Title: Conservation of DNA and Ligand Binding Properties of Retinoid X Receptor from the Placozoan *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 RXRα. 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.

INTRODUCTION

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

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

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

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

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

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

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

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

METHODS

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

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

Phylogeny of nuclear receptors in Family 2

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

Expression constructs for protein expression

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

177

178 *Ligand binding assays*

179 [11,12-^{3}H] 9-cis retinoic acid ("^{3}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 ^{35}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 ^{3}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 ^{3}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

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

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

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

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

RESULTS

T. adhaerens RXR

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

Phylogeny of nuclear receptors for Family 2

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

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

Ligand binding assays

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

RXR binding motifs

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

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

Predicted RXR binding sites and Gene Ontology annotation

In total, our bioinformatic pipeline identified 1,567 potential "strict" RXR binding sites (i.e., match consensus motif from top 10% of PBM probes), 557 of which contain the most common extended half-site (i.e, CAAAGGTCA), within the *T. adhaerens* genome (Figure 4, Table 1). Of these, 685 RXR sites were within 1000 bases of a protein coding region, with approximately 10% of these assigned a potential function (Supplemental Table 2). The largest scaffold (Scaffold_1) in the *T. adhaerens* genome assembly had the highest number of these RXR binding sites (N = 109), although, 18 other scaffolds (39% of those recovered) had a higher RXR binding site occurrence per nucleotide. A relatively small scaffold (Scaffold_2888, length = 1338 bp) had the most RXR sites at ~4.5 per 1kb, while the average across scaffolds is ~1.4 per 10 kb (Supplemental Table 1). We further searched the placozoan genome for half-sites across the whole genome, in addition to regions adjacent to the RXR sites identified with the PBM data. Interestingly, these searches revealed that the first position in the half-site had a bias of 3:1 for A over G (Figure 4). Comparisons with the human genome for these individual binding sites suggest no strong bias in representation when genome size is accounted for (i.e., human genome is 60 times larger).

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

ACKNOWLEDGEMENTS

We would like to thank Andreas Heyland (University of Guelph) for providing *T. adhaerens* and for assisting in production of the cDNA library. We would also like to thank Joe Ryan (Whitney Laboratory) for access to transcriptomes to search for nuclear receptors in sponges and cnidarians. Support for AMT was provided by the Tropical Research Initiative and an Internal Research and Development Award from the Woods Hole Oceanographic Institution. AMR was supported by NIH award R15GM114740. JM was supported by NSF award 1536530 to AMR. DM-P, BF and FMS were supported by NIH award R01DK094707 to FMS.

TABLES

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

Sequence motif for query (5' - 3')	Name	Unique matches within 1kb of gene*	Unique matches associated with RXR sites*	Matches in *T. adhaerens* genome overall	Unique matches in *H. sapiens* genome
CAAAGGTCA	Common top binder	219	557	557	36,079
C A/C A/G A/G GGTCA	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
AGGTCAnAGGTCA	DR1 (HS1-HS1)	0	1	3	1,059
GGGTCAnGGGTCA	DR1 (HS2-HS2)	0	0	0	3,187
AGGTCAnGGGTCA	DR1 (HS1-HS2)	2	2	3	715
GGGTCAnAGGTCA	DR1 (HS2-HS1)	0	2	3	715

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

FIGURE LEGENDS

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

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

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

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

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

SUPPLEMENTAL FIGURE LEGENDS

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

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

SUPPLEMENTAL TABLES

Supplemental Table 1. Number of RXR sites across each *Trichoplax adhaerens* genome scaffold.

Supplemental Table 2. Annotation of proteins within 2 kb of predicted RXR bindings sites in the *Trichoplax adhaerens* genome. Protein gene names and enzymatic pathways derived from homology-based annotation within Uniprot.

Supplemental Table 3. Full annotation associated with all predicted RXR regulatory targets within the *Trichoplax adhaerens* genome.

Supplemental Table 4. TaRXR processed PBM data.

Supplemental Table 5. HsRXR processed PBM data.

SUPPLEMENTAL FILE

Supplemental File 1. Python script for extracting 2 kb of genomic sequence around candidate RXR binding sites from the *Trichoplax adhaerens* genome.

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Figure 1

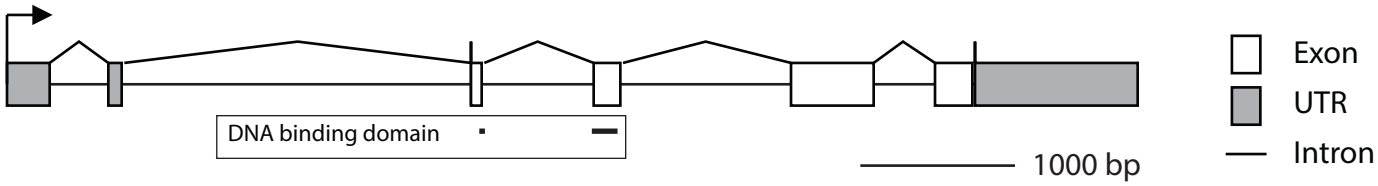
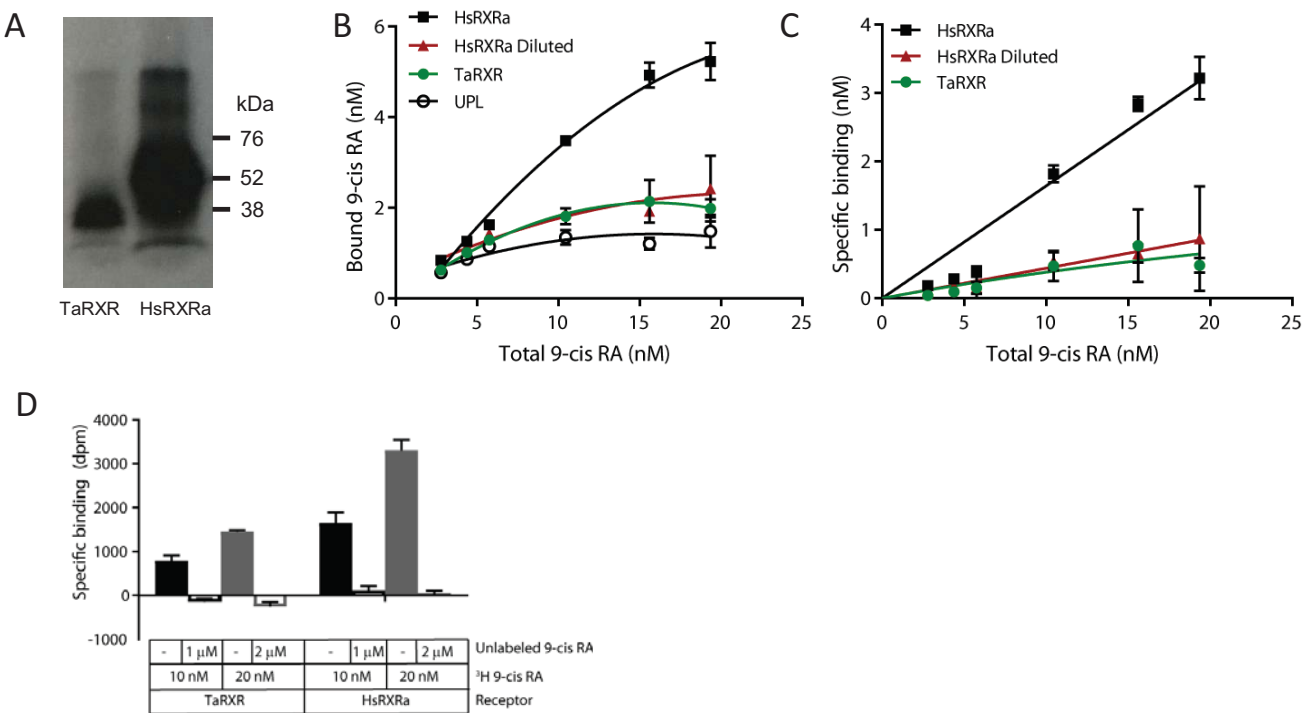


Figure 2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kDa
76
52
38
TaRXR
HsRXRa
B
HsRXRa
HsRXRa Diluted
TaRXR
UPL
Bound 9-cis RA (nM)
Total 9-cis RA (nM)
C
HsRXRa
HsRXRa Diluted
TaRXR
Specific binding (nM)
Total 9-cis RA (nM)
D
Specific binding (dpm)
- 1 µM - 2 µM - 1 µM - 2 µM
Unlabeled 9-cis RA
10 nM 20 nM 10 nM 20 nM
³H 9-cis RA
TaRXR HsRXRa
Receptor

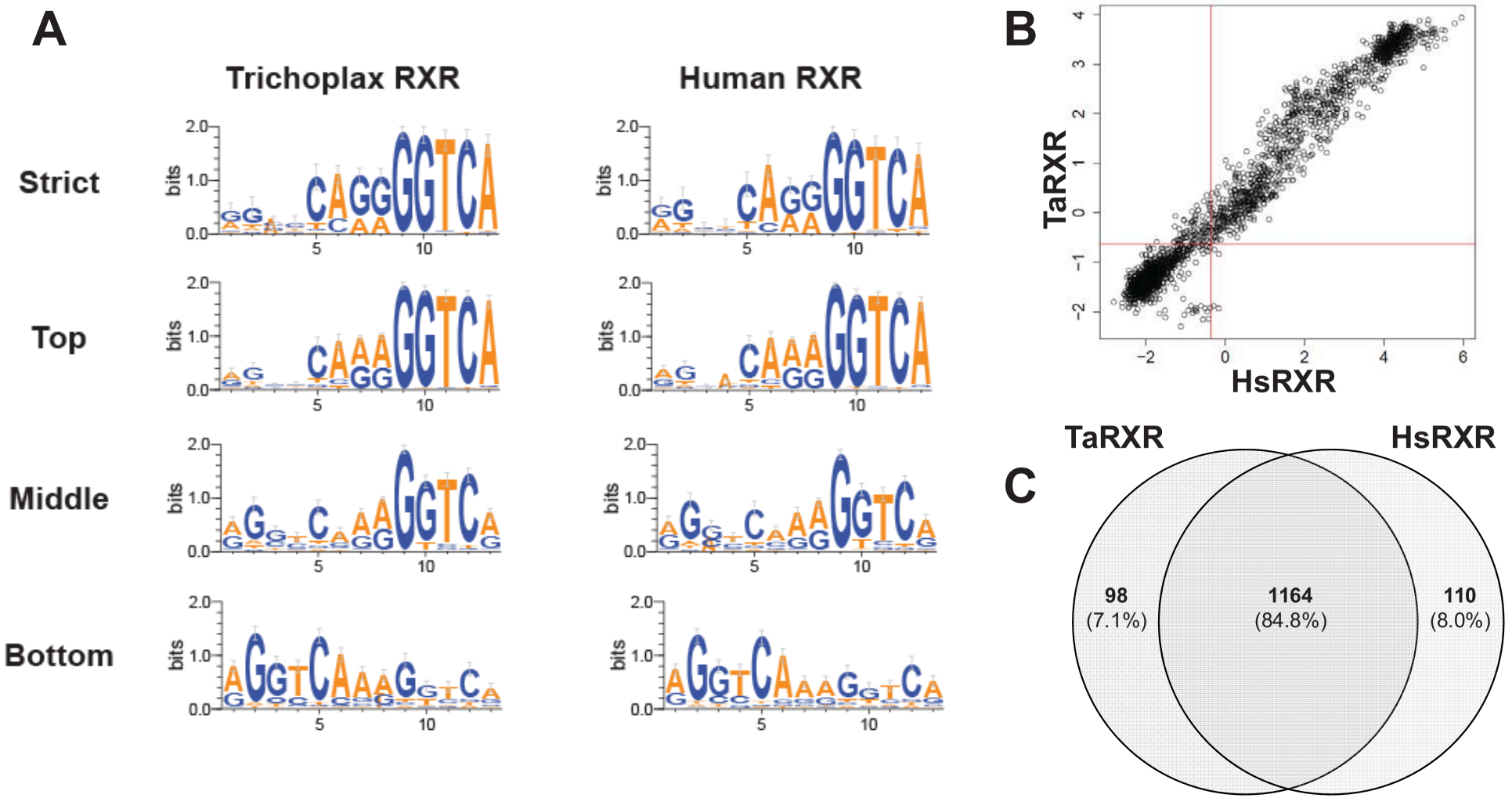
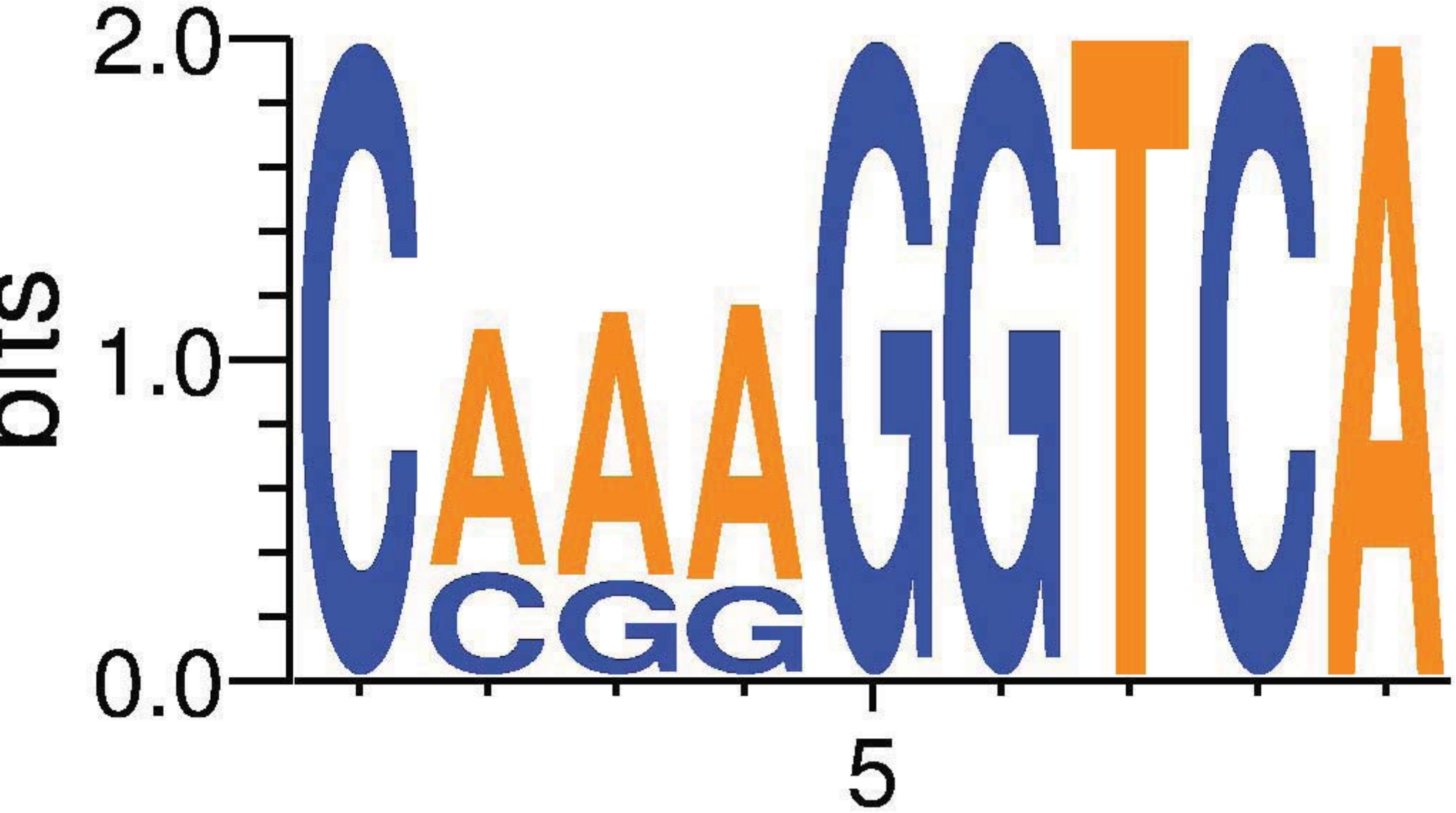
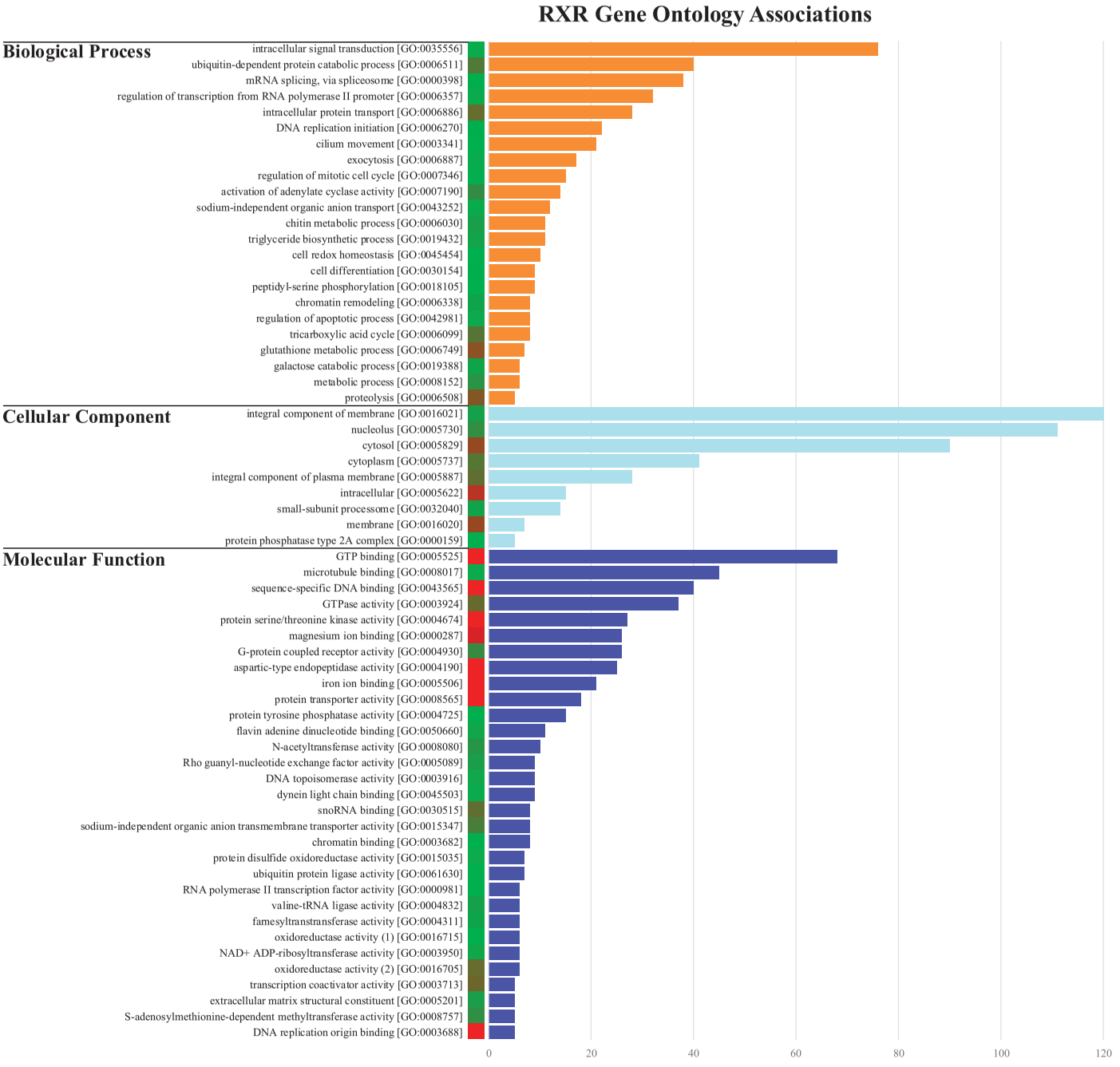

A
Trichoplax RXR
Human RXR
Strict
Top
Middle
Bottom
bits
B
TaRXR
HsRXR
C
TaRXR
HsRXR
98
(7.1%)
1164
(84.8%)
110
(8.0%)

Figure 4

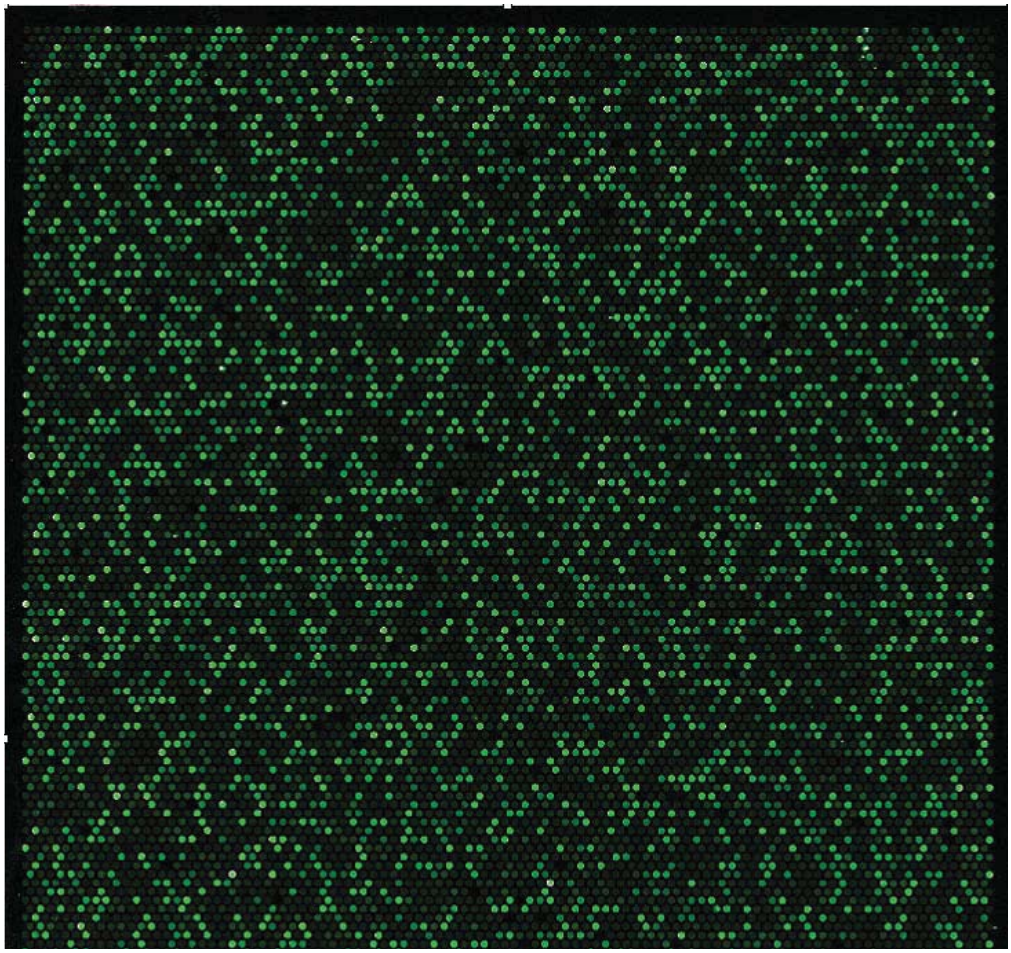



RXR Gene Ontology Associations
Biological Process
intracellular signal transduction [GO:0035556]
ubiquitin-dependent protein catabolic process [GO:0006511]
mRNA splicing, via spliceosome [GO:0000398]
regulation of transcription from RNA polymerase II promoter [GO:0006357]
intracellular protein transport [GO:0006886]
DNA replication initiation [GO:0006270]
cilium movement [GO:0003341]
exocytosis [GO:0006887]
regulation of mitotic cell cycle [GO:0007346]
activation of adenylate cyclase activity [GO:0007190]
sodium-independent organic anion transport [GO:0043252]
chitin metabolic process [GO:0006030]
triglyceride biosynthetic process [GO:0019432]
cell redox homeostasis [GO:0045454]
cell differentiation [GO:0030154]
peptidyl-serine phosphorylation [GO:0018105]
chromatin remodeling [GO:0006338]
regulation of apoptotic process [GO:0042981]
tricarboxylic acid cycle [GO:0006099]
glutathione metabolic process [GO:0006749]
galactose catabolic process [GO:0019388]
metabolic process [GO:0008152]
proteolysis [GO:0006508]
Cellular Component
integral component of membrane [GO:0016021]
nucleolus [GO:0005730]
cytosol [GO:0005829]
cytoplasm [GO:0005737]
integral component of plasma membrane [GO:0005887]
intracellular [GO:0005622]
small-subunit processome [GO:0032040]
membrane [GO:0016020]
protein phosphatase type 2A complex [GO:0000159]
Molecular Function
GTP binding [GO:0005525]
microtubule binding [GO:0008017]
sequence-specific DNA binding [GO:0043565]
GTPase activity [GO:0003924]
protein serine/threonine kinase activity [GO:0004674]
magnesium ion binding [GO:0000287]
G-protein coupled receptor activity [GO:0004930]
aspartic-type endopeptidase activity [GO:0004190]
iron ion binding [GO:0005506]
protein transporter activity [GO:0008565]
protein tyrosine phosphatase activity [GO:0004725]
flavin adenine dinucleotide binding [GO:0050660]
N-acetyltransferase activity [GO:0008080]
Rho guanyl-nucleotide exchange factor activity [GO:0005089]
DNA topoisomerase activity [GO:0003916]
dynein light chain binding [GO:0045503]
snoRNA binding [GO:0030515]
sodium-independent organic anion transmembrane transporter activity [GO:0015347]
chromatin binding [GO:0003682]
protein disulfide oxidoreductase activity [GO:0015035]
ubiquitin protein ligase activity [GO:0061630]
RNA polymerase II transcription factor activity [GO:0000981]
valine-tRNA ligase activity [GO:0004832]
famesyltranstransferase activity [GO:0004311]
oxidoreductase activity (1) [GO:0016715]
NAD+ ADP-ribosyltransferase activity [GO:0003950]
oxidoreductase activity (2) [GO:0016705]
transcription coactivator activity [GO:0003713]
extracellular matrix structural constituent [GO:0005201]
S-adenosylmethionine-dependent methyltransferase activity [GO:0008757]
DNA replication origin binding [GO:0003688]
0
20
40
60
80
100
120

```
import pandas
import os

Blastfile="/projects/areitze2_research/Regulation/Tripoplax/RXR_Extra_TES
T"
fnafile="/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(fnafile, '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_{}_{}_{}".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