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NR3E receptors in cnidarians : a new family of steroid receptor relatives extends the possible mechanisms for ligand binding

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Highlights :

- Cnidarians do not have vertebrate-type estrogen signaling.
- A cytoplasmic factor triggers nuclear translocation of hER α -GFP in *Hydra* epithelium.
- Medusozoan cnidarians have a specific type of steroid-related receptor, called NR3E.
- Anthozoan cnidarians have lost the NR3E receptor but can produce aromatic steroids.
- NR3E has a cnidarian-specific anchor that can bind to aromatic steroids in a novel way.

Abstract

Steroid hormone receptors are important regulators of development and physiology in bilaterian animals, but the role of steroid signaling in cnidarians has been contentious. Cnidarians produce steroids, including A-ring aromatic steroids with a side-chain, but these are probably made through pathways different than the one used by vertebrates to make their A-ring aromatic steroids. Here we present comparative genomic analyses indicating the presence of a previously undescribed nuclear receptor family within medusozoan cnidarians, that we propose to call NR3E. This family predates the diversification of ERR/ER/SR in bilaterians, indicating that the first NR3 evolved in the common ancestor of the placozoan and cnidarian-bilaterian with lineage-specific loss in the anthozoans, even though multiple species in this lineage have been shown to produce aromatic steroids, whose function remain unclear. We discovered serendipitously that a cytoplasmic factor within epidermal cells of transgenic *Hydra vulgaris* can trigger the nuclear translocation of heterologously expressed human ER α . This led us to hypothesize that aromatic steroids may also be present in the medusozoan cnidarian lineage, which includes *Hydra*, and may explain the translocation of human ER α . Docking experiments with paraestrol A, a cnidarian A-ring aromatic steroid, into the ligand-binding pocket of *Hydra* NR3E indicates that, if an aromatic steroid is indeed the true ligand, which remains to be demonstrated, it would bind to the pocket through a partially distinct mechanism from the manner in which estradiol binds to vertebrate ER.

Keywords : A-ring aromatic steroid, aromatization, steroid receptor, cnidarian

1. Introduction

Nuclear receptors are important regulators of life-history transitions in various phyla of bilaterian animals. They are well documented ligand-activated transcription factors in vertebrates, arthropods and nematodes. However, their role is largely unknown in other metazoan groups such as molluscs or annelids [1]. In cnidarians, following initial identification through PCR screens [2, 3], some nuclear receptors have been implicated as regulators of nervous system development [4, 5] and one

of them was recently shown to be involved in the molecular cascade triggering the polyp-to-jellyfish transition in the scyphozoan medusa *Aurelia aurita* [6]. However, the endogenous ligands, if any, for these cnidarian nuclear receptors are still unclear.

Strictly speaking, cnidarians do not have a bilaterian-like endocrine system, with signaling molecules transported by means of an internally circulating body fluid [7]. However, they do regulate their physiology in response to nutritional and environmental inputs, which also makes them sensitive to environmental pollution, and diffusible signaling molecules are involved in such processes [8, 9].

Historically, numerous efforts have been made to search for vertebrate-like signaling features in cnidarians, especially components of the estrogen signaling pathway, which, in vertebrates, is primarily mediated through the estrogen nuclear receptor belonging to the NR3 family. In particular, aromatization, the final step in the synthesis of estrogens from testosterone (Figure 1A), has been studied in multiple species. Early attempts to detect aromatase activity in cnidarian tissues (scleractinian corals and octocorals), using vertebrate precursors, were unsuccessful [10-12], but subsequently, aromatization activity has been reported in anemones and scleractinian corals [13-15]. Aromatization of exogenous androgen precursors by tissue homogenates from cnidarians or molluscs has been interpreted as evidence for the ability to endogenously synthesize vertebrate-type estrogens [16]. However, analyses of genomic data indicate that cnidarians cannot synthesize estrogens through the same pathways as vertebrates because essential components are lacking in cnidarians. Specifically the CYP19 aromatase is chordate-specific, and the CYP11A1 enzyme responsible for the early cholesterol side-chain cleavage is vertebrate-specific [17]. Moreover, there is evidence that corals can take up vertebrate steroids from the water column, especially around human polluted sites, so estrogen-like compounds detected in cnidarian tissues may have exogenous origins [18, 19]. These observations are also in line with the reinterpretation of the presence of vertebrate-type steroids in molluscs [20, 21]. Arthropod-like ecdysteroids and nematode-like dafachronic acids have also been isolated from corals [22, 23], but have not been viewed as evidence for ecdysozoan-type hormonal signaling in these cnidarians. In case of ecdysteroids, a defensive role and a dietary origin are the main interpretations that have been used to explain the presence and high abundance of those compounds [22]. Similarly, the presence and activity of

gonadotropin-like neuropeptides in cnidarians have been used to draw parallels between vertebrate and cnidarian reproductive signaling cascades [24]. However, here again, phylogenetic analyses indicate that the cnidarian peptides are equally similar to arthropod neuropeptides that do not act in reproductive signaling [25].

To date, ten distinct A-ring aromatized steroids have been identified from four different octocoral species belonging to different genera (*Alcyonium gracillimum*, *Capnella* sp., *Dendronephthya studei*, *Scleronephthya pallida*). Among these steroids are aromatized pregnanes and aromatized C9-C10 secosteroids, which are compounds that have core structures similar to progesterone and vitamin D, respectively [26]. A third group consists of A-ring aromatized steroids bearing a variety of side chains. These have been found in the Taiwanese octocoral *Dendronephthya studei* [27], see also Figure 1. Based on the co-occurrence of A-ring aromatic steroids and corresponding dienones (see compounds (1) and (3) on Figure 1), that bear an additional unsaturation (in yellow on Figure 1) compared to testosterone, we hypothesize that the aromatization reaction in those corals is distinct from that in vertebrates, as proposed for the two other groups [26].

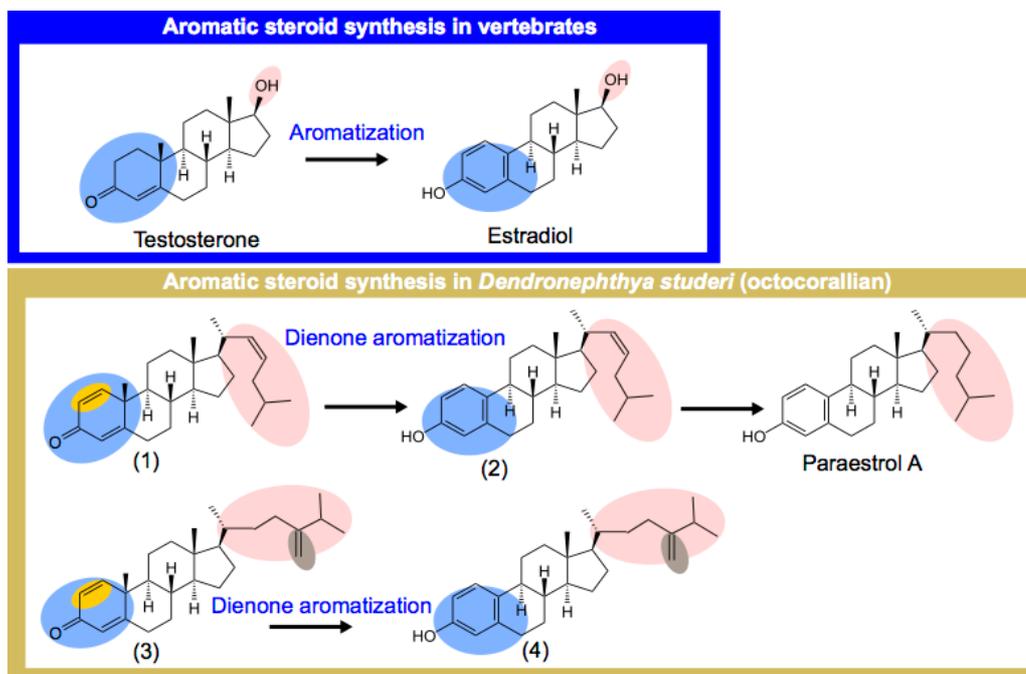


Figure 1. A model for A-ring aromatic steroid synthesis pathway in the octocoral cnidarian *Dendronephthya studeri*, based on the molecules described in Yan et al., 2011, and the knowledge of enzymatic pathways known from other organisms. (1): Cholesta-1,4,22-trien-3-one; (2): (22E)-19-Norcholesta-1,3,5(10),22-tetraen-3-ol; (3): 24-Methylenecholesta-1,4,22-trien-3-one; (4): 24-Methylene-19-norcholesta-1,3,5(10),22-tetraen-3-ol. Paraestrol A (19-norcholesta-1,3,5(10)-trien-3-ol) has been proposed as an ancestral steroid [28]. We have named « dienone aromatization » the proposed aromatization reaction to stress the difference from the vertebrate case where there is no delta1-2 double bond (highlighted in yellow) on the A ring.

Among the steroids present in *Dendronephthya studeri*, one compound has an identical structure to what we hypothesize to be the ancestral NR3 ligand in chordates. This compound indeed binds an ancestral steroid receptor with low affinity [28]. Due to the combination of data discussed above, we have previously interpreted paraestrol A (19-norcholesta-1,3,5(10)-trien-3-ol) and other aromatized steroids from corals as defensive compounds [28]. The diversity of steroids in cnidarians led us to reevaluate the distribution and potential signaling functions of NR3 in the phylum Cnidaria. Our data suggest that NR3 orthologs are present in three of the four major cnidarian lineages and that an endogenous ligand may be present based on heterologous expression of vertebrate ER α in *Hydra*. Together, our data suggest that some ring-A aromatic steroids might function as endogenous signaling molecules in cnidarians, particularly paraestrol A that has the potential to act as a ligand for cnidarian NR3 receptors.

2. Material and Methods

2.1. Sequence analysis

Cnidarian NR3 sequences were identified by tBLASTn and reciprocal BLASTp queries at NCBI GenBank using human ER α and *Trichoplax adhaerens* NR3 (previously identified [29]). In addition to NCBI data, putative NHRs were identified by tBLASTn searches of the *de novo* assembled transcriptomes of *Aurelia*, *Morbakka*, *Chironex*, *Tripedalia* and *Copula* (Khalturin, unpublished data). We required that identified sequences had to be complete or nearly complete predicted nuclear receptor proteins (i.e., both DNA and ligand binding domains) to facilitate phylogenetic analyses. All sequences were downloaded and, if necessary, translated into the correct reading frame. Collected sequences were aligned using Clustal Omega [30] and alignments were checked by eye and edited with Seaview [31]. A phylogenetic tree (Figure 2) was made using PHYML [32], a fast and accurate maximum likelihood heuristic method, using the LG model [33] with a gamma law. Reliability of nodes was assessed by likelihood-ratio test [34].

2.2. Heterologous *hER α -GFP* expression in Hydra

Nucleotide sequence of human estrogen receptor (*hER α*) was adjusted to fit the codon usage of *Hydra vulgaris* with the average GC content reduced to 33%. A synthetic gene (*shER α*) produced by GeneArt (Regensburg, Germany) was inserted into *Hydra* expression vector (ligAC-6) between the actin promoter and EGFP coding region using the SbfI and PacI restriction sites (see Figure 3a). The resulting plasmid ligAL-5 was propagated in *E. coli* and sequenced. DNA for microinjection was prepared using the Plasmid Midi Kit (QIAGEN). Construct DNA was microinjected into fertilized eggs of *Hydra vulgaris* strain AEP as described previously [35]. In about 70% of injected embryos EGFP-positive nuclei were detectable 48 hours after microinjection. Polyps started to hatch 2-3 weeks after microinjection and contained small patches of ectodermal epithelial cells with clear nuclear localisation of the shER α -EGFP protein. Polyps were cultivated under intensive feeding conditions and transgenic patches were enriched by selection of EGFP-positive buds and removal of non-transgenic areas. Within 3 months of selection a polyp culture where ~80% of ectodermal cells were transgenic was obtained (see Figure 3).

2.3. Homology modeling of NR3E and paraestrol A docking

Homology modeling was performed using the program Modeller [36] (version 9.18). The selected structural templates for the homology modeling were the crystal structure of ER α bound to 17 β -estradiol (PDB id: 1ERE) [37], ERR γ in the constitutively active conformation (PDB id: 1KV6) [38] and apo ERR α in its active conformation (PDB id: 3D24) [39]. Missing loops in the ER α structure, namely between residues 331-336 and 462-464, were built prior to homology modeling [40]. Sequence alignment of target and template was performed using the align2d procedure in Modeller, taking into account structural information from the template when constructing the alignment, resulting in 26.2, 29.5 and 25.8% of sequence identity for the modeling using ER α , ERR γ and ERR α respectively. A total of 50 models were generated and the structure with the best DOPE score [41], a statistical potential optimized for model assessment in Modeller, was selected for ligand docking. Examination of the best homology models based on ER α , ERR γ and ERR α revealed that the C-alpha traces nicely follow their respective crystal structure templates with an RMSD of 0.172, 0.289 and 0.293Å respectively after sequence-structure superposition using Pymol software and with 96.48, 95.87 and 96.36% of residues estimated in the Ramachandran favored space using Molprobit server [42]. We noticed that homology modelling of NR3E LBD based on the crystal structure of ERR γ , led to a slight local deformation in a helix turn belonging to helix H10. This is to maintain the structure/sequence conservation in the rest of the model, but this does not affect the ligand-binding pocket and thus does not impact the docking results.

Docking was performed with Autodock4 [43] using the Lamarckian genetic algorithm and consisted of 10 runs per search, with a maximum of 2.5 million energy evaluation per run and a population size of 150. Affinity maps for the receptor were computed using Autogrid4 prior to docking, using the default grid map spacing of 0.375Å. Paraestrol A was superimposed on the 17 β -estradiol ligand in the 1ERE structure, and the homology models on the ER α protein in the same structure. Based on this, residues in the models displaying steric clashes were selected as flexible residues in the docking procedure. In order to compare the estimated free energies of binding, a docking run was performed using the 17 β -estradiol ligand in the ER α crystal structure with the same docking

parameters. Interaction diagram was drawn using LIGPLOT v.4.5.3 [44]. All structure figures were prepared using PyMOL (The PyMOL Molecular Graphics System, Version 1.7.4.0 , Schrödinger LLC).

3. Results

3.1. *NR3E is a novel nuclear receptor type present in three of the four main cnidarian lineages*

We identified new nuclear receptor NR3 sequences from cnidarians that group together as a separate family at the base of the bilaterian NR3s, suggesting they are not particularly related to one of the bilaterian NR3 families (i.e., ER, ERR, or SR). This is also true for the *Trichoplax* sequence initially described as an ERR [29]. We have previously suggested, based on the uncertainty in the phylogenetic relationships between vertebrate ER and sequences from molluscs and annelids described as ERs to rename the protostome clade as NR3D, to avoid misleading inferences that estrogen would be their natural physiological ligand [45]. The most recent analyses of bilaterian NR3s, based on extensive sampling, are in agreement with this view, putting the molluscan and annelid NR3Ds at the base of a clade grouping the vertebrate NR3A and NR3C [46, 47]. Here, NR3A and NR3D group together in a weakly supported node, so we do not think this grouping contradicts these previous studies. Most importantly, the cnidarian sequences follow the known subdivisions between the three clades in which they are present : cubozoans, scyphozoans and hydrozoans (Figure 3). Therefore we propose to name this clade as NR3E. For the same reason, we propose that the *Trichoplax* ERR should be renamed NR3F.

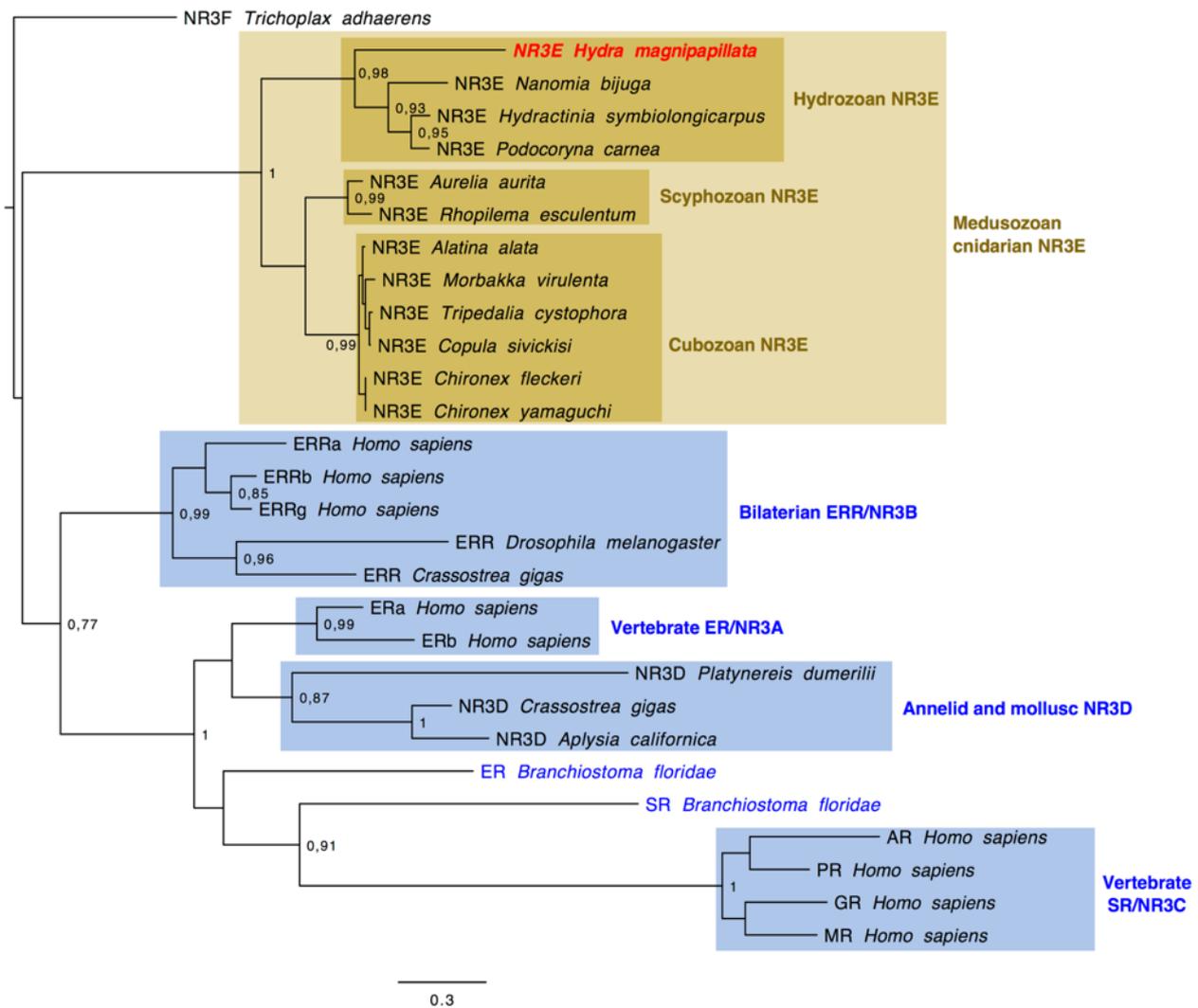


Figure 2. A new NR3E family at the base of the bilaterian ER/ERR/SR clade.

The tree was calculated using maximum-likelihood. Likelihood ratio test branch support values upper than 0.70 are indicated. Bilaterian sequences are shown in blue and cnidarian sequences in brown. The *Hydra* NR3E is highlighted in red. Mollusc and annelid NR3Ds correspond to proteins that were previously labelled as ERs [46]. Similar analyses using the LBD alone gave largely similar results and did not affect the placement of the cnidarian nuclear receptors.

3.2. Heterologous Human ER α -GFP construct translocates into the *Hydra* cytoplasm

In the context of functional studies based on transgenesis in *Hydra* [35], we made an inducible reporter system for *Hydra* based on human ER α . This was based on the assumption that the vertebrate estrogen signaling system does not naturally function within *Hydra*. The approach was to make transgenic animals where the translocation of transcription factors into the nucleus is

controlled by addition of estrogen (in the construct hER α would be fused to the gene of interest). Initially, a construct was made containing hER α fused to green fluorescent protein (hER α -GFP), which theoretically should stay in the cytoplasm in the absence of exogenous estrogen. Surprisingly, hER α -GFP was translocated to the nucleus in *Hydra* even without addition of estrogen (Figure 3). At the same time pure GFP protein never concentrates in the nuclei of transgenic *Hydra*.

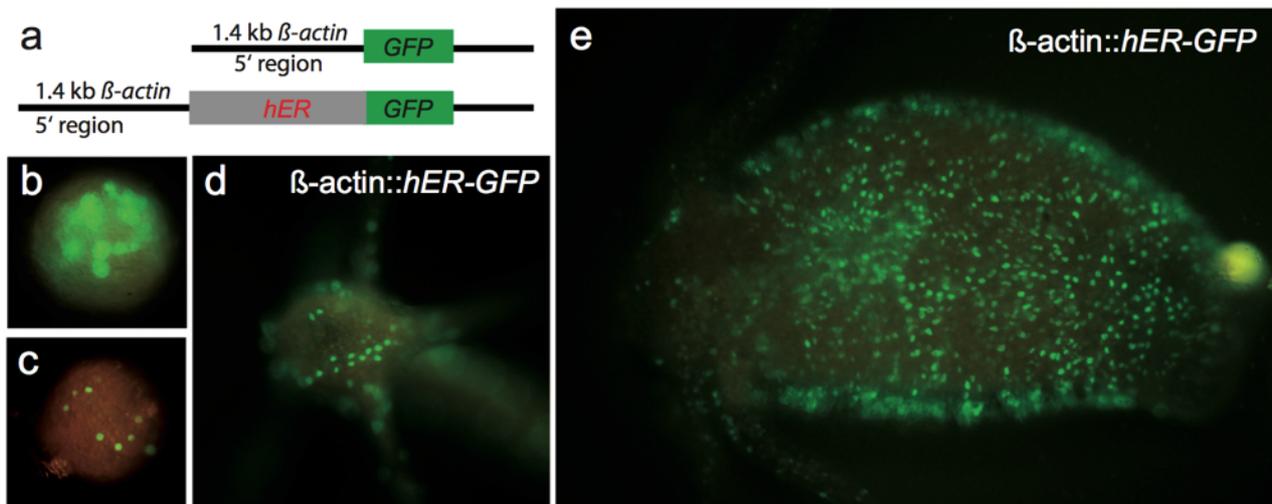


Figure 3. An endogenous cytoplasmic factor induces hER α -GFP translocation from cytoplasm to nucleus in *Hydra* epithelial cells. a) Schematic representation of transgenic constructs with GFP only and with human ER α -GFP driven by 1.4 kb of *Hydra* β -actin promoter. b) One week old *Hydra* embryo injected with the control construct. GFP positive blastomeres are visible. c) *Hydra* embryo injected with actin::ER α -GFP construct. Several GFP positive nuclei are visible and the cytoplasm of blastomeres is not GFP positive. d) Head of transgenic *Hydra* polyp. e) Body column of a transgenic polyp. Green spots are the nuclei of ectodermal epithelia cells which are filled with hER α -GFP without addition of any ligand.

There might be several explanations for the translocation of the hER α -GFP construct. For example, hsp90 and hsp70 of *Hydra* may not bind hER α -GFP properly and thus ER α would go into the nucleus by default. However, the high level of conservation between human and cnidarian Hsp70/90 speaks against "default" translocation. Another plausible scenario is the presence of a cytoplasmic factor in *Hydra* which can specifically bind the ligand-binding pocket of the human estrogen receptor and thus trigger nuclear translocation.

3.3. Docking of paraestrol A inside the ligand-binding pocket of ER α -, ERR γ - and ERR α -based homology models of NR3E

Docking experiments were performed to assess the potential binding of paraestrol A to *Hydra* NR3E. This was done using homology models for *Hydra* NR3E that were developed using the crystal structures of ER α , ERR γ and ERR α as templates. For comparison, we employed the same parameters (see Materials and Methods) for docking the 17 β -estradiol in the ER α crystal structure, estimated the corresponding binding free energy and compared it to that of paraestrol A in the different homology models. In the case of 17 β -estradiol in ER α , the conformation obtained for 17 β -estradiol was nearly identical to the one observed in the crystal structure (rmsd of 0.7Å) and the calculated binding free energy was estimated to be -10.32 kcal/mol.

Considering the *Hydra* NR3E ER α -based homology model, superimposition of paraestrol A onto the steroid core of 17 β -estradiol in the ER α -based homology model shows that paraestrol A fits inside the ligand-binding pocket without any steric clash with the surrounding residues. As a consequence, the initial conformations of the latter residues were maintained throughout the whole docking procedure. The best docking pose obtained had a binding free energy of -11.28 kcal/mol after 10 runs of docking attempts. These observations together with a comparison of the free energy with that of 17 β -estradiol in ER α suggest that paraestrol A would nicely fit inside the ligand-binding pocket of the ER α -based homology model with interactions as described below.

For the ERR γ -based model, ligand superimposition of paraestrol A onto the steroid core of 17 β -estradiol indicates that residues W80 and F35 are in steric clash with the ligand. We therefore considered them as flexible in the docking procedure, while the conformation of the other residues inside the binding cavity was kept as modeled initially. The binding free energy of the best docking pose was estimated to be -9.38 kcal/mol. As in the case of the ER α -based homology model, the results suggest that paraestrol A could be well-accommodated inside the ligand-binding pocket of the ERR γ -based homology model of *Hydra* NR3E LBD with an interaction pattern rather similar to the one observed for the docking in the ER α -based homology model

We further applied the same procedure to the ERR α -based homology model and observed that several residues, especially aromatic ones, were in steric clash with the paraestrol A initial conformation. Despite the fact that the residues in steric clash were allowed to be flexible in the

docking procedure, no reasonable docking pose was obtained, suggesting that successful docking of paraestrol A strongly depends on the nature of the homology model used and further supports the validity of the docking predictions obtained in the ER α - and ERR γ -based homology models.

3.4. Interactions of paraestrol A with residues inside the ligand-binding pocket

In the ER α -based homology model, paraestrol A is globally positioned in a similar manner as is 17 β -estradiol in its cognate receptor (Figure 4a). In fact, the aliphatic side chain of paraestrol A is oriented towards the helices H7 and H11, while the steroid core is positioned between helices H3 and H5 and rather close to the first strand of the β -sheet (Figure 4a). Several hydrophobic residues, including aromatic ones form stabilizing interactions with the ligand. In addition, a remarkable π - π interaction is observed between Trp80 in helix H5 and the steroid core (Supplemental Figure 1); this residue and the corresponding stabilizing interaction are specific to NR3E (Figure 5).

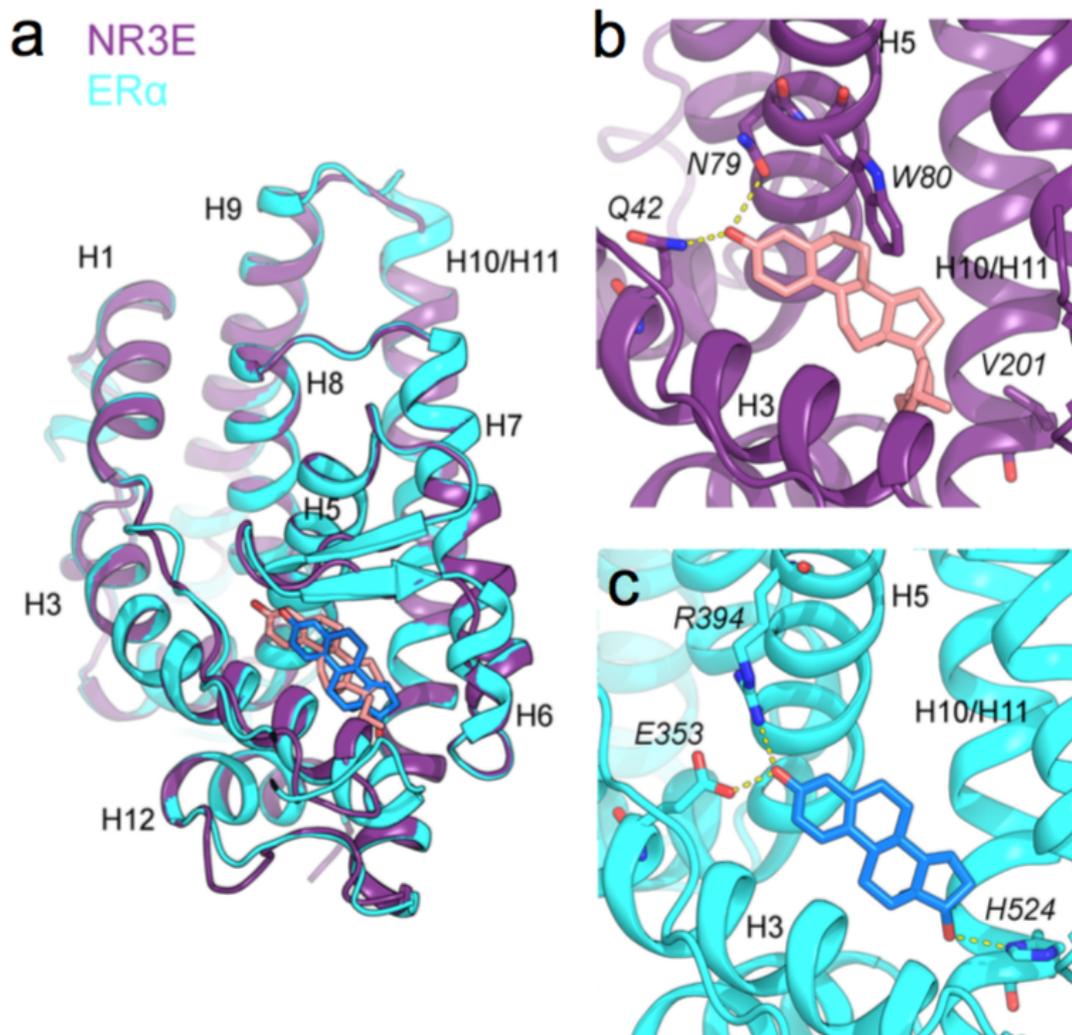


Figure 4. Paraestrol A docking into the ligand binding pocket of an estrogen receptor α homology-based model of *Hydra* NR3E. (a) Superposition of the NR3E structural model using ER α structure (in violet) where the paraestrol A is represented in pink, the ER α structure used as template (PDB id: 1ERE) is represented in cyan and the 17 β -estradiol in blue. (b) Detailed view of the binding pocket of the NR3E ER α -based model, highlighting interactions (in yellow dashed lines) between the paraestrol and residues Q42 and N79. W80 involved in the cnidarian-specific π - π interaction is also shown. (c) Detailed view of the binding pocket of ER α , highlighting interactions (in yellow dashed lines) between the 17 β -estradiol and residues E353, R394 and H524 (corresponding to V201 in NR3E model).

We further observe that the 3-OH group of paraestrol A is hydrogen-bonded to Asn79 (H5) with a donor-acceptor distance of 2.9Å, and examination of the surroundings of this interaction shows a potential additional hydrogen bond with Gln42 (H3) (Supplemental Figure 1). Both residues could form a clamp that would strongly position the ligand inside the pocket. To test this hypothesis, we started with the paraestrol A docked conformation in the ER α -based model and performed additional docking runs where both Gln42 and Asn79 were allowed to be flexible. Interestingly, the best docked conformation in this case showed structural adaptation of these two residues, which are then both implicated in hydrogen bond formation with the 3-OH group of paraestrol A (Figure 4b), with estimated binding free energy of -11.89 kcal/mol, and a similar π - π interaction with Trp80 in H5 as in the previous docking result.

	H3			H4-5		
NR3E Hydra magnipapillata	GTLTHKFGYGT	IGKQLIHIID	WAKRIPGYSS	LILSDQAVLL	QATWVELMVA	NWLFL
NR3F Trichoplax adhaerens	IRTTIICEM	VERE.VMV.T.	.C.N..V...	..S.L.VFMI	DLA.R
NR3E Hydractinia symbiolongicarpus	R...Q..SW.	VDQ..V...	...QL...CN	.VI....I..	..A..D.LAL	..VYH
NR3E Aurelia aurita	KPA.SSLCDI	V...AA..	...GL...ET	.T.N...I..	..G.I.MLLL	..I.Y
NR3E Rhopilema esculentum	-----LTLDI	V...VAA..	...GL...ET	.T.N...I..	..G.I.MLLL	..T.Y
NR3E Podocoryna carnea	R..VQN.CW.	VDQ..V...	...QL...CN	.VI....I..	..A..D.L.L	..VYH
NR3E Nanomia bijuga	E..IQ..CF.	VDQ..VR...	...QL...CN	.SI....I..	..A..D.L.L	..VYH
NR3E Chironex fleckeri	VIT.GYLCKQ	.S...VAV..	...GL...DT	.T.N...I..	..G.I.LLL	..V..
NR3E Alatina alata	VMT.GYLCKQ	...VAV..	...GL...DT	.T.N...I..	..G.I.LLL	..V..
NR3E Morbakka virulenta	TMT.GHLCKQ	..R..VAV..	...GL...DT	.T.N...I..	..G.I.LLL	..V..
NR3E Tripedalia cystophora	VMT.GYLCKQ	..R..VAV..	...GL...DT	.T.N...I..	..G.I.LLL	..V..
NR3E Copula sivickisi	VMT.GYLCKQ	..R..VAV..	...GL...DT	.T.N...I..	..G.I.LLL	..V..
NR3E Chironex yamaguchi	VIT.GYLCKQ	.S...VAV..	...GL...DT	.T.N...I..	..G.I.LLL	..V..
ERRa Homo sapiens	LPVATLCLD	FDREIVVT.S	...S...F..	.S...MSV.	.SV.M.VL.L	GVAQR
ERRb Homo sapiens	IKALTTLCDL	ADRE.VV..G	...H...F..	.S.G..MS.	..S.A.M.ILIL	GIVYR
ERRg Homo sapiens	IKALTTLCDL	ADRE.VV..G	...H...F..T	.S.A..MS..	..S.A.M.ILIL	GVVYR
ERR Drosophila melanogaster	NEILSVLSDI	YD.E.VSV.G	...Q...FID	.P.N..MK..	..VS.A.I.LTL	QLT.R
ERR Crassostrea gigas	VRLATVSDL	ADRE.VIT.S	...QV...FCT	.S...MN..	..HS.L.I.LCL	..LV.R
NR3D Crassostrea gigas	VH.LNSLVKL	AERE.V.L.N	..NV...TD	.S...VH.I	ECC.M..LLL	..CA.R
NR3D Aplysia californica	VH.LNTLIK	ADRE.VYL.N	..HV...TC	.T.G..VH.I	ECC.M..LLL	..CA.R
NR3D Platynereis dumerilii	YS.L.RLI.L	ADME.VDVVN	..VL...F.G	.E.R.RIAI.	ESC.M..LCI	GAAWR
ER Branchiostoma floridae	PE.IESVSSL	VDRE.TG..C	.G.K....K	.S.N..VL.M	ES..LD.LIL	DLVWC
ERa Homo sapiens	ASMMGLLTLN	ADRE.V.M.N	...V..FVD	.T.H..VH.	ECA.L.ILMI	GLVWR
ERb Homo sapiens	ASMMMSLTKL	AD.E.V.M.S	...K...FVE	.S.F..VR..	ESC.M.VLMM	GLMWR
SR Branchiostoma floridae	.Y.MALVTDL	ANREIEGLV.	..A..L...GM	.PMD..VN.I	RTV.LD.LML	GLVWR
GR Homo sapiens	WRIMTTLNML	G.R.V.AAVK	...A...FRN	.H.D..MT..	.YS.MF..AF	ALGWR
MR Homo sapiens	EN.LSTLNRL	A...M.QVVK	..VL...FKN	.P.E..IT.I	.YS.MC.SSF	ALSWR
AR Homo sapiens	AA.LSSLNEL	GER..V.VVK	..AL..FRN	.HVD..MAVI	.YS.MG...F	AMGWR
PR Homo sapiens	SS.LTSLNQL	GER..LSVVK	.S.SL..FRN	.HID..IT.I	.YS.MS...F	GLGWR

	H10-H11		
NR3E Hydra magnipapillata	-KENPLKFAK	IVLILPQLKY	LVNEVLKVLH ALNS
NR3F Trichoplax adhaerens	-----	-----	-----
NR3E Hydractinia symbiolongicarpus	.R...Q....	L.....	M..Q.IEY.Y TMKM
NR3E Aurelia aurita	.PDQ.Q....	.I.....	V..N.IEYFY RVKL
NR3E Rhopilema esculentum	.PDQ.Q....	.I.....C	V..N.IEYFY RVKL
NR3E Podocoryna carnea	.RD..Q....	L.....	M..Q.IEY.Y KMKM
NR3E Nanomia bijuga	.R.S.Q....	L.....	..R.IEL.T KMKM
NR3E Chironex fleckeri	.PDK.Q....	V..H.IEF.Y RVKL
NR3E Alatina alata	.PDK.Q....	V..H.IEF.Y RVKL
NR3E Morbakka virulenta	.PDK.Q....	V..H.IEF.Y RVKL
NR3E Tripedalia cystophora	.PDK.Q....	V..H.IEF.Y RVKL
NR3E Copula sivickisi	.PDK.Q....	V..H.IEF.Y RVKL
NR3E Chironex yamaguchi	.PDK.Q....	V..H.IEF.Y RVKL
ERRa Homo sapiens	GGAERRRAGR	LL.T..L.RQ	TAGK..AHFY GVKL
ERRb Homo sapiens	-.E.WRTG.	LL.T..L.RQ	TAAKAVQHfy SVKL
ERRg Homo sapiens	-.D.RRAG.	MLMT..L.RQ	TSTKAVQHfy NIKL
ERR Drosophila melanogaster	.SSAVSHQQQ	LL.L..S.RQ	ADDILRRFWR GIAR
ERR Crassostrea gigas	-.G.LRRLGH	LYML..A.NH	MKLLAKQYWF DVKK
NR3D Crassostrea gigas	HPD.VRHVPA	VL.L.THIRQ	AGERGIAFFQ R.K.
NR3D Aplysia californica	GFG.WRHAPS	.L.L.THIRQ	AGERGITYFQ K.KM
NR3D Platynereis dumerilii	SESSCVRM.Q	.LC...FARQ	VSLKAITH.F NMHN
ER Branchiostoma floridae	.ALGSRRP..	ML.L.SH.RQ	VSARASSH.G .VRN
ERa Homo sapiens	LQQHQRL.Q	LL...SHIRH	MS.KGMEH.Y SMKC
ERb Homo sapiens	SQQQSMRL.N	LLML.SHVRH	AS.KGMEH.L NMKC
SR Branchiostoma floridae	-.FS.GNI.R	LMM.VS.VRQ	SSLGVDH.N R.RG
GR Homo sapiens	SSQ.WQR.YQ	LTKL.DSMHE	V.ENL.NYCF QTFL
MR Homo sapiens	SGSQWR.YQ	LTKL.DSMHD	..SDL.EFCF YTFR
AR Homo sapiens	PTSCSRR.YQ	LTKL.DSVQP	IAR.LHQFTF D.LI
PR Homo sapiens	VVSSSQ.YQ	LTKL.DN.HD	..KQLHLYCL NTFI

Figure 5. Alignment of H3, H4-H5 and H10-H11 helices in the NR3 family, compared to NR3E from *Hydra*. Residues involved in estrogen binding in ER are highlighted in green. Homologous residues that are shared by the cnidarian NR3E and the vertebrate oxosteroid receptors (SRs) are highlighted in pink. The cnidarian-specific N79-W80 anchor is highlighted in brown. An alignment for the entire DNA-binding domain and ligand-binding domain is shown in Supplemental Figure 2. The helices from the ligand-binding domain are mapped according to the structure of human ER α [48]. Calculations of identity percentages for both domains are also provided in Supplemental Table 1.

The relevance of these observed interactions is emphasized by the remarkable conservation of residues W80, Q42 and N79 in cnidarian NR3 sequences (Figure 5). In fact, W80 is strictly conserved and specific to the cnidarian NR3E, while being replaced by M/L/V/I/C/A in bilaterian steroid receptors and ERRs as well as in *Trichoplax* NR3F. Furthermore, the anchoring residues Q42 and N79 are strictly conserved in all cnidarian NR3E sequences. Q42 is also present in human oxosteroid receptors, but replaced by E in human ERs and ERRs, as well as in *Trichoplax* NR3F. N79 is also lineage-specific, being strictly conserved in cnidarian NR3E, and replaced by G/D/A/Q/N in bilaterian steroid receptors and in *Trichoplax* NR3F. In human ER α bound to 17 β -estradiol, the 3-OH group is stabilized by an electrostatic anchor formed by two conserved residues E353 (H3) and R394 (H5) (Figure 4c). These residues are crucial for the positioning and the stabilization of the natural 17 β -estradiol ligand inside its cognate ligand-binding pocket. In the case of cnidarian NR3E, the anchor is made of Q42 and N79. In this case, the 3-OH group of paraestrol A acts both as an acceptor and a donor for these residues. An inspection of the multiple sequence alignment together with the superimposition of the homology model with the crystal structure indicate that Q42 in H3 is the cnidarian equivalent of human E353 that forms the first half of the electrostatic anchor. On the other hand, the second half of the anchor differs in the two cases. Residue N79 of *Hydra* NR3E is located one helix turn upstream of the position occupied by R394 and replaced by G in human ER α (Figure 4b and 4c). Altogether, we observed that the 3-OH functional group of paraestrol A is in an adequate environment to form complementary interactions with the conserved electrostatic Q(H3)/N(H5) anchor and further stabilized by π - π interaction with the cnidarian-specific W80 residue located in H5.

It is noteworthy that in our second set of docking experiments, the two best docked structures display hydrogen-bonding with Q42 and N79, however in the third one, the distance between 3-OH group of paraestrol A and the amide group of Q42 increases to 4.8Å, leading to a severe displacement of the ligand in the pocket, with the aliphatic side chain replacing the position of the aromatized A-ring. This structure supports the crucial role of the anchor in positioning of the ligand in the pocket, as strongly suggested by the sequence conservation of these residues.

The positioning of paraestrol A inside the ligand-binding pocket of the ERR γ -based homology model is similar as is its general interaction pattern with surrounding residues in the best docked

conformation with an estimated binding free energy of -9.38 kcal/mol. Even though the exact position of paraestrol A is slightly more towards the outside of the pocket and slightly shifted between helices H3 and H5, W80 still can form a π - π interaction and a hydrogen bond with Q(H3) (Supplemental Figures 3a and 3b). We then performed a similar docking experiment where this latter structure was used and both residues Q42 and N79 are allowed to be flexible in the search. Similarly to the previous results, adjustment of the side chains of these residues permits in the best model hydrogen bonding with the 3-OH group of the paraestrol, leading to a more favorable binding free energy of -11.50 kcal/mol (Supplemental Figure 3b). The three conserved residues that were shown to be crucial for the interaction of the ligand with the ER α -based homology model hence fulfill the same role in the ERR γ -based homology model.

Altogether, our docking studies of paraestrol A in NR3E homology models suggest that paraestrol A can be readily accommodated in the ER α -based model and depicts a binding free energy that is of the same order of magnitude as the one calculated for 17 β -estradiol in ER α . Paraestrol A can also fit inside the ligand-binding pocket of ERR γ , with comparable energy. In both cases, we have highlighted cnidarian-specific residues that interact with the ligand, among which two polar residues, Q(H3)/N(H5), that define a novel anchor that stabilizes paraestrol A inside the pocket.

4. Discussion

4.1. Novel cnidarian-specific candidate players for a steroid signaling pathway

All evidence to date has shown that the vertebrate-type estrogen-ER signaling pathway is not present in cnidarians [28]. However, recent findings, in the literature and the data presented in this study indicate the presence both of aromatic steroids and nuclear receptors within a novel subclade of the NR3 family (NR3E). Previous surveys of nuclear receptor diversity in cnidarian have largely studied anthozoans due to availability of sequence data as well as interest in possible effects of environmental pollution on reef-building corals. Our analysis here used the availability of sequence

data from throughout the phylum and revealed NR3 members in the other three cnidarian classes that form the medusozoan clade. The presence of these genes helps to better resolve the history of the NR3 family in animals because NR3 was present in the placozoan *Trichoplax*, absent from surveyed ctenophores and sponges, and until this study, inferred to absent from cnidarians. Our hypothesis is thus that NR3 evolved in the common ancestor of the placozoan and cnidarian-bilaterian with lineage-specific loss in the anthozoans.

Despite the presence of NR3 genes in cnidarians, it has not yet been demonstrated whether these aromatic steroids or any other compounds can serve as ligands for NR3E receptors. Interestingly, aromatic steroids have only been documented within a few species of anthozoans but NR3E genes are only present within the medusozoa (Hydrozoa, Cubozoa, Scyphozoa). Similarly, NR2B (RXR) homologs have been lost from the Anthozoa and are only present within Medusozoa. The loss of these two receptors from the anthozoan lineage is of considerable interest and may be related to the loss of the medusa (jellyfish) stage. This step implied the loss of metamorphosis between polyp and medusa, and/or the reduction of sensory organs (eyes). Regarding aromatic steroids in anthozoans, data about their quantitative variation under physiological conditions will be necessary to determine if they play a defensive function, or if they may be involved in endogenous intercellular signaling. If they do play any role in endogenous signaling, it could be mediated through binding to different nuclear receptors from other families than NR3, or to membrane receptors [49]. The translocation data on the shER α -GFP heterologous construct expressed in *Hydra* epithelial cells suggests that there may be a cytoplasmic factor that binds to shER α -GFP and triggers its translocation into the nucleus. Because *Hydra*, an hydrozoan, as other medusozoans, has an endogenous NR3E, which is the closest relative to ER and other vertebrate steroid receptors, we thought it would be important to explore its ability to bind an aromatic steroid which is present in another cnidarian, even from a distantly related species.

4.2. Cnidarian-specific molecular mechanisms for ligand binding in the NR3E pocket

Docking experiments with paraestrol A inside the ligand-binding pocket of *Hydra* NR3E indicate that this receptor might indeed bind an aromatic steroid. In this case, the binding would involve π - π interactions with a tryptophan residue, which is conserved in all cnidarian sequences, and a glutamine residue which, to date, was mainly correlated to the binding of oxosteroids, like

progesterone, androgens and corticoids. Thus, even the most estrogen-like aromatic steroid from cnidarians shows binding properties that are distinct from the mechanism of estradiol binding to the vertebrate estrogen receptor. This strongly suggests that identification of endogenous ligands for the cnidarian NR3E receptors and their concomitant functional characterization will enlarge our knowledge of the diversity ligand-binding interactions in play between steroid hormones and their cognate nuclear receptors. Over the last few years, a great deal of information has accumulated about physiologically relevant alternative ligands for the human estrogen receptors [50]. In particular, steroids without an aromatic A-ring were also reported to be able to bind ER. So far, all those described alternative steroid ligands have a hydroxy group on carbon 3, just like the hydroxy group on carbon 3 of estradiol. Interestingly, the distinction between binding of steroids with a 3-hydroxy group, like estrogens, and binding of steroid with a 3-oxo group, like androgens, progesterone and corticoids, has long been correlated to the distinction between the E and Q ligand-binding residues in the H3 part of the ligand-binding pocket, and interpreted as an evidence for more ancient origin of estrogen as a ligand relative to oxosteroids [51]. Docking experiments with NR3E and paraestrol A indicate that, structurally speaking, nothing prevents a 3-hydroxysteroid from binding the pocket through a Q residue, which is classically related to 3-oxosteroid binding. Therefore, the distinction between the two classes of molecules may not be so clear-cut, and even in vertebrates, we could find unexpected new selective estrogen receptor modulators by looking at endogenous 3-oxosteroids. Many of them are indeed present in the human body as precursors in bile acid synthesis [52].

4.3. Long distance chemical communication in the cnidarian body

Because they lack an internal circulating body fluid, cnidarians cannot have a vertebrate-like hormonal system. This does not necessarily preclude steroid-mediated intercellular signaling in those animals. Indeed, even in vertebrates, some steroids do not act through the canonical hormonal pathway. Classically, hormones are defined as internally circulating molecules, which excludes vertebrate bile acids based on this definition [53]. However, this view is changing with the acknowledgment of their functional role not only as facilitators of lipid digestion, but also as signaling molecules, that allow the coupling of the nutritional state with various parameters, from digestive physiology to behavioural traits such as the regulation of appetite [54]. This example

shows that there is no objective reason to strictly limit long-distance intercellular communication to the internal milieu. We thus hypothesize that in cnidarians, the digestive surfaces may be a major vehicle for signaling molecules, and that the steroids identified in these animals, if they really have a role in distant intercellular communication, could be transported in this way. The gut lumen is sometimes viewed as the continuation of the external milieu, because both are communicating through the oral openings. Thus, secretions of molecules in the gut are considered as exocrine secretions. The real chemical composition of some parts of the digestive tract is distinct from the external environment, including secretions and selective absorption processes from the digestive surfaces of the animal. The gut goes throughout the body for some cnidarians and is restricted to particular regions in others (e.g. mesenteries) and could be a carrier of signaling molecules between distant body parts or different ramets in colonial species (Figure 6). Anyways, caution is needed before extrapolating anatomical knowledge from bilaterians to cnidarians, because the homology between the germ layers is debated [55].

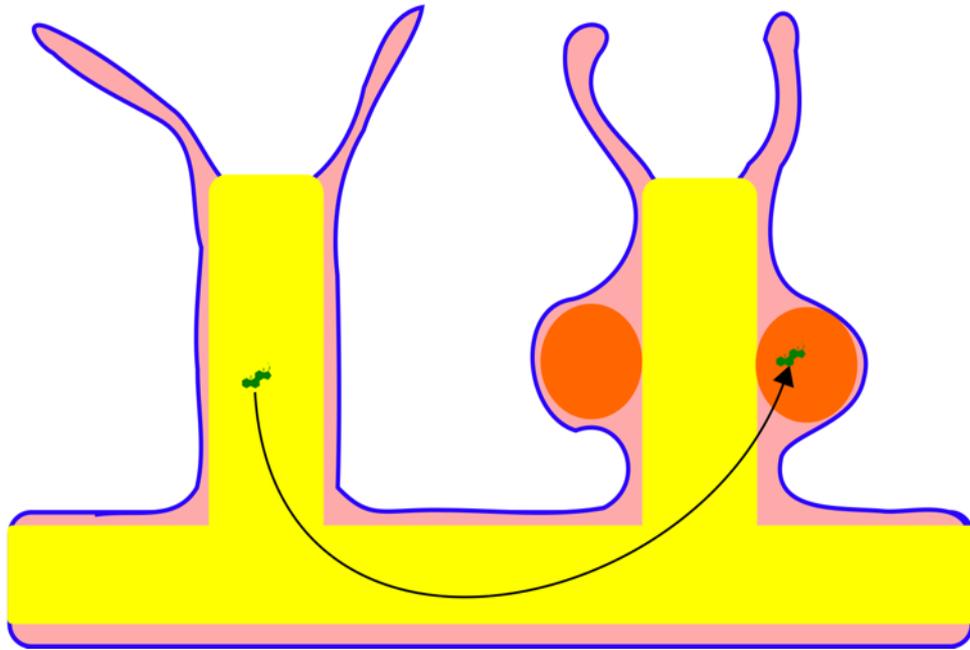


Figure 6. A possible pathway for steroid enterocrine signaling in cnidarians. Steroids, in green, could be produced from dietary sterols in the gut (in yellow) and go to the gonad (in orange) along with other nutrients. Gonads may be of ectodermal or endodermal origin, depending on the species. The pink tissue represents the mesoglea, which consists of a gelatinous matrix that contains collagen fibers and usually some cells. The mesoglea forms a hydrostatic skeleton, but does not contain a circulating body fluid or play a known role in circulation.

Regulation of reproduction and metamorphosis have already been proposed as potential roles for cnidarian nuclear hormone receptors. Regulation of energetic metabolism and growth is another prominent function of many nuclear receptors that has not yet been investigated in cnidarians. Indeed, within the NR3 family, ERR plays important and diverse roles in energetic homeostasis in both protostomes and deuterostomes [56, 57]. In cnidarians, regulation of energetic metabolism is of considerable interest, but the mechanisms are still poorly understood. Many species are tightly regulating their body size based on nutrition intake. For example, many anthozoan anemone species can survive several months without food, during which they can exhibit negative growth, consume stored lipids and reduce their metabolic rates [58-61]. Studies with the hydrozoan *Hydra* suggest an important role of autophagy during starvation. Starving hydras do not die, but simply shrink proportionally [62]. For both anemones and *Hydra*, growth rapidly resumes following feeding

(references cited in two preceding sentences and personal observations of authors). Hence, there must be some regulators and nuclear receptors are among the best candidates.

5. Conclusions

While molecular studies of steroid signaling in cnidarians are still in early stages, the fragmentary data are already sufficient to state that, despite some similarities to bilaterians, cnidarians have lineage-specific receptors and potential ligands. A detailed look at the mechanisms of steroid signaling and receptor function is certain to give new insights on the many different possible ways to achieve interactions between a receptor and a steroid ligand. Obviously, this also holds true for other chemical classes of possible ligands. In comparison to anthozoans, very few chemical analyses have targeted the steroid composition of medusozoan cnidarians [63-65]. There is growing awareness among the global metabolomics community that « time is ripe to focus on model organism metabolomes » [66]. Along these lines, metabolomic approaches have already proven extremely useful in understanding phenotypic plasticity in a mediterranean zoanthid coral [67]. We hope that our results will stimulate interest of natural product chemists in identifying potential endogenous ligands for cnidarian nuclear receptors and particularly in determining whether A ring aromatic sterols are present in *Hydra* and other medusozoan cnidarians, as well as other molecules that could function as endogenous ligands to cnidarian nuclear receptors. This will not only illuminate the field of cnidarian zoology but, more widely, the field of nuclear receptor pharmacology to uncover new mechanisms of action and novel signaling molecules.

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Supplemental information for

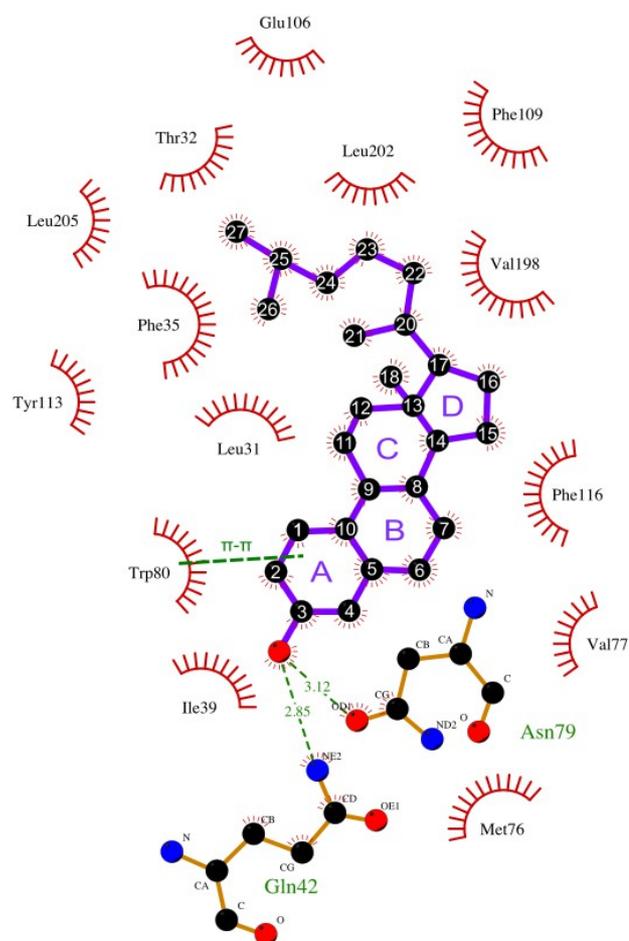
**NR3E receptors in cnidarians : a new family of steroid receptor relatives
extends the possible mechanisms for ligand binding**

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This PDF file includes :

- Supplemental Figure 1. NR3E-paraestrol A interaction diagram. P2
- Supplemental Figure 2. Full-length alignment of cnidarian NR3E with already known members of the NR3 family. p3-9
- Supplemental Table 1. Identity percentages of DBDs and LBDs when compared to NR3E from *Hydra*. p10
- Supplemental Figure 3. Paraestrol A docking into the ligand binding pocket of estrogen related receptor γ homology-based model of *Hydra* NR3E. p11



Supplemental Figure 1. NR3E-paraestrol A interaction diagram.

The interactions shown are those mediated by hydrogen bonds, hydrophobic contacts and π - π interaction. Hydrogen bonds with the amino acids from the NR3E binding pocket are indicated by dashed lines between the atoms involved, while hydrophobic contacts are represented by an arc with spokes radiating towards the paraestrol A atoms they contact. The contacted atoms are shown with spokes radiating back. The figure was drafted using LIGPLOT v.4.5.3 and manually edited to add the paraestrol A carbon and ring numbering according to the IUPAC sterol nomenclature.

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NR3E Hydra magnipapillata	-----	-----	-----	-----	-----	-----
NR3F Trichoplax adhaerensMTSTF....ASHS	G....TPIEP	V.....
NR3E Hydractinia symbiolongicarpus
NR3E Aurelia aurita
NR3E Rhopilema esculentum
NR3E Podocoryna carnea
NR3E Nanomia bijuga
NR3E Chironex fleckeri
NR3E Alatina alata
NR3E Morbakka virulenta
NR3E Tripedalia cystophora
NR3E Copula sivickisi
NR3E Chironex yamaguchi
ERRa Homo sapiens
ERRb Homo sapiens
ERRg Homo sapiens
ERR Drosophila melanogaster
ERR Crassostrea gigas
NR3D Crassostrea gigas
NR3D Aplysia californica
NR3D Platynereis dumerilii
ER Branchiostoma floridae
ERa Homo sapiensMTMTLHTKA	SGMALLHQIQ	G....NELEP	L.....
ERb Homo sapiensMDIKNSP	SSL.....NSPSS	Y.....
SR Branchiostoma floridaeMENQP	NTVTNPMTT.QGLQP	L.....
GR Homo sapiens	PD..TKPKIK	DNGDLVLSSP	SNVTLPQVKT	E.....K.....E
MR Homo sapiens	PIKQESTKHS	CSGTSFKGNP	TVNPFPMFDG	S.....YF.S	F.MDDKDYYS	LSGILGPPVP
AR Homo sapiens	TKGLEGESLG	CSGSAAAGSS	GTLELPSTLS	LYKSGALDEA	AAYQSRDYYN	FPLAL.....
PR Homo sapiens	PR.....SSP	CASSTPVAVG	DFPDCAYP.P	DAEPKDDAYP	LYSDFQPPAL

541

NR3E Hydra magnipapillata	-----	-----	-----	-----	-----MSDYN	RD-----
NR3F Trichoplax adhaerensTSASDQL	VFTSTAS...	.SR..LDKGV	DSGTTT.SDS	KN.....
NR3E Hydractinia symbiolongicarpus
NR3E Aurelia aurita
NR3E Rhopilema esculentum
NR3E Podocoryna carnea
NR3E Nanomia bijugaM	DSAELVVPFK	KR.....
NR3E Chironex fleckeri
NR3E Alatina alata
NR3E Morbakka virulenta
NR3E Tripedalia cystophora
NR3E Copula sivickisi
NR3E Chironex yamaguchi
ERRa Homo sapiens
ERRb Homo sapiens
ERRg Homo sapiensM	DSVELCLPES	FS...LHYE
ERR Drosophila melanogaster
ERR Crassostrea gigas
NR3D Crassostrea gigas-MEAK	YHDQ.QFEDA
NR3D Aplysia californica
NR3D Platynereis dumerilii
ER Branchiostoma floridae
ERa Homo sapiensNRPQLKIP..LERP.	.LGEVYL.SS	KPA..VYNYP
ERb Homo sapiensNCSQSILP..LE...	.HGSIIYIPSS	YVDS.HHEY
SR Branchiostoma floridaeAYQQGYI	VQQQQPS...	..QPYRLPSPI	QPEFVVISN.	FP.....VG
GR Homo sapiens	DFIELC....TPGVIKQE	KLGTVYCQAS	FPGANII..G
MR Homo sapiens	GFDGNCESG.	GFPVGIKQEP	DDGSYYPEAS	IPSSAIV..G
AR Homo sapiensAGPPPPP	P..PPHPHAR	IKLENPLDYG
PR Homo sapiens	KIKEEEEGAE	ASARSPRSYL	VAGANPAAFP	DFPLGPPPP.	..LPPRATPS	.PGEAAV..T

Supplemental Figure 2

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NR3E Hydra magnipapillata	-----	-----DSYVD	VECVH-----	-----QD	E-----INE	-----GDKN
NR3F Trichoplax adhaerensDNIT.LHN	S--.DRIQPL	P...LAGLPI	Y.....TAPTPAS
NR3E Hydractinia symbiolongicarpus	-----	---MD.....	-----	-----SEASHTA
NR3E Aurelia aurita	-----	-----	-----	-----	-----
NR3E Rhopilema esculentum	-----	-----	-----	-----	-----
NR3E Podocoryna carnea	-----	-----	-----	-----	-----
NR3E Nanomia bijugaNNL.AVFN	SNKMETDEVV	P..PFSSKSN	K.....SESES.
NR3E Chironex fleckeri	-----	-----	-----	-----	-----
NR3E Alatina alata	-----	-----	-----	-----	-----
NR3E Morbakka virulenta	-----	-----	-----	-----	-----
NR3E Tripedalia cystophora	-----	-----	-----	-----	-----
NR3E Copula sivickisi	-----	-----	-----	-----	-----
NR3E Chironex yamaguchi	-----	-----	-----	-----	-----
ERRa Homo sapiensMSSQ---.V	GIEPLYIKAE	PASPDSP.--	-----KSSE
ERRb Homo sapiensMSSD.RHLG	SS.GSFIKTE	PSSPSSGIDA	LSHHSP--SSSD
ERRg Homo sapiens	E.....	ELLC RMSNK.RHI.	SS.SSFIKTE	PSSPASLTDS	VNHHSP--GSSD
ERR Drosophila melanogaster	-----MS.	GVSILHIKQE	VDTPSASCFS	PSSKSTATQSTNG
ERR Crassostrea gigas	-----	---MD.DIIKME	PGSPLGLSRC	GHS.....	-----LS
NR3D Crassostrea gigas	L.....	TVIQ GLSDPGG-TV	CRILRESSAA	ASATGDEA--	..E...SMSESI
NR3D Aplysia californicaMVSPS---P	CMP.LPL...	.SGAGGGG--	..E...GTASAH
NR3D Platynereis dumerilii	-----	-----	-----	-----	-----
ER Branchiostoma floridae	-----	---MAYPDIHII	P..PQGQTPR	M.....TTPTKAA
Era Homo sapiens	E.....	GAAY EFNAAAA-AN	AQ-.YGQTGL	PYGPGEAAA	FGSN...GLGFPP
ERb Homo sapiens	A....	MTFY . . SPAV-MN	YSIPSNVTNL	EGGPGRQ.--	-----	-----
SR Branchiostoma floridae	N.....	MTQS QMSQ.-----	-----	PRGNDCAL.RN.SSTA
GR Homo sapiens	NK.....	.MSAI.VHG	-----S	TSGGQMYHY.	MNTASL.--S	QQ..QDQKPI
MR Homo sapiens	V.....	-----	-----	NSGGQSFHYR	IGAQTISLS	RSARDQSFQH
AR Homo sapiens	SAWAAAAQC	RYGDLA.LHG	A-----G	AAGP.....	-----	-----APPQ
PR Homo sapiens	AA.....	.PASA.VSS	A-----S	SSGSTLECLT	YKAEG.---APPQ

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NR3E Hydra magnipapillata	L-----	-----	-----	-----	-----	-----
NR3F Trichoplax adhaerens	-----	-----	-----	-----	-----
NR3E Hydractinia symbiolongicarpus	M.....	-----	-----	-----	-----	-----
NR3E Aurelia aurita	-.....	-----	-----	-----	-----	-----
NR3E Rhopilema esculentum	-.....	-----	-----	-----	-----	-----
NR3E Podocoryna carnea	-.....	-----	-----	-----	-----	-----
NR3E Nanomia bijuga	S.....	-----	-----	-----	-----	-----
NR3E Chironex fleckeri	-.....	-----	-----	-----	-----	-----
NR3E Alatina alata	-.....	-----	-----	-----	-----	-----
NR3E Morbakka virulenta	-.....	-----	-----	-----	-----	-----
NR3E Tripedalia cystophora	-.....	-----	-----	-----	-----	-----
NR3E Copula sivickisi	-.....	-----	-----	-----	-----	-----
NR3E Chironex yamaguchi	-.....	-----	-----	-----	-----	-----
ERRa Homo sapiens	-.....	T.ETE.....	-----	-----	-----	-----
ERRb Homo sapiens	-.....	A.SGGF..GL	A.....	-----	-----	LG.....
ERRg Homo sapiens	-.....	A.SGSY..SS	T.....	-----	-----	MN.....
ERR Drosophila melanogaster	-.....	KSSPSVSP.	ERQLCSSTT	S.....	-----	LS.....
ERR Crassostrea gigas	-.....	L.EESF..GR	D.....	-----	-----	VF.....
NR3D Crassostrea gigas	EKLV...PKE	E....SHGS	H.....	-----	-----	HS.....
NR3D Aplysia californica	QAA	T....STP	E.....	-----	AG.....
NR3D Platynereis dumerilii	-.....	-----	-----	-----	-----	-----
ER Branchiostoma floridae	.KQSPMSPVM	SVHGMRQNGR	-----	-----	-----	IQ.....
Era Homo sapiens	.NSVSPSPLM	L....LHPPP	Q.....	-----	-----	LS.....
ERb Homo sapiens	-...TTSPNV	L...WPTPG	H.....	-----	-----	LS.....
SR Branchiostoma floridae	.ISPQYFPFQ	VVRPYSPHR	P.....	-----	-----	-----
GR Homo sapiens	FNVIPPVPG	S..ENWNR.	-----	-----	Q	GSG...DDNL
MR Homo sapiens	.SSFPPVNTL	V..ESWKSH.	-----	-----	G	DLS...SRR.
AR Homo sapiens	-GSGPSAAA	S..SSWHTLF	TAEQGQLYGP	CGGGGGGGGG	GGGGGGGGGG	GGGGGEAGAV
PR Homo sapiens	QGPFAPPPCK	A..PGASGCL	LPRDGLPSTS	ASAA.....AA	GAA...PALY

Supplemental Figure 2 (continued)

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NR3E Hydra magnipapillata	--PINTSS--	-SNI-----	----PRVIE-	--DTIN-I--	-----I----	-----
NR3F Trichoplax adhaerens	..CGMLRP..	.K.Q.....Y----	..ASK....SIP..	...VVP....
NR3E Hydractinia symbiolongicarpus	...VLEKM..	----.....EVK..	..EQVK.V.E....VVP....
NR3E Aurelia auritaSALK.M.D....
NR3E Rhopilema esculentum	..-AFGRH..	..ESS.....LILTK.	..SALK.M.D....
NR3E Podocoryna carnea
NR3E Nanomia bijuga	..ANV....	----.....NNQFN.	..EDAQ.M.K....
NR3E Chironex fleckeri	..-MMD--.MQ.	..PT..Q.G....
NR3E Alatina alata
NR3E Morbakka virulenta	----MQ.	..ST..Q.G....
NR3E Tripedalia cystophora	..-MD--.LP.	..HT..Q.G....
NR3E Copula sivickisi	----MP.	..HT..Q.G....
NR3E Chironex yamaguchi	..-MD--.MQ.	..PT..Q.G....
ERRa Homo sapiens-PP.VA.LAPGP.
ERRb Homo sapiens	..T----HAN	GL--.....SPP.MF.AGAG..	...L..GG..
ERRg Homo sapiens	..G----HQN	GL--.....SPP.LY.PSAP..	...ILGGS..
ERR Drosophila melanogaster	..CDLHNVS	LNDG.....	----DS	L.KGSG.TSGGNG..	...GGGGG..
ERR Crassostrea gigas	..ADYNNDDY	G.DG.....SYQNS	A..NSG.NI.SPCS..	...IDSS..
NR3D Crassostrea gigas	..GMVPOGS	FLSM.....YMMHN	Q.VPSS.TTL	EFRDHDGR	...ILPLLDP
NR3D Aplysia californica	..SGTLPH..	----.....NFLMDV	H.GPGT.LGASP
NR3D Platynereis dumerilii	..-MADD..	..QG.....YT--.PQR..
ER Branchiostoma floridae	..NGMMQQ..	.HHH.....DILTL	H.NGGR..QVG..	...QVGD..
ERa Homo sapiens	..FLQPHGQ	QV--.....YYL.N	E.PSGY.T.	..VREAGPP.	...AF.YRPN
ERb Homo sapiens	..LVVH-RQ	L--.....HLYA.P	Q.KSPW.C.	..EARSLEH.	...TLPVNRE
SR Branchiostoma floridae	..-MADD..	..QP.....QAAM.YARNY	T..QNMEVRT	AVTDPQMGAW NRMQOPVYEE
GR Homo sapiens	TSL-----	GTLN.....	FPG---RTV	F.SNGY.SS
MR Homo sapiensSDGY.PV
AR Homo sapiens	APYGY.RPPQ	GLAQESDFT	APDVWYPGGM	VSRVPY.PSP	TCVKSEMGPW	MDSYSGPY..
PR Homo sapiens	PAL-----	GL.G.....	LPQLGYQAAV	L.KEGL.PQ.VYPPY..

DBD

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NR3E Hydra magnipapillata	-----	SSSPPSE-KG	LVQNKRPMTN	ARHAVPMLCL	VCTDKASGYH	YGVPS	CEGCK
NR3F Trichoplax adhaerens	AVIV.....	---SVPNLEH	GTFSQNQA.	..G.R...L.	...L..
NR3E Hydractinia symbiolongicarpusER..QP.R-	-ANLQSRLOS	NK.DQAF.V	..G.R...A.	...A..
NR3E Aurelia auritaK-----	----.SCPGY	KDGEM.F.V	..G.S...A.	...A..
NR3E Rhopilema esculentumK-----	----.SCPGF	KDGD.I.F.V	..G.S...A.	...A..
NR3E Podocoryna carnea	SKQDQ.F.V	..G.R...A.	...A..
NR3E Nanomia bijugaVKETNP.P-	-KAETP.TSE	.NTQRAF.V	..A.L...A.	...A..
NR3E Chironex fleckeriA-----	----CDTQES	RHGNS.F.V	..G.T...A.	...A..
NR3E Alatina alata
NR3E Morbakka virulentaT-----	----CDSQNP	STGNS.F.V	..G.T...A.	...A..
NR3E Tripedalia cystophoraT-----	----CDSQ.V	SHGSA.F.V	..G.T...A.	...A..
NR3E Copula sivickisiT-----	----CDSQ.V	SHGSA.F.V	..G.T...A.	...A..
NR3E Chironex yamaguchiA-----	----CDTQES	RHGNS.F.V	..G.T...A.	...A..
ERRa Homo sapiens	APTRCLPGHK	EEEDGEG.A.	PGEQGGKLV	LSSLPKR.	..G.V...A.	...A..
ERRb Homo sapiens	TPCRKS...	YEDCA.G.IM	EDSAIKCEYM	LNAIPKR.	..G.I...A.	...A..
ERRg Homo sapiens	GPVRKL...	YDDCS.T.IV	EDPQTKCEYM	LNSMPKR.	..G.I...A.	...A..
ERR Drosophila melanogaster	G...TSGG.	-----N.AT	NASAGAGSGS	V.DELRR.	..G.V...F.	...A..
ERR Crassostrea gigas	SPIDTSKLD	INNI--A.TS	ITNSINCDQE	NIELPKR.	..G.V...S.	...A..
NR3D Crassostrea gigas	S.....L	-----FA	DGRKCLKHGA	SA.GPNK.Q	..S.N...F.	...W..
NR3D Aplysia californica	S.....S	H-----	MK.EVGGAVP	GSSIQAK.Q	..S.N...F.	...W..
NR3D Platynereis dumerilii	SPEEEWGGQT	.EPS.IDLG.	HANADQLPSD	GDSKDVQK.Q	I.S.L...F.	...W..
ER Branchiostoma floridae	CPDQFSKGR.	D.PAS.G.SS	IEN.QPQVKE	LDNKARAV.R	..G.H...F.	...W..
ERa Homo sapiens	SDNRRQGGRE	R-----LA	STND.GS.AM	ESAKETRY.A	..N.Y...W.	...W..
ERb Homo sapiens	TLKRKVSNGR	C-----	---ASPVTGP	GSKRDAHF.A	..S.Y...W.	...W..
SR Branchiostoma floridae	SPQKGTGTEE	.APWTANQOV	KQAAVGTAS	PGSGHKPP.A	..HCPST.L.	...YA..
GR Homo sapiensP.MR.--DV	SSPPSSSS.A	TTGPP.K.	..S.E...C.	...LT.GS.
MR Homo sapiensLEYI.E-.NV	SSSTL.SVST	GSSRPSKI.	..G.E...C.	...VT.GS.
AR Homo sapiensGDMRLE-.TA	RDH--VLPID	YFPPQKT.	..I.G.E...C.	...ALT.GS.
PR Homo sapiensLNYLRP-.DS	-EASQS.QYS	FESLPQKI.	..I.G.E...C.	...LT.GS.

P-box

Supplemental Figure 2 (continued)

DBD

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NR3E Hydra magnipapillata	AF	FKRS	LQLK	QLNYK	PASN	N	TINKMSR	K	CCQA	CRLGKC	HAVGMSKECK	KVKKRDIR--
NR3F Trichoplax adhaerens	I.S-	SVA.T.	.SGS	R	KVD.QR..	Q.	FD...	IR.GV	RKNRNP	PGGRV
NR3E Hydractinia symbiolongicarpus	S.DS	DR.....	K.	.D.....	FL	GN.IKRP	KQE
NR3E Aurelia aurita	AT	D...R.	G..	K.D.....	R.	NE.....	FL	GN..IKK	PSR
NR3E Rhopilema esculentum	AT	D...R.	G..	K.D.....	R.	NE.....	VI	D-----	
NR3E Podocoryna carnea	S.ES	DR.....	Q.	.D.....	FL	GN.IKRA	KQD
NR3E Nanomia bijuga	S.EMG	D.....	K.	QD.....	FL	GN.IKRP	KPD
NR3E Chironex fleckeri	AA	D...R.	GN.	K.D.....	R.	.E.....	FL	GN..IKK	NAK
NR3E Alatina alata	AA	D...R.	GN.	K.D.....	R.	.E.....	FL	GN..IKK	STK
NR3E Tripedalia cystophora	AA	D...R.	GN.	K.D.....	R.	.E.....	FL	GN..IKK	NAK
NR3E Copula sivickisi	AA	D...R.	GN.	K.D.....	R.	.E.....	FL	GN..IKK	NAK
NR3E Chironex yamaguchi	AA	D...R.	GN.	K.D.....	R.	.E.....	FL	GN..IKK	NAK
ERRa Homo sapiens	TI.G-	SIE.S	E	E.T.RR.	A	FT.	LR...L.	GV	RLLDRV	GGRQ
ERRb Homo sapiens	TI.G-	NIE.S	E	E.T.RR.	S	FM.	LK...L.	GV	RLLDRV	GGRQ
ERRg Homo sapiens	TI.G-	NIE.S	E	E.T.RR.	S	FM.	LK...L.	GV	RLLDRV	GGRQ
ERR Drosophila melanogaster	TI.G-	NIE.T	E	E..RR.	A	FQ.	LLM..L.	GV	RLLDRV	GGRQ
ERR Crassostrea gigas	TI.G-	NIE.S	E	E.T.RR.	A	FQ.	LR...L.	GV	RLLDRV	GGRQ
NR3D Crassostrea gigas	I.G-	PVD.V	T	D.HR.	S	R.	YE...N.	GSQ	RKERKGS	SISM
NR3D Aplysia californica	I.G-	PVD.I	T	D.HR.	S	RR.	YE...N.	GSQ	RKE..NSG	NT
NR3D Platynereis dumerilii	D-	PVD.V	T	D.HR.	S	FR.	LE...M.	RRE	RRTTKVK	KSP
ER Branchiostoma floridae	I.QG	TD.I	GT	DRNR.	S	YR.	LM...T.	DGR	RSGE.RG	PRR
ERa Homo sapiens	I.G-	HND.M	T	D.NR.	S	R.	YE...M.	GGI	RKDR.GG	ML
ERb Homo sapiens	I.G-	HND.I	T	D.NR.	S	R.	YE...V.	CGS	RRERCY	LV
SR Branchiostoma floridae	S.	H.AHKR	AHP.V	N	V.DRRLK	N	P.	L.M...F.	EH	A.PN.	ATKKK
GR Homo sapiens	V.	AVEG-	H..L	AGR.	D	I.D.IR.	N	P.	LQA..NL	AR	.T.--	KIKG
MR Homo sapiens	V.	AVEG-	H..L	AGR.	D	I.D.IR.	N	P.	LQA..NL	GAR	.S..LG	KLKG
AR Homo sapiens	V.	AAEG-	KQK.L	ASR.	D	..D.FR.	N	PS	YEA..TL	GAR	.L..LGN	LKL
PR Homo sapiens	V.	AMEG-	H..L	AGR.	D	I.VD.IR.	N	P.	CQA..VL	GGR	.F..FN	KVRV

P-box

D-box

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NR3E Hydra magnipapillata	-----D-	----	TKSKNY	PRLNPDECI	ESVLT	-----	-----	-----	-----	-----	-----	-----
NR3F Trichoplax adhaerens	K...	YEKPT	S....	--VGQC	KSVTS..SF.	S.STASSFSQ	ADTDK.AEDN	SIQS..L	KN			
NR3E Hydractinia symbiolongicarpus	K...	QKQP	R---RK	SDKSS.EGFV	.STN.....
NR3E Aurelia aurita	K...	SK..KQ	KFDDDE.RK.	VEPTS.....
NR3E Rhopilema esculentum
NR3E Podocoryna carnea	K...	QKQP	R---RK	SDKSSSENFV	.STN.....
NR3E Nanomia bijuga	K...	DAAK.	QR..KT	E..ISES.NVV	.STN.....
NR3E Chironex fleckeri	G...	DGGKT	HKS.S	KLKQ	MSNDED.LKC	VEPTS.....
NR3E Alatina alata	-----	MANDDD.LKC	VEPTS.....
NR3E Morbakka virulenta	S...	DSGKN	HKA.S	KLKQ	MAHDDD.LKC	VEPTS.....
NR3E Tripedalia cystophora	G...	DSGKN	HKA.S	KLKQ	MSADDD.LKC	VEPTS.....
NR3E Copula sivickisi	G...	DSAKN	HKA.S	KLKQ	MSADDD.LKC	VEPTS.....
NR3E Chironex yamaguchi	G...	DGGKT	HKS.S	KLKQ	MSNDED.LKC	VEPTS.....
ERRa Homo sapiens	K...	YKRRP	EVD	-----	-P.PF.GFPF	AGP.AVA..G	GPRKTAAPVN	ALVSHLLV	V.			
ERRb Homo sapiens	K...	YKRRL	DSE	-----	-SSPYLSLQ	SPP--.....	AKKPLT	KIVSYLLVA.			
ERRg Homo sapiens	K...	YKRRR	DAE	-----	-NSPYLNPQL	VQP--.....	AKKPYN	KIVSHLLVA.			
ERR Drosophila melanogaster	K...	YRRNP	VSN	-----	-SYQTMQLLY	Q.N--.....	TTSLCDV	KILEVLNSY.			
ERR Crassostrea gigas	K...	YKRTV	DSG	-----	-PIVQQIFPM	IKK-ACV..E	STKSDFSSDN	KILTQLISIE				
NR3D Crassostrea gigas	SKPAATKRSR	ADSSDNTV	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
NR3D Aplysia californica	S.SLKGKRCR	ADSSDSAV	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
NR3D Platynereis dumerilii	G...	S..PE	EKITK.VRT	-PNTK.NPKE	-----KGPPVENN	GQSRRLVQLE				
ER Branchiostoma floridae	K...	RTHNQ	IDVSTADSC	KSSVS.LP--	--SSASAFDK	SRSASPTENN	SF.....
ERa Homo sapiens	K...	HKRQR	DDGEGRG-E	-----	-VGSA.....GD.....
ERb Homo sapiens	R...	RQRSA	DEQLHCAGK	-----	-AKRS.....	..GG.....
SR Branchiostoma floridae	P..KKT	KRSP	KRA.....	-KTSH..PPA	E---.....	..TPPEQQ	LIVSPTLP..					
GR Homo sapiens	I.....	QQA	TTG.---	VS-	QET-----	SENP-
MR Homo sapiens	I.....	HEE	QPQ.---	QQ-	QPPPP.PPPQ	SPEE-
AR Homo sapiens	Q.....	EEG	EAS.---	ST-	TSP-----	TEET-
PR Homo sapiens	V.....	RAL	DAV.---	A--	LPQPVGVPNE	SQA-

Supplemental Figure 2 (continued)

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NR3E Hydra magnipapillata	-----	-----	-----	-----	-----
NR3F Trichoplax adhaerens	EDK.....	..YIDVNCT.RL	PSTPPSPDKGY DIKILKEFIG
NR3E Hydractinia symbiolongicarpus
NR3E Aurelia aurita
NR3E Rhopilema esculentum
NR3E Podocoryna carnea
NR3E Nanomia bijuga
NR3E Chironex fleckeri
NR3E Alatina alata
NR3E Morbakka virulenta
NR3E Tripedalia cystophora
NR3E Copula sivickisi
NR3E Chironex yamaguchi
ERRa Homo sapiensEPEKL	YAMPDPAG..
ERRb Homo sapiensEPDKL	YAMPPPGM..
ERRg Homo sapiensEPEKI	YAMPDPTV..
ERR Drosophila melanogasterEPDAL	SVQTPPPQVH TTSITNDEAS
ERR Crassostrea gigas	Q.....QLDKL	YVNTESEI..
NR3D Crassostrea gigasT	SGSPNPAKSA RK.....
NR3D Aplysia californicaT	NINGASSKSS KR.....
NR3D Platynereis dumerilii	ETVAVESVKT	EAVSPQECSP	SGHTSQEDLS	VPMTSASQSF	NLTPPPSTSS ST....SSPS
ER Branchiostoma floridaeDS
ERa Homo sapiensMRAA	NLWPSPLMIK RS.....
ERb Homo sapiensH..	..AP.....R.....
SR Branchiostoma floridaeL	YN.....
GR Homo sapiensGN.K	TIVPAT.....
MR Homo sapiensGT.T	YIAPAKEPSV NT.....
AR Homo sapiensTQKL	TVS.....
PR Homo sapiensSQRF	TFSPG.....

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H1

H3

NR3E Hydra magnipapillata	-----	PIEIEHEN	IIEQIRLTHS	KL--YL----	---GLDE--	-TV--NGTL
NR3F Trichoplax adhaerens	SGEKYVIDQK	RDGKQ.IVND	V.KK.LEAEP	PM..LP....	...ACP.P..	EAEDSGIRT
NR3E Hydractinia symbiolongicarpusAC...I..	A.QS.TSL.A	E.....KPED..	CTIE.TR..
NR3E Aurelia auritaTQDA.L.K	L.RT.TSL.D	QE..KPS...	...LLPNV..	..EED.LKPA
NR3E Rhopilema esculentum	---
NR3E Podocoryna carneaAC..M.I..	A.QS.TAL..	EF.....KPED..	SSVR.TR..
NR3E Nanomia bijugaAC..M.L.S	M.QN.TTL.N	EM..C....	...KS.D..	RMEN.AE..
NR3E Chironex fleckeriTQDA.L.S	VMKA.VSLEN	ET..KEL...	...RVSQP..	DGVD.-VIT
NR3E Alatina alataTQDA.L.S	VVKA.ITLEN	ET..KEL...	...RVSQS..	EGAE.-VMT
NR3E Morbakka virulentaTQDA.L.S	VVKA.VTLEN	ET..KGL...	...RVCQS..	.D.TE.-TMT
NR3E Tripedalia cystophoraTQDA.L.S	VVKA.VTLET	ET..KEL...	...RVSQP..	DGSE.-VMT
NR3E Copula sivickisiTQDA.L.S	VVKA.VTLET	ET..KEL...	...RVSQP..	DGSD.-VMT
NR3E Chironex yamaguchiTQDA.L.S	VMKA.VSLEN	ET..KEL...	...RVSQP..	DGVD.-VIT
ERRa Homo sapiens	PD..GHLPA
ERRb Homo sapiens	PE..GDIKA
ERRg Homo sapiens	PD..SDIKA
ERR Drosophila melanogaster	SS.....	..SGS.KL.S	SV--VT---	---	...PNGTCIFQ..	NNNNNDPNEI
ERR Crassostrea gigas	LEGGEVRF
NR3D Crassostrea gigasS..	---QTVT	.LQA.NKAAL	PVLESHH...	---NH..
NR3D Aplysia californicaS..	---RSAS	.L.A.QKADL	PVMDSYH...	---NH..
NR3D Platynereis dumerilii	NGTSSLQ...	IDASTFKIDP	GL-P.DQP.P	LVVILEENDL	PPKICKEPLT	AESEETEYS.
ER Branchiostoma floridae	DGDSSTGREL	RTASHQRLKA	L.DA.DVKEG	EH..RGEENH	PTGQQAGN..	WQEIS.PE.
ERa Homo sapiensKKN.SLA	SLTADQ	MVSA.LDAEP	PILYSEY...	---P..
ERb Homo sapiensVRE.LLLDALSP	Q.LVLT	LEAEP.PHVLI-S	---RP..
SR Branchiostoma floridaePTVP	L.SH.VNIEP	NPILTGY...	---NP..
GR Homo sapiensPQLTPT	LVSL.EVIEP	EVLYAGY...	---S..
MR Homo sapiensALVPQL	STISRALTPS	PVMV.ENIEP	EIVYAGY...	---S..
AR Homo sapiensHIEGYEQPI	FLNV.EAIEP	GVVCAGH...	---N..
PR Homo sapiensQTIQLIPP	L.NL.MSIEP	DVIYAGH...	---N..

Supplemental Figure 2 (continued)

	1081	H3	H4-5
NR3E Hydra magnipapillata	THKFGYGTGK	QLIHIIDWAK	RIPGYSLLIL SDQAVLLOAT WVELMVANWL FLSITSTN-S
NR3F Trichoplax adhaerens	ITIIICEMVER	E.VMV.....T.C.N.V.....S.L.VFMIDLA.R.MPYD..K
NR3E Hydractinia symbiolongicarpus	.Q..SW.VDQ	.V.....	QL...CN.VI...I...A..D.LAL..V.YH.LYVEE.A
NR3E Aurelia aurita	.SSLCDIV..	...AA.....	GL...ET.T.N...I...G.I.MLLL..I.Y.LPH...K
NR3E Rhopilema esculentum	--LTLDIV..	.VAA.....	GL...ET.T.N...I...G.I.MLLL..T.Y.LSHK..K
NR3E Podocoryna carnea	VQN.CW.VDQ	.V.....	QL...CN.VI...I...A..D.L.L..V.YH.LCIDD.A
NR3E Nanomia bijuga	IQ..CF.VDQ	.VR.....	QL...CN.SI...I...A..D.L.L..V.YH.LHVSE.K
NR3E Chironex fleckeri	.GYLCKQ.S.	.VAV.....	GL...DT.T.N...I...G.I...LLL..V...ERI..K
NR3E Alatina alata	.GYLCKQ...	.VAV.....	GL...DT.T.N...I...G.I...LLL..V...ERI..K
NR3E Morbakka virulenta	.GHLCKQ..R	.VAV.....	GL...DT.T.N...I...G.I...LLL..V...ERI..K
NR3E Tripedalia cystophora	.GYLCKQ..R	.VAV.....	GL...D.T.N...I...G.I...LLL..V...ERF..K
NR3E Copula sivickisi	.GYLCKQ..R	.VAV.....	GL...DT.T.N...I...G.I...LLL..V...ERI..K
NR3E Chironex yamaguchi	.GYLCKQ.S.	.VAV.....	GL...DT.T.N...I...G.I...LLL..V...ERI..K
ERRa Homo sapiens	VATLCDLDFDR	EIVVT.S...	S...F...S...MSV.SV.M.VL.LGVA.QR.LPLQD.E
ERRb Homo sapiens	LTTLCDLADR	E.VV..G...	H...F...S.G..MS...SA.M.IILIGIV.YR.LPYDD.K
ERRg Homo sapiens	LTTLCDLADR	E.VV..G...	H...F.T.S.A..MS...SA.M.IILIGVV.YR.LSFED.E
ERR Drosophila melanogaster	LSVLSDIYD.	E.VSV.G...	Q...FID.P.N..MK...VS.A.ILTQLT.R.LPFNG.K
ERR Crassostrea gigas	LATVSDLADR	E.VIT.S...	QV..FCT.S...MN...HS.L.ILCL.LV.R.CPYNG.Y
NR3D Crassostrea gigas	LNSLVKLAER	E.V.L.N...	NV...TD.S...VH.IECC.M...LLL.CA.R.EHGG.K
NR3D Aplysia californica	LNTLIKLADR	E.VYL.N...	HV...TC.T.G..VH.IECC.M...LLL.CA.R.MEHG.R
NR3D Platynereis dumerilii	L.RLI.LADM	E.VDVVN...	VL..F.G.E.R.RIAI.ESC.M..LCIGAA.WR.RLN.T.F
ER Branchiostoma floridae	IESVSSLVDR	E.TG..C.G.	K....K.S.N..VL.MES..LD.LILDIV.WC..RHKG.E
ERa Homo sapiens	MGLLTNLADR	E.V.M.N...	.V..FVD.T.H..VH..ECA.L.ILMIGLV.WR.MEHPG.K
ERb Homo sapiens	MMSLTKLADR	E.V.M.S...	K...FVE.S.F..VR.ESC.M.VLMMGLM.WR..DHFG.K
SR Branchiostoma floridae	MALVTDLANR	E.IEGLV...A	.L...GM.PM.D.VN.IRTV.LD.LMLGLV.WR.MEHRG.E
GR Homo sapiens	MPTLNMIG.R	.V.AAVK...	A...FRN.H.D.MT...YS.MF..AFALG.WR.YRQSSAN
MR Homo sapiens	LSTLNRLA..	.M.QVVK...	VL..FKN.P.E..IT.I.YS.MC.SSFALS.WR.YKH..SQ
AR Homo sapiens	LSLNLGER	.V.VVK...	AL...FRN.HV.D..MAVI.YS.MG..FAMG.WR.F.NV.SR
PR Homo sapiens	LTSLNQLGER	.LSVVK.S.	SL...FRN.HI.D..IT.I.YS.MS...FGLG.WR.YKHVSGQ

	1141	H6	H7	H8
NR3E Hydra magnipapillata	LK-LSHTFDI	SK-----	QDST-ELGF	-GLIYDQFLA LVHRTKGYKL DEEISCFKA
NR3F Trichoplax adhaerens	.V.YACDMVM	GH.....	.KQ.R.AA.L	.DE.NRHAFE..TKYRSISM.KO.FA.L.R
NR3E Hydractinia symbiolongicarpus	I..F.KF.S	TY.....	NEAR...C	.EN..C.IFS.AN.A.K.NM...A.L..
NR3E Aurelia aurita	IQ.F.SNLIV	CE.....	ALGS..F.L	.ETVHH.LSS.IT.VQ..CI...FV.L..
NR3E Rhopilema esculentum	TQ.F.ANLIV	CE.....	.TLA..AF.L	.ETVHT.LSS.IA.VQ..GI..V.FL.L..
NR3E Podocoryna carnea	I..F.RY.S	TY.....	DEAM....	.EN..C.V.S.AN.A.K.NM...L..
NR3E Nanomia bijuga	IR...MV.SL	.F.....	.EAK.NI..	.EE..SYLIS..N.A.K.NI.V...I..
NR3E Chironex fleckeri	VA.F.ADVVV	DD.....	.RM.H.T.L	.DSV.S.L.P..T.VRS.G...FV.L..
NR3E Alatina alata	VA.F.SDVVV	DD.....	.RT.R.T.L	.DSV.S.L.P..T.VRS.G...FV.L..
NR3E Morbakka virulenta	VA.F.SDVVV	DD.....	.MT.R.T.L	.DSV.S.L.P..T.V.S.E...FV.L..
NR3E Tripedalia cystophora	VA.F.SDVVV	DD.....	.RS.R.T.L	.DSV.S.L.P..T.VRN.E...FV.L..
NR3E Copula sivickisi	VA.F.SDVVV	DD.....	.RS.R.T.L	.DSV.S.L.P..T.VRN.E...FV.L..
NR3E Chironex yamaguchi	VA.F.ADVVV	DD.....	.RM.H.T.L	.DSV.S.L.P..T.VRS.G...FV.L..
ERRa Homo sapiens	.A.FAEDLVL	DE.....	.EGAR.AA.L	.ELGAAL.Q..R.LQALR.ER..YVLL..
ERRb Homo sapiens	.V.YAEDYIM	DE.....	.EH.R.LA.L	.LEL.RAI.Q..R.Y.KL.V.EK..FVTL..
ERRg Homo sapiens	.V.YADDYIM	DE.....	.DQ.K.LA.L	.LDLNNAI.Q..KKY.SM..EK..FVTL..
ERR Drosophila melanogaster	.C.FATDVWM	DE.....	.HLAK..C.Y	.TEF.YHCVO.IAQ.MERISP.RR..YVLL..
ERR Crassostrea gigas	..FAEDLQL	.V.....	.DECK.LCHC	.SPELDGISRK.AKKFTDMEV.TK..YLLL..
NR3D Crassostrea gigas	SLAFAPDLVL	DR.....	.SSWS.TVEM	.TE.FE.VA.VSEQMMONH.HKD.LLLLQ.
NR3D Aplysia californica	TLVFAPD.HL	ER.....	.QWA.LT.M	.DVLE.VS.VSEQMLLHG.NK..LLLQ.
NR3D Platynereis dumerilii	QVNFAENLHF	NE.....	.ETAK.KAKM	.SS.VGEIWO.ISQOFRYLE.SNH.FMLLRV
ER Branchiostoma floridae	KLL..GGVLV	NRNTISNRRN	NSSGD.DMEV	.LEMC..I.S.IATKIFYEFD.QRR.YL.L..
ERa Homo sapiens	-LLFAPNLLL	DR.....	.NQCKCVE.M	.VE.F.ML..TSS.FRMN.OG..FV.L.S
ERb Homo sapiens	-LIFAPDLVL	DR.....	.DEGKCV.EI	.LE.F.ML..TTS.FREL..QHK.VL.V..
SR Branchiostoma floridae	WLVFAPDLLM	DR.....	.SLCR.LS.M	.EY.CTPM.E.FARQFADLQV.PQ.VYV.L..
GR Homo sapiens	.LCFAPDLI.	NE.....	.RM..LPCM	.YDQCKHM.Y.VSSELHRLQV.SY..YL.M.T
MR Homo sapiens	FLYFAPDLVF	NE.....	.EKM.H.QSAM	.YELCQGMHQ.ISLQFVRLQV.TF..YTIM.V
AR Homo sapiens	MLYFAPDLVF	NE.....	.YRM.H.KSRM	.YSQCVRMRH..SQEFGWLQI.TPQ.FL.M..
PR Homo sapiens	MLYFAPDLIL	NE.....	.RMK..SS.	.YSLCLTMWO.IPOEFVKLQV.SQ..FL.M.V

Supplemental Figure 2 (continued)

	H8		H9		H10-H11								
NR3E Hydra magnipapillata	IILTN	AETTTQ	LSRP	---	AKVHALSN	QFCSSLQYYT	KNRH	---	KE	NPLKFAKIVL			
NR3F Trichoplax adhaerens	LR	---	---	---	---	---	---	---	---	---			
NR3E Hydractinia symbiolongicarpus	.S.	.SSS	.AG	.	.E.	.ND.IT	T.S.T.	.NS	.	R.	.Q.	.L.	
NR3E Aurelia aurita	.G.L.	.DSRG	.TGA	.	.S.	.QD.V.	K.SLA.N.HI	SSQ	.	PD	Q.Q.	.I.	
NR3E Rhopilema esculentum	.G.L.	.DSRG	.TGS	.	.S.	.QE.VK	K.S.A.N.HI	SSH	.	PD	Q.Q.	.I.	
NR3E Podocoryna carnea	.G.	.SSS	.V.	.	.DN.	.NE.IT	T.S.T.	.SS	.	RD	.Q.	.L.	
NR3E Nanomia bijuga	.N.	.SS	.TTS	.	.D.	.NE.IQ	K.T.A.	.S	T.	.S.	R.	S.Q.	.L.
NR3E Chironex fleckeri	.G.M.	.DSCG	.GS	.	.RG.	.QE.VR	KYSMA.A.HI	SSY	.	PD	K.Q.	.	
NR3E Alatina alata	.G.M.	.DSCG	.GS	.	.RG.	.QE.VR	KYSMA.M.HI	STY	.	PD	K.Q.	.	
NR3E Morbakka virulenta	.G.M.	.DSCG	.GS	.	.RG.	.QE.VR	KYSMA.T.HI	STY	.	PD	K.Q.	.	
NR3E Tripedalia cystophora	.G.M.	.DSCG	.GS	.	.RG.	.QE.VR	KYSMA.T.HI	NTY	.	PD	K.Q.	.	
NR3E Copula sivickisi	.G.M.	.DSCG	.GS	.	.RG.	.QE.VR	KYSMA.T.HI	NTY	.	PD	K.Q.	.	
NR3E Chironex yamaguchi	.G.M.	.DSCG	.GS	.	.RG.	.QE.VR	KYSMA.A.HI	SSY	.	PD	K.Q.	.	
ERRa Homo sapiens	LA.A.	SDSVH	IEDA	.	.EA.	.EQ.RE	ALHEA.LE.E	AG	AGPGG	GA	ERRRAGRLL	.	
ERRb Homo sapiens	LA.A.	SDSMY	IEDL	.	.EA.	.QK.QD	LLHEA..D.E	LSQRH	.	.	.D.WRTG	LL.	
ERRg Homo sapiens	.A.A.	ASDMH	IEDV	.	.EA.	.QK.QD	VLHEA..D.E	AGQ.M.	.	.	.D.RRAG	MLM	
ERR Drosophila melanogaster	LL.A.	CD-IL	.DDQ	.	.SSLR.	FRD	TILN..NDVV	YLLRH	.	SS	AVSHQQQLL	.	
ERR Crassostrea gigas	MT.C.	ID-VA	IENTS	.	.EA.	.RQ.QD	KLQD..IE.V	.Y.YV.	.	-G	.LRRLGHLYM	.	
NR3D Crassostrea gigas	MV.V.	.VRR	.ASY	.	.NQIFNMQQ		SLLDAIVDTA	QK	-Y.	HPD	.VRHVPAVL	.	
NR3D Aplysia californica	TV.V.	.VRP	.DSF	.	.L.IQEMRQ		LILDVFMEVA	GRH-Q.	CFG	.	.WRHAPS.L.	.	
NR3D Platynereis dumerilii	VTML.	.SIR	.CL	.	.DAM.	KIRO	.YLEA.HFEC	GRFLGKIS	ES	SCVRM	.Q.LC	.	
ER Branchiostoma floridae	.T.VH	GSLKG	.ESD	.	.TQ.	RQ.QD	DLTDA.MDVC	SE	.	AL	GSRRP	.ML.	
ERa Homo sapiens	.L.	SGVYT	FLSSTLKSLE		EKDHI.	RVLD	KITDT.IHLM	AKA-GLTI	QQ	QHQL	QLL	.	
ERb Homo sapiens	M.L.	SSMYP	.VTATQDA	.	SSR.LAH.L.	AVTDA.VWVI	AKS-GISS	QQ	QSMRL	NLLM	.	.	
SR Branchiostoma floridae	LT.YT	TAVSR	.QDY	.	.RQ.	QR.QH	EINEA.AEAC	SSTFG	.	-F	S.GNI	RLMM	
GR Homo sapiens	LL.LS	SV--P	----	KDGLK	SQELFDEIRM	TYIKE.GKAI	VK.E.GNS	SO	.	WQR	YQLTK	.	
MR Homo sapiens	LL.LS	TI--P	----	KDGLK	SQ.AFEEEMRT	NYIKE.RKMV	TKCP.NNS	GO	.	SWQR	YQLTK	.	
AR Homo sapiens	LL.FS	II--P	----	VDGLK	NQKFFDE.RM	NYIKE.DRTI	ACKR.KNH	TS	.	SSRR	YQLTK	.	
PR Homo sapiens	LL.L.	TI--P	----	LEGLR	SQTQFEEMRS	SYIRE.IKAI	GL.Q.KGV	VVS	.	SSQR	YQLTK	.	

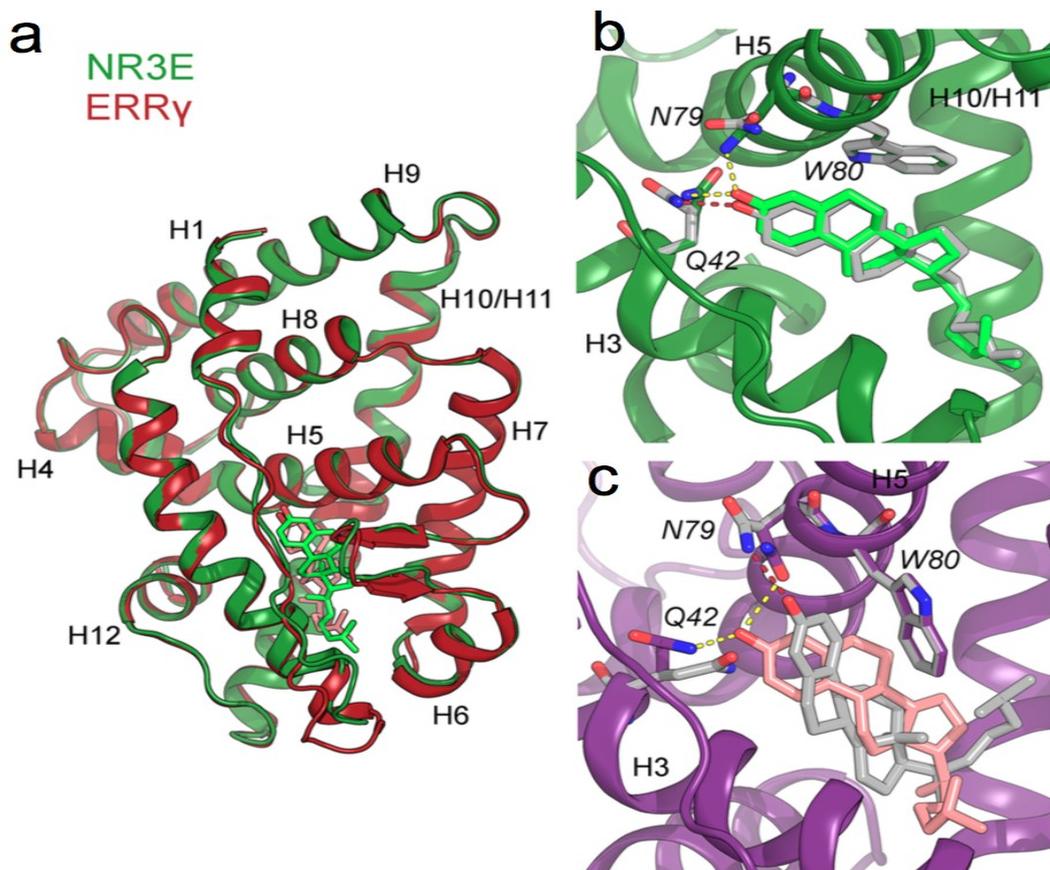
	H10-H11		H12									
NR3E Hydra magnipapillata	ILPQLKYL	EVLRVHLALN	SM-SEK	GAYL	SDLVVEMLDV	KNRQ	-----	-----				
NR3F Trichoplax adhaerensM.	Q.IEY	YTMK	MN.NSSES	N.	I.	.EA	.Q.L.				
NR3E Hydractinia symbiolongicarpusV.	N.IEY	FYRVK	IS.--SE	Q.	E.L.	.EA	.S.L.				
NR3E Aurelia auritaCV.	N.IEY	FYRVK	IS.--GE	Q.	E.L.	.EA	.S.L.				
NR3E Rhopilema esculentumM.	Q.IEY	YKMK	MH.ES	ESN.	I.	.EA	.Q.L.				
NR3E Podocoryna carneaR.	IEL	TKMK	MN.HQDK	CA.	I.	.EA	.Q.L.				
NR3E Nanomia bijugaV.	H.IEF	YRVK	IS.--GE	Q.	L.	.EA	.S.L.				
NR3E Chironex fleckeriV.	H.IEF	YRVK	IS.--GE	Q.	L.	.EA	.S.L.				
NR3E Alatina alataV.	H.IEF	YRVK	IS.--GE	Q.	L.	.EA	.S.L.				
NR3E Morbakka virulentaV.	H.IEF	YRVK	IS.--GE	Q.	L.	.EA	.S.L.				
NR3E Tripedalia cystophoraV.	H.IEF	YRVK	IS.--GE	Q.	L.	.EA	.S.L.				
NR3E Copula sivickisiV.	H.IEF	YRVK	IS.--GE	Q.	L.	.EA	.S.L.				
NR3E Chironex yamaguchiV.	H.IEF	YRVK	IS.--GE	Q.	L.	.EA	.S.L.				
ERRa Homo sapiens	T.L.	RQTAG	K.AHFYGVK	IE.--GK	VPM	HK.FL.	.EA	MMD				
ERRb Homo sapiens	T.L.	RQTAA	KAVQH	FYSVK	IQ.--GK	VPM	HK.FL.	.EA	VG.EQLRGS	PKDERMSSHD		
ERRg Homo sapiens	T.L.	RQTST	KAVQH	FYNIK	IE.--GK	VPM	HK.FL.	.EA	V--	-----		
ERR Drosophila melanogaster	L.S.	RQADD	ILRRF	WRGIA	RD.--EV	ITM	KK.FL.	.EP	LA	-----		
ERR Crassostrea gigas	L.A.	NHMKL	LAKQ	YWFVVK	KD.--GR	IMM	HK.FL.	.EA	DS	-----		
NR3D Crassostrea gigas	L.	THIRQAGE	RGIA	FFQR.K	IE.--GV	VTF	C.LK.	.A	QDFLEKKSSN	EGD		
NR3D Aplysia californica	L.	THIRQAGE	RGIT	YFQK.K	ME.--GC	VTF	C.LT.	.A	H.SSGERRRL	QQQQQQPQQ		
NR3D Platynereis dumerilii	..	FARQVSL	KAT	TH.FNMH	NQ.--HAV	VPV	G..A.	.VA	QKELMTNEDS	AKIIT		
ER Branchiostoma floridae	L.	SH.RQVSA	RASS	H.G.VR	NG.--LK	V.P	Y.ILLDI	.TD	QVSEGQRDQ	AGHVEVASSP		
ERa Homo sapiens	..	SHIRHMS	KGME	H.YSMK	CK.--NV	V.P	Y..LL.	.A	HRLHAPTSRG	GASVEETDQS		
ERb Homo sapiens	L.	SHVRHAS	KGME	H.LNMK	CK.--NV	V.P	Y..LL.	.NA	HVLRGCRSSI	TGSECSPAED		
SR Branchiostoma floridae	.VS.	VRQ.SS	LGVD	H.NR.R	GA.--ET	VSV	EG.LR.	IV.E	PP.ITETIEDS	PGSAEGNKTM		
GR Homo sapiens	L.	DSMHEV.E	NL.NY	CFQTF	LDK-TMS	IEF	PEMLA.	IIITN	QIPK	YSNGNIKK	
MR Homo sapiens	L.	DSMHD.	DL.E	FCFYTF	RESHALK	VVEF	PAML.	IIISD	QLPK	VESGNAKP	
AR Homo sapiens	L.	DSVQPIAR	.LH	FTFD.L	IKSHMVS	VDF	PEMMA.	IIIS	QVPK	ILSGKVPK	
PR Homo sapiens	L.	DN.HD.	.K	QLHL	YCLNFF	TQSRALS	VVEF	PEMMS.	VIIAA	QLPK	ILAGMVKP

Supplemental Figure 2. Full-length alignment of cnidarian NR3E with already known members of the NR3 family.

The DNA-binding domain (DBD) is highlighted with light grey. Within it, two interaction sites with DNA response elements (P-box and D-box) are boxed, and the nine conserved cysteine residues are highlighted in orange. The twelve helices of the ligand binding domain (LBD) are boxed based on the human ER α sequence. As in Figure 5, residues involved in estrogen binding in ER are highlighted in green. Homologous residues that are shared by the cnidarian NR3E and the vertebrate oxosteroid receptors (SRs) are highlighted in pink. The cnidarian-specific N79-W80 anchor is highlighted in brown.

	DBD	LBD
NR3E <i>Hydra magnipapillata</i>	--	--
NR3F <i>Trichoplax adhaerens</i>	66.7	28.8
NR3E <i>Hydractinia symbiolongicarpus</i>	85.1	51.2
NR3E <i>Aurelia aurita</i>	79.1	40.6
NR3E <i>Rhopilema esculentum</i>	79.1	41.7
NR3E <i>Podocoryna carnea</i>	85.1	52.1
NR3E <i>Nanomia bijuga</i>	83.6	49.3
NR3E <i>Chironex fleckeri</i>	79.1	42.2
NR3E <i>Alatina alata</i>	--	42.7
NR3E <i>Morbakka virulenta</i>	79.1	43.1
NR3E <i>Tripedalia cystophora</i>	79.1	42.2
NR3E <i>Copula sivickisi</i>	79.1	42.2
NR3E <i>Chironex yamaguchi</i>	79.1	42.2
ERRa <i>Homo sapiens</i>	68,2	24.4
ERRb <i>Homo sapiens</i>	66.7	26.8
ERRg <i>Homo sapiens</i>	66.7	26.3
ERR <i>Drosophila melanogaster</i>	65.2	21.2
ERR <i>Crassostrea gigas</i>	65.2	22.8
NR3D <i>Crassostrea gigas</i>	69.7	21.1
NR3D <i>Aplysia californica</i>	68,2	22.4
NR3D <i>Platynereis dumerilii</i>	69.7	19.7
ER <i>Branchiostoma floridae</i>	65.7	23.3
ERa <i>Homo sapiens</i>	71.2	22.8
ERb <i>Homo sapiens</i>	71.2	23.4
SR <i>Branchiostoma floridae</i>	53	18.2
GR <i>Homo sapiens</i>	56.1	19.4
MR <i>Homo sapiens</i>	57.6	17.5
AR <i>Homo sapiens</i>	51.5	16.6

Supplemental Table 1. Identity percentages of DBDs and LBDs when compared to NR3E from *Hydra*.



Supplemental Figure 3. Paraestrol A docking into the ligand binding pocket of estrogen related receptor γ homology-based model of *Hydra* NR3E. (a) Superposition of the NR3E structural model using ERR γ structure (in dark green) where the paraestrol A is represented in light green, the ERR γ structure used as template (PDB id: 3D24) is represented in red and for comparison the paraestrol A position found in the NR3E ER model in pink. (b) Detailed view of the ligand binding pocket of NR3E ERR γ -based model, highlighting interactions (in yellow dashed lines) between the paraestrol and residues Q42 and N79. Results of the first round of docking experiments are represented in grey, showing one hydrogen bond (in red dashed line) between paraestrol and residue Q42. (c) Detailed view of the binding pocket of NR3E ER α -based model, highlighting interactions (in yellow dashed lines) between the paraestrol and residues Q42 and N79. Results of the first round of docking experiments are represented in grey, showing one hydrogen bond (in red dashed line) between paraestrol and residue N79.