## **Supporting Information**

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**Fig. S1.** GST pull-down assays showing a strong interaction between Dax-1 and LRH-1. GST fusion proteins containing LRH-1 and SF-1 LBDs were incubated with *in vitro* transcribed and translated <sup>35</sup>S full-length mDax-1 for 30, 60, and 90 min at room temperature. For each time point, the amount of Dax-1 bound to GST control protein (lanes 2–4), GST-LRH-1 LBD (lanes 5–7), or GST-SF-1 LBD (lanes 8–10) was determined, as shown in comparison to 10% of total Dax-1 input (lane 1). Bound proteins were resolved by SDS-PAGE and visualized by autoradiography. Different expression time points for <sup>35</sup>S-Dax-1 (30, 60, and 90 min) were used to determine whether the total amount and concentration of accumulated protein affected its binding interactions with the protein partners tested.



**Fig. 52.** Dax-1 LBD is essential for binding interactions with LRH-1. (*A*) SDS-PAGE showing purification of the Dax-1 LBD:LRH-1 LBD complex. Lane 1: His<sub>6</sub>-tagged LRH-1 LBD with associated untagged Dax-1 fragment 205–472 eluted from Ni-NTA matrix. Lane 2: The protein sample after the N-terminal LRH-1 His<sub>6</sub>-tagged LRH-1 LBD with associated untagged Dax-1 fragment 205–472 eluted from Ni-NTA matrix. Lane 2: The protein sample after the N-terminal LRH-1 His<sub>6</sub>-tag was cleaved using TEV protease. Lanes 3–6: Purified Dax-1 LBD:LRH-1 LBD complex after size-exclusion chromatography [peak 2 in (*B*)]. Lanes 7–9: Excess LRH-1 LBD [peak 3 in (*B*)]. (*B*) Elutates from size exclusion chromatography. An FPLC Supedex 75 HiLoad 16/60 column was used for purification of the complex. Peak 1 corresponds to V<sub>free</sub> of the column, where the aggregated proteins (<1%) would appear. Peaks 2 and 3 correspond to the Dax-1 LBD:LRH-1 LBD complex [lanes 3–6 in (*A*)] and the excess LRH-1 LBD [lanes 7–9 in (*A*)]. (*C*) Longer Dax-1 fragments do not form a stable Dax-1:LRH-1 complex: SDS-PAGE showing interactions between LRH-1 LBD and Dax-1 fragment 138–472 (with one LXXLL repeat preceding the LBD). Lanes 1–2: Bacterial total and soluble protein fractions showing both His<sub>6</sub>-tagged LRH-1 LBD and the Dax-1 fragment expressed (indicated). Lane 3: Proteins bound to the affinity Ni-NTA matrix. Although a little amount of Dax-1 protein (upper band in lane 3) was copurified with His<sub>6</sub>-tagged LRH-1 LBD on the affinity matrix, a stable Dax-1:LRH-1 complex could not be obtained, because it dissociated during size-exclusion chromatography (*Right*). Lane 4 corresponds to the dissociated LRH-1 LBD. The peak corresponding to the LRH-1 LBD is shown on the elution profile (*Right*). The region preceding the peak corresponds to the dissociated LRH-1 LBD were mostly insoluble and did not bind to LRH-1. These findings suggest that the N-terminal region preceding the Dax-1 LBD might need additional binding partners for structuring. In the absence of th



**Fig. 53.** Biochemical characterization of the (Dax-1)<sub>2</sub>:LRH-1 heterotrimer. (*A*) Size-exclusion chromatography of the (Dax-1)<sub>2</sub>:LRH-1 heterotrimer. An FPLC Supedex 75 HiLoad 16/60 column was calibrated using different nuclear receptor LBD monomers, homodimers and heterodimers, and tetramers. Retention volumes corresponding to a nuclear receptor LBD dimer (60 kDa) and tetramer (120 kDa) are indicated by black and gray arrows, respectively. Peak 3 represents the LRH-1 LBD monomer (30 kDa). Based on this calibration, peak 2 (90 kDa) corresponds to the (Dax-1)<sub>2</sub>:LRH-1 heterotrimer. The complex maintains its trimeric monodispersed state at low (0.1 mg/ml;  $\approx 1 \mu$ M) and high (10 mg/ml;  $\approx 100 \mu$ M) concentrations, suggesting an association with a K<sub>d</sub> < 1  $\mu$ M. (*B*) SDS-PAGE of the purified (Dax-1)<sub>2</sub>:LRH-1 heterotrimer. The analyzed protein sample corresponds to peak 2 in (*A*). Note the 2:1 ratio between the Dax-1 (*Upper*) and LH-1 (*Lower*) components of the complex on the gel. (*C*) Surface plasmon resonance sensorgrams showing the formation and dissociation of the (Dax-1)<sub>2</sub>:LRH-1 heterotrime (Dax-1)<sub>2</sub>:LRH-1 heterotrime (Dax-1)<sub>2</sub>:LRH-1 complex. Samples of the Dax-1 protein (aa 205–472) at concentrations of 0.5, 1, 2, 4, 6, and 8  $\mu$ M were injected over the His<sub>6</sub>-tagged LRH-1 LBD immobilized on an Ni<sup>2+</sup> tri-NTA chip. The sensorgrams were corrected against responses over a blank reference surface, as well as against responses to injections of the 0  $\mu$ M Dax-1 samples. The experimental curves (with sharp transients and spikes removed) and fitted curves (for the one LRH-1 to one Dax-1 (*K*<sub>d</sub>) for the complex are indicated.



**Fig. 54.** Electron densities from a simulated annealing composite omit map for the  $(Dax-1)_2$ :LRH-1 structure. (*A*) The repression helix of Dax-1 docked into the primary AF-2 site of LRH-1. (*B*) A different view of the Dax-1 repression helix, showing the preceding and following helices, H3 and H4. For both figures, the electron density (shown as gold mesh) is displayed at a contour level of  $1\sigma$ . Residues P275, F277, I279, and P281 from the Dax-1 repression helix are indicated. Note that both the main and the side chains of the complex are well defined by the density. As reported in Table S1, the average temperature factor for the (Dax-1)\_2:LRH-1 structure is ~65 Å<sup>2</sup>. In considering the source of the apparently high B values, we suggest that this is best explained by the limited crystal contacts, which allow rigid body vibrations of the otherwise well-ordered Dax-1 and LRH-1 domains. This reasoning is consistent with the fact that the refinement statistics for the structure can be further improved when the TLS option accounting for anisotropic rigid body domain motions (CCP4 program REFMAC) is used in the refinement. Because the individual temperature factors for the (Dax-1)\_2:LRH-1 structure do not display any large fluctuations and are distributed uniformly around 65 Å<sup>2</sup>, the electron density is well defined for all parts of the structure, as shown here.



**Fig. S5.** Estimation of molecular weights of the Dax-1 LBD monomer and homodimer. (A) Comparison of the position of the peak corresponding to the Dax-1 homodimer (aa 205–472; 60 kDa; red) with the positions of the peaks corresponding to the (Dax-1)2:LRH-1 heterotrimer (90 kDa; dashed black) and the LRH-1 LBD monomer (30 kDa; dashed black). (*B*) In the absence of the 40 residues (aa 205–245) preceding the visible Dax-1 LBD, Dax-1 is a monomer. The position of the peak corresponding to the Dax-1 kDax-1 kDax-1 kDax-1 kDax-1 box-1 monomer (aa 245–472; 25 kDa; red) is compared with that of the peak corresponding to the Dax-1 homodimer (60 kDa; dashed black). A calibrated FPLC Supedex 75 column was used to determine the molecular weights of the Dax-1 fragments. (*C*) Schematic view of the Dax-1 assemblies. The visible structure of the Dax-1 LBD (aa 250–472; blue) is a monomer. Forty residues (205–245; dimerization domain; gray) N-terminal to the LBD allow the dimerization of Dax-1. LRH-1 LBD (yellow) binds to the Dax-1 homodimer and forms a heterotrimer without breaking the Dax-1 dimer.



**Fig. S6.** A magnified view of the second Dax-1 (blue)–LRH-1 (yellow) interface, showing the major structural elements. (A) A fragment of the complex structure in the vicinity of the LRH-1 ligand-binding pocket. The position of the second Dax-1 LBD in the complex places the side chain of semiconserved Q397 in loop L8–9 of Dax-1 at the distance of direct contact with the residues at the entrance to the ligand-binding pocket of LRH-1, which in rodent LRH-1 is gated by a salt bridge formed between conserved K539 and E440 (indicated). (B) Modeling of human LRH-1 LBD into the (Dax)]<sub>2</sub>:LRH-1 heterotrimer. The structure of human LRH-1 LBD into the (Dax)]<sub>2</sub>:LRH-1 heterotrimer. The structure of some as stick model and denoted as "PL") would replace the salt bridge and assume the same position in the three-dimensional space (indicated). Considering the existence of possible natural ligands for LRH-1, the placement of Dax-1 loop L8–9 in the vicinity of the LRH-1 ligand-binding pocket makes this loop a plausible candidate for a "sensor" of the LRH-1 ligand state and suggests that binding of the second Dax-1 might be controlled by a bound LRH-1 ligand *in vivo*.



**Fig. 57.** Effects of mutations at Dax-1–LRH-1 interfaces on the binding of Dax-1 to LRH-1. (*A*) Coexpressed wild-type and mutant Dax-1 LBD and His<sub>6</sub>-tagged LRH-1 LBD proteins detected by SDS-PAGE in the soluble fractions of bacterial lysates. Dax-1 RH and LRH-1 primary and secondary site mutants are expressed at comparable levels to those of wild-type proteins. (*B*) SDS-PAGE of proteins bound to the affinity Ni-NTA matrix. Wild- type Dax-1 is associated and copurified with His<sub>6</sub>-tagged LRH-1 LBD. Dax-1 RH mutants do not bind to LRH-1. Similarly, single mutations at the LRH-1 primary binding site completely abolish Dax-1 binding. In contrast, mutations at the LRH-1 secondary binding site permit binding. Dax-1 control mutations do not affect the binding between Dax-1 and LRH-1.



**Fig. S8.** SPR sensorgrams showing binding of the Dax-1 monomer to the LRH-1 LBD. Samples of the Dax-1 protein (aa 245–472) at concentrations of 0.5, 1, 2, 4, 6, 8, and  $\mu$ M were injected over the GST-tagged LRH-1 LBD immobilized on an anti-GST chip. Sensorgrams were corrected against responses over a blank reference surface, as well as against responses to injections of 0  $\mu$ M Dax-1 samples. The experimental curves (with sharp transients and spikes removed) and fitted curves (for the one-to-one binding model) are shown in black and red, respectively. The association and dissociation phases and the calculated equilibrium dissociation constant ( $K_d$ ) for the complex are indicated.

## Table S1. Data collection and refinement statistics

Crystallization

PNAS PNAS

Unit cell dimensions	
a, Å	103.3
b, Å	103.3
с, Å	117.4
Space group	P4 <sub>3</sub>
Molecules per asymmetric unit	1 (Dax-1) <sub>2</sub> :LRH-1 heterotrimer
Resolution, Å	3.0
Number of unique reflections	24,369
Completeness, %*	98.4 (96.9)
Data redundancy*	6.0 (3.5)
/or</td <td>18.4 (2.0)</td>	18.4 (2.0)
R <sub>symm</sub> , %* <sup>†</sup>	7.9 (30.6)
Refinement (25.0–3.0 Å)	
$\sigma$ -cutoff	None
R	23.4 (30.7)
R <sub>free</sub> <sup>‡</sup>	26.1 (34.3)
rmsd from ideal	
Bond length, A	0.012
Bond angle, °	1.68
Average B factor, Å <sup>2</sup>	65.7

\*The number in parentheses is for the last resolution shell (3.1–3.0). <sup>†</sup> $R_{symm} = \Sigma_h I_h - I / \Sigma_h I$ , where (I) is the mean intensity of reflection h. <sup>‡</sup> $R_{free}$  is for 5% of the total reflections.