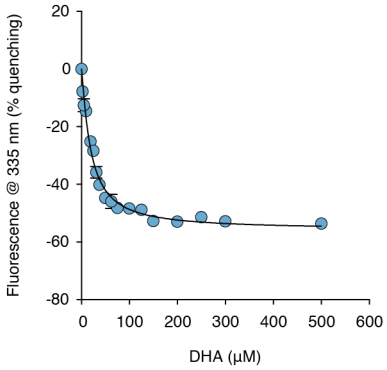

Figure S1. Steady-state tryptophan fluorescence reveals a concentration-dependent change in the fluorescence signal upon titration of DHA into RXRα LBD (K_d = 33±2 μM).

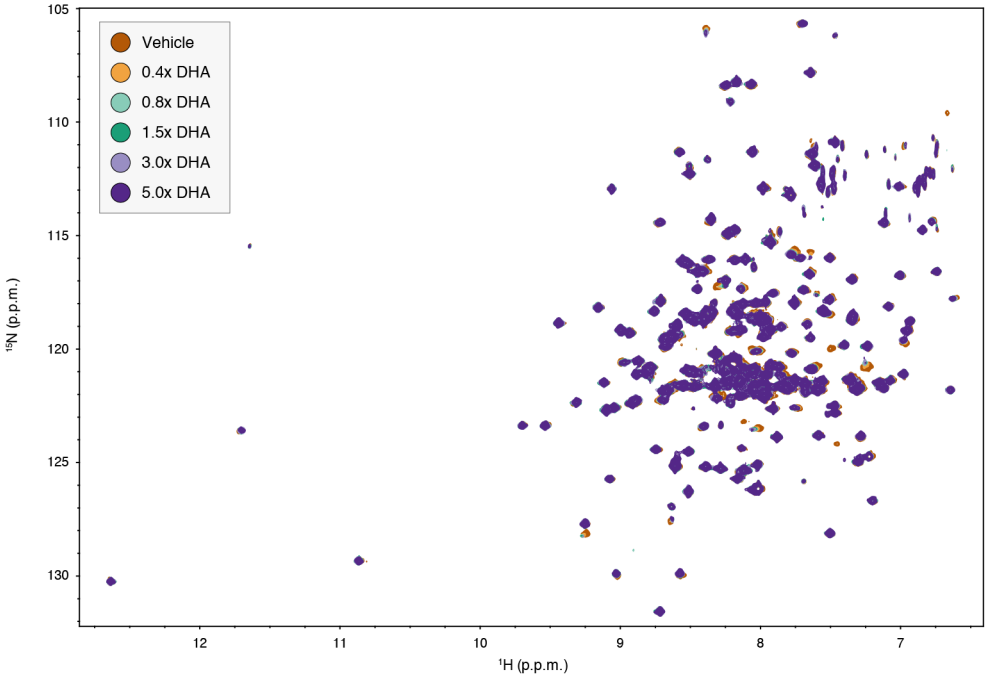

Figure S2. Overlay of 2D [^{1}H,^{15}N]-TROSY-HSQC NMR of ^{15}N-labeled Nurr1 LBD with increasing concentrations of DHA.

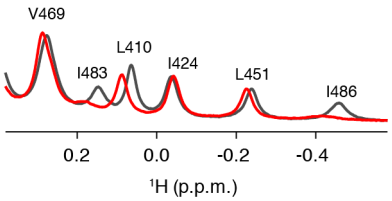

Figure S3. Overlay of 1D [^{1}H]-NMR data of the methyl region for Nurr1 LBD with (red) and without (black) addition of 5-fold molar excess arachidonic acid (AA).

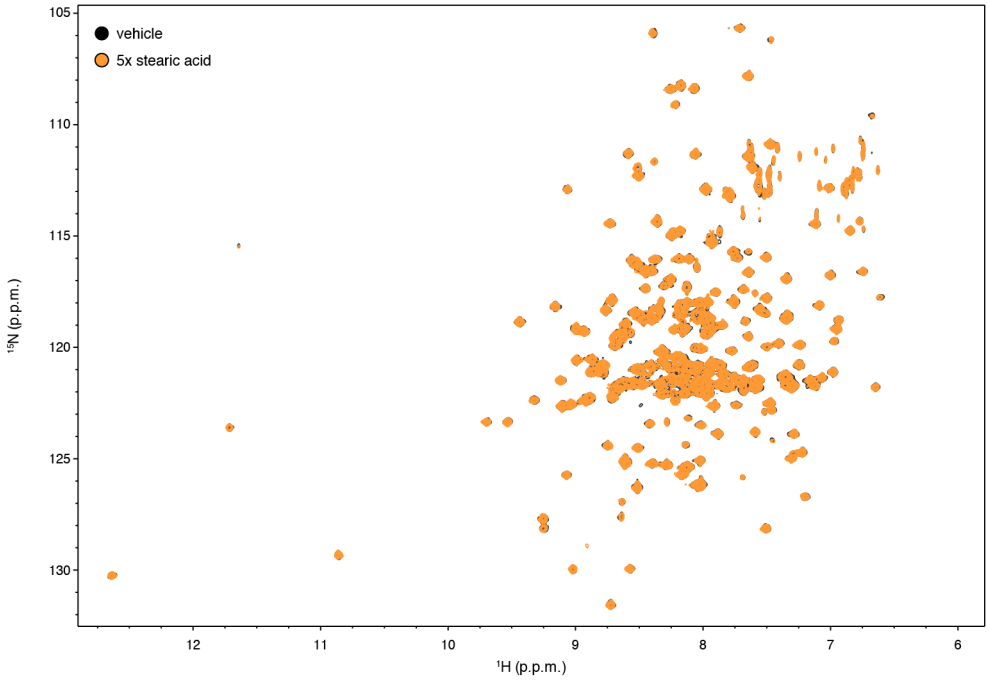

Figure S4. Overlay of 2D [^{1}H,^{15}N]-TROSY-HSQC NMR data for Nurr1 LBD with and without addition of 5-fold molar excess stearic acid (SA). These data complement tryptophan fluorescence spectroscopy data (Figure 2B) showing that SA does not bind to the Nurr1 LBD.

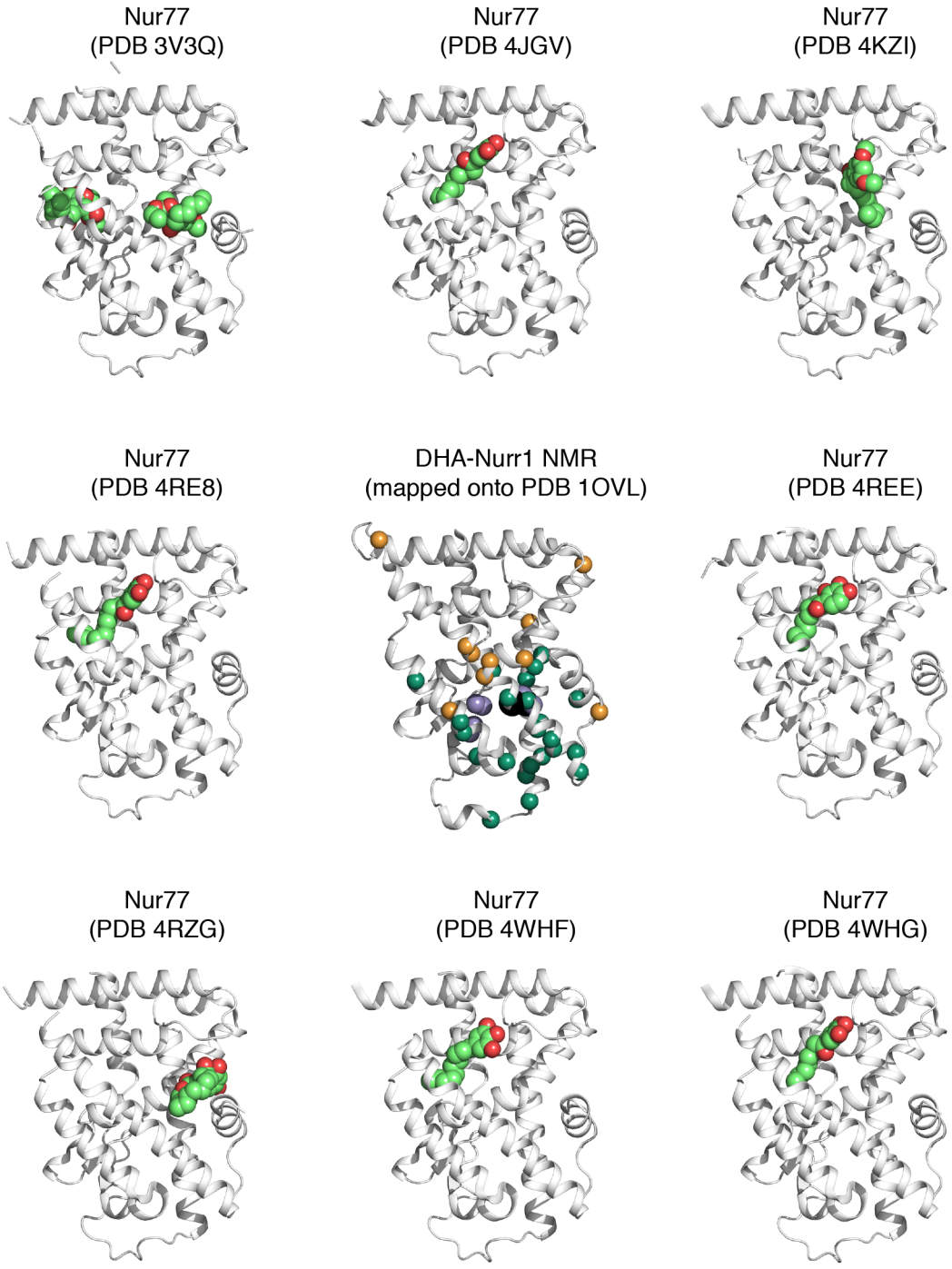

Figure S5. Comparison of Nur77 crystal structures bound to cytosporone B (CsnB) agonist analogs at the surface-exposed alternate ligand-binding sites with NMR data on the DHA-Nurr1 binding interaction. CsnB analogs are shown as green/red spheres. NMR data on DHA-Nurr1 interaction is colored as in Figure 4C.

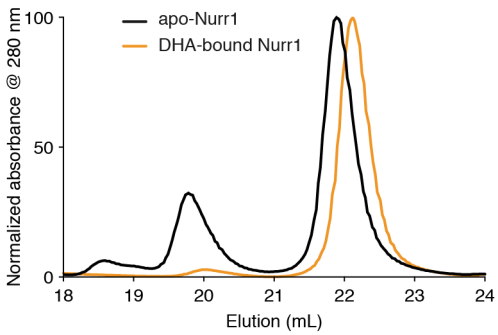

Figure S6. Size exclusion chromatography (SEC) of Nurr1 LBD in the presence and absence of DHA.

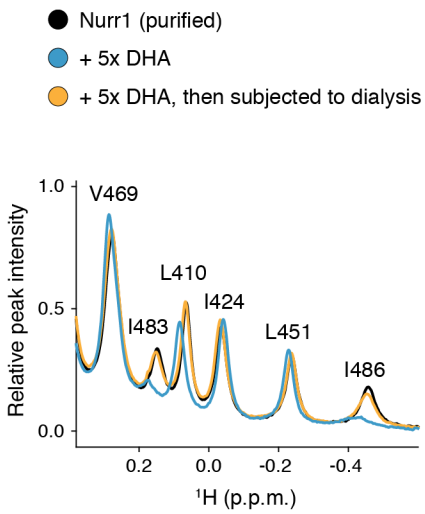

Figure S7. NMR data indicate that Nurr1 LBD protein purified from bacterial is not likely bound to an endogenous bacterial ligand. When compared to the purified Nurr1 LBD sample (black), binding of DHA to Nurr1 LBD (blue) results in chemical shift perturbations for Ile483 and Ile486, which are residues within the Nurr1 ligand-binding pocket (also see Figure 3B). When the DHA-bound sample (blue) is subjected to dialysis, the NMR spectrum of the dialyzed sample (orange) looks very similar to the purified sample (black) and different from the DHA-bound sample (blue). That the dialyzed DHA-bound sample (orange) reverts back to looking like the initial purified Nurr1 sample (black) indicates that both samples are likely not bound to a ligand.