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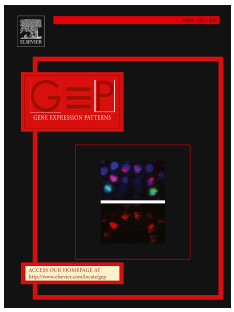

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PII: S1567-133X(18)30024-3

DOI: 10.1016/j.gep.2018.08.001

Reference: MODGEP 19030

To appear in: *Gene Expression Patterns*

Received Date: 17 February 2018

Revised Date: 2 July 2018

Accepted Date: 16 August 2018

Please cite this article as: Bonano, M., Martín, E., Moreno Ruiz Holgado, M.M., Silenzi Usandivaras, G.M., Ruiz De Bigliardo, G., Aybar, M.J., Molecular characterization of *wdr68* gene in embryonic development of *Xenopus laevis*, *Gene Expression Patterns* (2018), doi: 10.1016/j.gep.2018.08.001.

Molecular characterization of *wdr68* gene in embryonic development of *Xenopus laevis*

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ABSTRACT

WDR68, also known as DCAF7, is a WD40 repeated domain protein highly conserved in eukaryotic organisms in both plants and animals. This protein participates in numerous cellular processes and exerts its function through interaction with other proteins. In the present work, we isolated, sequenced and characterized cDNA corresponding to the *wdr68* gene in embryos of the amphibian *Xenopus laevis*. Syntenic analysis revealed high conservation of the genomic region containing the *WDR68* locus in amniotes. Nevertheless, in fishes and amphibians, we observed that the tandem genes surrounding *wdr68* undergoes certain rearrangements with respect to the organization found in amniotes. We also defined the temporal and spatial expression pattern of the *wdr68* gene in the development of *Xenopus laevis* through whole mount in situ hybridization and RT-PCR techniques. We observed that *wdr68* is ubiquitously expressed during early embryonic development but, during the neurula stage, it undergoes a strong expression in the neural tube and in the migratory cephalic streams of the neural crest. At the tailbud stages, it is strongly expressed in the cephalic region, particularly in otic and optic vesicles, in addition to branchial arches. In contrast, *wdr68* transcripts are localized in the somitic mesoderm in the trunk. The expression area that includes the migratory neural crest of the head and the branchial arches suggest that this gene would be involved in jaws formation, probably through a hierarchical relationship with the component genes of the e*ndothelin-1/endothelin receptor type A* cell signaling pathway.

KEY WORDS: expression pattern, *Xenopus laevis*, *wdr68* gene, synteny

INTRODUCTION

Trp-Asp (WD) repeat protein 68 (WDR68), also known as DCAF7 (Ddb1- and Cul4-associated factor 7) is a highly conserved protein throughout evolution that is associated with numerous important physiological functions (Miyata *et al*., 2014). It belongs to the superfamily of the WD40 repeat domain-containing proteins. The WD40 domain is composed of several copies of WD40 repeats. Each repeat is a fragment composed of 44-60 amino acids. It contains a variable region of 11–24 residues followed by a glycine-histidine (GH) dipeptide and harbors a tryptophan-aspartate (WD) dipeptide in the carboxy terminal end (Stirnimann *et al*., 2010). Between the GH and WD dipeptides, there is a conserved core sequence of approximately 40 residues. The number of WD repeats varies between 4 and 16 repeating units (Li and Roberts, 2001). WD40 repeats typically fold into a seven-bladed β-propeller ring. This tridimensional structure provides multiple surfaces to interact with various proteins, peptides or nucleic acids and contributes to the formation of large dynamic multi-protein complexes. The WD40 repeat facilitates protein-protein interactions and coordinates multiprotein complex assemblies where the repeating units serve as a rigid scaffold (Li and Roberts, 2001; Stirnimann *et al*., 2010).

The importance of this family of proteins is remarkable: the WD40 sequence is conserved in all analyzed eukaryotic species, they perform essential biological functions and it has been demostrated that several human diseases carry mutations in the WD repeated domains (Li and Roberts*,* 2001). Most proteins of this family have a regulatory function. The various sets of functions in which the WD40 repeat proteins participate include signal transduction, RNA synthesis/processing, chromatin assembly, vesicular trafficking, cytoskeletal assembly, cell cycle control and apoptosis (Benedict *et al*., 2000; Chow *et al*., 1996; Hamill *et al*., 1998; Li and Roberts, 2001; Stirnimann *et al*., 2010; Tyler *et al*., 1996; Weber *et al*., 1999; Yamamoto *et al*., 1998).

More than 200 cellular proteins that have a wide variety of crucial physiological implications bind WDR68 to carry out these functions. WDR68 can physically interact with several kinases, including two closely related members of the Dual-specificity tyrosine-regulated kinase gene family, Dyrk1a and Dyrk1b, MAPK/ERK kinase kinase 1 (MEKK1) and Cullin4-damage-specific DNA-binding protein 1 (CUL4-DDB1) (Miyata *et al*., 2014; Skurat *et al*., 2004; Wang *et al*., 2013).

WDR68 was first identified as the vertebrate homolog of the product of the *Petunia* gene *AN11*, which controls flower pigmentation through the activation of the pathway for anthocyanin biosynthesis (de Vetten *et al*., 1997; Mazmanian *et al.*, 2010). Highly conserved homologs have been identified in several species, including humans, with other functions. The protein sequence of WDR68 is identical in all mammals studied up to the present, with the same 342 amino acid residues (Miyata and Nishida, 2011; Miyata *et al.*, 2014). Among the biological functions assigned to this protein, its participation in the craniofacial development of vertebrates is remarkable, especially in the formation of the mandible.

Notably, the great evolutionary success of the vertebrates is partly due to the acquisition of a mandibular apparatus derived from the neural crest. The cell signaling pathways and the mechanisms implicated in the embryonic cartilaginous jaw formation have not been completely understood and several genes involved in this process remain unknown (Nissen *et al*., 2006).

Most tissues that form the head and neck of vertebrates are derived from the cranial neural crest; therefore, craniofacial malformations are due to an abnormal pattern of the cephalic neural crest and defective morphogenesis of this cell population.

Syndromes and congenital birth defects due improper development of the neural crest are called neurocristopathies. Examples of diseases caused by an inadequate neural crest formation and migration are DiGeorge syndrome, Waardenburg syndrome, Treacher-Collins and CHARGE syndrome and Hirschsprung disease (Mayor and Theveneau, 2013).

The ethical implications and limitations to study human genetic dysfunctions are overcome through the use of animal models which allow gene overexpression or blockage and their involvement in health and pathophysiology. In this sense, the amphibiam *Xenopus laevis* is an accepted animal model for genetic and development studies. Several reports about the genes that participate in *Xenopus* neural crest development have been published in the last decades (Agüero *et al*., 2012, Fernández *et al*., 2014; Huang and Saint-Jeannet, 2004; Meulemans and Bronner-Fraser, 2004; Simões-Costa and Bronner, 2015; Tríbulo *et al*., 2004; Vega López *et al*., 2015). However, other genes required for this crucial process have not been identified yet.

The neural crest is a population of multipotent and highly migratory cells unique to vertebrates. This transitory cell population is induced in the ectoderm during gastrulation and is specified at the interface between the neuroepithelium and the prospective epidermis (Mayor and Aybar, 2001). After the neural tube closes because of the progressive elevation of the neural folds towards the dorsal midline, the neural crest cells detach from the neuroepithelium, lose cell-cell adhesion and undergo cytoskeletal changes. This epithelial to mesenchymal transition allows them to migrate extensively in the developing embryo. Once they reach their final destinations, they differentiate into various derivatives (Glbert, 2006; Jones and Trainor, 2004; Rogers *et al*., 2012). Among the vast list of derivatives that emerge from the neural crest are the following: neurons and glia of the sympathetic and parasympathetic peripheral nervous system; epinephrine-producing cells of the adrenal gland; melanocytes; cartilages and bones of the face and neck; connective and adipose tissues and dermis of the head and the neck and outflow tract septum (Aybar and Mayor, 2002; Dupin *et al*., 2006; LaBonne and Bronner-Fraser, 2000; Le Douarin and Kalcheim, 1999; Rogers *et al*., 2012). Neural crest cells show similarities with stem cells and metastatic cancer cells. Because of that, this population of multipotent cells is a popular model system for studying cell/tissue interactions and signaling factors that influence cell fate decisions (Rogers *et al.*, 2012)

Neural crest development is governed by a complex gene network in which the components act sequentially. The activation of upstream genes induces or inhibits other downstream genes (Rogers *et al*., 2012). Several studies to evidence the hierarchical relationship between the genes of the different cell signaling pathways that regulate the formation, development, migration and differentiation of the neural crest have been successfully completed. However, up to the present, significant gaps remain in our knowledge with respect to certain genes expressed in the neural crest territory and their identity and/or function are still unknown. In 2008, we reported the participation of the *endothelin-1/endothelin receptor type A* (*edn-1/ednra*) at all stages of the neural crest development during *Xenopus laevis* embryogenesis, from its induction in the neural plate borders to the differentiation of craniofacial cartilages and melanocytes (Bonano *et al*., 2008). Nevertheless, in this organism, only a few genes have been identified as triggering the *edn-1/ednra* signal or as targets induced by this cell signaling pathway. It is also important to highlight the fact that craniofacial development in this amphibian is a poorly understood biological process.

In zebrafish, investigations have demonstrated that *wdr68* gene is required for the correct formation of the jaw, one of the main neural crest derivatives. *wdr68* acts upstream of the *edn-1/ednra* pathway and exerts this role through close association

with the DYRK1 protein in a highly conserved protein complex (Mazmanian *et al*., 2010; Nissen *et al.,* 2006).

Here we present a molecular characterization of the *wdr68* gene during the embryonic development of the amphibian *Xenopus laevis* and define its spatial and temporal expression pattern. The background of this gene provided by investigation in zebrafish makes it an interesting candidate as a transcript required for normal embryonic development because of its probable participation in the formation of the neural crest or its derivatives.

The study of the genes in *Xenopus laevis* is always interesting. The characterization of the genes that are duplicated in this allotetraploid organism is relevant because the redundant gene information could drive to a neofunctionalization or subfunctionalization of one of the homeologous copy (Session *et al*., 2016, Watanabe *et al*, 2017).

MATERIALS AND METHODS

Animal husbandry and embryo manipulation

The adult individuals of *Xenopus laevis* ("*african clawed toe frog*") were purchased from NASCO (Fort Atkinson, Wisconsin, USA). They were housed in static and dechlorinated water containers at a constant temperature of 17-19 °C. The water was completely changed three times per week. Embryos were obtained by natural mating after male and female hormonal stimulation with 500 IU and 750 UI, respectively, of human chorionic gonadotropin (Gonacor ® 5000, Ferring Pharmaceuticals). The protocols for fertilized eggs preparation and manipulation were performed as described by Sive *et al*. (Sive *et al*., 2000). Embryos were staged according to the Nieuwkoop and Faber's developmental table (1967).

Isolation, identification and sequencing of *Xenopus laevis wdr68* gene

The oligonucleotides to amplify *wdr68* sequence from *Xenopus laevis* were designed on the basis of cDNA sequences available in the NCBI, corresponding to a putative sequence of *Xenopus* (NM_001087389) and other from zebrafish (BC053157). The coding fragment corresponding to *wdr68* of *Xenopus* was amplified in two steps. The primers used were: *Xwdr68-F* 5´-CTG TGT TGG AAC AAG CAG GA -3´ and *Xwdr68-R* 5´-GCT CTC CTT CCT GTC ATT GG-3' (to amplify a 3´-terminus fragment of 463 bp in length) and *5´wdr68-F* 5´-GGTAACGGTGTCTGTAAAGC-3´ and *5´wdr68-R* 5´-CACTGCACATTGTTGATTTC-3´ (to amplify most of the coding sequence, producing a 992 bp-length fragment). Both fragments were digested by restriction endonuclease *Hinc*II (Promega, USA) and linked by *T4* DNA ligase (Invitrogen, USA). Finally, the sequence obtained through automated sequencing by capillary electrophoresis was uploaded on the NCBI under the access number KX910100.1.

RNA isolation and RT-PCR expression analysis

Total RNA isolation was performed from whole embryos at different developmental stages and from different embryonic tissues after microdissection using TRIzol® Reagent (Invitrogen, USA) according to manufacturer´s instructions. cDNAs were synthesized by M-MLV reverse transcriptase (Promega, USA) with oligo dT priming from 3 µg total RNA extracted. PCRs were performed with Taq Pegasus (PB-L, Argentina). The primers designed for this study were: *Xwdr68-F* 5´-CTG TGT TGG AAC AAG CAG GA -3´ and *Xwdr68-R* 5´-GCT CTC CTT CCT GTC ATT GG-3'. *ef1alfa* was used as a loading control by employing the following primers: *ef1alfa-F* 5´-CAGATTGGTGCTGGATATGC-3´ and *ef1alfa-R* 5´-CTGCCTTGATGACTCCTAG-3´. PCR amplification with these primers was performed over 27 cycles and the PCR

products were analyzed on 1.5% agarose gels. As a control, PCR was performed with RNA that had not been reverse-transcribed to check for DNA contamination.

In vitro transcription and *in situ* hybridization

Antisense probe containing Digoxigenin-11-UTP was prepared for *wdr68* (digested with *Hind*III from *pGEM-T Easy* vector, transcribed with *T7* RNA polymerase). Specimens were prepared, hybridized and stained according to the procedure previously described by Harland (1991) with modifications. Hybridized embryos were fixed in 4% formaldehyde in PBS.

Molecular cloning and transformation of competent *E. coli*

The obtained DNA fragment of 1171 bp, corresponding to the coding region and to a short non coding fragment of 112 bp, was cloned in the pGEM-T Easy (Promega) vector, in competent *E. coli* TOP10 (Invitrogen, USA). The bacterial transformation for the introduction of the plasmid with the insert was performed according to the protocol described by Sambrook and Russell (Sambrook and Russell, 2001), based on the induction of the plasma membrane permeability through heat shock and a high concentration of calcium chloride.

Homology and syntenic analysis

cDNAs and protein sequences corresponding to WDR68 from different species were obtained from NCBI basadates. Sequence alignment was carried out using Clustal Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic trees were drawn by means of Phylogeny application (http://www.phylogeny.fr/). Analysis of synteny or gene linear ordering in the genome was performed using Blast tools in Ensembl and Genomicus genome databases. *X. laevis dcaf7* was used as a query.

RESULTS

Identification and sequence analysis of WDR68 in *X. laevis*

Orthologous sequences have been identified in various animal and plant species. To evaluate the similarity of the WDR68 protein from different species, we performed an alignment of amino acid sequences. The sequences compared were: CAB45372 (*Arabidpsis thaliana*); AAC18914 (*Petunia* sp.); AAF50953 (*Drosophila melanogaster*); NP_956363 (*Danio rerio*); AAH48722 (*Mus musculus*), and the protein from *Xenopus laevis* characterized in the present work. We found that WDR68 is identical to homologous proteins from *Arabidopsis, Petunia*, fruit fly, zebrafish and mouse in values ranging from 52 to 99% and with similarity values between 68 and 99% (Table 1).

Table 1. Identity and similarity percentages of amino acid sequences between WDR68 from *X. laevis* and homologous proteins from other species.

		IDENTITY (%)					
SIMILARITY		***Arabidopsis***	***Petunia***	***Drosophila***	***D. rerio***	***X. laevis***	***M. musculus***
	Arabidopsis		78	53	52	52	52
	Petunia	81		54	52	53	53
	Drosophila	68	68		85	86	86
	D. rerio	69	67	91		99	98
	X. laevis	68	67	92	99		99
	M. musculus	68	67	91	99	99	

Amino acid sequences from WDR68 homologous proteins belonging to different vertebrate species (mouse, *Xenopus* and zebrafish), invertebrates (fruit fly) and plants (*Petunia sp*. and *Arabidopsis sp*.) were compared using the BLAST2 algorithm.

On the basis of the data obtained from the comparison of different WDR68 proteins, we built the unrooted phylogenetic tree (Figure 1) that illustrates the relatedness of these proteins.

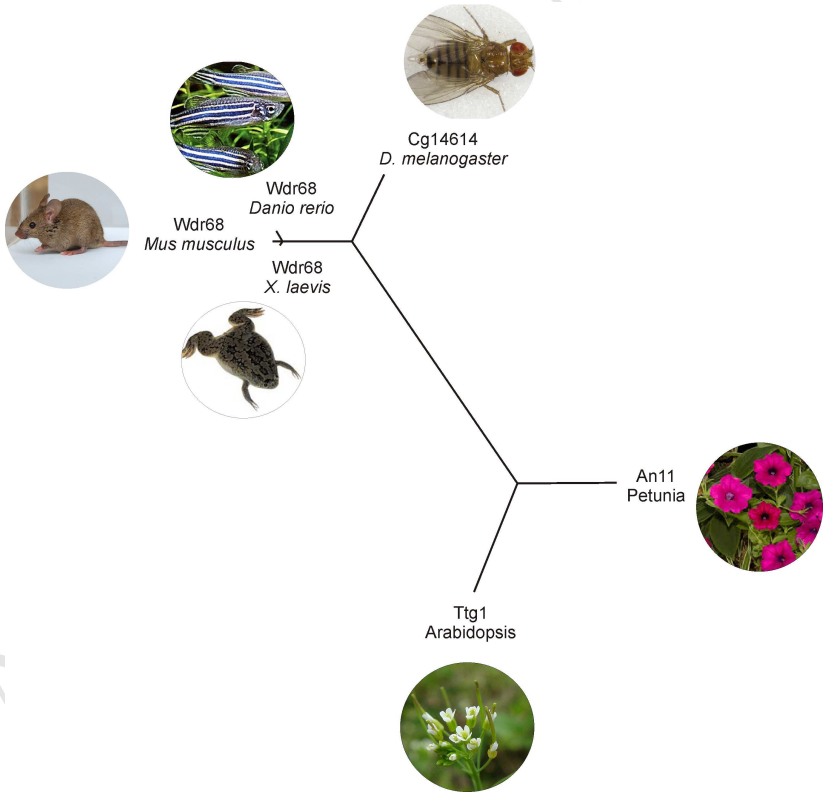

Figure 1. Unrooted phylogenetic tree showing the molecular relationship between WDR68 homologous proteins from different animals and plants, drawn by the TreeDyn algorithm from the data generated by CLUSTAL OMEGA.

The study and comparison of the WDR68 protein with other members of the WD40 protein family, and the bioinformatic prediction of its structure, revealed the topology of

the protein characterized in the present work (Figure 2). By using different bioinformatics applications, we were able to identify the WD40 domain, which is typical of this protein superfamily.

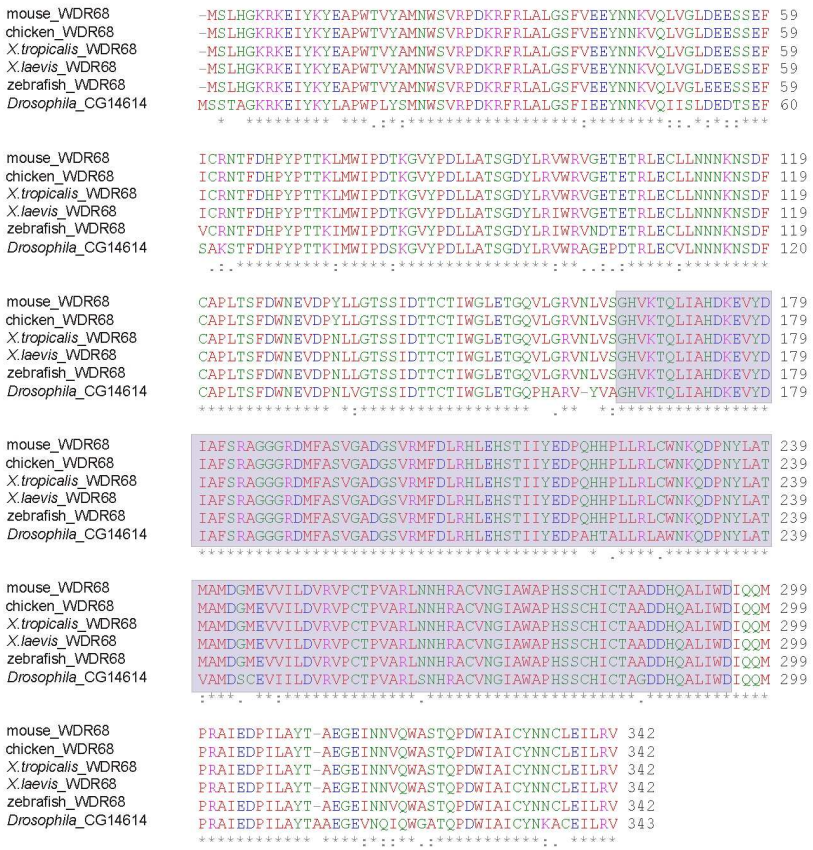

Figure 2. Alignment of WDR68 protein sequences. The sequence characterized in the present work, corresponding to *Xenopus laevis* (KX910100.1), was aligned with the sequences from *Xenopus tropicalis* (NP_989097), zebrafish (NP_956363), chicken (NP_001072972), mouse (AAH48722) and fruit fly (AAF50953). The comparison showed a high degree of conservation of the WD40 domain, indicated by a violet box. Asterisks indicate identical amino acids between the aligned sequences; colons indicate conserved substitutions; dot indicates semi conserved-substitutions.

A synteny analysis was carried out to establish a possible evolutionary pathway of the *WDR68* gene. The chromosomal regions containing the *WDR68* locus were explored in mammals (*Homo sapiens*, *Felis catus* and *Loxodonta africana*), birds (*Gallus gallus*), fishes (*Danio rerio* and *Oryzias latipes*) and amphibians (*Xenopus tropicalis* and *Xenopus laevis*). Figure 3 shows high conservation between the amniotes of the tandem of genes that include *WDR68*. However, an insertion of the *TACO1* gene is observed in mammals but not in birds. In fishes, however, even though there is conservation of the gene loci surrounding *WDR68*, certain changes occur in the linear order of the genes. These modifications could be due to an inversion and an insert of the *GFAP* gene. In the taxon of amphibians, an insertion of the *CRHR1.2* gene is observed but a conservation of the genetic loci is maintained. Nevertheless*, X. laevis* undergoes an inversion of some genes. This fact is in agreement with the localization of *WDR68* in chromosomal pair 9. According to the model proposed by Session *et al.* (2016), the ninth pair of homoeologues was generated by fusion of proto-chromosomes 9 and 10 that probably occurred before the allotetraploidization event of this organism around 17-18 Ma ago (Session *et al*., 2016). Moreover, the tandem of genes flanking *WDR68* locus is almost identical in both subgenomes of this allotetraploid amphibian.

In 2016, Session et al. provided evidence for the allotetraploid hypothesis and proposed that the allotetraploid genome arose via the interspecific hybridization of extinct diploid progenitors. Their genome suffered a partitioning into two homoeologous subgenomes called S and L that evolved asymmetrically: one of the two subgenomes experienced more intrachromosomal rearrangement, gene loss and changes in levels of gene expression and in histone and DNA methylation while the other preserved its ancestral condition (Session *et al*., 2016).

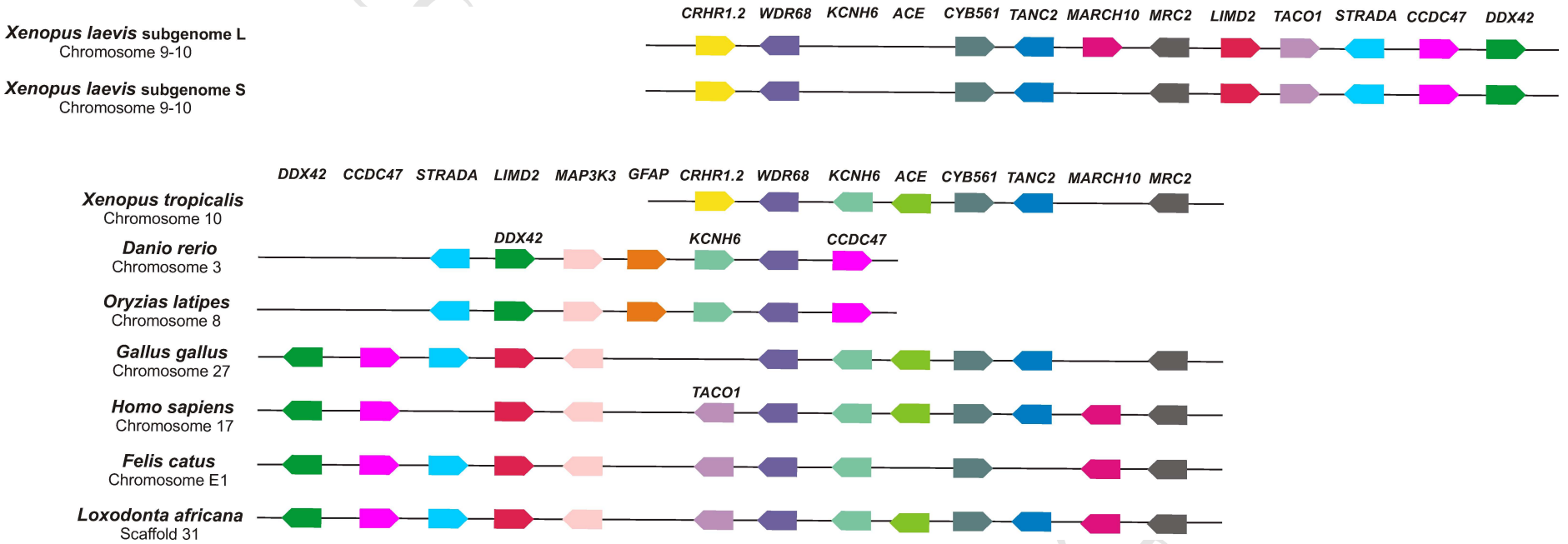

Figure 3. Analysis of the conserved syntenic regions containing the *WDR68* locus in amphibian genome: *X. laevis* (subgenome L and subgenome S) and *X. tropicalis*; in fishes: zebrafish and medaka; in birds: chicken; in three species of mammals: human, cat and elephant. Genes are represented by colored boxes while arrow indicates the orientation of the transcription unit. Boxes with the same color represent orthologous genes. Genomic regions were not drawn to scale in order to reduce complexity.

The *wdr68* gene is expressed ubiquitously during the early development of *Xenopus laevis*.

The spatial and temporal expression pattern of *wdr68* during the embryonic development of *Xenopus* was analyzed by whole-mount *in situ* hybridization (Figure 4). We detected a ubiquitous expression of *wdr68* at the early stages of development. As neurulation proceeds, the gene is expressed in the migratory streams of the cranial neural crest and in the neural tube. At the tail bud stage, some enrichment of transcripts in the cephalic region, in branchial arches and in otic vesicle could be observed and its localization in the developing somites is also apparent. In a transversal section at the trunk level, the expression is observed on the floor-plate of the neural tube and sideways, in the somites.

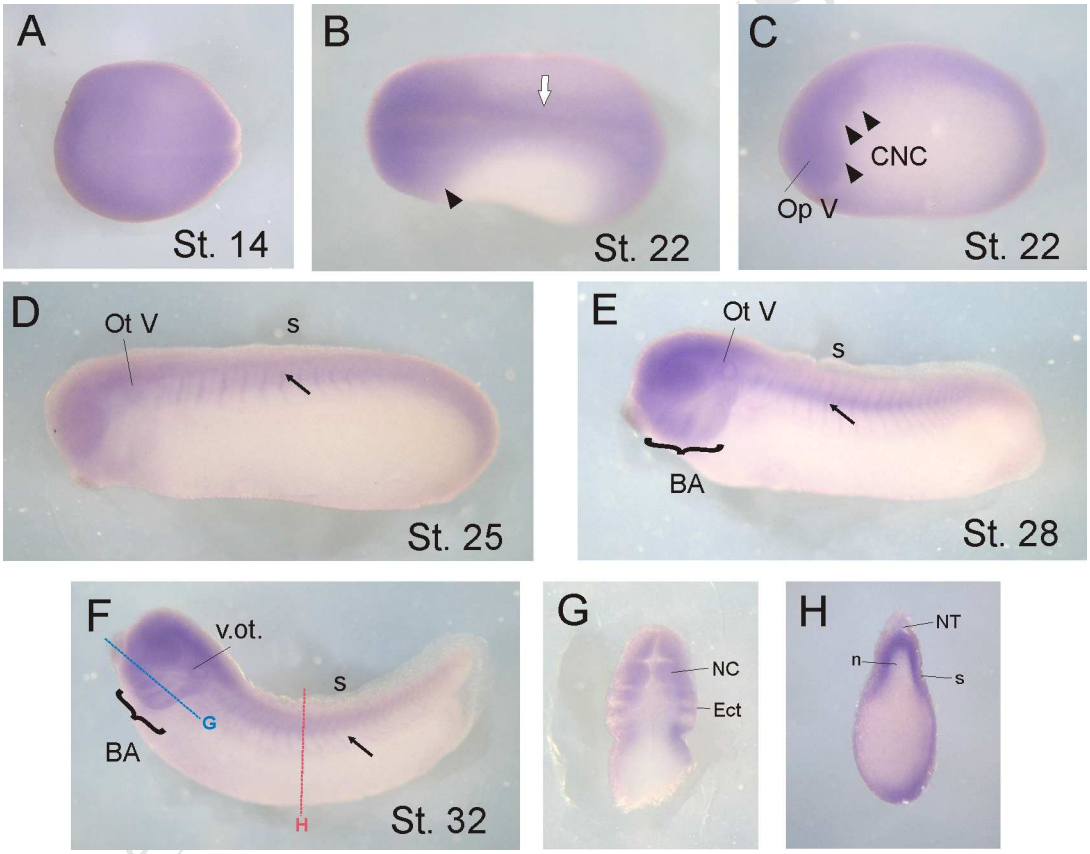

Figure 4. Expression pattern for the *wdr68* gene during *Xenopus* early development. (A, B) Dorsal view of embryo. (C-F) Side view. (G, H) Transverse sections. (A) *wdr68* expression is ubiquitous during early stages of development. (B, C) Since the late neurula stage, there is an enrichment in its expression in the head region (black arrowhead in B), in the migratory streams of the cranial neural crest (black arrowheads in C) and in the developing somites (white arrow). (D) At stage 25, *wdr68* transcripts are evident in the otic vesicle. In the somitic region (black arrow), they acquire a segmental pattern. (E, F) Since the tailbud stage, *wdr68* is strongly marked in the branchial arches (brackets). (G) Front section at the level of the branchial arches (blue broken line in F). Notice that the ectodermal cell population corresponding to the neural crest expresses *wdr68*. (H) Transverse section at the trunk level (red broken line in F). *wdr68* expression is evident on the floor of the neural tube and in somitic mesoderm. References: BA,

branchial arches; CNC, cranial neural crest; Ect, ectoderm; OpV, optic vesicle; OtV, otic vesicle; n, notochord; NC, neural crest; NT, neural tube.

Consistently with the data provided by the *in situ* hybridization analysis, the RT-PCR approach allowed the definition of the temporal expression pattern of *wdr68*. Transcripts are present at all early stages of development at equivalent levels (Figure 5) from fertilization onwards, hence first there is maternal supply of *wdr68* transcripts and, since the mid blastula transition, transcripts come from the zygote.

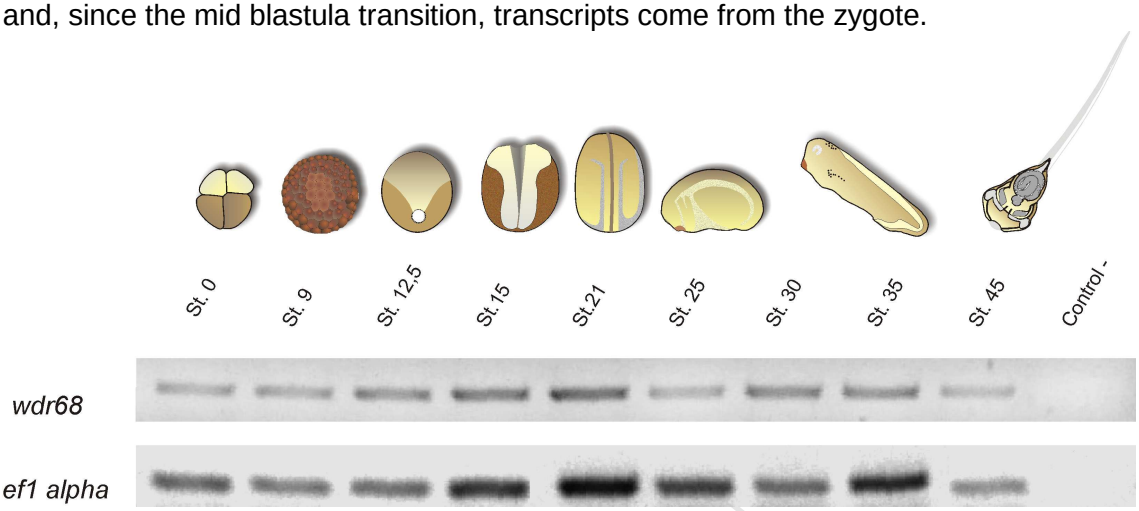

Figure 5. RT-PCR analysis of *wdr68* gene expression in early embryonic development of *Xenopus laevis*. A maternal source of *wdr68* transcripts can be observed. This molecular approach shows that the level of *wdr68* expression is quite similar throughout developmental stages: when zygotic expression starts (st. 9), along the neurulation (st 12,5-25), at the tailbud stage (st. 30-35) and at the tadpole stage (st. 45). *ef1 alpha* was used as loading control. A mix without DNA template was used as negative control.

We also analyzed the expression of *wdr68* at mid neurula by RT-PCR to reach a more accurate definition of the expression domains between the germ layers (Figure 6). The transcripts were detected in the three ectodermal domains: neural plate, neural folds and prospective epidermis. To a lesser extent, we noticed a mild expression in the paraxial mesoderm, underlying the neural crest.

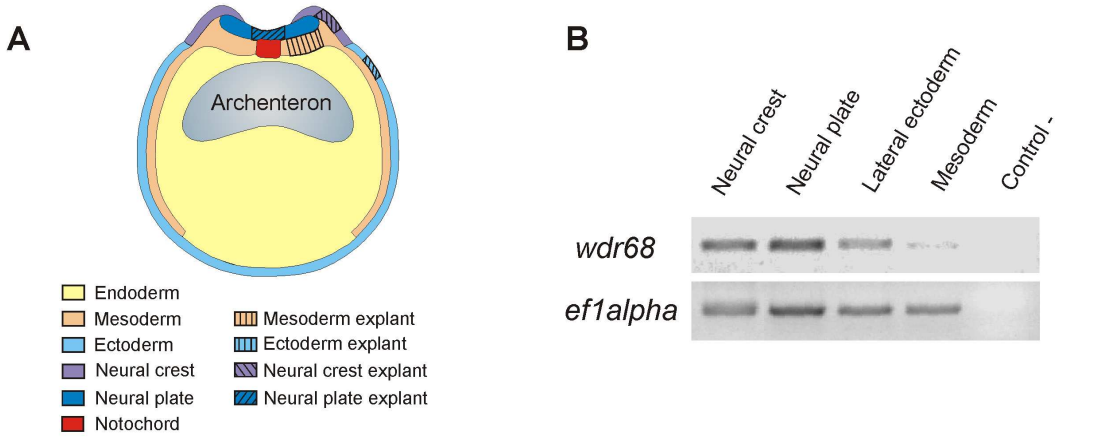

Figure 6. RT-PCR analysis showing the ubiquitous expression of *wdr68* at mid neurula stage. (A) Scheme representing a transverse section of a stage 16 embryo. Explants were dissected from ectodermal tissue, mesoderm, neural plate and neural crest in order to carry out total RNA purification for the subsequent RT-PCR. (B) Semiquantitative PCR showing a similar *wdr68* expression level in the three ectodermal domains of neural crest, neural plate and lateral ectoderm. A lower expression was detected in the paraxial mesoderm. A mix without DNA template was used as negative control. Expression of *ef1 alpha* was used as loading control.

wdr68 co expresses with some components of the *edn-1/ednra* cell signaling pathway

In zebrafish, it has been determined that the activation of the *wdr68* gene is essential for craniofacial development, upstream of *endothelin-1* (*edn-1*) gene, as well as for its target genes that drive jaw formation. (Nissen *et al*., 2006; Wang *et al*., 2013).

In *Xenopus*, the cell signaling pathway that activates *edn-1* has not yet been characterized. However, in a previous work, we identified the position of the *edrna,* the gene that encodes the endothelin receptor type A (the specific receptor of this cell signaling pathway) in the genetic cascade that specifies neural crest cell population (Bonano *et al*., 2008). Our findings showed that *ednra* belongs to the group of genes that participate in early development and is required for the induction and correct specification, migration and differentiation of the neural crest.

In order to establish a possible interaction between *wdr68* and the signal mediated by *edn-1/ednra*, we compared the expression patterns of the *wdr68* transcripts with some members of the *edn-1/ednra* cell signaling pathway determined by whole mount *in situ* hybridization (Figure 7). We noticed that, at mid neurula stages, there is a colocalization in the mesodermal layer of *wdr68* and *preproendothelin-1* (*ppet-1*), the gene that codifies the inactive precursor of the Edn-1 ligand. The *in situ* hybridization approach also reveals a matching location in the paraxial mesoderm between *ece-1* (*endothelin converting enzyme-1*) transcripts and those belonging to *wdr68* at neurula stages. At later stages, there is a coincident expression between *ednra* and *wdr68* transcripts at the embryonic cephalic region. Both genes express in otic and optic vesicles and in the branchial arches. Nevertheless, in the trunk, both genes show differences in their expression territories. These expression patterns at taibud stage suggest that *wdr68* participate in the development of the branchial arches and their derivatives possibly by interaction with *edn-1/ednra* cell signaling pathway.

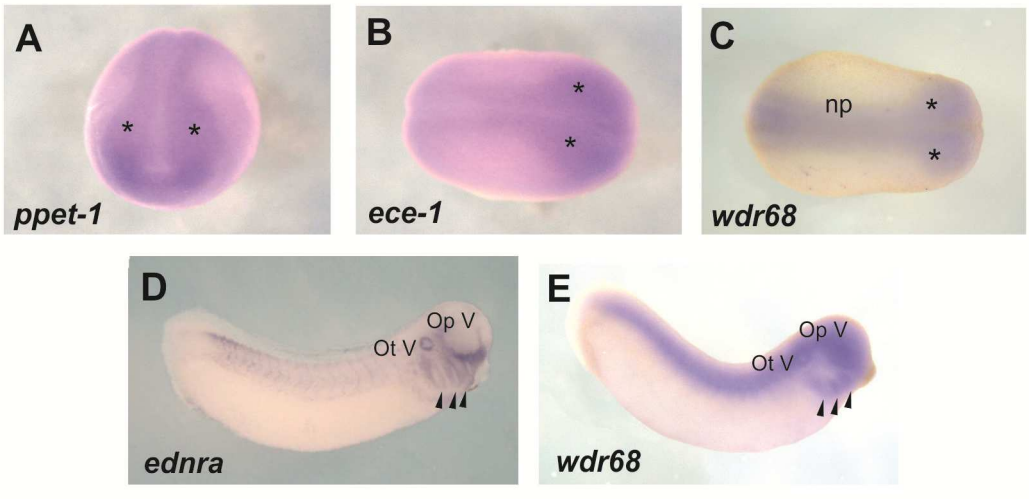

Figure 7. Comparison between embryonic expressions of *wdr68*, *ppet-1*, *ece-1* and *ednra* genes. (A) Antero dorsal view of mid- neurula stage. *ppet-1* transcripts are detected in the mesoderm underlying the neural crest (asterisks) (Bonano *et al*., 2008). (B) Dorsal view of an embryo at neurula stage showing *ece-1* expression in the lateral neural folds area (asterisks), in the ectoderm and mesoderm surrounding neural crest territory (Bonano et al., 2008). (C) Dorsal view of an embryo at neurula stage showing *wdr68* expression at the cephalic region and in somitic mesoderm. (D) Lateral view of tailbud stage embryos. *ednra* is expressed in the branchial arches (arrow heads), in otic and optic vesicles and also in the migratory truncal neural crest streams. (E) At tailbud stages, the head shows a strong expression of *wdr68*, mainly at the otic and optic vesicles and in the branchial arches. At the trunk level, *wdr68* is expressed in the somitic mesoderm. References: Op V, optic vesicle; Ot V, otic vesicle; n, notochord; np, neural plate; s, somites.

CONCLUSION

We isolated and identified a transcript corresponding to the *wdr68* (*dcaf7*) gene in the embryonic development of the amphibian *Xenopus laevis*. The cDNA has an open reading frame coding for 342 amino acid residues. Sequencing and the bioinformatic predictions confirm that the identified sequence is a member of the WD40 domains proteins superfamily, with high similarity with orthologous proteins in a wide variety or eukaryotic organisms including both plants and animals. These data reassert the high conservation of WDR68 throughout evolution.

We also analyzed the evolution of the *WDR68* gene in vertebrate genomes by synteny analysis. The genomic databases provided us with consistent information on the chromosome regions containing the orthologous of the *Xenopus laevis wdr68* gene to perform that study. The analysis of the genomic regions containing *WDR68* locus in the available genomes of some fishes, amphibians, birds and mammals revealed a high degree of syntenic conservation for at least 340 million years ago, since the Lower Carboniferous period, the estimated time for the emergence of amniotes. In fishes and amphibians there is a conservation of some neighboring loci to *WDR68*; nevertheless, genomic regions underwent some rearrangement. In fishes, the linear order of the genes suggests some inversions and the insertion of the *GFAP* gene. In amphibians, the insertion of the *CRHR1.2* gene immediately adjacent to the *WDR68* locus can be observed. Moreover, *X. laevis* displays an inverted genetic order with respect to amniotes.

It should be noticed that the recent paper of Session *et al*. (2016) allowed us to complete our analysis of the African clawed frog. The available sequences allowed the comparison of the syntenic organization in both subgenomes of this allotetraploid frog and to verify that it is very conserved. Before that work, information was fragmentary because *X. laevis* genome sequencing was not yet concluded. The authors provided evidence for the allotetraploid hypothesis and proposed that the allotetraploid genome arose via the interspecific hybridization of extinct diploid progenitors. Its genome suffered a partitioning into two homoeologous subgenomes called S and L that evolved asymmetrically: one of the two subgenomes experienced more intrachromosomal rearrangement, gene loss and changes in levels of gene expression and in histone and DNA methylation while the other preserved its ancestral condition (Session *et al.,* 2016).

On the other hand, the localization of the *wdr68* gene in chromosome 9 could explain the rearrangements underwent by the tandem of genes containing this locus. According to the current model of the origins of the *X. laevis* genome, the ninth pair of homoeologues is a fusion of proto-chromosomes homologous to their diploid ancestors that would have originated this allotetrapliod species by intraspecific hybridization (Session *et al*, 2016).

Our studies demonstrate that the *wdr68* gene is expressed since the very early development of the African clawed frog, first by maternal supply of its transcripts and then by the genetic expression of the embryo itself. Its expression area comprises the ectodermal and mesodermal layers at early neurula. As neurulation advances, the gene is more strongly expressed in the cephalic region, more specifically in the migratory streams of the neural crest, in otic and optic vesicles. In the truncal region of the body, it is located in the somitic mesoderm. During organogenesis, there is an enrichment of *wdr68* transcripts in the branchial arches, a transitory structure that generates the mandibular apparatus, an important evolutionary advantage acquired by the gnathostomates. Notably, its expression pattern shows some similarities with the corresponding one in zebrafish (Nissen *et al*., 2006). In this fish, a hierarchical relationship between *wdr68* and *edn-1* was corroborated. *wdr68* is required for the subsequent expression of *edn-1* and the effector genes that participate in jaw formation. In *Xenopus laevis*, the coincident localization of *wdr68* with other members of the *edn-1/ednra* cell signaling pathway suggests that a hierarchical relation could exist between the above genes. Nevertheless, this hypothesis requires gain- and loss-of-function studies to be validated.

ACKNOWLEDGEMENTS

We wish to thank the Instituto de Genética from the Fundación Miguel Lillo and Instituto de Biología “Dr. Francisco D. Barbieri”, Universidad Nacional de Tucumán, for the equipment provided. Our special thanks to Romel S. Sánchez and Guadalupe Barrionuevo for their valuable discussions of this work and their help with molecular cloning and synteny analisys. We are also grateful to Ms Virginia Mendez for her careful proofreading of the manuscript. This investigation was supported by grants from ANPCyT-Foncyt to M.J.A. (PICT2012-1224, PICT2013-1686), and by grants from CIUNT to M.J.A. (PIUNT 26/D408, PIUNT 26/D506).

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