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

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Abstract

Nurr1/NR4A2 is an orphan nuclear receptor, and currently there are no known natural ligands that bind Nurr1. A recent metabolomics study identified unsaturated fatty acids, including arachidonic acid and docosahexaenoic acid (DHA), that interact with the ligand-binding domain (LBD) of a related orphan receptor, Nur77/NR4A1. However, the binding location and whether these ligands bind other NR4A receptors were not defined. Here, we show that unsaturated fatty acids also interact with the Nurr1 LBD, and solution NMR spectroscopy reveals the binding epitope of DHA at its putative ligand-binding pocket. Biochemical assays reveal that DHA-bound Nurr1 interacts with high affinity with a peptide derived from PIASγ, a protein that interacts with Nurr1 in cellular extracts, and DHA also affects cellular Nurr1 transactivation. This work is the first structural report of a natural ligand binding to a canonical NR4A ligand-binding pocket, and indicates a natural ligand can bind and affect Nurr1 function.

Graphical abstract
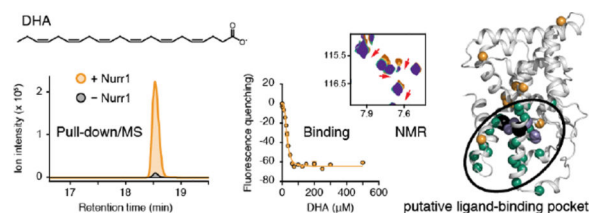
Keywords

Nuclear receptor (NR) transcription factors regulate many aspects of mammalian physiology, in part through binding small molecule ligands within a conserved "canonical" ligand-binding pocket located in the hydrophobic core of the ligand-binding domain (LBD). Ligand binding alters the LBD conformation and affects interaction of NR coregulators and other proteins resulting in alterations in chromatin and changes in gene expression. About half of the 48 human NRs were initially termed orphan NRs because their endogenous ligands were unknown.[1, 2] Some of these orphan NRs have since been "adopted" or "deorphanized" as natural ligands have been discovered. However, there remains a cohort of orphan NRs with unidentified natural ligands, including the NR4A receptors Nur77/NR4A1, Nurr1/NR4A2, and NOR-1/NR4A3. The NR4As are expressed in many tissues, including skeletal muscle, adipose, cardiovascular, kidney, liver, and central nervous system, often with aberrant expression in disease with roles in inflammation, arthritis, cancer, neurological and metabolic conditions including obesity, dyslipidemia, diabetes, and cardiovascular disease.[3–6]

Crystal structures of the LBDs of Nurr1 and Nur77 show no apparent cavity in the canonical pocket where non-orphan nuclear receptors bind natural ligands.[7, 8] Instead, their putative pockets are filled with bulky hydrophobic residues. Interestingly, a metabolomics study revealed that unsaturated fatty acids (UFAs) could be pulled down from Nur77 LBD protein incubated with a lipophilic metabolite extract from mouse brain and testes.[9] This work was inspired by the discovery of synthetic Nur77 agonists that were later shown to bind to surface exposed alternate ligand-binding sites.[10, 11] Analysis of two UFAs identified as the most abundantly enriched in the metabolomics workflow, arachidonic acid (AA) and docosahexaenoic acid (DHA), confirmed direct binding to Nur77. However, the structural basis of the UFA-Nur77 interaction, and whether UFAs can bind other NR4A receptors such as Nurr1, remain elusive.

To determine if Nurr1 can bind DHA, we performed a pull-down assay (Figure 1A) by incubating DHA with polyhistidine-tagged (6xHis) Nurr1 LBD bound to Ni^{2+}-NTA resin beads, or as a control with Ni^{2+}-NTA resin alone to account for background binding to the resin. After washing the resin, methanol was added to elute any bound DHA and the elution fraction was analyzed by liquid chromatography–mass spectrometry (LC-MS). A 25-fold enrichment of DHA was observed for Nurr1 LBD over control (Figure 1B). High-resolution mass spectrometry confirmed the identity of the LC peak as DHA (Figure 1C). These data

indicate that Nurr1 LBD is able to selectively bind and enrich DHA to a similar extent as Nur77.[9]

Nurr1 contains two tryptophan residues located in the LBD core and putative ligand-binding pocket. To confirm the LC-MS enrichment results and to test the direct interaction between Nurr1 LBD and DHA, we performed steady-state tryptophan fluorescence spectroscopy. Addition of DHA to Nurr1 LBD decreased tryptophan fluorescence emission demonstrating direct binding (Figure 2A). Furthermore, titration of the UFAs DHA and AA, but not the saturated fatty acid stearic acid (SA), caused a concentration-dependent decrease in fluorescence (Figure 2B). K_d values of DHA (30±1 μM) and AA (58±10 μM) to Nurr1 are similar to DHA binding to another NR known to bind DHA, RXRα (Supporting Information, Figure S1; K_d = 33±2 μM). These data indicate Nurr1 selectively binds UFAs, as observed for Nur77.[9]

Because the ligand-free/apo-Nur77 crystal structure lacks space for a ligand to bind within its putative canonical ligand-binding pocket, it was postulated that UFAs may bind to a non-traditional pocket.[9] For example, two non-canonical, alternate ligand-binding sites were observed on the surface of Nur77 in crystal structures bound to synthetic cytosporone B analogs.[12–14] Because our efforts to obtain a co-crystal structure of the DHA-Nurr1 LBD complex have not yet succeeded, we used solution NMR spectroscopy to map the structural binding epitope of DHA. Titration of DHA into ^{15}N-labeled Nurr1 LBD resulted in NMR peak shifting and reduction in peak intensity due to NMR resonance line broadening for specific peaks indicating localized binding effects (Figure 3A and Supporting Information, Figure S2). Line broadening here is a result of chemical exchange between apo and DHA-bound states due to the μM binding affinity, as well as changes in Nurr1 motions (dynamics) when bound to DHA.[15] DHA binding also affects methyl groups buried in the Nurr1 LBD core and putative pocket (Figure 3B; Leu410, Ile483, and Ile486), and similar effects are observed for AA binding to Nurr1 LBD (Supporting Information, Figure S3). In contrast, addition of SA, which does not bind Nurr1 as observed by tryptophan fluorescence (Figure 2B), revealed no NMR perturbations (Supporting Information, Figure S4).

To determine the location of DHA binding, we quantitated 2D NMR peak intensity changes and chemical shift perturbations (CSPs) (Figure 3C,D). Mapping these data onto the apo-Nurr1 LBD crystal structure[7] revealed that residues with the largest NMR perturbations localize to the putative ligand-binding pocket (Figure 3E). The perturbations are not wide-spread indicating DHA binding to Nurr1 preserves the overall LBD structure. Notably, NMR analysis of ligand binding to the canonical pocket of other NRs, including RXRα, PPARγ, and VDR similarly causes perturbations for residues in the interior core of the LBD (i.e., the canonical ligand-binding pocket).[16–22] By comparison, our NMR data on the DHA-Nurr1 interaction indicate that UFAs bind to the putative canonical ligand-binding pocket in Nurr1. Furthermore, DHA binding also perturbs NMR peaks of residues in the C-terminal helix 12 (e.g., Figure 3A; Ile588, Lys590), a region important for NR LBD-coregulator protein interaction.

Comparison of our DHA-Nurr1 NMR interaction data to available crystal structures of Nur77 bound to cytosporone B agonist analogs[12–14] indicates that UFAs do not bind to

Nurr1 at the same surface-exposed alternate sites (Supporting Information, Figure S5). Furthermore, unlike UFA binding to Nur77, which stabilized an oligomer species,[9] DHA binding to Nurr1 shifts the monomer-dimer equilibrium in favor of monomer (Supporting Information, Figure S6) resulting in one relatively modest CSP at the putative helix 11 dimer interface (Figure 3D,E; i.e., Asn547) indicating no substantial conformational change to this surface. However, CSPs to the Nurr1 ligand-binding pocket are more numerous. Notably, the DHANurr1 NMR interaction epitope is similar to the NMR interaction epitope recently mapped for amodiaquine, a synthetic Nurr1 agonist that binds Nurr1,[23] indicating overlapping natural and synthetic ligand-binding sites.

We next determined if an UFA binding to Nurr1 LBD could affect Nurr1 function. PIASγ is a SUMO-E3 ligase and coregulator of Nurr1 that was previously shown to bind Nurr1 LBD in whole cell extracts through an essential LXXLL coregulator motif sequence and repress Nurr1 transactivation.[24] We developed a fluorescence polarization (FP)-based biochemical assay using a peptide derived from the essential PIASγ LXXLL motif. Increasing amounts of Nurr1 LBD were added to a fixed concentration of the FITC-labeled PIASγ peptide (Figure 4A). In the absence of DHA, we observed very weak interaction with the PIASγ peptide. However, in the presence of DHA, a robust interaction is observed between Nurr1 LBD and the FITC-PIASγ peptide ($K_d = 3.5 \pm 0.8$ μM). To confirm these data using an orthogonal method, we developed a time-resolved fluorescence resonance energy transfer (TR-FRET) assay. Titration of DHA resulted in a concentration-dependent increase in the interaction between Nurr1 LBD and the FITCPIASγ peptide, confirming that DHA promotes the interaction with an EC_{50} of 25 ± 1.2 μM (Figure 5B), consistent with the 30 μM K_d determined by tryptophan fluorescence (Figure 2B).

To determine if DHA could affect Nurr1 transactivation, we used a cell-based luciferase reporter gene cotransfection assay that reports on transcription occurring from direct binding to its cognate DNA response element. We performed this assay in two cell lines, HEK293T (a general, commonly used cell line) and MN9D (used as a dopaminergic cellular model related to Nurr1 function). An expression plasmid encoding full-length Nurr1 was cotransfected with a reporter plasmid containing three copies of the NR4A monomeric DNA-response element (3xNBRE) upstream of the firefly luciferase gene Transfection of full-length Nurr1, but not an empty vector control expression plasmid, resulted in a significant increase in luciferase activity (Figure 4C). This demonstrates the 3xNBRE-*luciferase* reporter is specifically activated by Nurr1 overexpression. Cellular treatment with DHA and Nurr1 overexpression caused a statistically significant decrease in luciferase activity (Figure 4D) relative to the significant activation caused by Nurr1 overexpression alone (Figure 4C), indicating DHA can affect Nurr1 transactivation.

In conclusion, here we reveal that Nurr1 directly binds UFAs, which is the first demonstration of a natural ligand binding to Nurr1. This observation adds to the complex polypharmacology of UFAs, which bind other non-NR proteins and other NRs including RXRs, Nur77, FXR, LXRs, and PPARs, and affect their function also at μM concentrations. Our tryptophan fluorescence binding analysis shows that the affinity of DHA for Nurr1 and RXRα is essentially the same (30 μM and 33 μM, respectively). This observation, combined with our solution NMR studies that map the structural binding epitope of DHA to the

putative Nurr1 ligand- binding pocket, indicates DHA binding to Nurr1 is specific. Furthermore, given the high presence of UFAs and in particular DHA in tissues where Nurr1 is present, such as the brain and retina (40% and 60% of total UFAs, respectively), they are poised to potentially bind Nurr1 *in vivo*. One point to note is that our data show that DHA binding favors a monomeric LBD over a dimer species. However, the dimer species is of low abundance and may probably have low affinity, but this relatively modest equilibrium change could in principle have an effect on Nurr1 function. Another point to note is that it is possible that sub-stoichiometric amounts of a bacterial endogenous ligand bound to Nurr1 could in principle influence the binding studies. However, using NMR we have observed that subjecting DHA-bound Nurr1 to dialysis results in an NMR spectrum nearly identical to freshly purified Nurr1 (Supporting Information, Figure S7). Thus if there is an endogenous ligand bound to Nurr1 in bacteria, it likely dissociates out of the pocket resulting during purification in apo/ligand-free protein. These data also demonstrate that DHA-binding to the Nurr1 LBD is reversible.

Furthermore, our data also show that DHA-bound Nurr1 LBD, but not apo-Nurr1 LBD, binds with high affinity to an LXXLL peptide derived from PIASγ that essential for interaction with Nurr1.[24] The modest but statistically significant effect of DHA on Nurr1 transactivation could be the result of several consequences. It could be that DHA binding to Nurr1 may not substantially affect or perturb the already high cellular activation of DNA binding-dependent Nurr1 transcription. This could be the case whether or not Nurr1 binds an endogenous ligand present in cells. However, if there is a hypothetical endogenous ligand that binds and activates Nurr1 in cells, DHA may not compete with it for binding Nurr1. Finally, because PIASγ can SUMOylate Nurr1 independent of its ability to repress Nurr1 transctivation,[24, 25] future work is needed to explore roles of UFA binding in PIASγ-mediated functions of Nurr1 and other cellular outcomes such as gene expression.

Apo-Nurr1 and Nur77 crystal structures show no canonical binding pocket to which a natural ligand can bind as they have ~30–40 Å^3 putative pocket volumes.[26] However, structural studies have shown that the ligand-binding pockets of some adopted orphan NRs can undergo large conformational changes to bind natural ligands. A crystal structure of apo-REV-ERB revealed a collapsed pocket with no ligand-binding cavity (~50–90 Å^3).[27] However, a subsequent crystal structure revealed its pocket undergoes a large expansion to bind the porphyrin heme (~600 Å^3).[28] NMR studies revealed that the apo-REV-ERB ligand-binding pocket is dynamic on the μs-ms timescale,[29] confirming the collapsed apo-pocket captured by crystallography has the ability to expand in solution as part of the structural mechanism of binding heme. A previous NMR study on Nurr1 showed that residues comprising its putative ligand-binding pocket have shorter T_2 relaxation times indicating motion on the μs-ms NMR time-scale.[30] Like the REV-ERBs, this motion might be critical for the large conformational changes required for ligand binding to Nurr1, and future structural studies including crystallography are needed to reveal the molecular details of UFA binding to Nurr1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

ABBREVIATIONS

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Nurr1	nuclear receptor related 1 protein
NMR	nuclear magnetic resonance spectroscopy
DHA	docosahexaenoic acid
AA	arachidonic acid
SA	stearic acid
LBD	ligand-binding domain
UFA	unsaturated fatty acids
Nur77	nerve growth factor IB
NOR-1	neuron-derived orphan receptor 1
LC-MS	liquid chromatography–mass spectrometry
FITC	Fluorescein isothiocyanate
RXR	retinoid X receptor
PIASγ	protein inhibitor of activated STAT protein gamma

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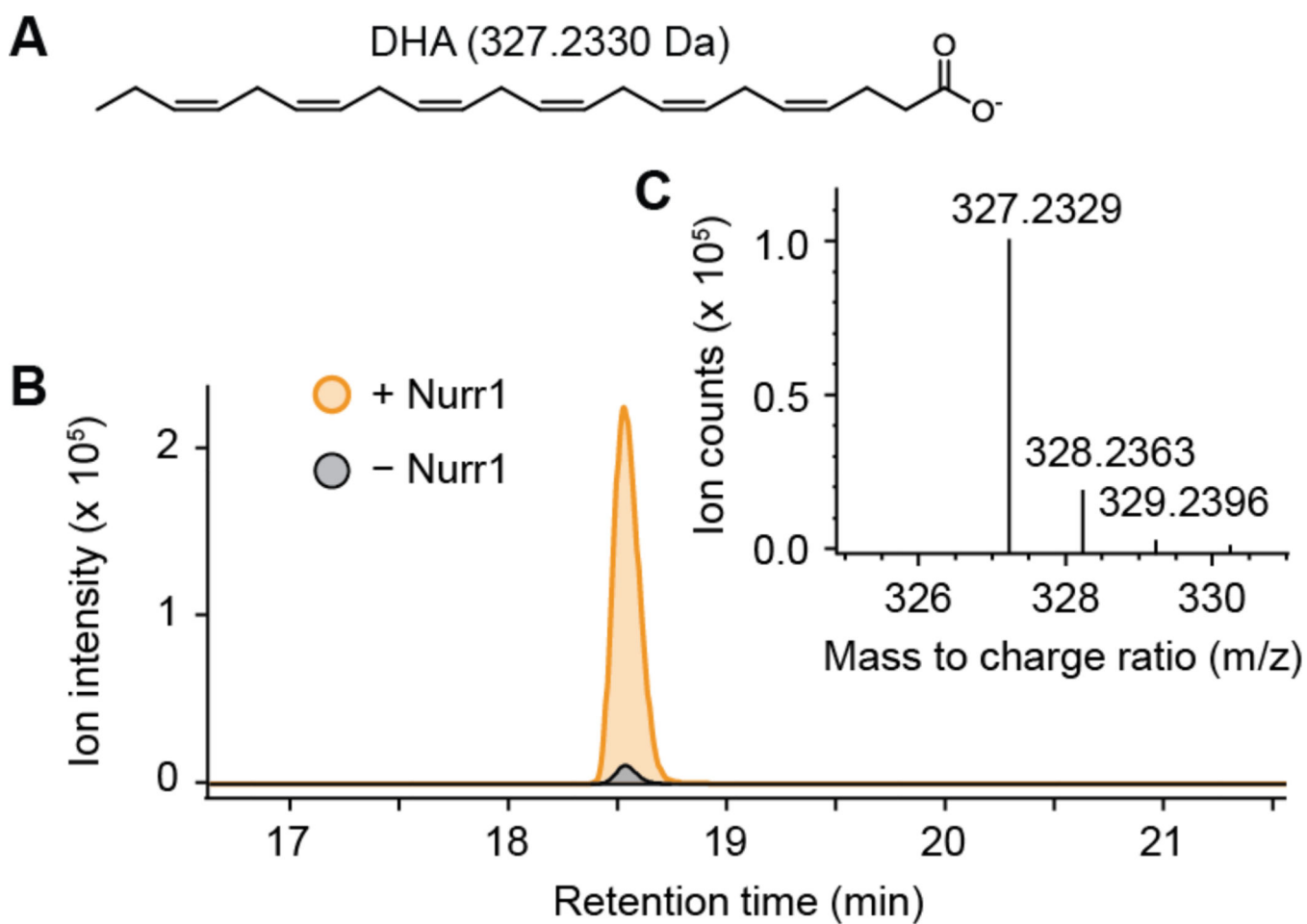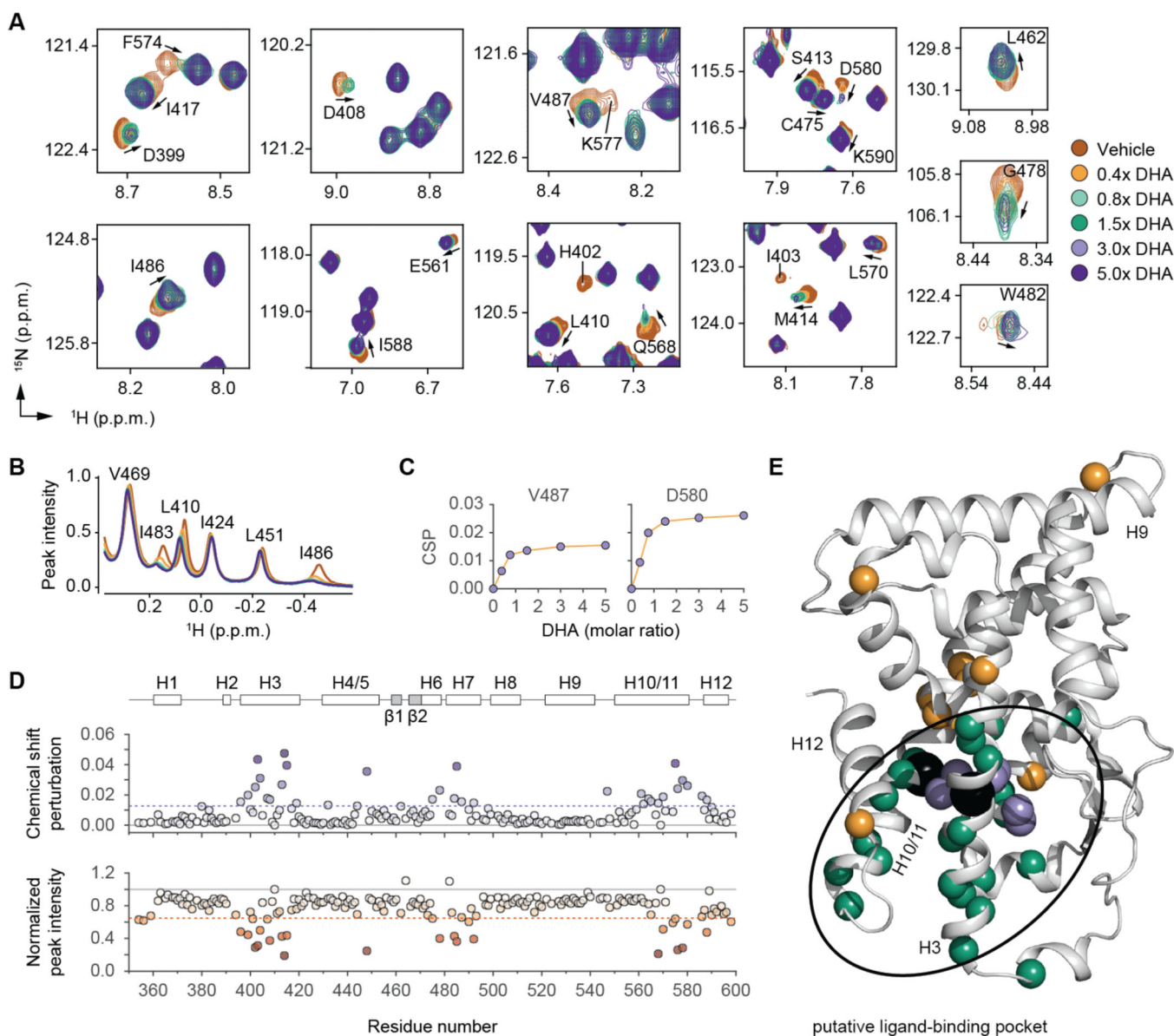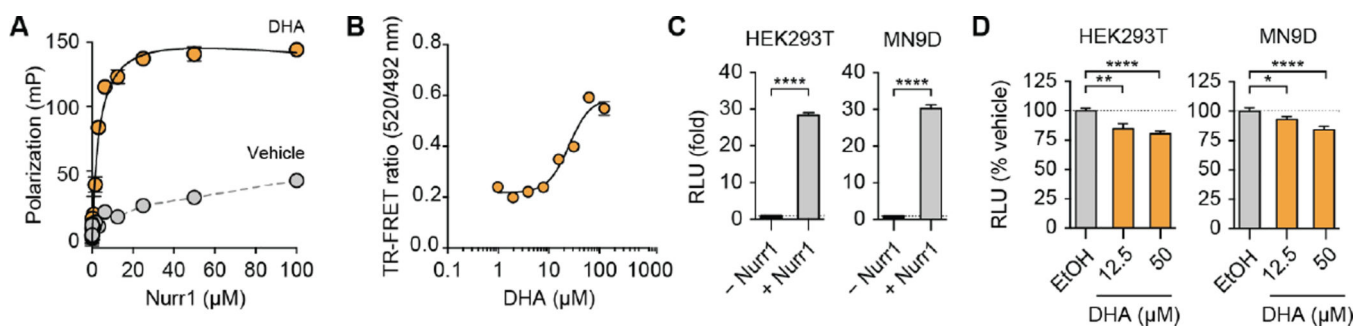
Figure 1.
DHA pull-down in the presence of 6xHis-Nurr1 LBD. (A) Structure of DHA. (B) LC-MS shows enrichment of DHA in the presence of Nurr1 LBD. (C) High-resolution MS confirmation of DHA.
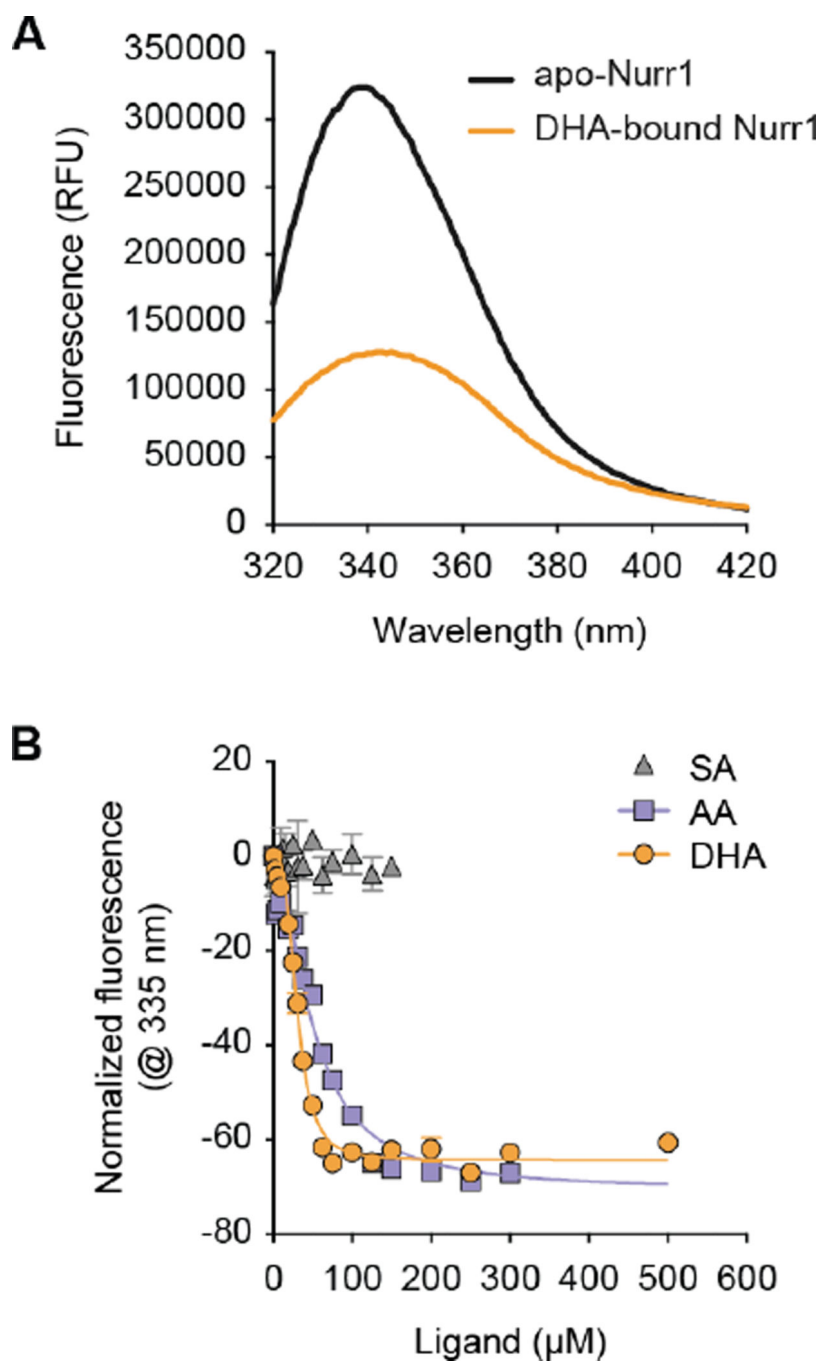
Figure 2.
Binding of UFAs to Nurr1 LBD. (A) Tryptophan fluorescence emission spectra of Nurr1 LBD ± DHA. (B) Titration of DHA and AA, but not SA, decreases Nurr1 fluorescence.

Figure 3.
NMR analysis of DHA binding to Nurr1. (A,B) DHA titration followed by 2D [^{1}H,^{15}N]-TROSY-HSQC NMR of backbone amides and (B) 1D [^{1}H]-NMR data of the methyl region. (C) Representative chemical shift perturbation (CSP). (D) Quantitation of 2D NMR peak intensities and CSPs. (E) NMR data mapped on the apo-Nurr1 LBD (PDB 1OVL)[7]. Spheres show residues with changes beyond dotted line thresholds in (D); backbone amides that disappear (black) or are perturbed (CSP only, orange; CSP and intensity, teal); methyl peaks perturbed (purple).

Figure 4.
Effect of DHA on Nurr1 function. (A) Fluorescence polarization (FP) assay shows increased binding of PIASγ peptide to Nurr1 LBD in the presence of DHA. (B) Time-resolved fluorescence resonance energy transfer (TR-FRET) assay shows a DHA concentration-dependent increase in the binding of PIASγ peptide to Nurr1 LBD. (C,D) Cell-based luciferase reporter gene assay analysis of Nurr1 transcription using a 3xNBRE-*luciferase* reporter. (C) Cells cotransfected with 3xNBRE reporter, with or without a full-length Nurr1 expression plasmid. (D) Cells cotransfected with 3xNBRE and a full-length Nurr1 expression plasmid treated with DHA or vehicle control (ethanol; EtOH).