

1 Title: Conservation of DNA and Ligand Binding Properties of Retinoid X Receptor from the Placozoan  
2 *Trichoplax adhaerens* to human  
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ABSTRACT

Nuclear receptors are a superfamily of transcription factors restricted to animals. These transcription factors regulate a wide variety of genes with diverse roles in cellular homeostasis, development, and physiology. The origin and specificity of ligand binding within lineages of nuclear receptors (e.g., subfamilies) continues to be a focus of investigation geared toward understanding how the functions of these proteins were shaped over evolutionary history. Among early-diverging animal lineages, the retinoid X receptor (RXR) is first detected in the placozoan, *Trichoplax adhaerens*. To gain insight into RXR evolution, we characterized ligand- and DNA-binding activity of the RXR from *T. adhaerens* (TaRXR). Like bilaterian RXRs, TaRXR specifically bound 9-cis-retinoic acid, which is consistent with a recently published result and supports a conclusion that the ancestral RXR bound ligand. DNA binding site specificity of TaRXR was determined through protein binding microarrays (PBMs) and compared with human RXR $\alpha$ . The binding sites for these two RXR proteins were broadly conserved (~85% shared high-affinity sequences within a targeted array), suggesting evolutionary constraint for the regulation of downstream genes. We searched for predicted binding motifs of the *T. adhaerens* genome within 1000 bases of annotated genes to identify potential regulatory targets. We identified 648 unique protein coding regions with predicted TaRXR binding sites that had diverse predicted functions, with enriched processes related to intracellular signal transduction and protein transport. Together, our data support hypotheses that the original RXR protein in animals bound a ligand with structural similarity to 9-cis-retinoic acid; the DNA motif recognized by RXR has changed little in more than 1 billion years of evolution; and the suite of processes regulated by this transcription factor diversified early in animal evolution.

## 47 INTRODUCTION

48 Nuclear receptors (NRs) are a diverse superfamily of metazoan transcription factors that regulate  
49 processes ranging from embryonic development to cellular differentiation and energetic metabolism  
50 (reviewed in Mangelsdorf, et al. 1995; Robinson-Rechavi, et al. 2003). Many NRs are activated by  
51 binding of specific ligands, such as steroids, thyroid hormones, retinoids, and fatty acids (Laudet, et al.  
52 1992; Beato, et al. 1995; Mangelsdorf, et al. 1995; Escriva, et al. 1997; Blumberg and Evans 1998). The  
53 remaining “orphan” NRs may be activated by unknown endogenous or environmental ligands, be  
54 activated through alternative mechanisms (*e.g.*, via phosphorylation), or possess constitutive activity  
55 (Giguere, et al. 1988; Power, et al. 1991; Marcus, et al. 1996; Escriva, et al. 1997). Activated NRs bind  
56 specific DNA response elements as monomers, homodimers, or heterodimers (reviewed by  
57 Khorasanizadeh and Rastinjad 2001). Upon binding to DNA response elements, NRs recruit coactivators  
58 or corepressors to modulate transcription of target genes. Progress toward elucidating the evolution of  
59 ligand diversity and specificity for NRs has accelerated in recent years, particularly through comparative  
60 studies involving more species from phylogenetically informative lineages. For example, integrated  
61 analyses of phylogeny, protein structure, and ligand affinity have resulted in novel and conflicting  
62 hypotheses for the evolution of ligands for the steroid hormone receptors (*e.g.*, the estrogen receptor (ER),  
63 and "steroid receptor" (SR)) in bilaterian animals (*e.g.*, Thornton 2003; Holzer, et al. 2017). Despite this  
64 progress, important theoretical and applied questions remain unanswered. Empirical identification of  
65 ligand specificity and ligand-mediated activation of NRs in aquatic animals remains a pressing concern  
66 due to the spectrum of pollutants that may function as potent mimics that disrupt endogenous NR  
67 signaling pathways.

68 Genes in the NR superfamily have been traditionally classified into six families, NR0 through  
69 NR6, by the Nuclear Receptors Nomenclature Committee (1999). Phylogenomic analyses of NR  
70 diversity over the past decade has shown that both protostomes and deuterostomes have at least one gene  
71 in each of these families, supporting the hypothesis that NRs had undergone extensive radiation prior to  
72 the divergence of these lineages. For example, Bertrand and colleagues (2004) analyzed the evolutionary

73 relationships among NRs from nine bilaterian genomes and inferred that 25 NRs likely existed in the  
74 Urbilaterian (ancestral bilaterian) with at least one gene from each of the six families. Additional PCR-  
75 based surveys of NRs and analyses of sequenced genomes have identified patterns of lineage-specific  
76 diversification and have also revealed lineage-specific losses of several NRs since the Urbilaterian  
77 ancestor, particularly in insects and nematodes (Taubert, et al. 2011; Fahrbach, et al. 2012).

78         Because the NR superfamily had already diversified prior to the divergence of the protostomes  
79 and deuterostomes, several analyses have aimed to characterize NRs within phyla that diverged early  
80 from the animal stem (Reitzel and Tarrant 2009; Bridgham, et al. 2010; Reitzel, et al. 2011). Such studies  
81 sought to understand not only when the NR families originated, but also how the function of specific NRs  
82 evolved and diversified. Together, these studies have revealed that the diversification of the NR  
83 superfamily was gradual, with the original NR likely to have been similar to HNF4 (NR2A), and  
84 diversification of the NR2 family and the origins of the NR3 and NR6 families pre-dating the appearance  
85 of the ancestral Urbilaterian. Studies conducted within early animal lineages to assess ligand specificity  
86 and modulation of NR activity by ligands support a hypothesis that ligands may have been functionally  
87 important in early NR evolution. Recent results from species in the phylum Ctenophora, the lineage  
88 likely to have first diverged from the animal stem, have shown that the only NRs present have only a  
89 conserved ligand binding domain (LBD) but not a DNA binding domain (DBD) (Reitzel, et al. 2011).  
90 Studies of two NRs (an HNF4 homolog and a second NR2 family member) from the sponge *Amphimedon*  
91 *queenslandica* revealed that these NRs both bound a fatty acid ligand, and one of these NRs was ligand-  
92 activated in reporter assays (Bridgham, et al. 2010). A number of other studies have shown that sponge  
93 extracts can function as ligands for vertebrate NRs (FXR and PXR) (Fiorucci, et al. 2012). Within the  
94 other non-bilaterian animal phyla, the Cnidaria and Placozoa, NR ligand binding has only been  
95 demonstrated for the retinoid X receptor, as further discussed below (Kostrouch, et al. 1998; Fuchs, et al.  
96 2014; Novotný, et al. 2017).

97         The retinoid X receptor (RXR, NR2B; USP in some insects) originated early in animal evolution  
98 with clear orthologs throughout the bilaterians as well as some cnidarians (medusozoans) and placozoans.

99 RXR has not been reported from sponges or ctenophores but NR diversity has only been characterized  
100 within a few species in each of these phyla. RXR is generally classified as a "type II" NR: it forms  
101 heterodimers, resides within the nucleus bound to DNA response elements, and becomes transcriptionally  
102 active upon ligand binding and associated release of corepressors (reviewed by Sever and Glass 2013). In  
103 bilaterians, RXR exhibits promiscuous heterodimerization with a number NR1 family proteins, including  
104 PPAR, RAR, FXR, PXR, VDR, and TR (reviewed by Lefebvre, et al. 2010; Evans and Mangelsdorf  
105 2014). The specific RXR heterodimerization partner influences the DNA binding behavior of the protein  
106 complex (Mader, et al. 1993; Laffitte, et al. 2000), thus influencing regulation of downstream genes. In  
107 addition, RXR has also been shown to bind as a tetramer and a monomer *in vitro*, although the  
108 physiological significance of this behavior has not been demonstrated (Kersten, et al. 1997; Kostrouch, et  
109 al. 1998). The combination of different partners and the corresponding differences in regulation of DNA  
110 correlate with the central roles for RXR in a diversity of cell functions from development to physiology  
111 (Evans and Mangelsdorf 2014). The ligands for RXR may be structurally diverse and influence the  
112 dimerization properties for this NR with other proteins. While it may not function as a physiological  
113 ligand, 9-cis-retinoic acid (9-cis-RA) binds RXR with high affinity and is the most commonly studied  
114 ligand (Heyman, et al. 1992). Studies in two cnidarian species, the box jellyfish *Tripedalia cystophora*  
115 (Kostrouch, et al. 1998) and the moon jelly *Aurelia aurita* (Fuchs, et al. 2014) have shown that the  
116 cnidarian RXR ortholog binds 9-cis-RA with high affinity and that ligand-based activation of RXR may  
117 be a central factor in mediating life history transitions in these species.

118 *Trichoplax adhaerens*, the originally described member of the phylum Placozoa, is a small,  
119 morphologically simple marine organism inhabiting pelagic marine environments around the world.  
120 Placozoa is an early-diverging phylum with a consensus position branching off the animal stem after  
121 sponges and ctenophores but prior to cnidarians, although its phylogenetic positions remains a subject of  
122 ongoing research (Nosenko, et al. 2013; Laumer, et al. 2017). Sequencing of the *T. adhaerens* genome in  
123 2008 provided a critical data set to discern the evolutionary history of particular gene families (Srivastava,  
124 et al. 2008), including NRs (Baker 2008). *Trichoplax adhaerens* has four NRs and is the first animal

125 lineage to have a well-supported RXR ortholog. Thus, studies of RXR from this species can provide a  
126 context for testing hypotheses regarding the antiquity of ligand and DNA binding within the NR2B  
127 subfamily. During development of this manuscript, it was reported that the RXR ortholog from *T.*  
128 *adhaerens* (TaRXR) can bind 9-cis-RA with high affinity (Novotny et al. 2017). Here, we further  
129 characterize the RXR ortholog from *T. adhaerens* including complementary studies of ligand binding, as  
130 well as new characterization of DNA binding and potential regulation of downstream genes. Because *T.*  
131 *adhaerens* lacks canonical RXR heterodimerization partners, these analyses of TaRXR binding also  
132 provide insights into the evolution of DNA binding by RXR before NR radiation in the bilaterians.

133

## 134 METHODS

135

### 136 *Annotation and cloning of T. adhaerens RXR (TaRXR)*

137 The NR complement from *T. adhaerens*, including an ortholog of RXR (JGI gene model 49897, TaRXR),  
138 was originally described by Baker (2008). The original annotation of the TaRXR was only a predicted  
139 gene model with no confirmation. We constructed a cDNA library (SMARTer RACE cDNA  
140 Amplification Kit, Clontech) with RNA isolated from a lab-reared colony of *T. adhaerens* in order to  
141 empirically determine the complete coding sequence for this gene. We used a nested primer design with  
142 two gene-specific "forward" primers (CGCGCCAAGTTGTGATGG and  
143 CCTGATGCCAAAGGATTGAATG) paired with the manufacturer-provided universal reverse primer to  
144 amplify the 3' end of TaRXR. Similarly, we used two gene-specific "reverse" primers  
145 (GGGATGTGCGATAGTAACCAA and TGCCGCAATTACTGAGAAAC) paired with the universal  
146 forward primer from the kit to amplify the 5' end. PCR amplicons were excised from 1% agarose gels,  
147 purified (QIAquick gel extraction, Qiagen), cloned (pGEM-T, Promega), and sequenced in both  
148 directions. Finally, we assembled the sequences *in silico* to produce a full-length transcript. We  
149 deposited the transcript into GenBank (Accession Number MG602679).

150

151 *Phylogeny of nuclear receptors in Family 2*

152 To more thoroughly assess the apparent absence of RXR from sponges (Phylum Porifera), we conducted  
153 BLAST-based searches of newly-available transcriptomes from 8 additional species that represented 4  
154 classes: Class Hexactinellida: *Aphrocallistes vastus*; Class Demospongiae: *Spongilla lacustris*, *Petrosia*  
155 *ficiformis*, *Pseudospongosorites suberitoides*, *Ircinia fasciculata* *Condrilla nucula*; Class  
156 Homoscleromorpha: *Corticium candelabrum*; Class Calcarea: *Sycon coactum* (Riesgo, et al. 2014).  
157 Transcriptomic databases were queried using NR sequences from *Trichoplax adhaerens* (NR2-4),  
158 *Tripedalia cystophora* (RXR) and *Amphimedon queenslandica* (NR1-2) as a search set. Searches were  
159 constrained to return the top 10 sequences using the tBLASTn algorithm with a threshold E-value of  $e^{-10}$ .  
160 These sequences were aligned with the query set using ClustalW, as implemented within BioEdit (Hall  
161 1999). The alignment was manually edited in the case of clear errors, and trimmed to contain only the  
162 well-conserved DBD and most of the LBD beginning in helix 3 (as in Reitzel, et al. 2011). Sequences  
163 shorter than 40 (DBD) or 100 residues (LBD) were deleted. Maximum likelihood analysis were  
164 conducted with RAxML (v7.2.7) using the CIPRES Science Gateway v 3.3 (Miller, et al. 2011). Support  
165 for nodes is indicated as a percentage of 1000 bootstraps. Trees were visualized using FigTree v1.1.2 and  
166 edited using Adobe Illustrator (colors, fonts, and additional labels).

167

168 *Expression constructs for protein expression*

169 Using the TaRXR transcript sequence we determined from our RACE products, we amplified the full-  
170 length open reading frame (Forward primer: GCTTGCAATGGAGGACAGATC, where underlined ATG is  
171 the start site; Reverse primer: CTGACCCACAATACAAGACAGC) and developed a protein expression  
172 construct using the Gateway cloning system (Thermo Fisher). We used pcDNA3.2/nV5-DEST  
173 (Invitrogen) generate a protein tagged with the v5 epitope at the amino terminus. We also obtained the  
174 human RXR $\alpha$  (HsRXR $\alpha$ ) in the pDONR221 vector from the Harvard Medical School (Clone  
175 HsCD00079702). We subcloned this into pcDNA3.2/V5-DEST (Invitrogen) but included the endogenous  
176 stop codon to generate an untagged protein.

177

178 *Ligand binding assays*

179 [11,12-<sup>3</sup>H] 9-cis retinoic acid (“<sup>3</sup>H 9-cis-RA” hereafter), 52.9 Ci/mmol was obtained from PerkinElmer.  
180 Assay conditions were similar to those previously described (Tarrant, et al. 2011). Briefly, RXR proteins  
181 from human and *T. adhaerens* were expressed using the TnT T7 Quick Coupled Reticulocyte Lysate  
182 System (Promega) using 2 µg of plasmid per 50 µl reaction. Expression was confirmed by synthesizing  
183 the protein in the presence of <sup>35</sup>S-labeled methionine. The labeled proteins were separated using SDS-  
184 PAGE, followed by fluorography (Amplify reagent, Amersham) and autoradiography. For binding  
185 studies, the proteins were expressed using unlabeled methionine and diluted 1:10 with TEG buffer (10  
186 mM Tris, 1.5 mM EDTA, 10% v/v sterile glycerol, 1 mM DTT). Unprogrammed lysate, (“UPL” an *in*  
187 *vitro* expression reaction conducted with an empty expression plasmid rather than a receptor) was used to  
188 assess non-specific binding. Where specified in the results, the human RXR was mixed with UPL to  
189 adjust for the difference in expression between RXR from the human and *T. adhaerens* plasmids. Diluted  
190 proteins were incubated in triplicate overnight in glass tubes at 4°C with <sup>3</sup>H 9-cis RA that was diluted in  
191 dimethylsulfoxide (2.5% solvent in assay) to give assay concentrations up to 25 nM. For competitive  
192 binding studies, triplicate glass tubes were incubated with 10 or 20 nM <sup>3</sup>H 9-cis RA with or without a  
193 100-fold excess of unlabeled 9-cis RA. The next morning, aliquots from each tube were incubated on ice  
194 (15 min, periodic vortexing) in a 1:1 mixture with 50 mg/ml dextran-coated charcoal. This mixture was  
195 then centrifuged at 14,000g for 1 min. A 40-µl aliquot of the supernatant was added to 4 ml of  
196 ScintiVerse II cocktail (Fisher Scientific) and counted on a Beckman 5000 liquid scintillation counter.

197

198 *Protein binding microarray data generation and identification of binding motifs*

199 DNA binding specificity of TaRXR was assessed using a custom protein binding microarray (PBM)  
200 ordered from Agilent Technologies (PBM2), which contains 15,000 spots (five replicates of ~3000  
201 unique sequences). Microarray design, composition and exposure conditions followed those described by



202 Boltin *et al.* (2010) and Fang *et al.* (2012). The v5-tagged TaRXR was expressed in Cos-7 cells. Eight  
203 hundred ng of crude nuclear extract were used for hybridization, followed by an overnight room  
204 temperature incubation in 1:100 dilution of anti-V5 antibody (mouse monoclonal, Invitrogen), and a 1 hr  
205 incubation in the secondary antibody (Dylight, Jackson ImmunoResearch). The slide was scanned at 543  
206 (Cy3) and 633 (Cy5) nm (Supplemental Figure 1). Data were extracted for analysis of signal intensity for  
207 each sequence motif. Position weight matrices (PWMs) were generated using Weblogo (Crooks, et al.  
208 2004). We compared the binding results for TaRXR with results from HsRXR $\alpha$  hybridized under the  
209 same conditions an identical microarray. Motif overlapping between *T. adhaerens* and human were  
210 compared using the program Venny (Oliveros 2007-2015).

211

#### 212 *Prediction of associated genes regulated by RXR and annotation based on function/GO*

213 We used a custom bioinformatic pipeline to identify genes potentially regulated by RXR binding sites in  
214 the *T. adhaerens* genome (version 1.0, release date 8/18/2014). Briefly, the genome was screened using  
215 grep commands for a strict top binder (AGGTCAAAGGTCA), a consensus sequence from the top 10% of  
216 bound sequences ("strict", C A/C A/G A/G GGTC A), and the most common extended half-site  
217 (CAAAGGTCA) from the PBM hybridization data. Seventy base-pair regions of the genome with  
218 associated RXR binding sites were extracted and used in a subsequent BLASTn search against the *T.*  
219 *adhaerens* genome. The position information from the BLASTn search results was then used in  
220 combination with a custom Python script (Supplemental File 1) to isolate 1000 bases upstream and  
221 downstream of each site on each genome scaffold. These 2 kb nucleotide sequences were then used in  
222 BLASTx searches to identify annotated predicted proteins within these regions. This strategy was used in  
223 place of searching around the currently annotated start sites in the reference genome because these gene  
224 models are largely unvalidated and the start sites are unknown. Proteins with e-values below 1E-10 and  
225 an identity score greater than 95% were retained to identify possible function based on gene ontology  
226 (GO) groups. GO terms were clustered in the program REVIGO (Supek, et al. 2011) with the whole  
227 UniProt database used to reference the size of each term, and semantic similarities were determined using

228 SimRel (Schlicker, et al. 2006). We also searched the *T. adhaerens* genome and the 2 kb windows for  
229 potential dimerization-specific RXR binding sites by using combinations of the two likely half-sites for  
230 this NR, AGGTCA and GGGTCA. We restricted these searches to DR1 sites only because these are  
231 common for the NR2 family. Lastly, we compared results from these searches with parallel queries using  
232 the human genome.

233

## 234 RESULTS

235

### 236 *T. adhaerens* RXR

237 *In silico* assembly of sequenced PCR and RACE clones resulted in a transcript of 2,514 bp encoding RXR  
238 from *T. adhaerens* (TaRXR), with the open reading frame coding for a protein of 345 amino acids from  
239 position 406-1443. Mapping this transcript to the reference genome showed that TaRXR is composed of  
240 six exons spanning 7,236 bp of genomic DNA (scaffold2: 5291710-5298946; Figure 1). The first two  
241 exons exclusively encode 5' UTR, and the predicted translational start site is located on exon 3. The  
242 amino acids corresponding to the DNA binding domain (DBD) are located on exons 3 and 4, and the  
243 ligand binding domain (LBD) is located on exons 5 and 6. The stop site for TaRXR is located on exon 6,  
244 which is followed by 1,188 bp of 3' UTR with a poly(A) site at position 2,464. The DBD and LBD of  
245 TaRXR are both well conserved when compared with RXR sequences from other animals (~80% and  
246 ~65-75%, respectively).

247

### 248 *Phylogeny of nuclear receptors for Family 2*

249 Queries of sponge transcriptomes with a set of six NRs resulted in largely overlapping results that differed  
250 primarily in the ordering of sequences returned (i.e., their relative e-values). Most sequences appeared to  
251 be incomplete, frequently missing either the DBD or LBD. For this reason, separate likelihood-based  
252 analyses were conducted for the DBD alone, LBD alone and DBD plus LBD. Results were qualitatively  
253 similar between these three analyses, so only the DBD plus LBD analyses are shown and used in all

254 future analysis and discussion. Overall, analyses of sponge NRs resulted in a strongly supported clade that  
255 included the previously described HNF4 from *A. queenslandica* and was most similar to HNF4  
256 (Supplemental Figure 2). The cnidarian and placozoan RXR sequences (from *T. cystophora* and *T.*  
257 *adhaerens*, respectively) were grouped together with strong support. Poor support was obtained for most  
258 other nodes, and no sponge genes emerged as clear RXR homologs.

259

#### 260 *Ligand binding assays*

261 When expressed using *in vitro* transcription and translation and <sup>35</sup>S-labeled methionine, the HsRXR $\alpha$  and  
262 TaRXR plasmids each produced a dominant band of the appropriate size, but the human plasmid yielded  
263 4.2-fold more protein (Figure 2A). Both the human and *T. adhaerens* RXRs consistently bound tritiated  
264 9-cis RA, in excess of binding to the “unprogrammed lysate” (UPL) control (Figure 2B, paired t-test on  
265 means  $p = 0.007$ ). However, in this assay system, after subtracting this nonspecific binding, saturation  
266 was not observed, even at ligand concentrations up to 20 nM (Figure 2C). This may be due to high  
267 binding of 9-cis-RA to other proteins in the lysate mixture, resulting in ligand depletion. Thus, these assay  
268 conditions are not appropriate for measuring binding affinity and capacity, and can only give qualitative  
269 insight into ligand binding. In competitive binding assays, co-incubation with a 100-fold excess of  
270 unlabeled 9 cis-RA effectively decreased the bound ligand to background levels (Figure 2D).

271

#### 272 *RXR binding motifs*

273 Custom PBMs were used to identify DNA binding motifs for TaRXR and to then compare these motifs  
274 with those bound by human RXR $\alpha$ . Bound sequences were ranked according to the signal intensity for  
275 each species. TaRXR bound a total of 1266 sequences (42%). The strongest binders contained a strongly  
276 conserved sequence (GGTCA) in the last five positions of the 3' half site and relatively well-conserved  
277 sequences in the four positions before these (Figure 3A). Weaker binding was measured for both halves  
278 of the DR1 sequence (middle and bottom binders), suggesting TaRXR may bind as a dimer despite a  
279 preference for the 3' half site. Human RXR $\alpha$  has been previously shown to bind a similar number of

280 unique sequences (n = 1285) with the PWM showing predominant binding as a homodimer to two half  
281 sites as well as to the 3' half site in this PBM design (Fang, et al. 2012). In contrast, our results here using  
282 the same protein suggest strongest binding to the 3' half site; the difference is an artefactual one  
283 dependent on the precise number of sequences used to generate the PWM. The single top binding  
284 sequence for both human and TaRXR is an exact, full DR1 (AGGTCAAAGGTCA), suggesting that  
285 indeed both RXRs could be binding as homodimers. Binding motifs for human and *T. adhaerens* RXRs  
286 closely resemble one another for each grouping of binding strength. Overall specificity for binding is  
287 broadly correlated between the human and placozoan RXRs (Figure 3B). The similar specificity for  
288 binding by each RXR protein was mirrored by a large overlap in shared sequences bound in the PBM  
289 comparisons (Figure 3C). Approximately 84% of bound sequences are shared despite the vast  
290 evolutionary distance between these species.

291

#### 292 *Predicted RXR binding sites and Gene Ontology annotation*

293 In total, our bioinformatic pipeline identified 1,567 potential "strict" RXR binding sites (i.e., match  
294 consensus motif from top 10% of PBM probes), 557 of which contain the most common extended half-  
295 site (i.e., CAAAGGTCA), within the *T. adhaerens* genome (Figure 4, Table 1). Of these, 685 RXR sites  
296 were within 1000 bases of a protein coding region, with approximately 10% of these assigned a potential  
297 function (Supplemental Table 2). The largest scaffold (Scaffold\_1) in the *T. adhaerens* genome assembly  
298 had the highest number of these RXR binding sites (N = 109), although, 18 other scaffolds (39% of those  
299 recovered) had a higher RXR binding site occurrence per nucleotide. A relatively small scaffold  
300 (Scaffold\_2888, length = 1338 bp) had the most RXR sites at ~4.5 per 1kb, while the average across  
301 scaffolds is ~1.4 per 10 kb (Supplemental Table 1). We further searched the placozoan genome for half-  
302 sites across the whole genome, in addition to regions adjacent to the RXR sites identified with the PBM  
303 data. Interestingly, these searches revealed that the first position in the half-site had a bias of 3:1 for A  
304 over G (Figure 4). Comparisons with the human genome for these individual binding sites suggest no  
305 strong bias in representation when genome size is accounted for (i.e., human genome is 60 times larger).

306 Since potential dimer activity was detected in the PBM we searched for DR1 sites using the two common  
307 half-sites. Surprisingly, we only identified nine total, two of which were within 1 kb of an annotated gene  
308 (Table 1).

309 Many of the proteins near a predicted RXR binding site (Table 1) within the *T. adhaerens*  
310 genome could not be annotated using BLAST-based similarity searches against other taxa (574 of 648  
311 proteins annotated only as “uncharacterized” or “predicted proteins”, Supplemental Table 2). Despite  
312 this, many of these proteins still contained GO information on UNIPROT, recovering 1,584 GO terms  
313 associated with the full set of 597 unique predicted proteins (Supplemental Table 3). The most abundant  
314 GO terms within the Biological Process domain were associated with *intracellular signal transduction*  
315 [GO:0035556], *ubiquitin-dependent protein catabolic process* [GO:0006511], and *mRNA splicing via*  
316 *spliceosome* [GO:0000398]. The Cellular Component domain recovered the smallest number of GO  
317 terms, but included three of the most frequent terms: *integral component of membrane* [GO:0016021],  
318 *nucleolus* [GO:0005730], and *cytosol* [GO:005829]. The Molecular Function domain had the largest  
319 number of individual GO terms associated with predicted RXR target proteins, including *GTP binding*  
320 [GO:0005525], *microtubule binding* [GO:0008017], *sequence-specific DNA binding* [GO:0043565], and  
321 *GTPase activity* [GO:0003924] (Figure 5).

322

## 323 DISCUSSION

324

325 Studies of nuclear receptor diversity and function from early-diverging animal phyla provide essential  
326 comparative data to determine historical patterns of gene evolution with particular molecular and  
327 physiological activities. NR-specific functions, including ligand-specific binding and cell-specific  
328 expression, are now well characterized in many bilaterian groups. Current understanding of the  
329 evolutionary origin of these genes and their ancestral functions has been heavily influenced by studies of  
330 individual NRs using a few species from early-diverging animal phyla. NRs in sponges and ctenophores  
331 and a majority of NRs from the cnidarians and placozoans are confidently placed in the NR2 family,

332 suggesting that the ancestral NR resembled these proteins, particularly HNF4. RXR, as a member of  
333 NR2, also evolved quite early in animal evolution, likely at the ancestor of the Parahoxozoa, a group that  
334 includes Plazozoa, Cnidaria and Bilateria (terminology after Ryan, et al. 2010). Our analyses of NR  
335 complements from additional sponge species did not reveal any RXR homologs within sponges and  
336 consequently does not change the inferred timing for the evolution of RXR (Supplemental Figure 2).  
337 Wiens *et al.* (2003) previously reported that exposure to micromolar concentrations of all-trans retinoic  
338 acid induces tissue regression and up-regulates NR expression in the sponge *Suberites domoncula*. Given  
339 the absence of a clear retinoid receptor from the sponge lineage, the mechanism for these effects is still  
340 unclear.

341 Ligand binding assays within both placozoans (Novotný, et al. 2017 and this study) and cnidarians  
342 (Kostrouch, et al. 1998; Fuchs, et al. 2014) clearly support the hypothesis that the ancestral RXR bound  
343 ligand(s). This contrasts with vertebrate-type steroid receptors, in which the origins of ligand binding and  
344 activation are less clear (Thornton 2003; Holzer, et al. 2017). Within both protostome and deuterostome  
345 lineages, RXR homologs from many species are able to bind 9-cis RA. However the capacity for 9-cis  
346 RA binding has been lost from several lineages, most notably in the case of the insect ultraspiracle  
347 proteins (Iwema, et al. 2007), but also from the urochordate *Halocynthia roretzi* (Maeng, et al. 2012) and  
348 some RXR subtypes from the zebrafish *Danio rerio* (Jones, et al. 1995). As in other species, 9-cis RA is a  
349 useful pharmacological reagent to study the function of TaRXR, but it is not necessarily a physiological  
350 ligand. Identification of physiological ligands for RXR is a subject of active study (e.g., Rühl, et al. 2015  
351 and references therein), and additional insight could be gained from assessing the activity of candidate  
352 ligands with RXRs from early-diverging animal lineages.

353 Evolutionary changes in the DNA binding domains of transcription factors are one molecular  
354 mechanism that can create shifts in the regulation of gene networks. Nonsynonymous mutations in these  
355 domains and other parts of the protein may result in amino acid substitutions that influence the particular  
356 sequence motifs bound (e.g., Balczarek, et al. 1997; Fang, et al. 2012; Sen, et al. 2013; Barrera, et al.  
357 2016). Identification of NR binding sites has revealed subfamily-specific patterns of preferential binding

358 to half sites with different spacing and orientation (e.g., Fang, et al. 2012). DNA binding motifs for  
359 TaRXR were highly similar to human RXR $\alpha$  with more than 84% of binding sites conserved. TaRXR  
360 bound DNA sequences that contained a majority of the conserved half-site AGGTCA, with only the most  
361 5' position showing variation (A or G). The most strongly bound motif contained the canonical half-site.  
362 These results suggest extreme conservation of motifs for RXR orthologs over vast evolutionary distance.  
363 Indeed, binding sites for NR2 family members are generally conserved due to conservation of the contact  
364 amino acids in the DNA binding domain. However, subtle variations in the DNA binding motif for  
365 transcription factors can result in shifts in gene expression in particular cells types dependent on co-  
366 factors (Nakagawa, et al. 2013; Cheatle Jarvela, et al. 2014; Cary, et al. 2017).

367 Our queries of the *T. adhaerens* genome for potential RXR binding sites within 1 kb of the  
368 annotated genes suggested more than 600 potential downstream genes (of the total 11,514 predicted genes  
369 in the genome) could be regulated by this transcription factor. Of these, 192 predicted RXR binding sites  
370 were found adjacent to a predicted protein's start codon; however, most of those proteins are  
371 uncharacterized. Of those with predicted functions, there were four locations within the *T. adhaerens*  
372 genome where RXR binding sites were found adjacent to start sites to the Eukaryotic translation initiation  
373 factor 3 (eIF-3) complex (B3RIH4) and two sites which may contain start sites for Acyl-coenzyme A  
374 oxidase (B3S9Y8). Recent research by Novotný, et al. (2017) showed that 9-cis-RA causes shifts in NR  
375 transcription, results in growth arrest in *T. adhaerens*, and induces expression of L-malate-NADP+  
376 oxidoreductase. The annotations in the *T. adhaerens* genome remain largely unverified, thus our  
377 interpretations from these binding sites are certainly preliminary. Furthermore, these binding motifs may  
378 be shared with the other NR2 family genes in *T. adhaerens* (HNF4 and COUP-TF-like), as well as the  
379 ERR-like gene within the NR3 family. Future research to characterize the specific binding sites for these  
380 other three NRs and compare them to TaRXR would identify the potential degree of overlap for NR  
381 regulatory targets in this placozoan species.

382 It is of interest to determine whether TaRXR binds as a dimer or a monomer. The preference for a  
383 single half site from the PBM data suggest that Ta RXR may bind as a monomer. Particular bilaterian

384 NRs have been shown to preferentially or exclusively bind to DNA as a monomer; e.g., NGFI-B (Meinke  
385 and Sigler 1999), TR (Quack and Carlberg 2001), and RORs (Schröder, et al. 1996). However, dimeric  
386 binding cannot necessarily be ascertained by PBMs. We also found a single half site preference for  
387 human RXR in our analysis, and mammalian RXRs are well-established to bind DNA as dimers,  
388 especially as heterodimers. Another perhaps more important question is, if TaRXR does bind DNA as a  
389 dimer, does it bind as a homo- or a heterodimer? There are just 4 NR genes in *T. adhaerens*. In addition  
390 to RXR, there is HNF4, which has been well established as an obligate homodimer that cannot  
391 heterodimerize with RXR in mammalian systems (Jiang, et al. 1995; Bogan, et al. 2000) and an ERR,  
392 which classically binds as a monomer (Johnston, et al. 1997). The last NR is COUP-TF-like, which is  
393 known to heterodimerize with RXR in bilaterians (e.g., Kliewer, et al. 1992). Co-immunoprecipitation  
394 studies to determine the potential dimerization activity of *T. adhaerens* NRs would be essential to  
395 determine how RXR may form protein-protein interactions. Identification of the first instance of  
396 heterodimerization among NRs is an important issue and requires additional studies.

397 Our study of ligand and DNA binding by TaRXR supports hypotheses that (1) ligand binding was  
398 present at the origin of RXR in animals, and (2) the DNA binding motif has changed little over more than  
399 1 billion years of independent evolution. Although studies of steroid receptors have suggested that ligand  
400 binding may or may not have evolved after the evolution of the NR3 family (Eick and Thornton 2011;  
401 Markov and Laudet 2011), comparative studies of HNF4 in sponges suggest that specific ligand binding  
402 also was present early in animal evolution (Bridgham, et al. 2010). These data with NRs correspond with  
403 other transcription factors studied in early diverging phyla that have shown ligand specificity was present  
404 early animal evolution, e.g., canonical and non-canonical Wnt signaling (Lee, et al. 2006; Rigo-  
405 Watermeier, et al. 2012). Continued comparisons of transcription factor diversification and binding  
406 specificity will illuminate how ligand-regulated gene networks evolved in early animal evolution.

407

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409



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416 R01DK094707 to FMS.

## 416 TABLES

417

418 Table 1. Distribution of predicted RXR binding sites in the *T. adhaerens* genome and within 1 kb of an  
 419 annotated gene. Identification of sites for possible RXR dimer binding was restricted to DR1-type binding  
 420 sites composed of two half-sites that match the dominant sequences from PBM data (Figure 3A).

421

Sequence motif for query (5' - 3')	Name	Unique matches within 1kb of gene*	Unique matches associated with RXR sites*	Matches in <i>T. adhaerens</i> genome overall	Unique matches in <i>H. sapiens</i> genome
CAAAGGTCA	Common top binder	219	557	557	36,079
C A/C A/G A/G GGTC A	Top 10%	685	1,567	1,567	96,323
AGGTCA	Half-site 1 (HS1)	834	2,005	23,389	2,060,590
GGGTCA	Half-site 2 (HS2)	323	721	8,256	1,064,378
AGGTCA <sub>n</sub> AGGTCA	DR1 (HS1-HS1)	0	1	3	1,059
GGGTCA <sub>n</sub> GGGTCA	DR1 (HS2-HS2)	0	0	0	3,187
AGGTCA <sub>n</sub> GGGTCA	DR1 (HS1-HS2)	2	2	3	715
GGGTCA <sub>n</sub> AGGTCA	DR1 (HS2-HS1)	0	2	3	715

422 \*Values for all Half-site and DR1-type binding sites were estimated by screening 1KB regions flanking  
 423 predicted RXR binding sites.

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432 FIGURE LEGENDS

433

434 Figure 1. Gene structure for *Trichoplax adhaerens* RXR (TaRXR).

435

436 Figure 2. Characterization of <sup>3</sup>H 9-cis retinoic acid (<sup>3</sup>H 9-cis RA) by the *Trichoplax adhaerens* RXR  
437 (TaRXR) in comparison with human RXR $\alpha$  (HsRXR $\alpha$ ). (A) Proteins expressed in the presence of <sup>35</sup>S  
438 methionine produced dominant bands of the expected size (TaRXR 39.4 kDa, HsRXR $\alpha$ : 56.7 kDa). (B)  
439 Total binding of HsRXR, TaRXR and unprogrammed lysate (UPL, non-specific) to varying concentrations  
440 of <sup>3</sup>H 9-cis RA. Quadratic fitted curves shown. Mean and standard deviation are shown. Red symbols  
441 indicate the human RXR $\alpha$  diluted with UPL to produce an equivalent level of expressed protein. (C)  
442 Specific binding of HsRXR and TaRXR to <sup>3</sup>H 9-cis RA, calculated from the data shown in B by subtracting  
443 the binding to the UPL. Non-linear curve fit one-site specific binding, as implemented within GraphPad  
444 Prism. (D) Competitive binding of <sup>3</sup>H 9-cis RA to TaRXR and HsRXR after subtracting non-specific  
445 binding to UPL.

446

447 Figure 3. Binding site specificity of TaRXR and comparisons with HsRXR $\alpha$  assessed by protein binding  
448 microarray (PBM). (A) Weblogo summarizing the top 10% of sequences bound (strict), top 33% (top),  
449 middle 33% (middle), and bottom 33% (bottom) for TaRXR and HsRXR $\alpha$  bound sequences. (B) Overall  
450 specificity of TaRXR and HsRXR $\alpha$  over all sites bound is highly correlated. (C) Binding sites for these  
451 two RXR proteins are largely shared, with ~91% overlap of bound sequences.

452

453 Figure 4. Weblogo of each nucleotide across the 1,567 potential RXR binding sites identified in the the *T.*  
454 *adhaerens* genome.

455

456 Figure 5. Gene Ontology analysis of proteins identified through REVIGO with RXR binding sites within  
457 1 kb of the start site. Values represent the abundance of selected GO terms; the full list of GO terms can  
458 be found in Supplemental Table 3. Colored column adjacent to sequence IDs correspond to REVIGO  
459 frequencies scores, with red representing GO terms that are more abundant/generic and green representing  
460 less abundant/rare GO terms.

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463 SUPPLEMENTAL FIGURE LEGENDS

464

465 Supplemental Figure 1. Image of the PBM post-hybridization with TaRXR.

466

467 Supplemental Figure 2. Maximum likelihood tree of Poriferan (sponge) NRs in relation to NR diversity  
468 from other animals. Sequence names from newly analyzed sponge transcriptomes are abbreviated with  
469 the first letter of the genus and species (Class Hexactinellida: *Aphrocallistes vastus*; Class Demospongiae:  
470 *Spongilla lacustris*, *Petrosia ficiformis*, *Pseudospongosorites suberitoides*, *Ircinia fasciculata*, *Condrilla*  
471 *nucula*; Class Homoscleromorpha: *Corticium candelabrum*; Class Calcarea: *Sycon coactum*) followed by  
472 a contig number. Numbers represent a percentage of 1000 bootstrap replicates; for ease of visualization  
473 only values above 50 are shown. Asterisks indicate sequences that were part of the query set in BLAST-  
474 based searches. Colored lines indicate groups with previously described NRs from the sponge  
475 *Amphimedon queenslandica* Class Demospongiae).

476

477 SUPPLEMENTAL TABLES

478

479 Supplemental Table 1. Number of RXR sites across each *Trichoplax adhaerens* genome scaffold.  
480  
481 Supplemental Table 2. Annotation of proteins within 2 kb of predicted RXR bindings sites in the  
482 *Trichoplax adhaerens* genome. Protein gene names and enzymatic pathways derived from homology-  
483 based annotation within Uniprot.  
484  
485 Supplemental Table 3. Full annotation associated with all predicted RXR regulatory targets within the  
486 *Trichoplax adhaerens* genome.  
487  
488 Supplemental Table 4. TaRXR processed PBM data.  
489  
490 Supplemental Table 5. HsRXR processed PBM data.  
491  
492 SUPPLEMENTAL FILE  
493  
494 Supplemental File 1. Python script for extracting 2 kb of genomic sequence around candidate RXR  
495 binding sites from the *Trichoplax adhaerens* genome.  
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500 REFERENCES

- 501
- 502 Baker ME. 2008. *Trichoplax*, the simplest known animal, contains an estrogen-related receptor but no  
503 estrogen receptor: Implications for estrogen receptor evolution. *Biochemical and Biophysical Research*  
504 *Communications* 375.
- 505 Balczarek KA, Lai ZC, Kumar S. 1997. Evolution of functional diversification of the paired box (Pax) DNA-  
506 binding domains. *Molecular Biology and Evolution* 14:829-842.
- 507 Barrera LA, Vedenko A, Kurland JV, Rogers JM, Gisselbrecht SS, Rossin EJ, Woodard J, Mariani L, Kock KH,  
508 Inukai S, et al. 2016. Survey of variation in human transcription factors reveals prevalent DNA binding  
509 changes. *Science* 351:1450.
- 510 Beato M, Herrlich P, Schutz G. 1995. Steroid hormone receptors: many actors in search of a plot. *Cell*  
511 83:851-857.
- 512 Bertrand S, Brunet F, Escriva H, Parmentier G, Laudet V, Robinson-Rechavi M. 2004. Evolutionary  
513 genomics of nuclear receptors: from twenty-five ancestral genes to derived endocrine systems.  
514 *Molecular Biology and Evolution* 21:1923-1937.
- 515 Blumberg B, Evans R. 1998. Orphan nuclear receptors--new ligands and new possibilities. *Genes &*  
516 *Development* 12:3149-3155.
- 517 Bogan AA, Dallas-Yang Q, Ruse MD, Maeda Y, Jiang G, Nepomuceno L, Scanlan TS, Cohen FE, Sladek FM.  
518 2000. Analysis of protein dimerization and ligand binding of orphan receptor HNF4 $\alpha$ . *Journal of*  
519 *Molecular Biology* 302:831-851.
- 520 Bolotin E, Liao H, Ta TC, Yang C, Hwang-Verslues W, Evans JR, Jiang T, Sladek FM. 2010. Integrated  
521 approach for the identification of human hepatocyte nuclear factor 4 $\alpha$  target genes using protein  
522 binding microarrays. *Hepatology* 51:642-653.
- 523 Bridgham JT, Eick GN, Larroux C, Deshpande K, Harms MJ, Gauthier MEA, Ortlund EA, Degnan BM,  
524 Thornton JW. 2010. Protein evolution by molecular tinkering: diversification of the nuclear receptor  
525 superfamily from a ligand-dependent ancestor. *PLOS Biology* 8:e1000497.
- 526 Cary GA, Cheatle Jarvela AM, Francolini RD, Hinman VF. 2017. Genome-wide use of high- and low-  
527 affinity Tbrain transcription factor binding sites during echinoderm development. *Proceedings of the*  
528 *National Academy of Sciences* 114:5854-5861.
- 529 Cheatle Jarvela AM, Brubaker L, Vedenko A, Gupta A, Armitage BA, Bulyk ML, Hinman VF. 2014. Modular  
530 evolution of DNA-binding preference of a tbrain transcription factor provides a mechanism for  
531 modifying gene regulatory networks. *Molecular Biology and Evolution* 31:2672-2688.
- 532 Crooks GE, Hon G, Chandonia J-M, Brenner SE. 2004. WebLogo: A sequence logo generator. *Genome*  
533 *Research* 14:1188-1190.
- 534 Eick GN, Thornton JW. 2011. Evolution of steroid receptors from an estrogen-sensitive ancestral  
535 receptor. *Molecular and Cellular Endocrinology* 334:31-38.
- 536 Escriva H, Safi R, Hanni C, Langlois M-C, Saumitou-Laprade P, Stehelin D, Capron A, Pierce R, Laudet V.  
537 1997. Ligand binding was acquired during the evolution of nuclear receptors. *Proceedings of the*  
538 *National Academy of Science, USA* 94:6803-6808.
- 539 Evans RM, Mangelsdorf DJ. 2014. Nuclear receptors, RXR, and the big bang. *Cell* 157:255-266.
- 540 Fahrbach SE, Smagghe G, Velarde RA. 2012. Insect nuclear receptors. *Annual Review of Entomology*  
541 57:83-106.
- 542 Fang B, Mane-Padros D, Bolotin E, Jiang T, Sladek FM. 2012. Identification of a binding motif specific to  
543 HNF4 by comparative analysis of multiple nuclear receptors. *Nucleic Acids Research* 40:5343-5356.
- 544 Fiorucci S, Distrutti E, Bifulco G, D'Auria MV, Zampella A. 2012. Marine sponge steroids as nuclear  
545 receptor ligands. *Trends in Pharmacological Sciences* 33:591-601.

546 Fuchs B, Wang W, Graspentner S, Li Y, Insua S, Herbst E-M, Dirksen P, Böhm A-M, Hemmrich G,  
547 Sommer F, et al. 2014. Regulation of polyp-to-jellyfish transition in *Aurelia aurita*. *Current Biology*  
548 24:263-273.

549 Giguere V, Yang N, Segui P, Evans R. 1988. Identification of a new class of steroid hormone receptors.  
550 *Nature* 331:91-94.

551 Hall TA. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for  
552 Windows 95/98/NT. *Nuclear Acids Symposium Series* 41:95-98.

553 Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, Thaller C. 1992. 9-cis retinoic acid is  
554 a high affinity ligand for the retinoid X receptor. *Cell* 68:397-406.

555 Holzer G, Markov GV, Laudet V. 2017. Evolution of nuclear receptors and ligand signaling: Toward a soft  
556 key-lock model? In: Douglas F, Sophia T, editors. *Current Topics in Developmental Biology*: Academic  
557 Press. p. 1-38.

558 Iwema T, Billas IM, Beck Y, Bonneton F, Nierengarten H, Chaumot A, Richards G, Laudet V, Moras D.  
559 2007. Structural and functional characterization of a novel type of ligand-independent RXR-USP  
560 receptor. *The EMBO Journal* 26:3770-3782.

561 Jiang G, Nepomuceno L, Hopkins K, Sladek FM. 1995. Exclusive homodimerization of the orphan  
562 receptor hepatocyte nuclear factor 4 defines a new subclass of nuclear receptors. *Molecular and Cellular*  
563 *Biology* 15:5131-5143.

564 Johnston SD, Liu X, Zuo F, Eisenbraun TL, Wiley SR, Kraus RJ, Mertz JE. 1997. Estrogen-related receptor  
565  $\alpha 1$  functionally binds as a monomer to extended half-site sequences including ones contained within  
566 estrogen-response elements. *Molecular Endocrinology* 11:342-352.

567 Jones BB, Ohno CK, Allenby G, Boffa MB, Levin AA, Grippo JF, Petkovich M. 1995. New retinoid X  
568 receptor subtypes in zebra fish (*Danio rerio*) differentially modulate transcription and do not bind 9-cis  
569 retinoic acid. *Molecular and Cellular Biology* 15:5226-5234.

570 Kersten S, Gronemeyer H, Noy N. 1997. The DNA binding pattern of the retinoid X receptor is regulated  
571 by ligand-dependent modulation of its oligomeric state. *Journal of Biological Chemistry* 272:12771-  
572 12777.

573 Khorasanizadeh S, Rastinjad F. 2001. Nuclear-receptor interactions on DNA-response elements. *Trends*  
574 *in Biochemical Sciences* 26:384-390.

575 Kliewer SA, Umesono K, Heyman RA, Mangelsdorf DJ, Dyck JA, Evans RM. 1992. Retinoid X receptor-  
576 COUP-TF interactions modulate retinoic acid signaling. *Proceedings of the National Academy of Sciences*  
577 89:1448-1452.

578 Kostrouch Z, Kostrouchova M, Love W, Jannini E, Piatigorsky J, Rall J. 1998. Retinoic acid X receptor in  
579 the diploblast, *Tripedalia cystophora*. *Proceedings of the National Academy of Science, USA* 95.

580 Laffitte BA, Kast HR, Nguyen CM, Zavacki AM, Moore DD, Edwards PA. 2000. Identification of the DNA  
581 binding specificity and potential target genes for the farnesoid X-activated receptor. *Journal of Biological*  
582 *Chemistry* 275:10638-10647.

583 Laudet V, Hanni V, Coll J, Catzeflis F, Stehelin D. 1992. Evolution of the nuclear receptor gene  
584 superfamily. *The EMBO Journal* 11:1003-1013.

585 Laumer CE, Gruber-Vodicka H, Hadfield MG, Pearse VB, Riesgo A, Marioni JC, Giribet G. 2017. Placozoans  
586 are eumetazoans related to Cnidaria. *bioRxiv*.

587 Lee PN, Pang K, Matus DQ, Martindale MQ. 2006. A WNT of things to come: Evolution of Wnt signaling  
588 and polarity in cnidarians. *Seminars in Cell & Developmental Biology* 17:157-167.

589 Lefebvre P, Benomar Y, Staels B. 2010. Retinoid X receptors: common heterodimerization partners with  
590 distinct functions. *Trends in Endocrinology & Metabolism* 21:676-683.

591 Mader S, Chen JY, Chen Z, White J, Chambon P, Gronemeyer H. 1993. The patterns of binding of RAR,  
592 RXR and TR homo- and heterodimers to direct repeats are dictated by the binding specificities of the  
593 DNA binding domains. *The EMBO Journal* 12:5029-5041.

594 Maeng S, Lee JH, Choi S-C, Kim MA, Shin YK, Sohn YC. 2012. The retinoid X receptor in a marine  
595 invertebrate chordate: Evolutionary insights from urochordates. *General and Comparative*  
596 *Endocrinology* 178:380-390.

597 Mangelsdorf D, Thummel C, Beato M, Herrlich P, Schatz G, Umesono K, Blumberg B, Chambon P, Evans  
598 R. 1995. The nuclear receptor superfamily: the second decade. *Cell* 83:835-839.

599 Marcus S, Winrow C, Capone J, Rachubinski R. 1996. A p56lck ligand serves as a coactivator of an orphan  
600 nuclear hormone receptor. *Journal of Biological Chemistry* 271:27197-27200.

601 Markov GV, Laudet V. 2011. Origin and evolution of the ligand-binding ability of nuclear receptors.  
602 *Molecular and Cellular Endocrinology* 334:21-30.

603 Meinke G, Sigler PB. 1999. DNA-binding mechanism of the monomeric orphan nuclear receptor NGFI-B.  
604 *Nature Structural & Molecular Biology* 6:471-477.

605 Miller MA, Pfeiffer W, Schwartz T. 2011. The CIPRES science gateway: a community resource for  
606 phylogenetic analyses. *Proceedings of the 2011 TeraGrid Conference: Extreme Digital Discovery*; Salt  
607 Lake City, Utah: 2016785: ACM. p. 1-8.

608 Nakagawa S, Gisselbrecht SS, Rogers JM, Hartl DL, Bulyk ML. 2013. DNA-binding specificity changes in  
609 the evolution of forkhead transcription factors. *Proceedings of the National Academy of Sciences*  
610 110:12349-12354.

611 Nosenko T, Schreiber F, Adamska M, Adamski M, Eitel M, Hammel J, Maldonado M, Müller WEG, Nickel  
612 M, Schierwater B, et al. 2013. Deep metazoan phylogeny: When different genes tell different stories.  
613 *Molecular Phylogenetics and Evolution* 67:223-233.

614 Novotný JP, Chughtai AA, Kostrouchová M, Kostrouchová V, Kostrouch D, Kaššák F, Kaňa R, Schierwater  
615 B, Kostrouchová M, Kostrouch Z. 2017. *Trichoplax adhaerens* reveals a network of nuclear receptors  
616 sensitive to 9-cis-retinoic acid at the base of metazoan evolution. *PeerJ* 5:e3789.

617 Nuclear Receptors Nomenclature Committee. 1999. A unified nomenclature system for the nuclear  
618 receptor superfamily. *Cell* 97:161-163.

619 Oliveros JC. 2007-2015. Venny. An interactive tool for comparing lists with Venn's diagrams.  
620 <http://bioinfogp.cnb.csic.es/tools/venny/index.html>.

621 Power R, Lydon J, Conneely O, O'Malley B. 1991. Dopamine activation of an orphan of the steroid  
622 receptor superfamily. *Science* 252:1546-1548.

623 Quack M, Carlberg C. 2001. Single thyroid hormone receptor monomers are competent for co-activator-  
624 mediated transactivation. *Biochemical Journal* 360:387-393.

625 Reitzel AM, Pang K, Ryan JF, Mullikin JC, Martindale MQ, Baxeavanis AD, Tarrant AM. 2011. Nuclear  
626 receptors from the ctenophore *Mnemiopsis leidyi* lack a zinc-finger DNA-binding domain: lineage-  
627 specific loss or ancestral condition in the emergence of the nuclear receptor superfamily? *EvoDevo* 2:3.

628 Reitzel AM, Tarrant AM. 2009. Nuclear receptor complement of the cnidarian *Nematostella vectensis*:  
629 phylogenetic relationships and developmental expression patterns. *BMC Evolutionary Biology* 9:230.

630 Riesgo A, Farrar N, Windsor PJ, Giribet G, Leys SP. 2014. The analysis of eight transcriptomes from all  
631 poriferan classes reveals surprising genetic complexity in sponges. *Molecular Biology and Evolution*  
632 31:1102-1120.

633 Rigo-Watermeier T, Kraft B, Ritthaler M, Wallkamm V, Holstein T, Wedlich D. 2012. Functional  
634 conservation of *Nematostella* Wnts in canonical and noncanonical Wnt-signaling. *Biology Open* 1:43-51.

635 Robinson-Rechavi M, Escriva Garcia H, Laudet V. 2003. The nuclear receptor superfamily. *Journal of Cell*  
636 *Science* 116:585-586.

637 Rühl R, Krzyzosiak A, Niewiadomska-Cimicka A, Rochel N, Szeles L, Vaz B, Wietrzyk-Schindler M, Álvarez  
638 S, Szklenar M, Nagy L, et al. 2015. 9-cis-13,14-dihydroretinoic acid is an endogenous retinoid acting as  
639 RXR ligand in mice. *PLOS Genetics* 11:e1005213.

640 Schlicker A, Domingues FS, Rahnenführer J, Lengauer T. 2006. A new measure for functional similarity of  
641 gene products based on Gene Ontology. *BMC Bioinformatics* 7:302.

642 Schröder M, Danielsson C, Wiesenberg I, Carlberg C. 1996. Identification of natural monomeric response  
643 elements of the nuclear receptor RZR/ROR. They also bind COUP-TF homodimers. The Journal of  
644 Biological Chemistry 271:19732-19736.

645 Sen P, Yang Y, Navarro C, Silva I, Szafranski P, Kolodziejska KE, Dharmadhikari AV, Mostafa H, Kozakewich  
646 H, Kearney D, et al. 2013. Novel FOXF1 mutations in sporadic and familial cases of alveolar capillary  
647 dysplasia with misaligned pulmonary veins imply a role for its DNA binding domain. Human Mutation  
648 34:801-811.

649 Sever R, Glass CK. 2013. Signaling by nuclear receptors. Cold Spring Harbor Perspectives in Biology 5.  
650 Srivastava M, Begovic E, Chapman J, Putnam NH, Hellsten U, Kawashima T, Kuo A, Mitros T, Salamov A,  
651 Carpenter ML, et al. 2008. The Trichoplax genome and the nature of placozoans. Nature 454.

652 Supek F, Bošnjak M, Škunca N, Šmuc T. 2011. REVIGO summarizes and visualizes long lists of gene  
653 ontology terms. PLoS One 6:e21800.

654 Tarrant AM, Behrendt L, Stegeman JJ, Verslycke T. 2011. Ecdysteroid receptor from the American lobster  
655 *Homarus americanus*: EcR/RXR isoform cloning and ligand-binding properties. General and Comparative  
656 Endocrinology 173:346-355.

657 Taubert S, Ward JD, Yamamoto KR. 2011. Nuclear hormone receptors in nematodes: Evolution and  
658 function. Molecular and Cellular Endocrinology 334:49-55.

659 Thornton JW. 2003. Nuclear receptor diversity: phylogeny, evolution and endocrine disruption. Pure and  
660 Applied Chemistry 75.

661 Wiens M, Batel R, Korzhev M, Müller W. 2003. Retinoid X receptor and retinoic acid response in the  
662 marine sponge *Suberites domuncula*. Journal of Experimental Biology 206:3261-3271.

663



Figure 1

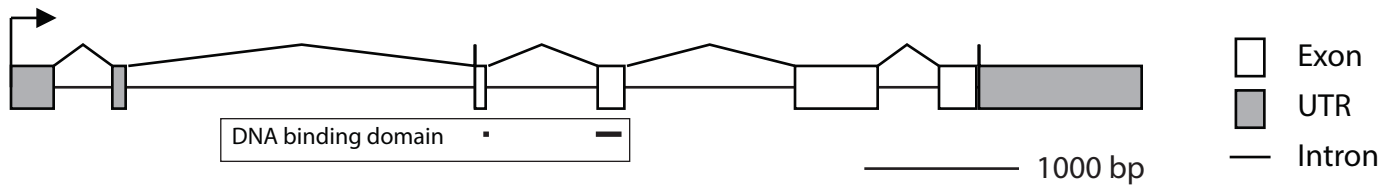


Figure 2

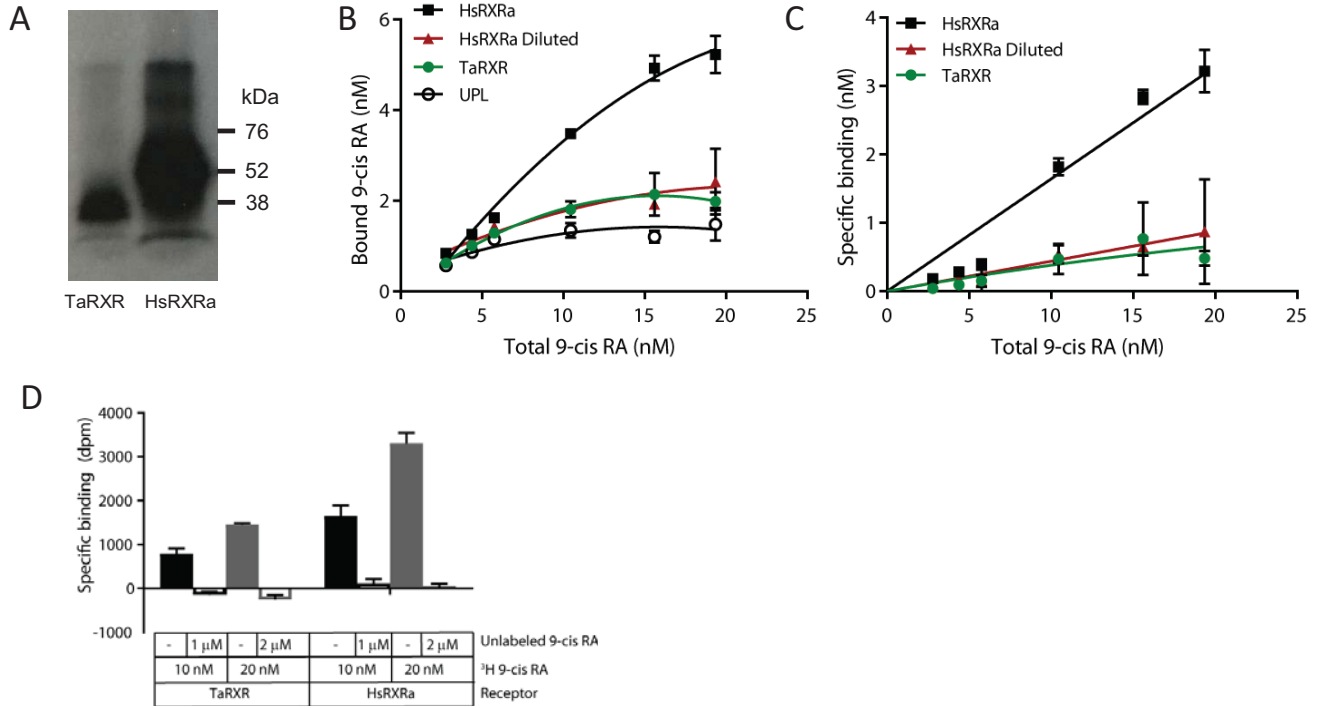


Figure 3

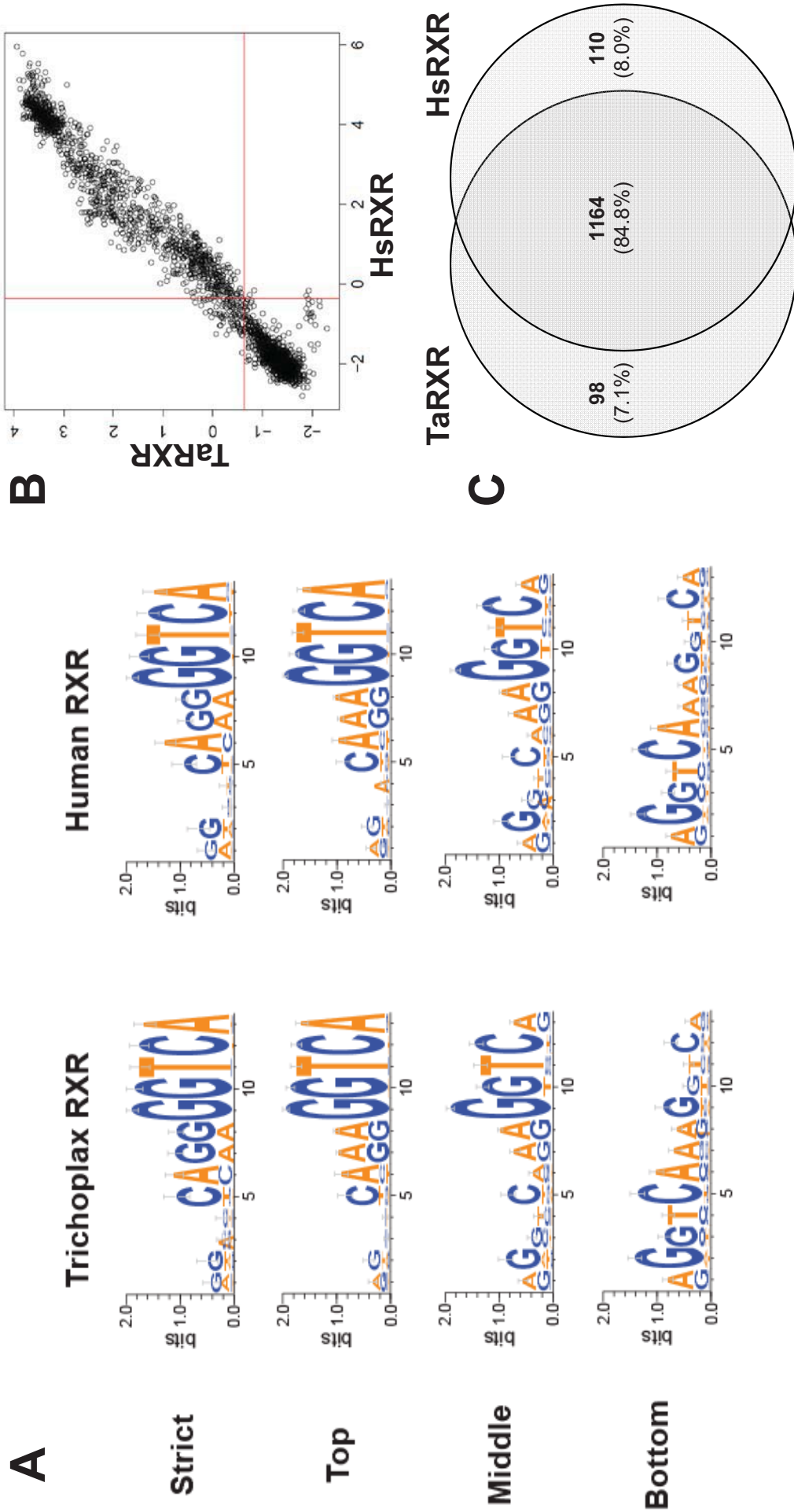
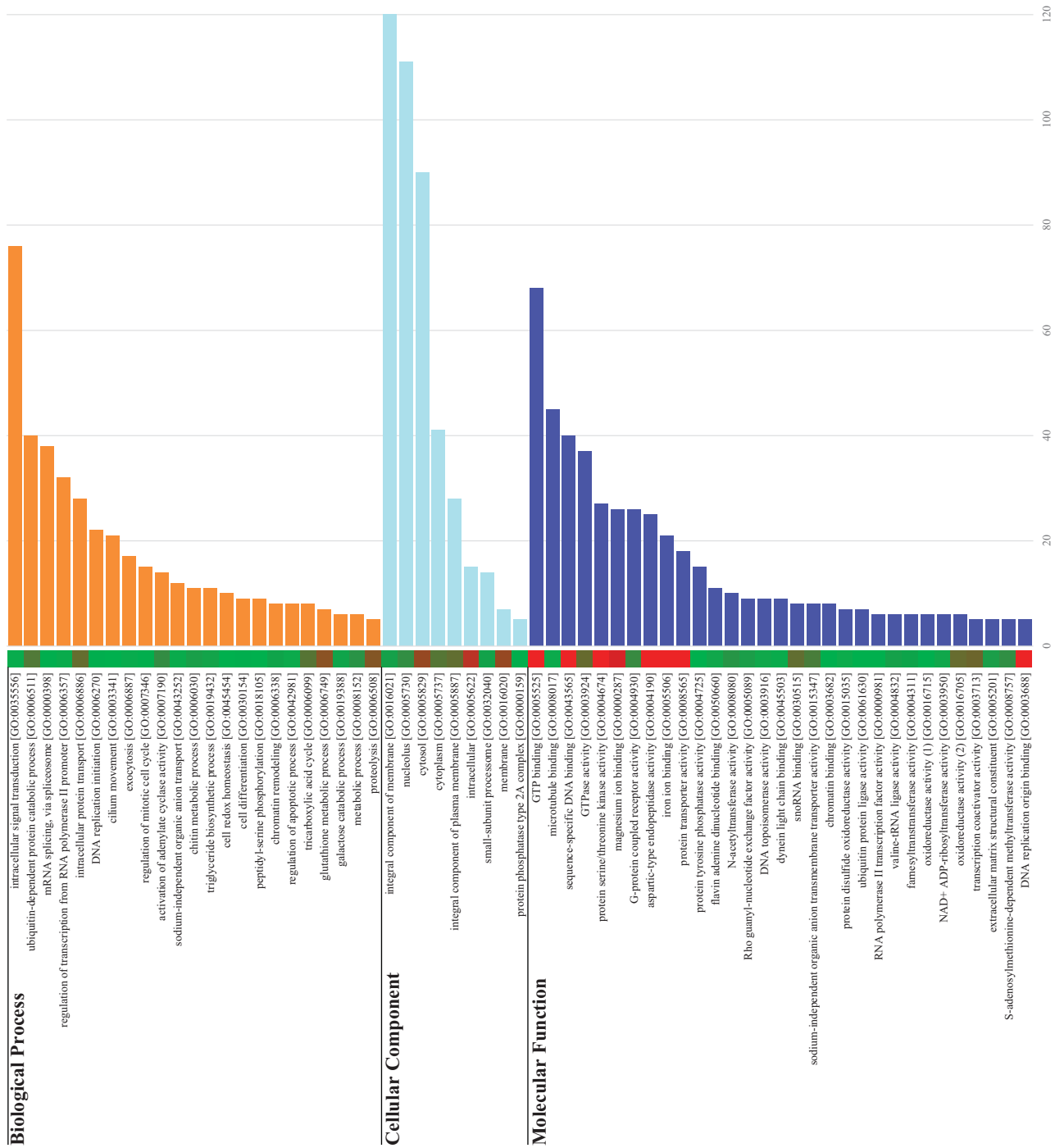


Figure 4



## RXR Gene Ontology Associations



```

import pandas
import os

Blastfile="/projects/areitze2_research/Regulation/Tripoplax/RXR_Extra_TES
T"
fnafilename="/projects/areitze2_research/Regulation/Tripoplax/Triad1_genomic_
scaffolds.fasta"

file=pandas.read_csv(Blastfile, sep="\t")
ids=set(file['chr'])
ids1=[]
start={}
end={}
for i in ids:
    j=i.strip("scaffold_")
    start[j]=file['UP'][file['chr']==i]
    end[j]=file['DOWN'][file['chr']==i]
result={}
result1=[]
with open(fnafilename, 'r') as k:
    for line in k:
        if line.startswith(">"):
            count=line.split("_")[1]
            result[int(count)-1]=''.join(map(str, result1))
            result1=[]
        if not line.startswith(">"):
            result1.append(line.strip("\n"))

for i in ids:
    i=i.strip("scaffold_")
    ids1.append(i)

for i in list(result):
    if not str(i) in ids1:
        del result[i]

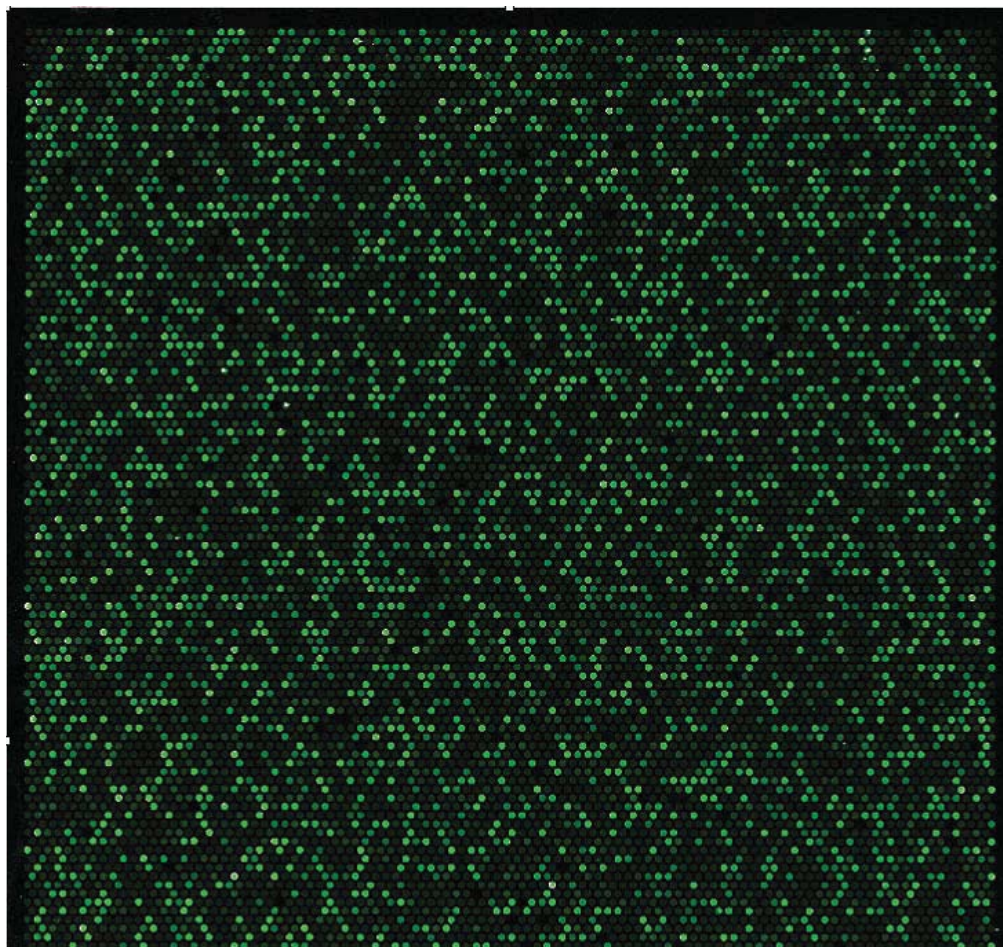
finaldict = {key:(start[key], end[key]) for key in start}

with
open("/projects/areitze2_research/Regulation/Tripoplax/RXR_Extra_Lines_ex
tractedfile.fna", 'w') as j:
    for i in result:
        temp1=finaldict[str(i)][0]
        temp2=finaldict[str(i)][1]
        for item in zip(temp1, temp2):
            j.write(">scaffold_{0}_{1}_{2}".format(i, item[0],
item[1]))
            j.write("\n")
            j.write(result[i][item[0]:item[1]])
            j.write("\n")

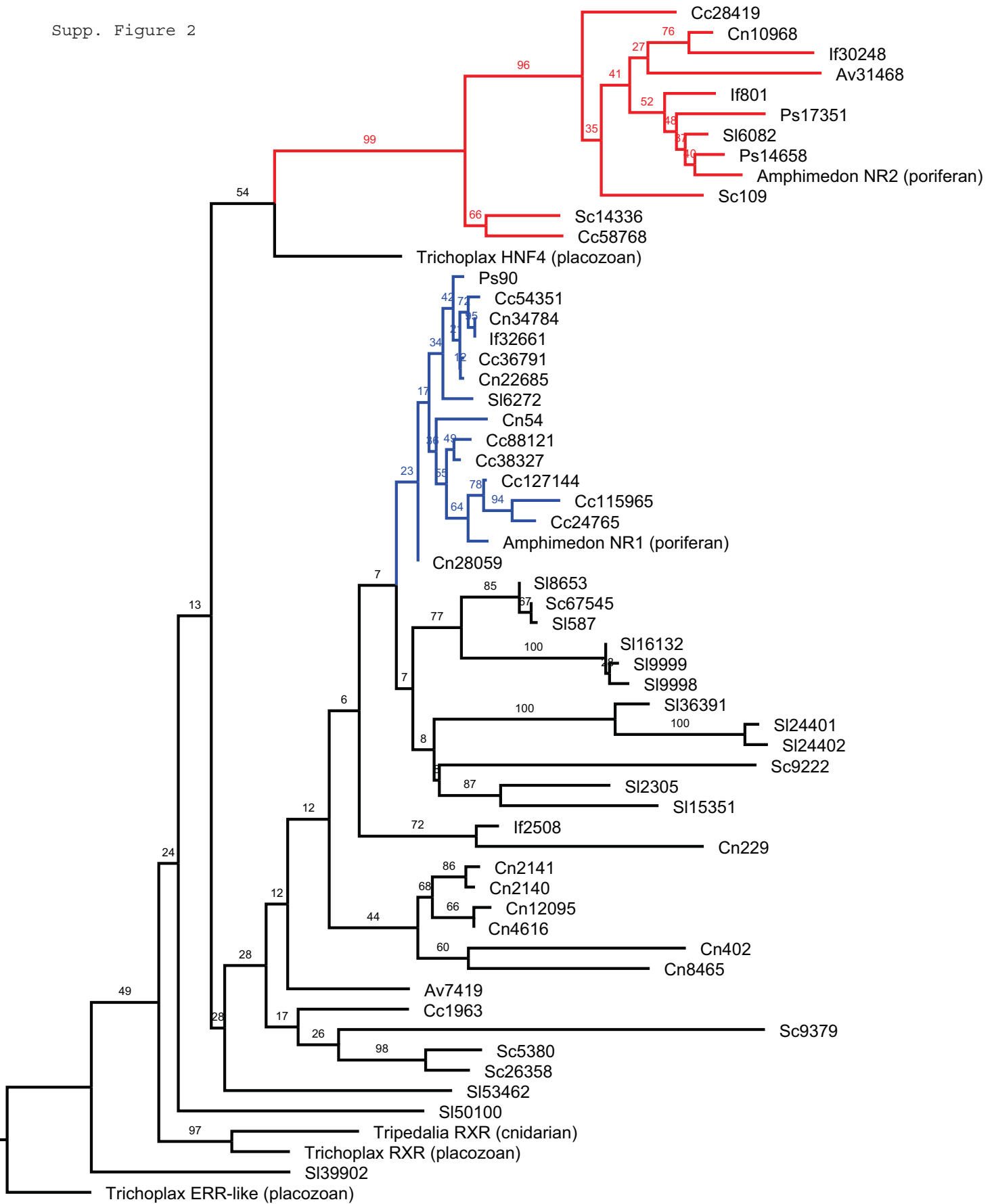
```

PBM-2 Ancient NRs  
Slide# 252717510009  
Subarrays 1234

**Grid #1 TaRXR (800ng)**  
**Anti V5 1/100**  
**GaM Dylight Cy3 1/50**  
**Gain 530**  
**Contrast 53/57**



Supp. Figure 2



0.3