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An Enhancer Near ISL1 and an Ultraconserved Exon of PCBP2 are Derived From a Retroposon

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Hundreds of highly conserved distal cis-regulatory elements have been characterized to date in vertebrate genomes[1]. Many thousands more are predicted based on comparative genomics[2,3]. Yet, in stark contrast to the genes they regulate, virtually none of these regions can be traced using sequence similarity in invertebrates, leaving their evolutionary origin obscure. Here we show that a class of conserved, primarily non-coding regions in tetrapods originated from a novel short interspersed repetitive element (SINE) retroposon family that was active in Sarcopterygii (lobe-finned fishes and terrestrial vertebrates) in the Silurian at least 410 Mya[4], and, remarkably, appears to be recently active in the "living fossil" Indonesian coelacanth, *Latimeria menadoensis*. We show that one copy is a distal enhancer, located 500kb from the neuro-developmental gene ISL1. Several others represent new, possibly regulatory, alternatively spliced exons in the middle of pre-existing Sarcopterygian genes. One of these is the >200bp ultraconserved region[5], 100% identical in mammals, and 80% identical to the coelacanth SINE, that contains a 31aa alternatively spliced exon of the mRNA processing gene PCBP2[6]. These add to a growing list of examples[7] in which relics of transposable elements have acquired a function that serves their host, a process termed "exaptation"[8], and provide an origin for at least some of the highly-conserved vertebrate-specific genomic sequences recently discovered using comparative genomics[2,3].

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One of the most evolutionarily constrained regions in mammalian genomes is ultraconserved element uc.338[5], a mammal-specific 223bp region perfectly conserved between human, mouse and rat, overlapping a short protein coding exon of PCBP2[6]. This small region was observed to have multiple paralogs within the human genome, overlapping protein coding exons of otherwise unrelated genes, as well as conserved intronic and intergenic regions[9] (Fig. S1). Unexpectedly, this region also has multiple homologs in coelacanth that are closer in sequence to the human ultraconserved element than many of its human paralogs (Fig. 1c).

Further scrutiny of the 1Mb of sequence available from the Indonesian coelacanth reveals that the match is contained there in a 480bp genomic repeat. A total of 59 closely related copies are found in all four different genomic regions sequenced to date (Table S1). Using all copies, we reconstructed a consensus coelacanth sequence of this repeat (Fig. S2). Despite the huge evolutionary separation between the two species, the coelacanth repeat consensus is 80% identical over 360bp to the human region containing the ultraconserved element. We could find no significant similarity between the coelacanth sequence and any known repeat. However, SINE families are often generated by the fortuitous retroposition of a tRNA sequence into a location where it can provide a Pol III promoter for a retrotranscriptionally capable transcript[10,11]. Indeed, the 3' end of the coelacanth consensus has a clear poly-A region, the 5' end is similar to the vertebrate Serine tRNA, conserving the A and B boxes of an internal Pol III promoter, and the sequence is free of internal oligothymidylate tracts (Fig. 1a). This, combined with the high copy number (extrapolated to $\sim 10^5$ copies genome-wide), low divergence between copies, and evidence in the most conserved instances of target site duplication, indicates that the *Latimeria menadoensis* sequences define a recently active SINE family, which we term the LF-SINE (for "living fossil" or lobe-finned fishes SINE). Interestingly, this family shares a weak 65bp signature with two superfamilies of known SINEs (Text S1).

Diverged traces of LF-SINE copies are found in all available tetrapod genome drafts (Table S3), as well as in miscellaneous DNA sequences from a related coelacanth species, and an array of widely diverged amniotes (Table S4). We cannot detect significant LF-SINE matches in what little lungfish DNA sequence is currently available (under 300kb), or in genome drafts of available ray-finned fish or invertebrates (Table S2). Nor is there any sequence similarity evidence suggesting non-hereditary (horizontal) transfer from any other DNA source in the public repositories (Text S2). Thus it appears that this SINE family was generated by a tRNA retroposition in an ancestral Sarcopterygii species, and is specific to this clade (Fig. 2).

There are altogether only several hundred recognizable partial copies of the LF-SINE in tetrapods for which we have genome drafts: 245 in human, 235 in dog, 394 in opossum (*Monodelphis domestica*), 699 in chicken, and 26 in frog (*Xenopus tropicalis*) (Table S3, electronic supplement). These copies form orthology

groups, in which each ortholog is in the same relative location with respect to the surrounding genes in all tetrapods where it is present (Fig. 1c). Each such group represents a single LF-SINE retroposition that occurred before the common ancestor of the species in the group. As expected from a recently active SINE, none of the 59 coelacanth instances has a human ortholog. However, multiple tetrapodal instances, including 29 in human (Fig. S15), match the entire span of the reconstructed coelacanth SINE, including portions of its poly-A tail, thus providing direct evidence for retroposition activity in the tetrapod lineage. This analysis establishes that virtually all retroposition events that generated mammalian LF-SINE instances predate the divergence of placental mammals and marsupials, and at least several of them, possibly all, predate the divergence of amniotes and amphibians. Examination of ortholgy groups, even using a very conservative test, indicates that a majority of human instances and their orthologs have evolved significantly slower than would be expected assuming neutrality (Text S3). This suggests that most detectable instances of the LF-SINE in tetrapods have been exapted into cellular roles benefiting the host, and subjecting them to purifying selection. In some cases the exapted tetrapod instance is remarkably close to the coelacanth SINE (up to 80% identity), suggesting that the active "living fossil" SINE has changed very little over >410My of independent evolution. The dispersion of coelacanth instances over many subclades in the evolutionary tree for these elements (Fig. 1c) precludes the possibility of recent horizontal transfer from tetrapods to coelacanth.

The majority of human LF-SINE instances are either intergenic (163/245, 66%) or intronic (68, 28%), and a smaller subset (14, 6%) overlap documented human exons, according to current annotations. Among intergenic instances, 107 are >100kb away from any known gene. We cannot find transcriptional evidence or predictions suggesting the human LF-SINEs are still active as small non-coding RNAs, or involved in antisense regulatory transcripts. However, LF-SINE instances are preferentially found next to genes involved in transcriptional regulation, and neuronal development, suggesting exaptation in the form of distal cis-regulatory regions (Text S4).

To test this hypothesis we picked a likely candidate for enhancer activity and tested it *in vivo* using mouse transient transgenics. The ISL1 gene encodes a LIM homeobox transcription factor that is required for the differentiation of motor neurons[12], and is expressed in motor and sensory neurons during vertebrate embryogenesis[13]. An ISL1 proximal LF-SINE instance is significantly conserved between mammals, chicken and frog. It lies 488kb downstream of ISL1, in a 1.4Mb gene desert that is home to two confirmed distal enhancers conserved down to ray-finned fish[13]. The relative ordering and proximity of ISL1, the confirmed enhancers and the LF-SINE instance representing an ancient organization that is invariant in frog, chicken, opossum, mouse, and human (Fig. S8) led us to test this sequence for enhancer activity.

The human ISL1 proximal LF-SINE instance was cloned upstream of a mouse minimal heat shock 68 promoter coupled to the beta-galactosidase reporter gene and injected into the pronuclei of fertilized mouse oocytes. The resulting embryos were analyzed at embryonic day 11.5 (E11.5) by whole embryo staining for beta-galactosidase activity (see methods). Eight out of nine independent ISL1 transgenic embryos showed consistent expression in the head and spinal cord region, outer ridge of the developing limbs (the dorsal apical ectodermal ridge, or AER), and genital eminence; in addition, 4/9 embryos showed staining in the trigeminal ganglion (Fig. 3). Horizontal sections demonstrate specific co-localization of the ISL1 proximal LF-SINE driven beta-galactosidase reporter and murine Isl1 RNA in neural tissues (Fig. 4). In the head region of these embryos we found overlap in the developing hindbrain and thalamus (Fig. 4a-b) as well as cranial motor neurons such as the trigeminal (V) and facial/acoustic (VII/VIII) ganglia (Fig. 4c-d). Similar sections in the thoracic region of these embryos show expression in the dorsal root ganglia and ventral horn of the spinal cord (Fig. 4e-f). These expression patterns clearly recapitulate aspects of murine Isl1 expression in developing motor neurons at this developmental stage[13,14]. Interestingly, the novel and two previously confirmed enhancers drive a very similar pattern of reporter gene expression at E11.5, suggesting that their distinct roles occur at a different timepoint, perhaps later in development[13]. Our combined functional and evolutionary analysis suggest that this LF-SINE instance was exapted as an ISL1 enhancer prior to the divergence of the tetrapods and still functions in this capacity today. This constitutes a proof that mobile elements give birth to distal enhancers.

The ISL1 proximal LF-SINE instance and the instance overlapping ultraconserved region uc.338 have conserved a very similar portion of the ancient LF-SINE (Fig. 1). Yet, mysteriously, one serves as a distal enhancer, and the other as an alternatively spliced exon. To gain a better understanding of exonization, we examined all 19 LF-SINE instances that were exapted into protein coding mRNAs (Table 1). The encoded proteins are unrelated. Only a single pair of them shares a structural (helicase) domain. Without exception the LF-SINE-derived exon is antisense to the original LF-SINE transcript. In 17/19 cases it forms a complete new exon in the middle of the coding region. Only canonical splice sites are used, one of which is similar, yet distinct from a recently studied counterpart in primate specific Alu-SINEs[15]. Exapted exons start in all three possible reading frames. Nearly all (16/17) are alternatively-spliced, leaving the original functional isoforms intact while evolution optimizes the function of the new isoform[16]. They often (11/17) introduce an early stop codon into the reading frame, predicted to trigger nonsense-mediated decay[17]. In many cases the most evolutionarily conserved regions are the LF-SINE derived intronic regions immediately flanking the exons, suggesting the presence of exapted regulatory elements in these regions. Combined, these observations

do not suggest a common protein structural modification induced by exonization of the LF-SINE, but rather that there may be frequent exaptation of the LF-SINE to regulate the level of protein, including in SMARCA4 (BRG1)[18], LRP1B[19], and PCBP2 where the two major splice-variants differ only by including or excluding the ultraconserved exon, which may be involved in cellular localization[20], dimerization[21] and post-transcriptional autoregulation[22] (Text S5).

After discovering mobile DNA elements, Barbara McClintock suggested that they were fundamentally involved in gene regulation[23], an idea further developed by Britten and Davidson who speculated on the benefit of obtaining similar control regions for a "battery" of co-regulated genes via exaptation[24]. At least 50% of our genome originates from characterized transposon-derived DNA[25]. While the early systematic theories of their role in gene regulation were not confirmed, it does appear possible that because these elements optimize their interaction with the host machinery under strong, virus-like evolutionary pressures they are a particularly fecund source of evolutionary innovations, including novel gene regulatory elements, and these are at times exapted by the host to improve its own fitness[7]. If so, it is possible that many more of the >1 million conserved vertebrate genomic elements within our genome originated from ancient retroposon families. Supporting this hypothesis we find thousands of paralog families of highly conserved non-coding sequences in the human genome[9], and many individual exons with multiple non-coding paralogs (e.g., Fig. S12), that are as yet unexplained (Text S6). Indeed, if a significant portion of repeats predating the human-mouse split, used to quantify neutral evolution, were found to be functional[26], the current estimate that at least 5% of the human genome is under purifying selection[3] will need to be revised.

The SINE families that are active in the eight tetrapods for which we have obtained draft genomes to date have all been restricted to specific clades, indicating rather recent origins. Thus it would appear that in tetrapods there is a rather rapid turnover of active SINE families on an evolutionary time scale. The Indonesian coelacanth may be different, in that its LF-SINEs, and its independently discovered DeuSINEs[27], have apparently remained active for more than 400 million years with very little change. In this sense, at least, the coelacanth may really be a living molecular fossil, and one that is quite useful in decoding key events in our own evolution.

Methods

The ISL1 proximal LF-SINE instance was amplified from human DNA (BD Biosciences Clontech) by PCR USING the following primers: F, 5'-AACATCTTGAAAAGAAGATCTAAGC-3' R, 5'-AAGCTGCTTT-TAAAACTGTATCTTC-3'. The amplified DNA was cloned into the Hsp68-lacZ vector[28]. The con-

struct was then purified and injected into pronuclei as previously described[29]. Embryos were harvested at embryonic day 11.5, and transgenic embryos were identified by PCR of beta-galactosidase (lacZ), using DNA from yolk sac and the following lacZ primers: F, 5'-TTTCCATGTTGCCACTCGC-3' R, 5'-AACGGCTTGCCGTTCAGCA-3'. Expression of beta-galactosidase was assayed in all embryos, using 5-bromo-4-chloro-3-indolyl-b-Dgalactoside (X-Gal; Sigma), as described previously[29].

The ISL1 proximal LF-SINE transient transgenics were further analyzed by examining 20 micron horizontal sections made from cryopreserved embryos. Sections were counterstained with neutral red (Sigma, 0.3% w/v in PBS) to visualize the tissues. For in situ RNA hybridizations a murine Isl1-containing plasmid (IMAGE #3419119) was used to make sense and antisense digoxigenin labeled RNA probes. In situ hybridizations on wild type embryonic day 11.5 embryos were done as previously described[30]. Stained sections were photographed using a PowerShot G6 digital camera (Canon) mounted onto a dissecting microscope.

The computational methods are described in the supplementary material.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Table 1: LF-SINE Occurrences Overlapping Documented Exons in Tetrapods

Species	Gene	Function	Exon No.	Alt-Spl.	CDS/ UTR	Start Frame	Insert F.Shift	Ins. Stop	Trig. NMD
H,M,p	PCBP2	mRNA stability and shuttling	9	+	C	0	-	-	-
H,M,p	SMARCA4	SWI/SNF related matrix associated	27	+	C	0	-	-	-
H,m,p,c	EEF1B2	translation elongation factor	3	+	C	1	-	+	+[a]
H,M,p	TCERG1	transcription factor	22	+	C	1	+	+	+[a]
H,M,p	PTDSR	promote cell differentiation	5	+	C	1	+	+	+
H,M,p,c	RORA	DNA binding nuclear receptor	3	+	C	2	+	+	+
H,m,p,c	GRID1	neurotransmitter receptor	1	+	5'U				
H,M,p,c	ATF2	transcription factor	14	+	C	0	+	+	+[a]
H,m,p	FLJ22833	nucleic acid binding	4	+	C	1	+	+	+
H,M,p	ARHGAP6	GTPase activator	13	+	C	1	-	+	+
H,M,p,C	KIAA1409	unknown	34	-	C	2	-	-	-
H,M,p	NT5C2	5'-nucleotidase	9	+	C	1	+	+	+
H,m,p,c,f	LRP1B	low density lipoprotein receptor relate	90	+	C	0	-	-	-
H,m,p	DHX30	Unwind double-stranded nucleic acids	4	+	C	2	-	-	-
h,m,p,C	gg-DMTF1	transcription factor	12	+	C	1	+	+	+
C	gg-PPP2R2C	subunit of protein phosphatase	2	+	C	2	+	+	+
C	gg-SHFM1	subunit of 26S proteasome	3	+	3'U[b]				
F	xt-MBNL1	RNA binding / development	4	+	C	0	-	-	-
F	JGI-49280	unknown	5	+	C	0	+	+	+[a]

[a] Stop codon is the very first fully encoded codon.

[b] Instance is embedded inside a larger 3' UTR region.

Species: H - Human, M - Mouse, P - Possum, C - Chicken, F - Frog. Lowercase characters signify no current evidence of transcription. NMD - nonsense mediated decay. Predicted where the inserted stop codon appears more than 50 nucleotides upstream of the final splice junction[17]. Also see Table S6.

List of Figures

1 **Coelacanth SINE, human ultraconserved PCBP2 exon and ISL1 proximal enhancer share a common origin.** (a) Anatomy of the LF-SINE and its relation to an exapted tetrapodal distal enhancer near ISL1, and the ultraconserved exon of PCBP2, exonized from the reverse strand. (b) Alignment of multiple species instances of the PCBP2 exonized element, and ISL1 proximal enhancer to the reconstructed coelacanth SINE. Filled squares (matches) and white spaces (tetrapodal inserts) are with respect to the coelacanth sequence. (c) A maximum-likelihood joint phylogeny of LF-SINE instances from multiple species. The orthologous copies are shown to form monophyletic subtrees, while the additional instances serve to demonstrate the remarkable overall similarity between human and coelacanth instances. 11

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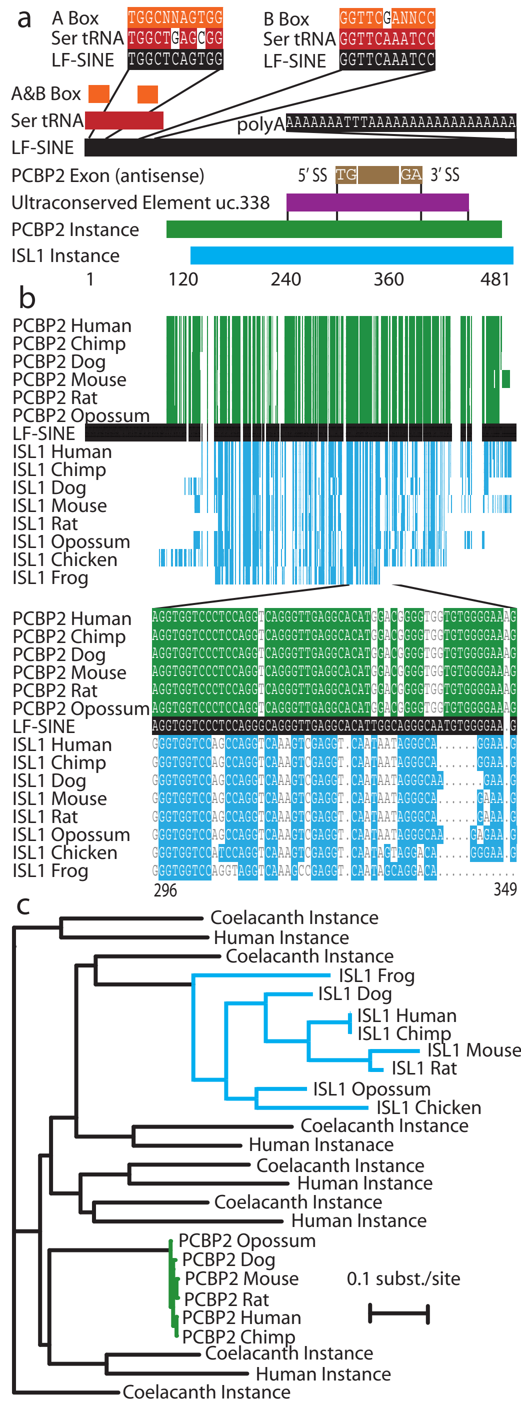
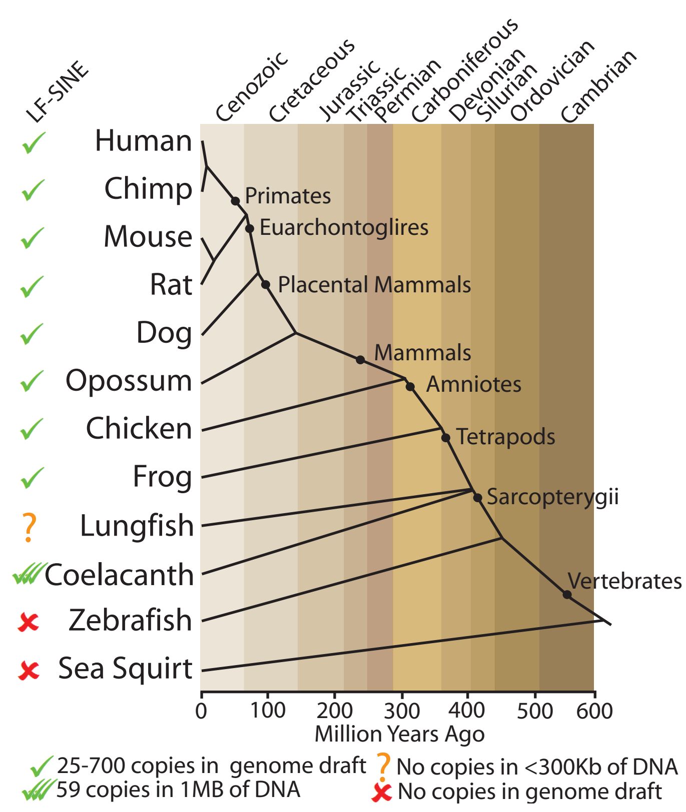


Figure 1:



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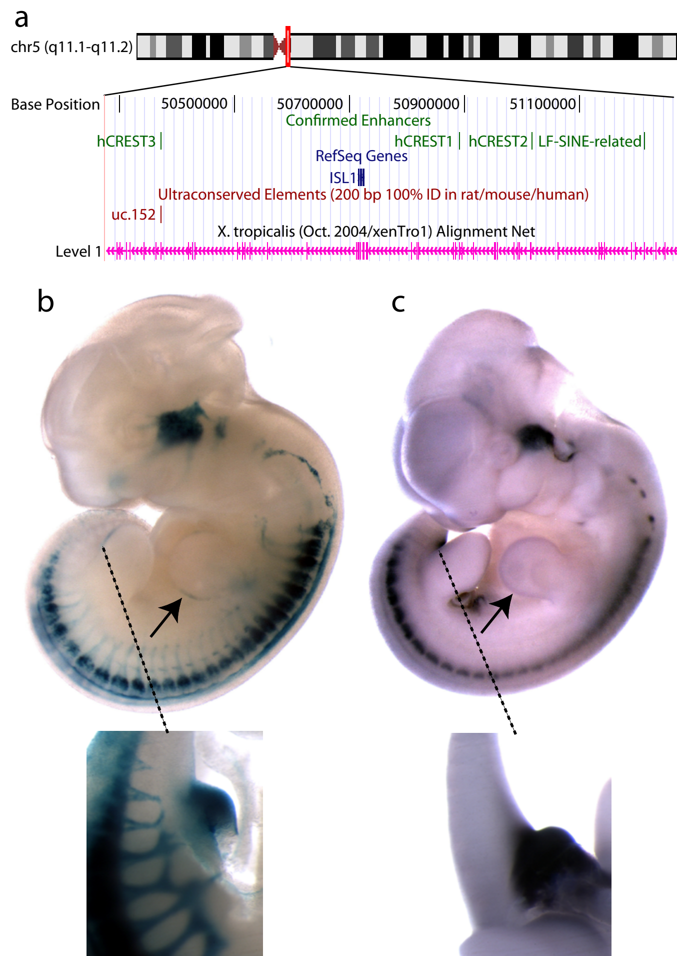

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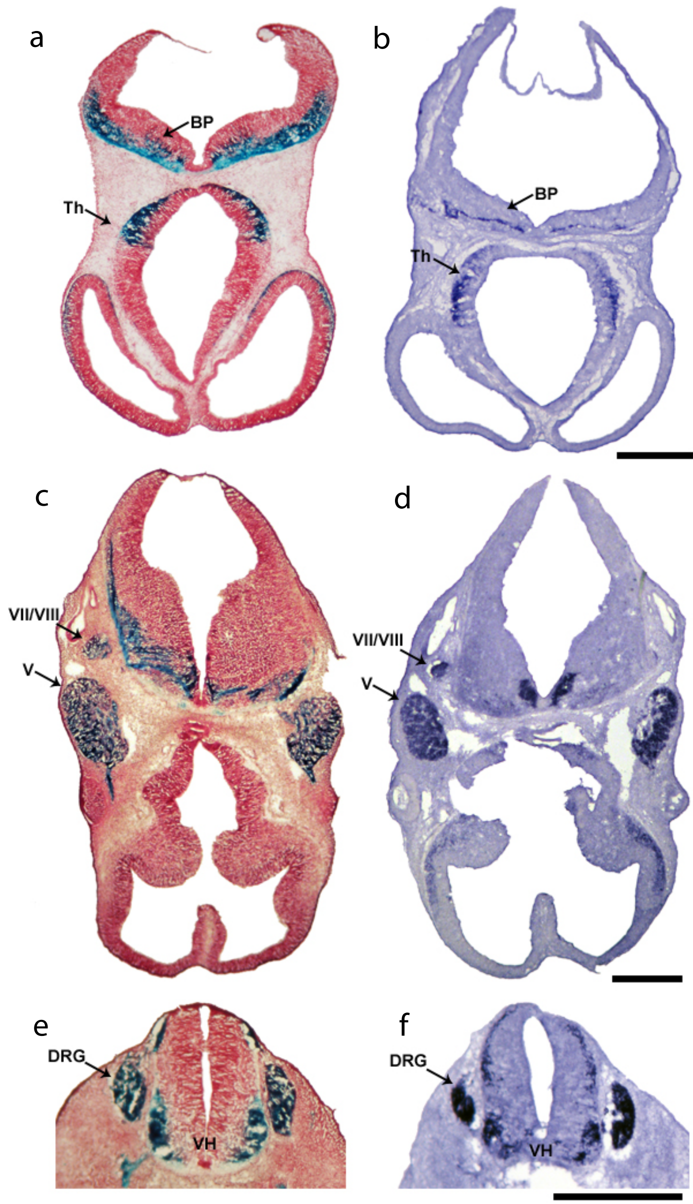

Figure 4: