Origin and evolutionary landscape of \textit{Nr2f} transcription factors across Metazoa

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Abstract

Background

Nuclear Receptor Subfamily 2 Group F (\textit{Nr2f}) orphan nuclear hormone transcription factors (TFs) are fundamental regulators of many developmental processes in invertebrates and vertebrates. Despite the importance of these TFs throughout metazoan development, previous work has not clearly outlined their evolutionary history.

Results

We integrated molecular phylogeny with comparisons of intron/exon structure, domain architecture, and syntenic conservation to define critical evolutionary events that distinguish the \textit{Nr2f} gene family in Metazoa. Our data indicate that a single ancestral eumetazoan \textit{Nr2f} gene predated six main Bilateria subfamilies, which include single \textit{Nr2f} homologs, here referred to as \textit{Nr2f1/2/5/6}, that are present in invertebrate protostomes and deuterostomes, \textit{Nr2f1/2} homologs in agnathans, and \textit{Nr2f1, Nr2f2, Nr2f5, and Nr2f6} orthologs that are found in gnathostomes. Four cnidarian \textit{Nr2f1/2/5/6} and three agnathan \textit{Nr2f1/2} members are each due to independent expansions, while the vertebrate \textit{Nr2f1/Nr2f2} and \textit{Nr2f5/Nr2f6} members each form paralogous groups that arose from the established series of whole-genome duplications (WGDs). \textit{Nr2f6} members are the most divergent \textit{Nr2f} subfamily in gnathostomes. Interestingly, in contrast to the other gnathostome \textit{Nr2f} subfamilies, \textit{Nr2f5} has been independently lost in numerous vertebrate lineages. Furthermore, our analysis shows there are differential expansions and losses of \textit{Nr2f} genes in teleosts following their additional rounds of WGDs.

Conclusion

Overall, our analysis of \textit{Nr2f} gene evolution helps to reveal the origins and previously unrecognized relationships of this ancient TF family, which may allow for greater insights into the conservation of \textit{Nr2f} functions that shape Metazoan body plans.
Introduction

Nuclear hormone receptors (NRs) form a large, ancient superfamily of transcription factors (TFs) found in all Metazoa [1]. While NR functions are often dictated by interactions with specific ligands, including steroids, thyroid hormones, and retinoids [2, 3], the ligands for many NRs, called orphan NRs, are still not known [4]. Nuclear Receptor Subfamily 2 Group F Members (Nr2fs), initially named Chicken ovalbumin upstream promoter-transcription factors (Coup-TFs) due to their ability to bind the COUP element of the ovalbumin gene [5–7], are some of the most highly studied orphan NRs. Despite an overall expansion of the NR superfamily [1, 2], invertebrate phyla appear to have predominantly retained a single Nr2f gene. Only one Nr2f member is present in the protostome Drosophila melanogaster (fly), early-branching deuterostome Strongylocentrotus purpuratus (sea urchin) [8, 9], and invertebrate chordates Branchiostoma floridae (amphioxus) and Ciona robusta (sea squirt) [10, 11]. However, the number of Nr2f genes in early-branching metazoans is presently less clear. In cnidarians, one Nr2f has been reported in Hydractinia echinata [12, 13], while multiple have been reported in Nematostella and Hydra vulgaris [14, 15]. In contrast to most invertebrates, vertebrates have exhibited a significant expansion of the Nr2f family, with gnathostomes having multiple Nr2f genes. Furthermore, teleosts possess additional Nr2f Ohnologs (duplicates originating from whole-genome duplication (WGD)) [16], most likely reflecting the additional WGDs that have occurred in the teleost lineage [17, 18].

Nr2f proteins are highly conserved at the sequence level throughout Metazoa [19]. From the N-terminus to the C-terminus, all Nr2f proteins have six domains (Fig 1): an A/B domain, which contains the activating function-1 (AF-1) domain; the C domain, which contains the DNA-binding domain (DBD); the D domain (a linker); the E domain, which is comprised of the ligand-binding domain (LBD) and an AF-2 domain; and the F domain (C-terminal) [20]. While the A/B domains are the most divergent in sequence, strikingly, the DBDs and LBDs of Nr2f members even from distantly related species (e.g. fly, sea urchin, frog, zebrafish, mouse, and human) are ~94% identical [21]. The extremely high degree of conservation among several species implies the preservation of critical roles for Nr2f in development and differentiation [21, 22]. Moreover, requirements for Nr2f genes have been found in organs of all three germ layers during embryogenesis [14, 22]. For instance, the Drosophila Nr2f homolog, called seven up (svp), is required for retinal, dorsal vessel, and liver development [23, 24]. Furthermore, Nr2f TFs in vertebrates appear to both have acquired diverse and retained redundant functions. For instance, in mice, Nr2f1 is predominantly required for neural development with a role in regulation of premigratory and migratory neural crest cells in the developing hindbrain [25, 26]. However, the mouse Nr2f2 gene is required for differentiation of mesodermal derivatives, including atrial cardiomyocytes of the heart and venous endothelial cells [22, 27, 28]. An example of redundancy is zebrafish nr2f1a and nr2f2, which are both required for proper ventricular cardiomyocyte and cranial muscle specification [29].

![Fig 1. Schematic of conserved domain architecture of Nr2f TFs. A/B (N-terminal variable domain with transactivating AF-1 domain), C (DBD, which contains two Zinc finger (Znf) motifs), D (a linker domain), E (LBD plus transactivating AF-2 domain), and F (C-terminal).](https://doi.org/10.1371/journal.pone.0254282.g001)
While Nr2f proteins were initially identified as transcriptional activators of chicken ovalbumin gene [5], they have since been shown to function directly as both transcriptional activators and repressors in several developmental contexts [22, 30, 31]. Nr2fs can bind a range of different response elements [32–34] and in signaling reporter assays can compete with and inhibit retinoic acid receptors [35]. *In vivo* they bind numerous targets that reflect their various requirements in the specific tissues. For instance, Nr2f1 KO mice also have inner ear defects [36]. In the mouse inner ear, direct targets of Nr2f1 include fatty acid binding protein 7 (FABP7), cellular retinoic acid binding protein 1 (CRABP1) [37], microRNA-140 (miR-140), and Krüppel-like 9 (Klf9) [37, 38]. In adipogenesis, Nr2f2 directly represses peroxisome proliferator-activated receptor γ (PPARγ) downstream of canonical Wnt/β-catenin signaling [39]. In the mammalian heart, Nr2f2 is thought to directly orchestrate a regulatory network that facilitates atrial cardiomyocyte identity through concurrently promoting Tbx5 and repressing Irx4 and Hey2, the latter of which promote ventricular cardiomyocyte identity [40]. Thus, Nr2fs can activate and repress a range of direct targets related to their functions in specific tissues.

Despite the conservation and clear importance of this gene family to numerous developmental processes in Metazoa, we still do not completely understand the evolution of Nr2f TFs. Here, we investigated Nr2f family evolution through a combination of phylogenetic, domain architecture, intron/exon structure, and genomic synteny analyses. Our data show that the single Nr2f gene found in placozoans, represents the ancestral Nr2f to those found in cnidarians, protostomes, and deuterostomes. Importantly, a single Nr2f homolog, which we have named Nr2f1/2/5/6, is present in the majority of invertebrates, while most vertebrate genomes contain Nr2f1, Nr2f2, Nr2f5, and Nr2f6 orthologs, which are derived from established rounds of WGDs [41, 42]. Interestingly, the invertebrate Nr2f1/2/5/6 and agnathan Nr2f1/2 homologs have retained the greatest similarity with vertebrate Nr2f1 and Nr2f2 paralogs. With respect to the vertebrate Nr2f5 and Nr2f6 paralogs, Nr2f5 genes have been independently lost in some cartilaginous fish and amniote lineages, while the Nr2f6 subfamily is the most divergent with respect to sequence and genomic structure. Overall, our data clarify the relationships among Nr2f genes within Metazoa and define the expansion, divergence, and independent loss of extant Nr2f genes in vertebrates, which will allow us to make meaningful inferences about the conserved developmental functions of this family that have helped mold animal body plans.

**Results**

**Phylogenetic reconstruction of Nr2f evolution in animals**

Although previous work has investigated the homology of some Nr2fs within metazoans, these analyses were primarily focused on their relationship to other NRs and were limited by the comparatively little genomic information at the time [1, 3, 14, 43, 44]. Therefore, the relatively few Nr2f family members examined in the previous analysis did not provide a specific and detailed understanding of Nr2f evolution. To garner a better understanding of how the Nr2f family has evolved in animals, we performed a phylogenetic analysis using 153 Nr2f proteins with representatives from placozoans to mammals (Fig 2; S1 File). Early-branching metazoan models *Amphimedon queenslandica* (sponge) and *Mnemiopsis leidyi* (ctenophore) were not included, as we did not find putative Nr2f orthologs based on current databases, consistent with published phylogenetic studies of the NR superfamily [13, 15]. The placozoan *Trichoplax adhaerens* Nr2f, which was previously shown to cluster with vertebrate Nr2fs in phylogenetic analyses [45], was used as the outgroup in a maximum-likelihood (ML) phylogenetic tree. Protein sequences from groups that caused long branch artifacts due to significant divergence were not included in the phylogenetic trees (S2 File). This phylogenetic analysis provided
evidence for the existence of distinct Nr2f subfamilies (Fig 2). Moreover, the same relationships were also supported using a Bayesian model selection (S1 Fig). Present information allowed us to identify four Nr2fs in the cnidaria Nemastoma vectensis and Acropora millepora, three in Hydra vulgaris, and one for Hydractinia echinata. However, while identifiable as Nr2fs, an A. millepora, the H. vulgaris, and the H. echinata Nr2fs caused long-branch artifacts and were
consequently excluded (S2 File). Interestingly, the tree incorporating the *N. vectensis* and remaining *A. millepora* Nr2f members, which we now call Nr2f1/2/5/6a-d based on their relationship to Bilateria Nr2fs, were found at the base of the eumetazoan Nr2f proteins and are likely the result of gene duplications within cnidaria [15] (Fig 2). The protostome and deuterostome Nr2f sequences clustered into six subfamilies, which we have called Nr2f1/2/5/6, Nr2f1/2, Nr2f1, Nr2f2, Nr2f5, and Nr2f6. Single Nr2f1/2/5/6 subfamily genes, which are highly conserved, yet evolutionary divergent from the Nr2f1/2/5/6 genes present in early-branching eumetazoan, were found in invertebrate protostomes, invertebrate deuterostomes (hemichordates, echinoderms), and invertebrate chordates (amphioxus, tunicates). An older nomenclature proposal suggested that the *Drosophila* Nr2f (*svp*) should be designated Nr2f3 [19], implying the other invertebrate Nr2fs should follow this nomenclature. However, this designation seems to obfuscate the homology of these genes revealed here and imply a different evolutionary relationship, as there is no distinct Nr2f3 subfamily. Thus, we propose using Nr2f1/2/5/6 in this manuscript to refer to the invertebrate Nr2fs to reinforce their evolutionary relationship within the Nr2f family. The invertebrate Nr2f1/2/5/6 group is more closely related to the branch that includes Nr2f1/2s from the agnathan (lamprey and hagfish) and vertebrate Nr2f1 and Nr2f2 proteins than the vertebrate Nr2f5 and Nr2f6 subfamilies (Fig 2). The clustering of the invertebrate Nr2f1/2/5/6 and agnathan Nr2f1/2 proteins with Nr2f1 and Nr2f2 of gnathostomes suggests that these paralogous gnathostome genes arose from distinct duplicative events during vertebrate evolution [41, 42]. In addition, the three agnathan Nr2f proteins found in Sea lamprey (*Petromyzon marinus*) and hagfish (*Eptatretus burgeri*) (Fig 2), which we have called Nr2f1/2A, Nr2f1/2B, and Nr2f1/2C, diverge and cluster together at the base of the vertebrate Nr2f1 and Nr2f2 proteins (Fig 2), supporting that the duplications leading to these proteins in agnathans were distinct from those that gave rise to the Nr2f paralogs in gnathostomes.

Our analysis also shows that Nr2f5 and Nr2f6 form a separate branch and are sisters groups, implying that they are paralogous and derived from the second of the vertebrate WGDs [41, 42]. Importantly, while all gnathostomes examined have retained Nr2f1, Nr2f2 and Nr2f6, current genomic data support that Nr2f5 has been independently lost by multiple vertebrate groups. Cartilaginous fish, including Whale shark (*Rinchodon typus*) [46] and the Great white shark (*Carcharodon carcharias*) [47], have retained Nr2f5 genes, while they are absent in chimaera [48] and skates (S3 File). In amniotes, Nr2f5 genes were found in reptiles, such as American alligator (*Alligator mississippiensis*), gecko (*Gekko japonicus*), and the Green sea turtle (*Chelonia mydas*) (Fig 2; S1 Fig), but absent from the Chinese sea turtle (*Pelodiscus sinensis*), as well as birds and mammals (S3 File). Although previous work had also designated a *Xenopus* laevis Nr2f4 [19], our data indicate there is no evidence for a separate Nr2f4 subfamily and that this gene should be called Nr2f5. Comparing the vertebrate Nr2f1/Nr2f2 and Nr2f5/Nr2f6 clusters, the branching and distances from our phylogenetic trees indicate that Nr2f1 and Nr2f2 are more highly conserved, while Nr2f6 TFs comprise the most divergent vertebrate Nr2f subfamily (Fig 2; S1 Fig).

To analyze the impact of additional WGDs on Nr2f genes, which took place in teleosts [17, 18], and specifically, in salmonids [49], we surveyed the Nr2f proteins of 12 teleost species (Fig 2; S1 Fig). Consistent with the WGDs in these species, there was a tremendous expansion of the Nr2f family in this clade, although it was accompanied by differential Nr2f paralog losses in some species (Fig 2; S1 Fig). To further interrogate the evolution of the Nr2f proteins, we examined alignments of the highly conserved zinc-fingers (Znf) within their DNA-binding domains (DBDs) using representatives from each subfamily (Fig 3). Although there is a high degree of conservation in all the examined Nr2fs, the amino acid changes in the DBDs parallels
**Fig 3. Zinc finger (Znf) motifs within the DBD of the Nr2f family.** Alignments of first (I) and second (II) Znfs found in Nr2f TFs. Yellow represents highly conserved amino acids throughout all species. White indicates amino acids that are not conserved. Turquoise and blue indicate amino acid changes that are conserved within Znf I of Nr2f5 and -6, respectively. The valine change found in some Nr2f6 LBDs is also found in the placozoan and cnidaria Nr2fs. Magenta and red indicate amino acid changes at the same residue that are conserved within Znf II of Nr2f5 and Nr2f6, respectively. A glycine residue is also found at the same position in some cnidaria and invertebrate Nr2fs. Green indicates a conserved change found in most Nr2f5 and Nr2f6 Znf IIs.

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the phylogenetic results of the whole proteins. The Nr2f1/2/5/6 proteins of early-branching eumetazoans showed a high degree of variability and multiple differences with respect to Nr2f1/2/5/6 DBDs of protostome and deuterostome invertebrates and the Nr2f DBDs in vertebrates. There is high similarity between Nr2f1/2, Nr2f1 and Nr2f2 DBDs in agnathans and gnathostomes, whereas Nr2f5 and Nr2f6 DBDs of gnathostomes exhibited specific changes that are consistent with their positions in the phylogenetic trees (Figs 2 and 3). Interestingly, single amino acid changes found in most Nr2f5 and Nr2f6 proteins are also found in some early-branching eumetazoans and invertebrate Nr2fs. However, the functional significance of these changes, if any, is not clear. Thus, our phylogenetic reconstruction of Nr2f genes in metazoans overall shows the presence of single orthologs in invertebrates and a significant expansion of the family in vertebrates that is punctuated with independent losses of Nr2f5 in some cartilaginous fishes and amniotes.

**Nr2f genes have conserved intron codes**

To complement the phylogenetic analysis of Nr2f genes, we first analyzed the conservation of Nr2f intron/exon structure [50–52]. Intron/exon junctions from early-branching eumetazoans and vertebrates matching the transcripts and the translated proteins were mapped and given a score for the intron phases (S4 File), with 0, 1 and 2 introns falling before the first, second and third bases of a codon, respectively. The introns were then mapped on a protein alignment comprising the highly conserved Nr2f protein DBDs and LBDs (S4 File). We found that two “phase 1” introns (one within the 3’ end of the DBD and one within the LBD) are preserved in all the extant Nr2f subfamilies (Fig 4A; S4 File). However, Nr2f6 genes also have a “phase 2” intron inside the second zinc-finger domain belonging to the DBD (Fig 4A; S4 File). The conservation of intron/exon junctions in the examined Nr2f genes allows two groups to be distinguished: one constituted by Nr2f, Nr2f1/2/5/6, Nr2f1/2, Nr2f1, Nr2f2, Nr2f5, and one comprising only vertebrate Nr2f6 (Fig 4B), implying this unique intron/exon boundary originated after the duplication event that generated Nr2f5 and Nr2f6. Thus, our analysis of intron/exon boundaries demonstrates the existence of a highly conserved intron code throughout eumetazoan Nr2f family members and the divergence of Nr2f6 genes following the second WGD.

**Synteny analysis defines differential duplications and losses in the Nr2f family**

In order to confirm the specific homologies indicated from the phylogenetic analysis, we next carried out an examination of synteny within the Nr2f genomic environments. With respect to representatives of the more ancient Nr2f genes, we did not find evidence of synteny between the single Nr2f in the placozoan *T. adhaerens* and the multiple Nr2fs in cnidarians. However, the location of the four Nr2f genes in *N. vectensis* and *A. millepora* genomes indicates they were likely derived from an initial duplication event followed by a tandem duplication event (Fig 5). Interestingly, *Mef2* and *Rbm8* homologs were associated with Nr2f1/2/5/6b in *N. vectensis* and *A. millepora*, while an *Arrdc* homolog is associated with Nr2f1/2/5/6a in *N. vectensis*. In vertebrates, *Mef2* paralogs (*Mef2c, Mef2b, Mef2b*) are associated with *Nr2f1*, *Nr2f2*, and *Nr2f6*, *Arrdc* paralogs (*Arrdc3, Arrdc4, Txnip/Arrdc6*) are associated with *Nr2f1*, *Nr2f2*, and *Nr2f5*, and *Rbm8a* is associated with *Nr2f5* (Figs 6–8), implying an ancient association of these genes within eumetazoan genomes.

In invertebrates, despite the synteny suggested between *Nr2f, Mef2, Arrdc*, and *Rbm8* genes in cnidaria and vertebrates, we only found limited preservation of the *Nr2f1/2/5/6* loci between two slow-evolving deuterostomes: the amphioxus (*B. belcheri*) [53] and the hemichordate
However, the limited synteny still corroborates the existence of the invertebrate Nr2f1/2/5/6 cluster shown in the phylogenetic trees (Fig 2; S1 Fig). Furthermore, the only remaining synteny between Nr2f1/2/5/6 in invertebrates and vertebrate orthologs appears to be the linkage between UNCA5A and NR2F2 of primates and Unc45a and

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**Fig 4. Intron code of the Nr2f family in metazoa.** (A) Protein alignment showing conservation of intron/exon structures within the DBDs (black) and LBDs (red) of Nr2f members. Znfs in the DBDs are underlined. Phase 0 introns—yellow, phase 1 introns—green, and phase 2 introns—turquoise. Asterisks indicate 100% amino acid conservation. Colons indicate high levels (>90%) amino acid conservation. Periods indicate moderate levels (50–89%) of amino acid conservation. (B) Schematization of intron/exon boundaries of Nr2f genes as they relate the Nr2f protein DBD and LBDs. Black box indicates DBD. Purple boxes represent the zinc-finger motifs within the DBD. Red boxes indicate the LBD. Colored bars indicate the conserved Nr2f Phase 1 introns (green) and the Nr2f-specific Phase 2 intron (turquoise).

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(S. kowalevskii) [54] (S2 Fig).
Nr2f1/2/5/6 of the tunicate *C. robusta* (S3 Fig), which is considered the closest living relative of vertebrates [55]. Focusing on *Nr2f1* and *Nr2f2* in the genomes of gnathostomes, including Great white sharks, coelacanths, spotted gars, zebrafish, chickens, and humans, we found a high degree of synteny for *Nr2f1* and *Nr2f2* loci and conservation of the location of flanking genes among these taxa (Fig 6). Specifically, *Nr2f1* and *Nr2f2* genes exhibited remarkably conserved syntenic environments, clustering with putative orthologs belonging to other families. *Lysmd3, Arrdc3, Mctp1* and *Mef2a* flank *Nr2f1* orthologs, while *Nr2f2* orthologs are flanked by *Lysmd4, Arrdc4, Mctp2* and *Mef2c* paralogs. Furthermore, in teleosts like zebrafish, two *Nr2f1* Ohnologs (nr2f1a and nr2f1b) also shared significant conservation of paralogous genes (Fig 6), which is consistent with an origin from the teleost-specific genome duplication (TSGD) [17, 18]. However, the *nr2f1b* gene has been lost by several teleost species (Fig 2; S1 Fig). Although the genomic information is somewhat fragmented, orthologs of flanking genes found in gnathostome *Nr2f1* and *Nr2f2*, such as *Arrdc2/3, Lysmd3, Fam172a*, were also found near each of the three Sea lamprey *Nr2f1/2* genes and the hagfish *Nr2f1/2C* gene (S4 Fig), which is consistent with these genes arising from genome duplication(s) within the agnathan lineage [56]. Together, these results suggest that *Nr2f1* and *Nr2f2* of gnathostomes have a common origin and are derived from a WGD event [41, 42].

Examining *Nr2f5* loci in representative gnathostomes showed a high degree of conservation in both species that have retained and lost the gene. The adjacent genomic environments in the majority of examined *Nr2f5* loci have retained an association with *Rbm8a* (Fig 7), whose homolog in cnidarians flanks *Nr2f1/2/5/6b* (Fig 5). The synteny is generally not shared with gnathostome *Nr2f1* and *Nr2f2* orthologs (Fig 6). However, the *Nr2f5* loci in coelacanth and amphibians have retained *Txnip* (Fig 7), which is also named *Arrdc6*. As aforementioned,
Arrdc family members flank the *N. vectensis* Nr2f1/2/5/6a (Fig 5) and both Nr2f1 and Nr2f2 genes (Fig 6). Interestingly, amniotes that have lost Nr2f5 (representatives including Chinese soft-shell turtles, chickens, and humans) (Fig 7; S3 File) have largely preserved the flanking genomic loci that are present in cartilaginous fish, zebrafish, coelacanth, frogs, and Green sea turtles (Fig 7). In contrast, the absence of Nr2f5 in some cartilaginous fish, such as *C. milii*, correlates with the lack of the entire locus. Within the Actinopterygii (ray-finned fishes), the synteny of genes has been lost only on one side of the Nr2f5 loci (Fig 7). With respect to the lamprey, its Nr2f1/2C ortholog is flanked by a Bola1 ortholog, as well as orthologs of genes that flank gnathostome Nr2f1 and Nr2f2 (Fig 6). Within gnathostomes, the Nr2f6 loci were highly conserved from cartilaginous fish to mammals, although there were significant gene losses surrounding *nr2f6a* and *nr2f6b* loci in zebrafish and one side of the Nr2f6 locus in

**Fig 6. Synteny analysis of vertebrate Nr2f1 and Nr2f2 genes.** Schematization of conserved genomic environments of gnathostome Nr2f1 and Nr2f2 genes (red rectangles) in selected species with relative chromosomes/scaffolds. Flanking orthologous genes are represented employing rectangles of the same color. Arrows indicate transcription orientation.

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Fig 7. Synteny analysis of vertebrate Nr2f5 genes. Schematization of conserved genomic environments of gnathostome Nr2f5 genes (red rectangles) in selected species with relative chromosomes/scaffolds. Flanking orthologous genes are represented using rectangles of the same color. Arrows indicate transcription orientation.

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Fig 8. Synteny analysis of vertebrate Nr2f6 genes. Schematization of conserved genomic environments of gnathostome Nr2f6 genes (red rectangles) in selected species with relative chromosomes/scaffolds. Flanking orthologous genes are represented using the same color code. Arrows indicate transcription orientation.

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humans. Furthermore, the presence of conserved orthologs (ano8a and ano8b, plvapa and plvapb) flanking nr2f6a and nr2f6b zebrafish genes suggested that they originated from the TSGD. Together, these findings show that despite the greater divergence of the Nr2f5 and Nr2f6 within vertebrates the genomic environments have retained some synteny and surrounding Nr2f5 and Nr2f6 loci are highly conserved within gnathostomes.

**Effects of TSGD on the Nr2f gene repertoire**

We next wanted to measure the impact of the series of additional WGDs that have occurred in teleosts on Nr2f gene number (Fig 2; S1 Fig). For this comparison, we examined all the Nr2f loci in zebrafish, the Asian arowana (S. formosus), which is documented to retain duplicates [57], and the Atlantic salmon (S. salar), which has a salmonid-specific genome duplication (SSGD) [49]. We found that each of these teleosts retained two Nr2f1 Ohnologs (Fig 9), suggesting they either were not duplicated or that one pair of Ohnologs was lost in salmonids. Zebrafish lost one nr2f2 Ohnolog, maintaining only the nr2f2a ortholog, while Asian arowana retained two Nr2f2 Ohnologs. Salmonids have 3 Nr2f2 genes (Nr2f2a1, Nr2f2a2, and Nr2f2b1), due to a loss of the one of the Nr2f2b Ohnologs following their additional WGD. With respect to Nr2f5, only Atlantic salmon showed two copies, implying these were generated during the SSGD event, as suggested by the presence of two Nr2f5 Ohnologs in other salmonids (Oncorhynchus spp., Coregonus clupeaformis) (S5 File). Finally, zebrafish and Asian arowana each possess two Nr2f6 genes, while Atlantic salmon has 3 similar to what is found in the Nr2f2 subfamily (Fig 9). Inspecting other teleost Nr2f gene family repertoires (Fig 2; S1 Fig), we

![Fig 9. Synteny analysis of Nr2f genes in teleosts.](https://doi.org/10.1371/journal.pone.0254282.g009)
found that the Channel catfish (Ictalurus punctatus), Red-bellied piranha (Pygocentrus nattereri), cavefish (Astyanax mexicanus) and Sheepshead minnow (Cyprinodon variegatus) all retained only Nr2fb. The Sheepshead minnow and Princess cichlid (Neolamprologus brichardi) also lost Nr2f5. However, other cichlids like Nile tilapia (Oreochromis niloticus) and Zebra mbuna (Maylandia zebra) did not lose Nr2f5 (S5 File). Intriguingly, the Monterey platfish (Xiphophorus couchianus) is the only gnathostome without any Nr2f1 paralogs, differing from its sibling species, the common platifish (X. maculatus), which possesses Nr2f1a. Therefore, teleosts show an expansion of Nr2f genes following TSGD and SSGD, which were followed by high variability in species-specific losses of Nr2f Ohnologs.

**Discussion**

We have performed an examination of Nr2f gene evolution in metazoans. Our analysis corroborates previous work showing that Nr2f genes are present in some representative early-branching eumetazoans (placozoans and cnidarians) [15, 58], but that they are absent in early-branching metazoans, i.e. sponges and ctenophores [15, 58]. Importantly, our data support a model in which a single Nr2f gene, which is present in a representative placozoan, predated a Nr2f1/2/5/6 subfamily found in cnidaria and six Bilateria subfamilies that include Nr2f1/2/5/6 (found in invertebrate protostome and deuterostomes), Nr2f1/2 (found in agnathans), and Nr2f1, Nr2f2, Nr2f5, and Nr2f6 (found in vertebrates; Fig 10). Single, conserved Nr2f1/2/5/6 genes are predominantly found throughout invertebrate protostomes and deuterostomes and have even been retained in species traditionally considered gene losers, such as the tunicates [52, 59, 60]. There has been significant expansion and retention of Nr2fs in gnathostomes, particularly in teleosts. Although initial analysis in lampreys suggested they may possess only one Nr2f gene [61], our evolutionary assessment shows that extant agnathans have three Nr2f members, which appear to have originated in part from an agnathan WGD event [56]. Interestingly, the single Nr2f1/2/5/6 proteins in invertebrates are also highly conserved at the

Fig 10. Model summarizing the evolutionary events of the Nr2f family in Metazoa. A single Nr2f of placozoans (white box) represents the ancestor of extant Nr2fs. There were duplicative events specific to cnidaria leading to the expansion of Nr2f1/2/5/6 (pink circle). Invertebrate protostomes and deuterostomes have predominantly retained a single Nr2f1/2/5/6 homolog. There were duplicative events specific to agnathans leading to an expansion of Nr2f1/2 genes (orange circle). WGDs within vertebrates (green circles) generated the four Nr2f genes found in vertebrates, with Nr2f1/Nr2f2 being paralogous and Nr2f5/Nr2f6 being paralogous. Nr2f5 has been independently lost in multiple vertebrate groups (red circle). It is lost in some cartilaginous fish and turtles (reptilian amniotes), and is absent in avian and mammalian amniotes. Teleosts have additional Nr2f Ohnologs due to TSGDs (blue circles) and SSGDs (yellow circles).
sequence level and cluster with the Nr2f1/2 proteins in agnathans and Nr2f1 and Nr2f2 proteins in gnathostomes. Furthermore, our data support a parsimonious view that Nr2f1 and Nr2f2 are paralogous and Nr2f5 and Nr2f6 are paralogous, consistent with each of the Nr2f1/2 and Nr2f5/6 branches being created from an initial WGD [41, 42]. Within gnathostomes, the genomic environments of each the Nr2f1, Nr2f2, Nr2f5, and Nr2f6 orthologs have retained significant synteny of their loci [16, 21, 22]. Remarkably, while limited synteny exists between the Nr2f1/2 and Nr2f5/6 branches and within the Nr2f5/6 branch, members of these families have retained association with Mef2, Arrdc, and Rbm8 homologs within their genomic environments, which is also found in cnidaria. However, this genomic association was not found in other examined invertebrate genomes. Our analysis also shows the Nr2f5 subfamily is the smallest in vertebrates, having been independently lost in multiple gnathostomes (some cartilaginous fishes, amniotes—some reptiles, absent in birds and mammals) (Fig 10). In contrast to Nr2f5, the Nr2f6 subfamily has been retained by all the evaluated gnathostomes, despite being the most divergent at the sequence level, with respect to synteny, and intron/exon structure.

Although overall there has been relatively limited comparative analysis of Nr2f gene expression beyond major model organisms, integrating our phylogenetic assessment with available expression and functional analyses of the Nr2f members in evolutionarily distant animals [12, 14] presently supports a hypothesis that Nr2f expression originated in neural tissue and regulation of neuronal differentiation may be the most ancient Nr2f function. Foremost, the two Nr2f members (both Nr2f1/2/5/6c) of the diploblastic cnidaria H. vulgaris and H. echinata thus far examined appear to be expressed in neurons and have requirements in neurogenesis [12, 14]. Clearly, the expression of the additional Nr2f cnidarian homologs that have been identified needs to be examined and if found to be expressed in endoderm would alter this hypothesis. Nevertheless, the function of Nr2f1/2/5/6 orthologs of protostome invertebrates nematodes and flies have been extensively studied in neural tissues and neural sensory cell differentiation [8, 62, 63]. In invertebrate deuterostomes, the single Nr2f1/2/5/6 orthologs are expressed in neural tissue of sea urchin (Strongylocentrotus purpuratus), amphioxus, and sea squirt embryos [11, 64–66]. Recent functional analysis of the Mediterranean sea urchin (Paracentrotus lividus) Nr2f1/2/5/6 shows that it is required for the development of neural and ectodermal derivatives [67]. A Nr2f1/2/5/6 ortholog from the agnathan River lamprey (Lampetra fluviatilis) is also expressed in the developing nervous system [61]. However, our identification of three Nr2f1/2 members in agnathans suggests that additional expression and potentially functional analysis should be performed in the Sea lamprey (P. marinus) and/or hagfish (E. burgeri) to understand the conservation of the different agnathan paralogs compared to Nr2fs in vertebrates. Both Nr2f1 and Nr2f2 orthologs share overlapping central nervous system (CNS) expression in mouse and zebrafish [16, 21, 68]. However, nr2f1a and nr2f2 are both expressed more extensively in neural tissue of zebrafish embryos, while Nr2f1 is predominantly expressed in neural tissues of mice [21, 22]. Nr2f5 is expressed in neural tissue and derivatives, including in the eyes of zebrafish and newts [68–70]. Nr2f6 genes have conserved expression within the central nervous system of mammals [16], as well as both zebrafish nr2f6 Ohnologs. Thus, all Nr2fs examined are expressed in neural tissue, with experiments in cnidaria and invertebrates presently supporting their ancestral requirements may be in neural cell differentiation.

While we propose that Nr2f6 may have originated with requirements in neural differentiation, they are also required for the development of mesodermal and endodermal-derived tissues through Bilateria. Thus, it is interesting to consider some of these requirements in light of our phylogenetic analysis. In addition to neural differentiation, Nr2f homologs are necessary for copulation control in nematodes [71] and heart vessel specification in flies [23, 24]. Furthermore, the recent work with the Mediterranean sea urchin suggests that it is required for
the development of mesendodermal derivatives [67]. The functions of \textit{Nr2f1} and \textit{Nr2f2} genes have been intensely investigated in vertebrate models and they are required for proper human development [22, 31, 72]. Both expression and functional analysis of \textit{Nr2f1} and \textit{Nr2f2} genes in vertebrates show that they have acquired distinct developmental roles during evolution. Following overlapping expression early in mouse embryos, murine \textit{Nr2f1} and \textit{Nr2f2} become predominantly expressed in neural and mesendodermal tissues, respectively [21, 22]. Analysis of these \textit{Nr2f} genes in mice and zebrafish support the functional divergence of these proteins. Murine \textit{Nr2f1} KO s have glial differentiation defects [73], while \textit{Nr2f2} is required for proper development of many mesendodermal-derived tissues, including atrial chamber and arterial-venous differentiation [40, 74]. Intriguingly, mouse \textit{Nr2f2} and zebrafish \textit{nr2f1a} are functional homologs with respect to heart development, as both are required for atrial differentiation [75], further supporting the common evolutionary origins of these paralogs. While zebrafish \textit{nr2f2} is not required for early atrial or vein development [29], \textit{Nr2f1} and \textit{Nr2f2} TFs do appear to have redundant requirements, for instance promoting atrial cardiomyocyte differentiation in human embryonic stem cells [76, 77]. It is interesting that the single \textit{Nr2f1/2/5/6 (svp/Nr2f3)} homolog of flies is also required for dorsal vessel (heart) development [23]. However, if these similar roles in mesodermally-derived heart tissues reflect homologous requirements within Bilateria for cardiac differentiation requires functional studies from many additional model organisms [67]. With respect to analysis of the expansion of \textit{Nr2f1} and \textit{Nr2f2} Ohnologs in teleosts, \textit{Nr2f1b} actually has been lost in the majority of surveyed teleosts. \textit{Nr2f1b} zebrafish mutants are viable [78] and surprisingly do not exhibit redundancy with \textit{nr2f1a} in atrial cardiomyocyte differentiation [29], but do exhibit some redundancy with multiple other \textit{Nr2f} genes in neural crest cells that promote jaw development [78]. Virtually all the analyzed gnathostome genomes have a single \textit{Nr2f2} gene, excluding the teleosts \textit{S. formosus} (2) and \textit{S. salar} (3), implying there may be some dosage sensitivity that favors the retention of single orthologs in gnathostomes.

With respect to the function of \textit{Nr2f5} and \textit{Nr2f6} genes, zebrafish \textit{nr2f5} mutants are viable, yet like zebrafish \textit{nr2f1b} mutants they function redundantly with other \textit{nr2f} genes for proper upper-jaw development [78]. While expression and functional analysis from other organisms that have retained \textit{Nr2f5} (coelacanth, spotted gar, and frog) may provide insights into conservation of \textit{Nr2f5} orthologs, the independent loss of \textit{Nr2f5} genes in multiple vertebrate lineages, as well as the lack of overt requirements alone in zebrafish, suggests that \textit{Nr2f5} orthologs likely have retained minimal developmental requirements and its loss can be tolerated. Murine \textit{Nr2f6} KO mice have forebrain defects. Specifically, these mutants show a loss of neurons that regulate the circadian clock genes [79]. However, \textit{Nr2f6} also has a critical role in lymphocyte differentiation and T-cell mediated tumor surveillance, suggesting requirements in mesodermally-derived tissues and neofunctionalization in adaptive immunity [80, 81]. Altogether, minimally, expression and functional data support requirements for \textit{Nr2f6}s in all three germ layers of Bilateria. However, the conservation of these requirements and if they reflect homologous roles in the different germ layers throughout Bilateria is not yet as clear.

In examining the evolution of the \textit{Nr2f} TFs, it is also worthwhile to note that in early-branching eumetazoans through invertebrate chordates and gnathostomes there is conserved responsiveness to retinoic acid (RA) signaling [82], a critical molecule involved early patterning of vertebrate embryos [83–85], implying this relationship may form the core of an ancient gene regulatory network. \textit{Nr2f} genes from placozoans [58] and the invertebrate chordates \textit{Ciona} and amphioxus are all RA-responsive [11, 64]. Furthermore, in vertebrates, where the earliest requirement for RA is posteriorization of the embryo [86], virtually all the \textit{Nr2f} genes have been shown to be responsive to RA signaling in developmental contexts involving all three germ layers. Specifically, RA signaling has been shown to positively regulate all the \textit{Nr2f}s
in zebrafish in the developing zebrafish endoderm [87], the CNS [68], and anterior lateral plate mesoderm (ALPM) [29]. RA signaling also positively regulates Nr2f1, Nr2f2 and Nr2f6 in mice [88, 89], and NR2F1 and NR2F2 in humans [90, 91]. Nr2fs can inhibit RA signaling in some contexts, suggesting it may form a negative feedback loop. One role Nr2fs may play is through direct competition with retinoic acid receptors (RARs) in binding retinoic acid response elements (RAREs) [21]. Moreover, it has been shown that the cnidarian Nr2f1/2/5/6c possesses the ability to inhibit RA signaling in in vitro signaling assays [14]. Thus, the responsiveness of the Nr2f family to RA may have evolved very early and has been highly maintained through the diversification of multiple vertebrate Nr2f genes, implying there is high selection to maintain this relationship.

Conclusions

Overall, our evolutionary assessment sheds new light on the events that have shaped the extant Nr2f family in Metazoa. The phylogenetic analysis defines the individual Nr2f subfamilies and their relationships across metazoan phyla, which complements available expression and functional data presently supporting an origin of their requirements in the development of neural tissue. Interestingly, the functions of Nr2f proteins are found to regulate development of all germ layers of Bilateria. The detailed evolutionary understanding of the Nr2f gene family we now have will allow us to infer more meaningful conclusions about the origins and conserved requirements of Nr2f genes in normal metazoan development and their role in sculpting diverse body plans.

Methods

Ethics statement

Ethical approval is not required. No animals were used in this study.

Genome database searches and phylogenetic reconstruction

Homo sapiens NR2F protein sequences were employed as queries in BLASTp and tBLASTn in genome databases of selected species (NCBI, Ensembl, Ensembl Metazoa, SkateBase [92], ANISEED [93]). The entire dataset of protein sequences for domain architecture was analyzed by using the domain database provided by Expasy, named PROSITE [94] and then, manually annotated. All the surveyed sequences were verified to be Nr2f proteins through analysis of DBDs and LBDs (S6 File). The analysis was weighted with 30 species from agnathans to pri-mates to take into account the impact of multiple WGDs in vertebrates [41, 42] and in teleosts [17, 18]. Orthology of the Nr2f members was initially assessed by using a reciprocal best blast hit (RBBH) approach employing default parameters and corroborated by phylogenetic analyses. Protein alignment for phylogeny was generated using L-INS-i (accurate; for alignment of <200 sequences) on MAFFT [95, 96] (S7 File). The phylogenetic reconstruction of Fig 2 was performed on the entire protein sequences and based on maximum-likelihood (ML) inferences calculated with PhyML 3.0 [97], employing automatic Akaike Information Criterion (AIC) by Smart Model Substitution (SMS) [98], which selected the JTT+G+F model employing discrete gamma distribution in categories. All parameters (gamma shape = 0.7; proportion of invariants (fixed) = 0.000) were established from the dataset. Branch support was provided by aLRT [99]. The phylogeny of S1 Fig was carried out employing Bayesian Information Criterion (BIC) by SMS, which sorted the JTT+G+F model using discrete gamma distribution in categories. All parameters (gamma shape = 0.7; proportion of invariants (fixed) = 0.000) were established from the dataset, with branch support calculated employing aBayes method [100].
Accession numbers and protein sequences used for phylogenetic tree reconstructions are provided in S1, S6, and S7 Files, while those excluded for their divergence are listed in S2 File. Common and Latin names for species used in this study are listed in S8 File.

Analysis of intron/exon structures and phases
Gene structures were deduced after merging the genomic sequences with ESTs when available, as previously described [50–52]. Introns were classified as phase 0, phase 1, and phase 2, according to their positions with respect to the protein-reading frame. The amino-acid residues with the conserved introns were manually mapped on a ClustalX alignment [101] of selected Nr2f proteins (S9 File).

Evaluation of synteny
We evaluated the presence/absence of synteny examining the chromosomes on public genome databases (NCBI, Ensembl, Ensembl Metazoa, ANISEED [93]). We verified the existence of duplicates using Genomicus [102] and Vertebrate Ohnologs [103]. The window considered for the locus analyses was twenty flanking genes. Genes that were not conserved were excluded from the analysis. All the genes were represented employing colored rectangles, using the same color for all Nr2f genes (red).

Supporting information
S1 Fig. Phylogenetic tree of the Nr2f family, using Bayesian Information Criterion (BIC). The same color code as Fig 2 is used. Values at the branches indicate replicates obtained employing the aBayes method.
(TIF)

S2 Fig. Synteny analysis of Nr2f1/2/5/6 genes found invertebrates. Schematic of limited conservation for Nr2f1/2/5/6 loci between the hemichordate S. kowalevskii and amphioxus B. belcheri. Black arrows indicate transcription orientation.
(TIF)

S3 Fig. Unc45–Nr2f gene duplet preservation. Schematic of Unc45–Nr2f duplet conservation in genomes of ascidians (Ciona) and primates. The duplet is absent in other vertebrate models, including zebrafish and mouse.
(TIF)

S4 Fig. Synteny analysis of Nr2f1/2 genes in agnathans. Schematic of lamprey (P. marinus) Nr2f1/2 loci with relative chromosomes and available genomic data from the hagfish (E. burgeri). Genomic data could only be obtained for the hagfish Nr2f1/2C gene. Same color code of Figs 6–8 is used. Flanking genes are in common with gnathostomes, with Arrdc2 and Arrdc3 (green) that form a conserved duplet with Nr2f1/2B and Nr2f1/2C. Nr2f1/2C is adjacent to Fam172a in both lamprey and hagfish. Arrows indicate transcription orientation.
(TIF)

S1 File. List of all protein sequences employed in Nr2f phylogenetic tree with accession numbers.
(TXT)

S2 File. List of protein sequences excluded from Nr2f phylogenetic tree due to their high degree of divergence.
(TXT)
S3 File. List of examined species whose genomes lacked Nr2f5 with their common names, Latin names, and phyla. (XLSX)

S4 File. Intron/Exon structure of Nr2f genes in Metazoa. Alignment of specific and conserved intron/exon boundaries within the Zinc finger motifs of DBD (underlined) and LBDs (red). The intron phases have been depicted using color code: Phase 0 (yellow), Phase 1 (green), Phase 2 (turquoise). (DOCX)

S5 File. Sequences of additional salmonid and cichlid Nr2f5 proteins. (TXT)

S6 File. Nr2f domain architectures during metazoan evolution. Sequences used in analysis with DBDs (yellow) and LBDs (green) domains in metazoan Nr2f proteins indicated. the Zinc-finger motifs within the DBDs are underlined. (DOCX)

S7 File. MAFFT alignment of protein sequences used for phylogenetic analysis of Fig 2 and S1 Fig. (TXT)

S8 File. List of species used for our evolutionary analyses with their common names, Latin names, and phyla. (XLSX)

S9 File. Selected Nr2f transcripts and translations used for analysis with the positions and phases of intron/exon boundaries indicated. (DOCX)

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