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Dact-4 is a *Xenopus laevis* Spemann organizer gene related to the Dapper/Frodo antagonist of β-catenin family of proteins

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ABSTRACT

Dact/Dapper/Frodo members belong to an evolutionarily conserved family of Dishevelled-binding proteins present in mammals, birds, amphibians and fishes that are involved in the regulation of Wnt and TGF-β signaling. In addition to the three established genes (Dact1-3) that compose the Dact family, a fourth paralogue group of related proteins has been recently identified and named Dact-4. Interestingly, Dact-4 is the most rapidly evolving gene of the entire family, as it displays very low homology with other Dact proteins and has lost key conserved domains. Dact-4 is not present in mammals, but weakly conserved homologs were found in reptiles and fishes. Recent RNAseq from our group identified new genes specifically expressed in the *Xenopus laevis* Spemann organizer. Among these, *LOC*100170590 mRNA encoded a protein sharing weak homology with a coelacanth Dact-like protein member. Here, by analyzing protein phylogeny and synteny, we show that this organizer gene corresponds to *Dact-4*. We report that *Dact-4* is expressed in the *Xenopus* blastula pre-organizer region in addition to the gastrula organizer, as well as in placodes, eyes, neural tube, presomitic mesoderm and pronephros. Dact-4-Flag microinjection experiments suggest it is a nucleocytoplasmic protein, as are the other Dact paralogues.

1. Introduction

Since the completion of the *Xenopus laevis* genome (Session et al., 2016), many studies have investigated global gene expression dynamics during early frog embryo development. For example, genome-wide approaches identified transcriptional targets of the Wnt/β-catenin pathway during *Xenopus* gastrulation (Kjolby and Harland, 2017), and how Wnt and FGF signaling pathways integrate at the promoter level to regulate gene expression (Kjolby et al., 2019). We used RNA-sequencing approaches to analyze the transcriptomes of the dorsal and ventral regions of the *Xenopus* gastrula, which allowed to define a dorsal gene signature dependent on the early Wnt/β-catenin signal (Ding et al., 2017a, 2017b). Notably, studies on the Spemann organizer dorsal inductive center (Spemann, 1938; Spemann and Mangold, 1924) over the years have generated a saturation screen of all genes expressed in *Xenopus* gastrula organizer (De Robertis, 2009). As a result of our recent RNA-seq studies, additional novel Spemann organizer genes were identified, including *Pkdcc*, a secreted tyrosine kinase homolog (Ding et al., 2017a), *Angptl4*, a secreted protein involved in Wnt inhibition (Kirsch et al., 2017), and *Bighead*, a novel secreted Wnt inhibitor that acts through Lrp6 endocytosis (Ding et al., 2018).

Additional organizer-specific genes were also identified, but remain poorly characterized. Among these, *LOC100170590* (*Xenopus tropicalis* nomenclature; LOC734651 for *X. laevis*) was found to encode for a protein with weak amino acid similarities to a protein of coelacanth fish (Ding et al., 2017b). Basic Local Alignment Search Tool (BLAST) analysis of the amino acid sequence revealed additional weakly homologous genes among reptiles and amphibians, but not mammals. Importantly, the coelacanth sequence had similarities to the Dapper antagonist of β-catenin (Dact) family of adaptor proteins. Dapper/Dact proteins were identified in *Xenopus* as signaling proteins involved in the regulation of Wnt pathway (Brott and Sokol, 2005). In *Xenopus*, the original isolate of Dact-1 was designated Frodo (functional regulator of Dsh in ontogenesis), a maternal protein expressed in the Spemann organizer and involved in neural and eye development (Gloy et al., 2002). Frodo was shown to bind to the Wnt regulator Dvl, promoting Wnt signaling as assayed by secondary axis induction (Gloy et al., 2002). Interestingly, inhibiton of Wnt activity was also linked to Dact-1, through destabilization of β-catenin, which is required for notochord and head development in *Xenopus* (Cheyette et al., 2002). Binding to Dvl required the Frodo/Dact-1 C-terminal domain, which contains a PDZ-binding motif (Thr-Thr-Val). In addition, the first 120 N-terminal amino acids contain

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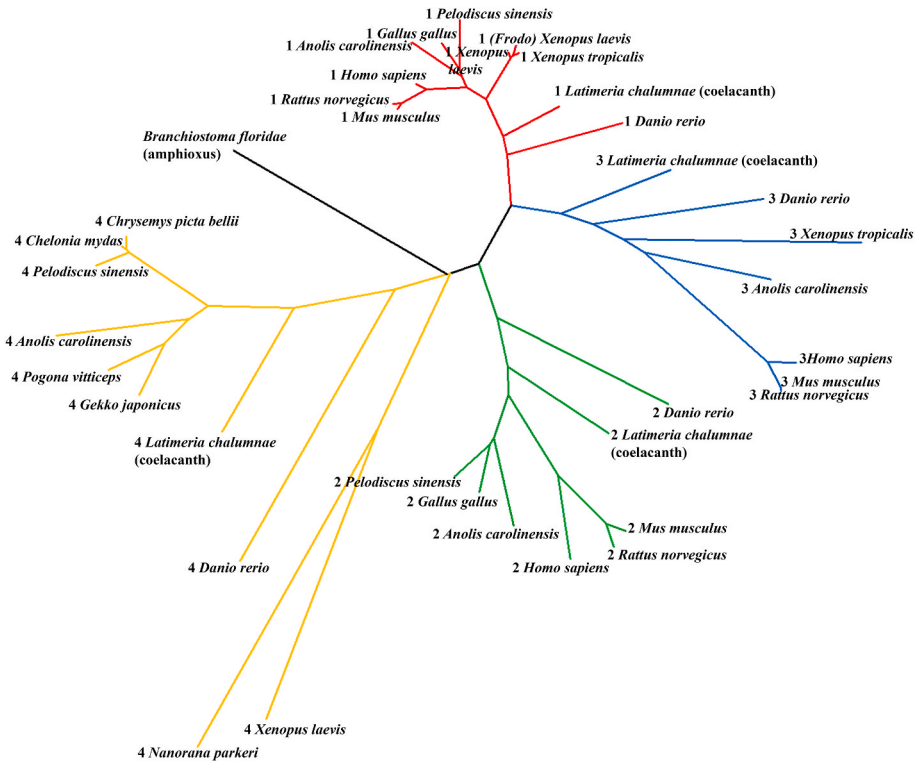

Fig. 1. Phylogenetic analysis of the Dact paralogous genes. Evolutionary analyses were conducted in MEGA-X (Kumar et al., 2018). The evolutionary history was inferred by using the Maximum Likelihood method and the Jones, Taylor and Thornton (JTT) matrix-based model (Jones et al., 1992). The phylogenetic tree was constructed using the Neighbor-Joining method, using 250 bootstrap values (Felsenstein, 1985). This analysis involved 37 amino acid sequences, and the tree was rooted on the amphioxus (*B. floridae*) Dact sequence. *Anolis* is the green anole lizard; *Pogona* is an agamid lizard also known as bearded dragon; *Chrysemys* is the painted turtle; *Chelonia* is the green sea turtle; *Pelodiscus* is the Chinese softshell turtle; *Gekko* is the Japanese gecko; *Nanorana* is a species of frog from the family of Dicroglossidae, whose whole genome has been sequenced (Sun et al., 2015). See Supplementary Materials Table 1 for the sequences utilized.

a leucine-zipper in the coiled-coil domain with significant similarity to dystrophins. These domains are prominent features conserved in all the three distinctive paralogues that form the Dact protein family (Dact1-3) (Schubert et al., 2014). However, recent phylogenetic analysis has shown the presence of another group of proteins, loosely related to Dacts but missing the key N-terminal leucine zipper and C-terminal

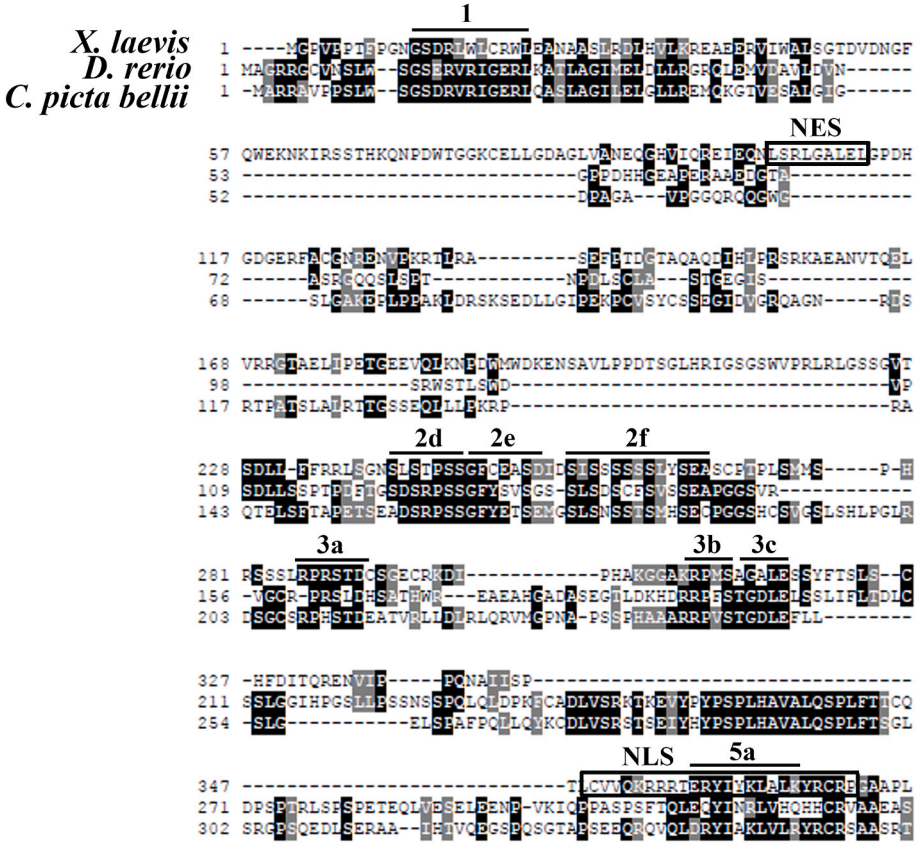

Fig. 2. Conservation of *Xenopus laevis* LOC734651, zebrafish and painted turtle Dact-4 amino acid sequences. Deduced amino acid sequences for *Xenopus*, zebrafish and painted turtle (*Chrysemys picta bellii*) were aligned with Clustal Omega. To assess sequence conservation, the Clustal O alignment was processed with Boxshade. Black shades indicate amino acid identity, while grey shades indicate amino acid similarity. Only the alignment for the first 350 *Xenopus* LOC734651/Dact-4 residues are shown here, where conservation is highest. The Dact-4 adaptor proteins tend to evolve rapidly. Evolutionary conserved amino acid motifs (1, 2d-f, 3a-c, 5a) typical of Dact-4 paralogs are indicated following the same nomenclature as described in Schubert et al. (2014). Nuclear export signal (NES) and nuclear localization signal (NLS) were identified with NetNES (http://www.cbs.dtu.dk/services/NetNES/) (la Cour et al., 2004), and NLS Mapper (nls-mapper.iab.keio.ac.jp) (Kosugi et al., 2009) and are indicated by boxes in the amino acid sequence.

PDZ-binding domains (Schubert et al., 2014). Because of this, it has been proposed that Dact-4 proteins might counteract the activity of other Dacts (Schubert et al., 2014), although functional evidence is still missing. The four Dact paralogues resulted from the two whole-genome duplications (WGD) that occurred in the fish ancestors of all jawed vertebrates (Dehal and Boore, 2005). These two duplications are considered to have been crucial for the extraordinary evolutionary success of vertebrate animals. From detailed phylogenetic analyses it has been proposed that Dact1 and 3 form a separate group from Dact2 and 4, with the Dact1/3 subfamily originating from the first vertebrate WGD, and Dact2/4 paralogues from the second WGD (Schubert et al., 2014). Here, based on phylogenetic, sequence and genomic synteny analyses we show that LOC734651 is the amphibian homolog of the elusive *Dact-4* gene.

2. Results

2.1. Phylogenetic and sequence analysis of LOC734651

RNA-seq screens led to the identification of a novel uncharacterized gene, LOC734651, specifically expressed in the *Xenopus laevis* organizer (Ding et al., 2017b). No signal peptide sequences were found through sequence prediction software, suggesting that LOC734651 may represent a cytoplasmic protein. A previous BLAST analysis of the *Xenopus* sequence revealed the presence of weak homologs, like a gene in the coealacanth fish *Latimeria* that shares 27% identity with LOC734651 at the amino acid level (Ding et al., 2017b). Importantly, the *Latimeria* homolog belongs to the recently identified family of Dact-4 proteins (Schubert et al., 2014). Other homologs included sequences from the frog *Nanorana parkeri* and a number of reptiles (Ding et al., 2017b), all identified as Dact-like homologs. Analysis of the primary amino acid sequence of LOC734651 revealed that both the conserved N-terminal Leucine zipper domain and the C-terminal PDZ-binding motifs were lost, as was the case for Dact-4 (Schubert et al., 2014). Interestingly, Dact4 sequences were only found in anapsid and diapsid reptiles, in *Latimeria* and in actinopterygians, with the protein sequence often very divergent (Schubert et al., 2014). Although these initial analyses did not identify any Dact-4 sequence in other amphibians, the genetic locus was very well conserved in *Xenopus tropicalis* (LOC100170590).

These observations prompted us to re-examine the LOC734651 protein sequence to determine whether it represented the elusive amphibian Dact-4 ortholog. We performed a phylogenetic analysis by comparing the *X. laevis* LOC734651 sequence to the known amino acid sequences of Dact-1, Dact-2 and Dact-3 from different species, as well as identified Dact-4 sequences (Schubert et al., 2014). As shown in the phylogenetic tree in Fig. 1, Dact-1 and 3 formed a metagroup of related proteins, while the Dact-4 group was more closely related to Dact-2, as proposed by Schubert et al. (2014). Interestingly, LOC734651 clustered within the Dact-4 group, suggesting an evolutionary conservation with the Dact-4 type sequences (Fig. 1). Phylogenetic analysis using both MEGA-X (Kumar et al., 2018) and Phylogeny. Fr (Dereeper et al., 2008) platforms gave similar results, showing that *X. laevis* LOC734651 consistently grouped with Dact-4 paralogs (Fig. S1 and S2). Amino acid sequence alignment revealed that *Xenopus* LOC734651 shared moderate sequence similarities with two known members of the Dact-4 group, zebrafish Dact-4 and painted turtle (*Chrysemys picta bellii*) Dact-4, especially within the first 300 amino acids, with an overall identity index of 27% (Fig. 2). Interestingly, analysis of the *Xenopus* amino acid sequence revealed the presence of predicted nuclear export signal (NES) and nuclear localization signal (NLS), as well as the presence of several short motifs (Fig. 2), also found in other Dact-4 members (Schubert et al., 2014).

2.2. Synteny analysis of LOC734651

In the genome, *Dact-4* genes are invariably linked to *Tetratricopeptide Repeat Domain 9C* (*Ttc9c*), a protein up-regulated by progesterone (Cao et al., 2006) and with *Map1lc3*, a protein involved in autophagy (Tanida et al., 2004). In reptiles, Ttc9 was also linked to *Hnrnpul2*, while *Map1lc3c* associated with *Zbtb3* and *Polr2g* (Schubert et al., 2014). In teleost fish Dact-4 is also flanked by *Ttc9c* and *Map1lc3*, but the order of the other genes was less conserved (Schubert et al., 2014). Based on the sequences available on Ensembl and on Xenbase, we reconstructed and compared the conservation of the genetic loci between the genomes of *Xenopus laevis*, zebrafish (*Danio rerio*) and the painted turtle *Chrysemys picta bellii*. Fig. 3 shows that the synteny between *Xenopus* and *Chrysemys* was very well conserved, with LOC734651 (also identified with the Xenbase gene ID XB22041727) flanked by *Ttc9c* and *Map1lc3* in chromosome 4L (Fig. 3). Gene order conservation with the turtle included also *Zbtb3*, *Polr2g* (linked to *Map1lc3*), and *Hnrnpul2*, *Gng3* and *Bscl2* (linked to *Tt9c*). However, zebrafish *Dact-4*, located in chromosome 14, showed only conservation of the flanking *Ttc9c* and *Map1lc3* genes (Fig. 3). Altogether, the results from phylogenetic, protein alignment and synteny analyses suggest that LOC734651 is the *Xenopus Dact-4* ortholog.

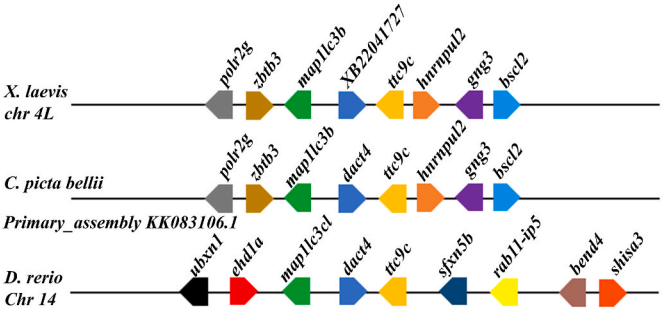

Fig. 3. Synteny analysis between *Xenopus*, zebrafish and painted turtle genomic loci. The syntenic order of Dact-4 neighboring genes was assessed by analyzing primary assembled contig sequences available on Xenbase and Ensembl. For those transcripts that were not annotated in the genomes, the deduced amino acid sequence was obtained and then submitted to BLAST for identification. Genes are shown as colored blocks, with their apex showing transcriptional orientation (sense or antisense strand). Homologous genes in different species are indicated in the same color.

2.3. Xenopus Dact 4 is a nucleo-cytoplasmic protein expressed in the Spemann organizer

Next, we investigated the expression pattern of *Dact-4* transcripts in *Xenopus* embryos. Reverse-Transcription Polymerase Chain Reaction (RT-PCR) showed that *Dact*-4 mRNA is already present in the *Xenopus* egg and in 16-cell embryos (Fig. 4A), when zygotic transcription has not yet started, indicating the presence of maternally deposited mRNA. Transcripts persisted until late embryonic stages, suggesting function throughout development. At stage 10, which coincides with the beginning of *Xenopus* gastrulation, RT-PCR showed *Dact*-4 mRNA enrichment in the gastrula dorsal side, where the Spemann organizer is located (Fig. 4B).

Formation of the Spemann organizer requires the Wnt/β-catenin signaling. Inhibition of early Wnt signaling in embryos injected with β-catenin morpholino (Heasman et al., 2000) completely eliminated expression of Chordin, a prototypical zygotic organizer gene (Sasai et al., 1994), and reduced *Dact-4* expression at stage 10, as assessed by real-time quantitative PCR (Fig. 4C). Residual maternal *Dact*-4 mRNA may account for the incomplete knock-down upon morpholino-mediated β-catenin depletion. Alternatively, *Dact-4* transcription may be under the control of other signaling pathways, in addition to Wnt/β-catenin.

To analyze the *Dact-4* expression pattern, whole-mount *in situ* hybridization was performed on *Xenopus* embryos fixed at different

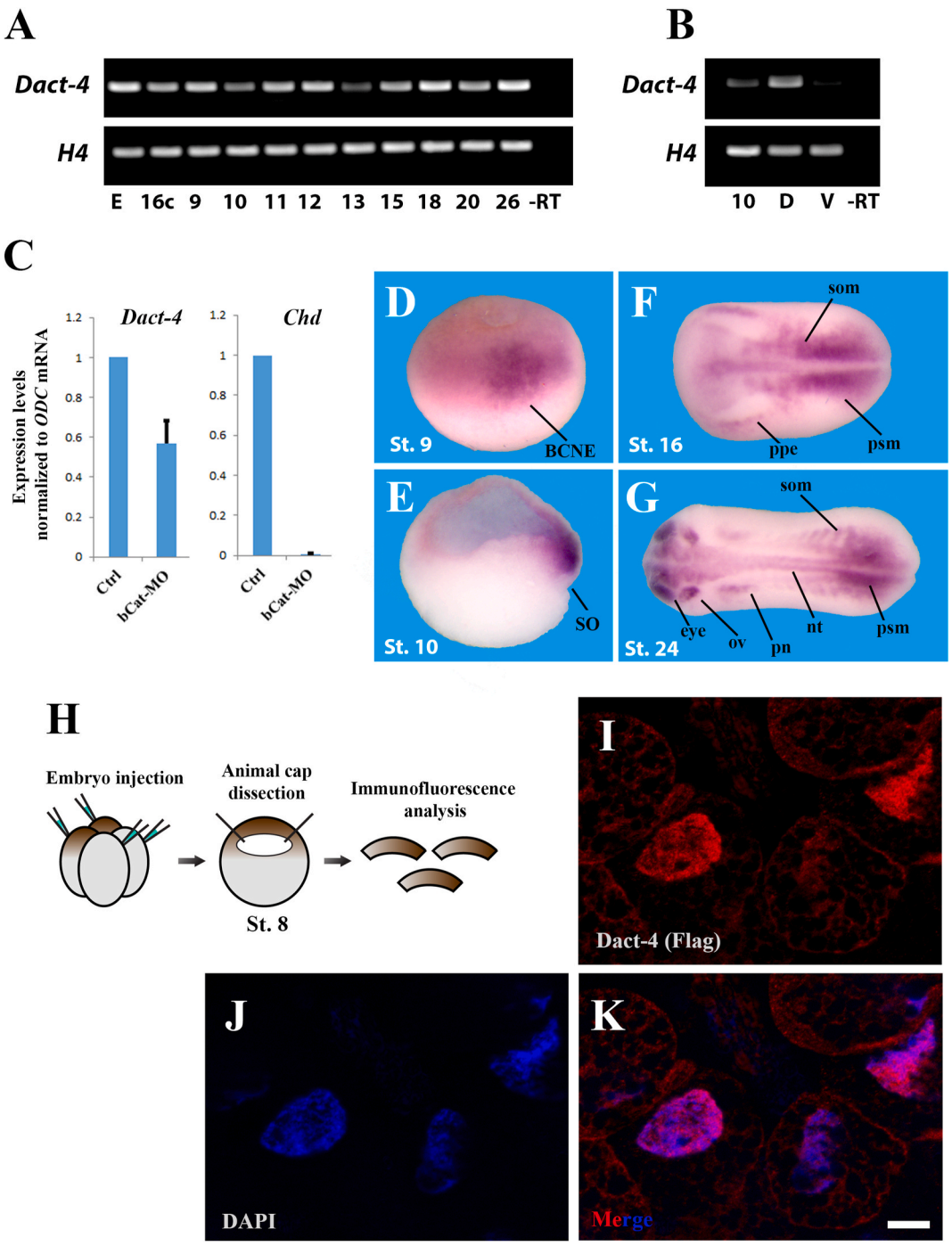

Fig. 4. Developmental expression of the *Xenopus laevis* Dact-4 Spemann organizer gene. (A) RT-PCR showing expression of Dact-4 mRNA at selected developmental stages. Expression at egg (E) and 16-cell (16c) stages indicates the presence of maternal transcripts; Histone 4 (H4) was used as loading control. (B) RT-PCR performed on dorsal (D) and ventral (V) marginal zones dissected from stage 10 *Xenopus* gastrula embryos. Dact-4 transcripts are strongly expressed on the dorsal side. Stage 10 whole embryos were used as positive controls and histone H4 as a loading control. -RT was used as a negative control. (C) *Xenopus* - embryos were injected at the 2-cell stage with β-Catenin morpholino (MO) and assessed for *Dact-4* and *Chordin* mRNA expression through RT-qPCR, at stage 10. β-catenin MO reduces *Dact-4* expression by 40% in injected embryos. *Chordin*, whose expression relies on early Wnt/β-catenin signals and lacks maternal expression, was used as a positive control. Expression levels were normalized to ornithine decarboxylase (ODC) transcripts. The experiment was performed in biological triplicate, and average values are shown. Error bars represent standard deviation. (D–G) whole-mount *in situ* hybridizations at stages 9 (blastula), 10 (gastrula), 16 (neurula), and 24 (tailbud), showing Dact-4 expression pattern at different developmental stages. The embryo shown in (E) was bisected along the Animal-Vegetal axis using a surgical scalpel, splitting the Organizer region in two halves. The half-gastrula embryos were then subjected to *in situ* hybridization. BCNE, blastula Chordin-Noggin expressing center; nt, neural tube; ov, otic vesicle; pn, pronephros; ppe, pre-placodal ectoderm; psm, pre-somitic mesoderm; SO, Spemann organizer; som, somite. (H) Schematic showing the procedure for *Xenopus* animal cap immunofluorescence (IF). Embryos were injected at 4-cell stage with Dact-4-Flag mRNA, cultured until stage 8, and animal caps were dissected and processed for IF. (I–K) Dissected animal caps were stained with anti-flag antibody, to determine the subcellular localization of injected Dact-4-Flag. Nuclei were counterstained with Dapi. Note that Dact-4, like other paralogues of the family, localizes both to the nucleus and the cytoplasm. Scale bar represents 10 μm.

developmental stages. At blastula stage 9, *Dact-4* expression was observed dorsally (Fig. 4D), in the region corresponding to the blastula Chordin and Noggin expressing center (BCNE), a region previously shown to mark the future brain and organizer regions (Kuroda et al., 2004). At gastrula stage 10, mRNA was found in the Spemann organizer (Fig. 4E) (Ding et al., 2017b). Later, *Dact*-4 mRNA was found in the pre-somitic mesoderm and the presumptive placodal ectoderm (Fig. 4F and Fig. S3A), as well as the eye, the neural tube, otic vesicles, pronephros, presomitic mesoderm, somites and around the proctodeum (Fig. 4G and Fig. S3B, C).

To determine the subcellular localization of *Dact-4*, we injected mRNA encoding a flag-tagged chimeric construct into an animal blastomere of 4-cell stage embryos. At stage 8, animal caps were dissected and processed for immunostaining using an anti-flag antibody (Fig. 4H). Immunofluorescence revealed that Dact-4 protein was distributed both in the cytoplasm and the nucleus (Fig. 4I–K and Fig. S4A-C), as is the case for other paralogues, such as Dact1 (Gao et al., 2008). Similar results were obtained from immunofluorescence assays performed on HeLa cells, transfected with flag-tagged Dact4 (Fig. S4D-F).

3. Discussion

Two rounds of whole genome duplication occurred early during vertebrate evolution, expanding the complexity and evolutionary possibilities of the vertebrate genome (Dehal and Boore, 2005). This generated four Dact paralogue genes, all of which are still present in some extant species. For example, both sarcopterygian and actinopterygian fishes have four distinct Dact genes (Schubert et al., 2014). During the vertebrate second whole-genome duplication, Dact-1/3 were generated from one ancestor gene, and Dact-2/4 from the other one (Schubert et al., 2014). Along the evolution of the tetrapod lineage, some individual Dact adaptor proteins were lost and some others diverged greatly in sequence. For example, all mammals have lost the Dact-4 gene, while frogs have lost Dact-2. Although initially it was thought that Dact-4 was lost in *Xenopus*, the work presented here suggests this is not the case. Indeed, our phylogenetic analysis shows that the *Xenopus* LOC734651 is a member of the Dact-4 subfamily. Although its amino acid sequence is quite divergent from other Dact-4 members, we could still identify motifs that are characteristic of Dact-4 orthologs and were conserved during evolution. While the molecular mechanisms of Dact-4 have not been dissected yet, we speculate that these conserved motifs may be important for Dact-4 function. Furthermore, our genomic synteny analysis shows that the locus surrounding *Dact-4* is well conserved in *Xenopus*, including genes that have been described to be invariably associated with Dact-4, like Ttc9c and Map1lc3 (Schubert et al., 2014). Conservation appeared greater between *Xenopus* and the painted turtle than in zebrafish, perhaps suggesting that the genetic locus surrounding Dact4 underwent more extensive rearrangements during teleost evolution. In conclusion, our phylogenetic, amino acid sequence and synteny data provide multiple lines of evidence that the amphibian *Xenopus laevis* retains a Dact-4 ortholog.

An interesting observation emerges from studies reporting the expression domains of different Dact paralogs. For example, the results presented here indicate that *Xenopus Dact-4* is expressed in the Spemann organizer, and is later found in the eye, neural tube, somitic mesoderm, pronephros and tailbud (Fig. 4D–G), like *Frodo/Dact-1* (Cheyette et al., 2002; Gloy et al., 2002). Zebrafish Dact-1 and -2, on the other hand, are also expressed in the embryonic shield (a region corresponding to the frog organizer), the developing neural tissue, the forming retina, the paraxial mesoderm (somatic and pre-somitic mesoderm) and the pronephros (Gillhouse et al., 2004). Similarly, a comparative analysis of Dact paralogs expression patterns revealed the presence of zebrafish Dact4 and Dact4R transcripts in the brain, eye, olfactory vesicles and pronephros, as well as expression in the presomitic mesoderm and somites for mouse Dact-1, -2 and -3, chicken Dact-1 and -2, and *Xenopus* Dact-1 and -3 (Schubert et al., 2014).

The similarity of these embryonic expression patterns suggests the presence of common regulatory mechanisms, which were maintained during the evolution of the different Dact paralogs. It has been suggested that the conserved expression in tissues undergoing extensive morphogenetic movements reflects the role of Dact proteins in regulating cell shape and motility (Hunter et al., 2006). Perhaps, Dact-4 shares a similar regulatory function during *Xenopus* gastrulation. Our immunostaining assays also show that Dact-4 is a nucleo-cytoplasmic protein, as it has been observed for other Dacts. Nuclear export and import signals were found in the primary structure, but further biochemical characterization and functional analysis are needed to understand how Dact-4 subcellular localization is regulated. It has been proposed that since Dact-4 proteins lack a PDZ binding domain, they may antagonize the activity of other Dact paralogs (Schubert et al., 2014). Dact1-3 proteins have the ability to form dimers with themselves or with each other (homo- or hetero-dimers) (Kivimäe et al., 2011), and it is then possible that Dact-4 associate with the other three paralogs, acting in a dominant negative fashion. Future work will assess the role of Dact-4 during development, and whether this protein regulates signaling pathways such as Wnt.

4. Materials and methods

4.1. Animal husbandry and in vitro fertilization

All animal experiments were performed in accordance to UCLA guidelines for animal welfare. *X. laevis* frogs were purchased from the Nasco Company. A sperm suspension was obtained from testicles manually dissected from male frogs, and crushed in 1 ml of 1x Marc's Modified Ringers (MMR, 0.1 M NaCl, 2.0 mM KCl, 1 mM MgSO4, 2 mM CaCl2, 5 mM HEPES, pH 7.4). Ovulation of female frogs was induced the night before experimentation by injecting 800 Units of human chorionic gonadotropin (HCG). The day after, frogs were let to spontaneously lay eggs in a high-salt solution (0.12x MMR). Laid eggs were collected and fertilized with 200–300 μl of the sperm suspension. To remove the jelly-coat, fertilized eggs were treated with a 2% cysteine in 0.1x MMR solution pH 7.8, for about 7 min at room temperature (RT). Dejellied embryos were then cultured in 0.1x Marc's modified Ringer's solution and staged according to Nieuwkoop and Faber (1967).

4.2. Whole mount in situ hybridization

Whole-mount *in situ* hybridizations were performed as described at http://www.hhmi.ucla.edu/derobertis. Briefly, for *in situs* of hemisected gastrula embryos, embryos were fixed, cut into halves with sharp surgical blades and processed for *in situ* hybridization. Full-length *Xenopus Dact-4* was amplified by PCR from gastrula embryo cDNA, and cloned into pCS2 vector. Sequence was confirmed by Sanger sequencing. To obtain a digoxigenin-labeled antisense probe, pCS2-Dact-4 was linearized with BstBI restriction enzyme (New England Biolabs) and purified with a PureLink PCR purification kit (Invitrogen). In vitro transcription was performed using T7 polymerase (Roche) and a Dig-labeling mix (Roche), using the purified linearized plasmid as a template. Probes obtained from several reactions were pooled and purified using the phenol/chloroform extraction method. Pure Dact-4 antisense probe was quantified using a Nanodrop spectrophotometer and used at a concentration of 100–200 ng/ml.

4.3. Immunostaining on Xenopus animal caps and HeLa cells

Full length Dact-4 cDNA was obtained as described above, and cloned (without its STOP codon) into a home-made pCS2 vector containing Gateway sequences and an in-frame C-terminal 3xFlag epitope. mRNA was obtained from in vitro transcription with a mMessage Sp6 kit (Ambion), using a purified pCS2-Dact-4-3xFlag template previously linearized with NotI restriction enzyme (New England Biolabs). For animal cap immunostaining, 100 pg of Flag-tagged Dact-4 mRNA were

Table 1
Primers used in this study.

Primers for RT-PCR and RT-qPCR (5' – 3')	
Dact-4 forward	GCCCTTGAGTCTTCTTAT
Dact-4 reverse	CAGACATGAGAGAGATGG
H4 forward	CGGGATAACATTCAGGGTATCACT
H4 reverse	ATCCATGGCGGTAACTGTCTTCCT
Chordin forward	GTTGTACATTTGGTGGGAA
Chordin reverse	ACTCAGATAAGAGCGATCA
ODC forward	CAGCTAGCTGTGGTGTGG
ODC reverse	CAACATGGAAACTCACACC
Primers for Dact-4 cloning (5' – 3'; Gateway sequence underlined)	
Dact-4 forward	GGGGACAAGTTTGTACAAAAAAGCAG
	GCTTAGTGTTCCGatgggccctgtccccccaacc
Dact-4 reverse	GGGGACCACTTTGTACAAGAAAG
	CTGGGTcatgaagcttattcaaccctttttgac

injected animally into each blastomere of 4-cell embryos. Animal caps were dissected at stage 8 using watchmaker forceps, and immediately fixed in paraformaldehyde (PFA) 4% in PBS. Animal caps were then washed three times in PBS, 5′ each wash, permeabilized in PBS +0.1% Triton X-100 for 15′ at R.T., blocked in PBS +3% BSA for 1h at R.T., and incubated with anti-flag antibody (Sigma, M2 mouse monoclonal, F1804) diluted 1:250 in blocking buffer overnight. Next day, animal caps were washed again 3 times 5' each with PBS, incubated 2h at RT with secondary antibody and mounted with Vectashield mounting medium containing DAPI for nuclear staining. For the mounting step, animal caps were flipped upside-down so that the sensory (white) layer was exposed to the objective. For immunofluorescence on HeLa, cells were cultured on glass coverslips in 12-well plates, and transfected with 1 μg of pCS2-Dact-4-3xFlag using Lipofectamine 2000 (Invitrogen). 48 h after transfection, cells were washed with PBS and fixed with PFA 4% in PBS, for 15 min at room temperature. Fixed HeLa cells were then processed for immunostaining following a protocol similar to that described above.

4.4. RT-PCR

For Reverse Transcription – Polymerase Chain Reaction (RT-PCR) and Reverse Transcription – quantitative Polymerase Chain Reaction (RT-qPCR) experiments, total RNA was extracted from *Xenopus* embryos using RNeasy kit (Qiagen) following manufacturer's instructions, and cDNA synthesis and PCR assays were performed as previously described (Colozza and De Robertis, 2014). For the experiment shown in Fig. 4C, uninjected controls and embryos injected four times vegetally with β-catenin morpholino were cultured until stage 10, when they were collected and processed for mRNA extraction. Primer sequences are available in Table 1.

4.5. Phylogenetic analysis, amino acid sequence alignments and synteny reconstruction

The amino acid sequence for *Xenopus* LOC100170590/Dact-4 was used to search for homologs through the Basic Local Alignment Search Tool (BLAST). A variety of sequences were collected and analyzed along with other Dact-4 sequences previously described. The amino acid sequences were used to construct a phylogenetic tree using the MEGA-X software and its built-in MUSCLE sequence alignment tool. Tree reliability is tested with the Bootstrap method, which indicates how often the branching pattern occurs if the tree is constructed for a certain number of reiterations. The percentage of times each branching pattern occurs is projected on the tree branches. For amino acid alignments we used the Clustal Omega software available online (https://www.ebi.ac.uk/Tools/msa/clustalo/). These alignments were then further analyzed with Boxshade (https://embnet.vital-it.ch/software/BOX_form.html), a program that assigns color shades to indicate how similar two sequences are. Black shading indicates residue identity on that position, while grey shading indicates residue similarity. Synteny between *Xenopus*, *Danio rerio* and *Chrysemys picta bellii* Datc-4 loci were reconstructed using sequence information available on Xenbase (*Xenopus* ENTREZ ID: *LOC100170590*, which in the genome browser is indicated as XB-GENE-22041729) and Ensembl. For zebrafish ENSDART00000125970 and for painted turtle ENSCPBG00000017965 indicated the *Dact-4* orthologs, and the associated genomic regions were analyzed. Non-annotated sequences were processed with BLAST for identification.

Author contributions

GC carried out the experiments and data analysis. GC and EMDR conceived and designed the research and wrote the manuscript.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gep.2020.119153.

References

Brott, B.K., Sokol, S.Y., 2005. Frodo proteins: modulators of Wnt signaling in vertebrate development. Differentiation 2005 73 (7), 323–329. https://doi.org/10.1111/j.1432-0436.2005.00032.x.

Cao, S., Iyer, J.K., Lin, V., 2006. Identification of tetratricopeptide repeat domain 9, a hormonally regulated protein. Biochem. Biophys. Res. Commun. 345 (1), 310–317. https://doi.org/10.1016/j.bbrc.2006.04.091.

Cheyette, B.N., Waxman, J.S., Miller, J.R., Takemaru, K., Sheldahl, L.C., Khlebtsova, N., Fox, E.P., Earnest, T., Moon, R.T., 2002. Dapper, a Dishevelled-associated antagonist of beta-catenin and JNK signaling, is required for notochord formation. Dev. Cell 2 (4), 449–461. https://doi.org/10.1016/s1534-5807(02)00140-5.

Colozza, G., De Robertis, E.M., 2014. Maternal syntabulin is required for dorsal axis formation and is a germ plasm component in Xenopus. Differentiation 88 (1), 17–26. https://doi.org/10.1016/j.diff.2014.03.002.

Dehal, P., Boore, J.L., 2005. Two rounds of whole genome duplication in the ancestral vertebrate. PLoS Biol. 3 (10), e314. https://doi.org/10.1371/journal.pbio.0030314.

De Robertis, E.M., 2009. Spemann's organizer and the self-regulation of embryonic fields. Mech. Dev. 126, 925–941. https://doi.org/10.1016/j.mod.2009.08.004.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.-F., Guindon, S., Lefort, V., Lescot, M., Claverie, J.-M., Gascuel, O., 2008. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res. 36, 465–469. https://doi.org/10.1093/nar/gkn180.

Ding, Y., Colozza, G., Zhang, K., Moriyama, Y., Ploper, D., Sosa, E.A., Benitez, M.D., De Robertis, E.M., 2017a. Genome-wide analysis of dorsal and ventral transcriptomes of the Xenopus laevis gastrula. Dev. Biol. 426 (2), 176–187. https://doi.org/10.1016/j.ydbio.2016.02.032.

Ding, Y., Ploper, D., Sosa, E.A., Colozza, G., Moriyama, Y., Benitez, M.D., Zhang, K., Merkurjev, D., De Robertis, E.M., 2017b. Spemann organizer transcriptome induction by early beta-catenin, Wnt, Nodal, and Siamois signals in Xenopus laevis. Proc. Natl. Acad. Sci. U.S.A. 114 (15), E3081–E3090. https://doi.org/10.1073/pnas.1700766114.

Ding, Y., Colozza, G., Sosa, E.A., Moriyama, Y., Rundle, S., Salwinski, L., De Robertis, E. M., 2018. Bighead is a Wnt antagonist secreted by the Xenopus Spemann organizer that promotes Lrp6 endocytosis. Proc. Natl. Acad. Sci. U.S.A. 115 (39), E9135–E9144. https://doi.org/10.1073/pnas.1812117115.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x.

Gao, X., Wen, J., Zhang, L., Li, X., Ning, Y., Meng, A., Chen, Y.G., 2008. Dapper1 is a nucleocytoplasmic shuttling protein that negatively modulates Wnt signaling in the nucleus. J. Biol. Chem. 283 (51), 35679–35688. https://doi.org/10.1074/jbc.M804088200.

Gillhouse, M., Nyholm, M.W., Hikasa, H., Sokol, S.Y., Grinblat, Y., 2004. Two Frodo/Dapper homologs are expressed in the developing brain and mesoderm of zebrafish. Dev. Dynam. 230 (3), 403–409. https://doi.org/10.1002/dvdy.20060.

Gloy, J., Hikasa, H., Sokol, S.Y., 2002. Frodo interacts with Dishevelled to transduce Wnt signals. Nat. Cell Biol. 4 (5), 351–357. https://doi.org/10.1038/ncb784.

Heasman, J., Kofron, M., Wylie, C., 2000. Beta-catenin signaling activity dissected in the early Xenopus embryo: a novel antisense approach. Dev. Biol. 222 (1), 124–134. https://doi.org/10.1006/dbio.2000.9720.

Hunter, N.L., Hikasa, H., Dymecki, S.M., Sokol, S.Y., 2006. Vertebrate homologues of Frodo are dynamically expressed during embryonic development in tissues undergoing extensive morphogenetic movements. Dev. Dynam. 235 (1), 279–284. https://doi.org/10.1002/dvdy.20609.

Jones, D.T., Taylor, W.R., Thornton, J.M., 1992. The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. 8, 275–282. https://doi.org/10.1093/bioinformatics/8.3.275.

Kirsch, N., Chang, L.S., Koch, S., Glinka, A., Dolde, C., Colozza, G., Benitez, M.D., De Robertis, E.M., Niehrs, C., 2017. Angiopoietin-like 4 is a Wnt signaling antagonist that promotes LRP6 turnover. Dev. Cell 43 (1). https://doi.org/10.1016/j.devcel.2017.09.011, 71-82 e76.

Kivimäe, S., Yang, X.Y., Cheyette, B.N., 2011. All Dact (Dapper/Frodo) scaffold proteins dimerize and exhibit conserved interactions with Vangl, Dvl, and serine/threonine kinases. BMC Biochem. 30 https://doi.org/10.1186/1471-2091-12-33, 12:33.

Kjolby, R.A.S., Harland, R.M., 2017. Genome-wide identification of Wnt/beta-catenin transcriptional targets during Xenopus gastrulation. Dev. Biol. 426 (2), 165–175. https://doi.org/10.1016/j.ydbio.2016.03.021.

Kjolby, R.A.S., Truchado-Garcia, M., Iruvanti, S., Harland, R.M., 2019. Integration of Wnt and FGF signaling in the Xenopus gastrula at TCF and Ets binding sites shows the importance of short-range repression by TCF in patterning the marginal zone. Development 146 (15). https://doi.org/10.1242/dev.179580.

Kosugi, S., Hasebe, M., Tomita, M., Yanagawa, H., 2009. Systematic identification of yeast cell cycle-dependent nucleocytoplasmic shuttling proteins by prediction of composite motifs. Proc. Natl. Acad. Sci. U.S.A. 106, 10171–10176. https://doi.org/10.1073/pnas.0900604106.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. Mega X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35 (6), 1547–1549. https://doi.org/10.1093/molbev/msy096.

Kuroda, H., Wessely, O., De Robertis, E.M., 2004. Neural induction in Xenopus: requirement for ectodermal and endomesodermal signals via Chordin, Noggin, beta-Catenin, and Cerberus. PLoS Biol. 2 (5), E92. https://doi.org/10.1371/journal.pbio.0020092.

la Cour, T., Kiemer, L., Mølgaard, A., Gupta, R., Skriver, K., Brunak, S., 2004. Analysis and prediction of leucine-rich nuclear export signals. Protein Eng. Des. Sel. 17, 527–536. https://doi.org/10.1093/protein/gzh062.

Nieuwkoop, P.D., Faber, J., 1967. Normal Table of Xenopus laevis (Daudin): A Systematic and Chronological Survey of the Development from the Fertilized Egg till the End of Metamorphosis. North-Holland Publishing Co., Amsterdam), reprinted 1994 (Garland Publishing, Inc., New York.

Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L.K., De Robertis, E.M., 1994. Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. Cell 79 (5), 779–790. https://doi.org/10.1016/0092-8674(94)90068-x.

Schubert, F.R., Sobreira, D.R., Janousek, R.G., Alvares, L.E., Dietrich, S., 2014. Dact genes are chordate specific regulators at the intersection of Wnt and Tgf-beta signaling pathways. BMC Evol. Biol. 14, 157. https://doi.org/10.1186/1471-2148-14-157.

Session, A.M., et al., 2016. Genome evolution in the allotetraploid frog Xenopus laevis. Nature 538 (7625), 336–343. https://doi.org/10.1038/nature19840.

Spemann, H., 1938. Embryonic Development and Induction Yale Univ Press, New Haven (CT).

Spemann, H., Mangold, H., 1924. Induction of embryonic primordia by implantation of organizers from a different species. Roux Arch. Entwicklungsmech. Org 100, 599–638.

Sun, Y.B., et al., 2015. Whole-genome sequence of the Tibetan frog Nanorana parkeri and the comparative evolution of tetrapod genomes. Proc. Natl. Acad. Sci. U. S. A. 112 (11), E1257–E1262. https://doi.org/10.1073/pnas.1501764112.

Tanida, I., Ueno, T., Kominami, E., 2004. Human light chain 3/MAP1LC3B is cleaved at its carboxyl-terminal Met121 to expose Gly120 for lipidation and targeting to autophagosomal membranes. J. Biol. Chem. 279 (46), 47704–47710. https://doi.org/10.1074/jbc.M407016200.