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Retinoic acid negatively regulates *dact3b* expression in the hindbrain of zebrafish embryos

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Abstract

Wnt signaling plays important roles in normal development as well as pathophysiological conditions. The Dapper antagonist of β -catenin (Dact) proteins are modulators of both canonical and non-canonical Wnt signaling via direct interactions with Dishevelled (Dvl) and Van Gogh like-2 (Vangl2). Here, we report the dynamic expression patterns of two zebrafish *dact3* paralogs during early embryonic development. Our whole mount *in situ* hybridization (WISH) analysis indicates that specific *dact3a* expression starts by the tailbud stage in adaxial cells. Later, it is expressed in the anterior lateral plate mesoderm, somites, migrating cranial neural crest, and hindbrain neurons. By comparison, *dact3b* expression initiates on the dorsal side at the dome stage and soon after is expressed in the dorsal forerunner cells (DFCs) during gastrulation. At later stages, *dact3b* expression becomes restricted to the branchial neurons of the hindbrain and to the 2nd pharyngeal arch. To investigate how zebrafish *dact3* gene expression is regulated, we manipulated retinoic acid (RA) signaling during development and found it negatively regulates *dact3b* in the hindbrain. Our study is the first to document the expression of the paralogous zebrafish *dact3* genes during early development and demonstrate *dact3b* can be regulated by RA signaling. Therefore, our study opens up new avenues to study Dact3 function in the development of multiple tissues and suggests a previously unappreciated cross regulation of Wnt signaling by RA signaling in the developing vertebrate hindbrain.

Keywords

Dact; Dishevelled; Wnt signaling; retinoic acid signaling; vertebrate development

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1. Introduction

Wnt signaling regulates animal development and homeostasis through directing numerous fundamental cellular processes including cell specification, proliferation, differentiation, migration, and stem cell self-renewal (Clevers and Nusse, 2012; Logan and Nusse, 2004; MacDonald et al., 2009). The Dapper antagonist of β -catenin (Dact) protein family was initially isolated from yeast-two hybrid screens with the Dishevelled (Dvl) PDZ domain as bait (Cheyette et al., 2002; Gloy et al., 2002). Since then, three Dact family members Dact1, Dact2, and Dact3 have been reported in vertebrates. All three have been reported in humans and mice, Dact1 and Dact2 have been reported in zebrafish and chicken (Alvares et al., 2009), and Dact1 has been reported in *Xenopus* (Cheyette et al., 2002; Gloy et al., 2002). Structurally, the Dact proteins share a number of conserved domains (Waxman et al., 2004). However, it is the conserved N-terminal leucine zipper domain (LZD) and C-terminal PDZ-binding (PDZB) domain that have been implicated in different Wnt-related functions (Brott and Sokol, 2005; Cheyette et al., 2002; Gloy et al., 2002; Waxman et al., 2004). The N-terminal LZD of Dact1 has been shown to interact with LEF/TCF proteins and is involved in homo- and heterodimerization of Dact proteins (Hikasa and Sokol, 2004; Kivimäe et al., 2011). While the PDZ-binding domain is necessary and sufficient for interaction with Dvl (Gloy et al., 2002), more recent studies have indicated that this domain is important for facilitating interactions with several other proteins, including Vangl2, PKA, PKC, CK1 δ/ϵ , and p120ctn (Kivimäe et al., 2011). Thus, from the many interactions with regulators of Wnt signaling, in particular Dvl, Dact function in cellular signaling and proper embryonic development is likely very much dependent on the nature of the proteins it interacts with in a given context.

Dact1 orthologs have been the best studied of this protein family. In *Xenopus*, Dact1 can inhibit both β -catenin (canonical branch) and JNK (non-canonical branch) downstream of Dvl with a requirement in proper notochord and head development (Cheyette et al., 2002; Gloy et al., 2002). Additionally, *dact1* was shown to mediate lysosome-inhibitor regulated Dvl degradation (Zhang et al., 2006). With respect to regulation of canonical Wnt signaling, Dact1 can also act more downstream of Dvl. Dact1 associates with TCF3 independent of β -catenin in *Xenopus* (Hikasa and Sokol, 2004), inhibits β -cat/LEF interactions in the nucleus, and promotes HDAC recruitment to LEF promoters (Gao et al., 2008). Interestingly, Dact1 can also act as a positive regulator of canonical Wnt signaling (Gloy et al., 2002; Park et al., 2006; Suriben et al., 2009; Waxman, 2005; Yang et al., 2013). Gain-of-function studies have found that co-injection of Dvl and Dact1 can promote canonical Wnt signaling. In zebrafish, depletion of Dact1 can enhance anteriorization due to the loss of canonical Wnt signaling in a *wnt8* hypomorphic background. While the molecular mechanism behind this context dependent switch in function of Dact proteins in canonical Wnt signaling remains elusive, phosphorylation status of Dact1 has been suggested to be a decisive factor (Teran et al., 2009). Although studies in non-mammalian vertebrates primarily suggested that Dact1 proteins function to regulate canonical Wnt signaling, Dact1 knockout (KO) mice show severe urogenital defects and posterior body malformations, which has been associated with impaired Wnt-PCP signaling (Suriben et al., 2009; Wen et al., 2010). In humans, *Dact1* mutations have been found in patients with neural tube defects (NTD) (Shi et al., 2012) and

altered expression is found in many types of cancers (Astolfi et al., 2010; Yang et al., 2010; Yau et al., 2005; Yin et al., 2013; Yuan et al., 2012). Therefore, *Dact1* has a multitude of functions regulating different types of Wnt signaling in developmental and disease contexts.

Similar to *Dact1*, *Dact2* regulates different Wnt signals and has been proposed to also regulate TGF- β signaling (Su et al., 2007). During early zebrafish development, *Dact2* regulates proper convergent extension movements. However, in mice, *Dact2* functions as a negative regulator of canonical Wnt signaling in tooth development via interacting with and repressing *pitx2* expression (Li et al., 2013). *Dact2* has also been shown to attenuate TGF- β /Nodal signaling by facilitating lysosomal degradation of type I receptors ALK4 and ALK5 during mesoderm induction (Lee et al., 2010; Su et al., 2007; Zhang et al., 2004). Although *Dact2* null mice are viable, they exhibit accelerated wound healing due to enhanced TGF- β signaling (Meng et al., 2008).

To this point, *Dact3* has been the least studied *Dact* member. Its expression has only been described for mice and it had been suggested that it may only be specific to mammals (Fisher et al., 2006). Epigenetic repression of *Dact3* has been shown to be associated with increased canonical Wnt signaling in colorectal cancer, suggesting in this context *Dact3* acts as a negative regulator of canonical Wnt signaling (Jiang et al., 2008). *Dact3* null mice did not display any obvious morphological abnormalities, but they have a slightly smaller body weight than wild type (WT) siblings indicating *Dact3* has a role in postnatal development in mice (Xue et al., 2013). Furthermore, unilateral ureteral obstruction in *Dact3* knockout mice produced noticeable kidney fibrosis along with increased Dvl2 and β -catenin, expression of several Wntresponsive fibrotic, and epithelial-to-mesenchymal transition marker genes (Xue et al., 2013). Together with a previous report, these observations support that *Dact3* can act as a canonical Wnt signaling antagonist (Jiang et al., 2008).

We wanted to better understand the evolution of the *Dact* family of proteins in vertebrates. Although the expression and function of *dact1* and *dact2* have been previously reported in zebrafish (Waxman, 2005), the identity and putative expression pattern of any zebrafish *Dact3* orthologs has not been determined. Here, we report the expression of two zebrafish *Dact3* paralogs, *dact3a* and *dact3b*, during early development. Our finding also indicates that retinoic acid (RA) signaling negatively regulates *dact3b* expression in the zebrafish hindbrain, suggesting a previously unexplored regulation of Wnt signaling by RA signaling in the vertebrate hindbrain.

2. Results and discussions

2.1 Comparison of *Dact* protein sequences

Examining the current zebrafish genome (Zv9; ensemble.org), we found that there are two predicted zebrafish *dact3-like* paralogs. The first predicted zebrafish *dact3-like* gene (XM_005172584) is located on Chromosome (Chr) 10, has 4 exons, and encodes a 586 amino acid protein. The second *dact3-like* gene (XM_001340960) has 5 exons encoding a 533 amino acid protein. In order to determine the level of conservation of zebrafish *dact3-like* genes with the mammalian *dact3* orthologs, we performed sequence alignments using Clustal W for human (Hs), mice (Mm) and zebrafish (Dr) *Dact3*s. The zebrafish *dact3-like*

gene on Chr 10 shares greater homology with both human and mice Dact3s compared to the *dact3-like* gene on Chr 18, so we now refer to them as *dact3a* and *dact3b*, respectively (Fig. 1A, B). While the overall amount of conservation is not high among the Dact3 proteins, there was greater conservation of an N-terminal coiled domain (CD1) and leucine zipper domains (LZD) (Waxman et al., 2004). Despite the overall higher conservation of *dact3a* with the mammalian Dact3 genes, its LZD was actually less conserved than *dact3b*. The C-terminal PDZ-binding (PDZB) domain for Dact3 homologs is completely conserved (Fig. 1A). Comparing the four zebrafish Dact homologs, we found greater conservation of sequence between zebrafish Dact1 and Dact2 than between either zebrafish Dact3 paralog (Fig. 2A, B). The alignments between the four zebrafish Dact proteins indicate a strongly conserved PDZ-binding domain, except for a threonine (T) to leucine (L) change in Dact2 sequence. Interestingly, the N-terminal CD1 domain again is more highly conserved than the LZD domain, which is not as easily identifiable (Fig. 2A) in the alignments of the zebrafish Dact proteins. The high conservation of the PDZ-binding domain is understandable, as deletion studies have reported that it is essential for interactions with multiple proteins (Cheyette et al., 2002). Therefore, this analysis of zebrafish Dact3a and Dact3b shows that Dact3 proteins are conserved in non-mammalian vertebrates.

2.3 *Dact3a* is expressed in the somites, neural crest cells and hindbrain neurons

To analyze the spatio-temporal expression patterns of *dact3a* and *dact3b*, we performed whole mount *in situ* hybridization (WISH) and reverse transcriptase-PCR (RT-PCR) at stages of zebrafish development starting from the dome stage until 48 hours post fertilization (hpf). Although we could detect *dact3a* expression with RT-PCR by the shield (SH) stage (Fig. 3), with WISH we were not able to detect specific *dact3a* expression until the tailbud (TB) stage, when it appeared in adaxial mesodermal cells (Fig. 4D). Initial expression of *dact3a* is seen much later than *dact1* and *dact2*, which appears to be present as early as the high stage with dorsal-specific expression (Waxman, 2005). At the 8 somite (s) stage, *dact3a* expression is observed in the anterior lateral plate mesoderm (ALPM) and prominently in the adaxial cells of the somites (Fig. 4B, E). By the 18s stage, *dact3a* is expressed in the posterior, newly forming somites, the 3 (mandibular, hyoid and branchial) streams of migrating cranial neural crest cells (Diogo et al., 2007; Schilling and Kimmel, 1997; Sperber et al., 2008), the anterior brain, hindbrain neurons, and spinal cord neurons (Fig. 4C, F). Flat mounting of the probed embryos at 18s revealed that *dact3a* is expressed in the telencephalon and lines the inner periphery of eye. At this stage, *dact3a* is also expressed in the precursors to the cranial branchiomotor neurons (Fig. 5A) and by 26 hpf in the trigeminal (V) the facial (VII) neurons (Fig. 5B) (Auclair et al., 1996; Chandrasekhar, 2003; Studer et al., 1995). The expression in the hindbrain persists through 48 hpf (Fig. 4H, I and Fig. 5C, D), while the *dact3a* expression in the cranial cartilage is consistent with it being derived from the cranial neural crest (Fig. 4J) (Schilling and Kimmel, 1997). By 36hpf, *dact3a* expression also initiated in the pectoral fin bud (Fig. 4H). At 48 hpf, it is also interesting to notice that the pectoral fin bud expression of *dact3a* is present in two separate domains, consistent with expression in the pectoral muscle (Fig. 5D) (Ahn and Ho, 2008; Thorsen and Hale, 2005). By comparison, zebrafish *dact2* is also expressed in the pectoral fin bud (Waxman et al., 2004)

2.4 *Dact3b* is expressed in the dorsal forerunner cells and brain

In contrast to *dact3a*, which did not have specific expression until the TB stage, localized *dact3b* expression begins at the dome stage in a cluster of cells at the dorsal blastoderm (Fig. 6A, E), very similar to what has been reported for zebrafish *dact1* (Waxman, 2005). The dorsal *dact3b* expression maintained through 40% epiboly (Fig. 6B, F). Compared to zebrafish *dact1* and *dact2* at the same developmental stages, the dorsal region expressing *dact3b* is narrower and the onset of expression is delayed (Waxman, 2005). By 60% epiboly, this scattered dorsal expressing cell population starts to reduce and become concentrated in only a few cells at the dorsal blastoderm margin (Fig. 6C, G). These dorsal expressing cells appear to be the dorsal forerunner cell population (DFC), because they are located below the blastoderm margin (Fig. 6C, G). Although the DFCs give rise to Kupffer's vesicle (Cooper and D'Amico, 1996), we did not observe *dact3b* expression in the Kupffer's vesicle (data not shown), suggesting the DFC expression is not maintained in these cells after gastrulation. By the end of gastrulation at the TB stage, *dact3b* expression waned, as we did not detect specific expression via WISH or significant transcripts via RT-PCR (Fig. 3 and data not shown). Furthermore, even though *dact3b* expression increased by the 8s stage, we did not detect noticeable specific *dact3b* expression until late somitogenesis stages (Figs. 3 and 6D, H). The lack of expression from the end of gastrulation through early somitogenesis contrasts with zebrafish *dact1*, *dact2*, and *dact3a*, which are all expressed throughout these stages in the somites, tailbud, brain, and spinal cord (Figs. 4 and 5) (Gillhouse et al., 2004).

Beginning at the 18s stage, specific *dact3b* expression initiates in the hindbrain (Fig. 6H). As development proceeds, this expression persists and expands posteriorly by 24hpf (Fig. 6I). To examine the relative location of *dact3b* in the hindbrain, we performed double WISH at 24hpf with *krox-20*. This revealed *dact3b* expression resided in r2 through r6, with more medial expression in r3–r6 (Fig. 7A). At this stage, *dact3b* is also found in the 2nd pharyngeal arch (Fig. 7A). By 36hpf, we found expression begins in the anterior tectum/midbrain region (Fig. 6I and Fig. 7B) and extended up to the midbrain-hindbrain boundary. As development proceeds, the intensity of *dact3b* expression in these neural tissues becomes stronger through 48 hpf (Fig. 6K and 7C), with hindbrain expression in a striped pattern consistent with cranial branchiomotor neurons (Chandrasekhar et al., 1997) (Fig. 7C). Interestingly, the hindbrain expression patterns for *dact3b* is more anterior compared to *dact3a* at the comparable stages (Fig. 5).

Comparing the previously reported expression patterns of zebrafish *dact1* and *dact2* to *dact3a* and *dact3b*, respectively, revealed that prior to gastrulation, *dact3b* is more similar to zebrafish *dact1* and *dact2* as they all share dorsally localized expression. After gastrulation, *dact3a* expression is more similar to *dact1* and *dact2* expression than its paralog *dact3b*, as *dact3a* shares broader expression in the somites, brain, and lateral plate mesoderm (Waxman et al., 2004). Although all four *dact* genes are expressed in the brain during development, *dact1* and *dact2* brain specific expression begins as early as the TB stage, while both *dact3a* and *dact3b* expression initiates after mid-somitogenesis stages.

In comparing zebrafish *dact3* paralog expression to the expression of the single mouse *Dact3* gene, *dact3a* expression is more similar to the mouse *Dact3* than *dact3b*. The mouse

Dact3 is expressed in the somites, fore- and hind limb buds, otic vesicle, aortic arches, craniofacial mesenchyme, tail bud and central nervous system (Fisher et al., 2006). Likewise, *dact3a* is expressed in the somites, pectoral fin bud, cranial neural crest cells, and hindbrain neurons. Zebrafish *dact3b*, however, is not as broadly expressed as the mouse *Dact3*. While zebrafish *dact3b* is expressed only in the 2nd pharyngeal arch, mouse *Dact3* and zebrafish *dact3a* are both expressed throughout all the pharyngeal arches. Interestingly, although the mouse *Dact3* was not reported in the brain during development, it is prominently expressed in the adult brain and specifically in the cerebral cortex. We find specific regional expression of both *dact3a* and *dact3b* in the zebrafish brain during development that is reminiscent of the expression in the adult mouse.

2.5 RA signaling negatively regulates *dact3* expression in the hindbrain

We next wanted to understand if any of the major developmental signaling pathways, including canonical Wnt, FGF, BMP, and RA signaling regulate either *dact3a* and *dact3b* expression in the zebrafish hindbrain. To modulate RA signaling, we treated the embryos beginning at the 16s stage and 24 hpf with RA and diethylaminobenzaldehyde (DEAB), a retinaldehyde dehydrogenase inhibitor that inhibits RA synthesis (Russo et al., 1988). Following treatment, embryos were fixed at 24hpf and 30hpf, respectively, and analyzed for *dact3b* expression. Examination of the embryos treated with exogenous RA revealed that at both stages RA induced a significant decrease in *dact3b* expression (Fig. 8A, B). Conversely, treatment with DEAB resulted in expansion of *dact3b* expression within the hindbrain and extension posteriorly into the spinal cord (Fig. 8A, C). In the 30hpf embryos, treatment with RA also diminished the pharyngeal expression (Fig. 8D, E). Similar effects were not seen on the *dact3a* hindbrain expression when RA signaling was manipulated at these stages nor did we find that RA signaling affected *dact3a* expression at earlier stages (data not shown). Manipulation of Wnt, FGF, and BMP signaling pathways utilizing heat-shock inducible transgenic fish lines at similar stages did not affect *dact3b* expression in the hindbrain (data not shown). Together, our data suggest that RA signaling negatively regulates *dact3b* expression in the anterior hindbrain, but not *dact3a* in the posterior hindbrain, in zebrafish. Cross talk between Wnt and RA signaling has been studied in a variety of developmental contexts, including hematopoietic stem cell development, chondrocyte formation, and adipogenesis (Chanda et al., 2013; Kim et al., 2013; Yasuhara et al., 2010). Our present findings suggest a previously unappreciated access point for the cross-regulation of Wnt signaling by RA signaling and provide a basis of future investigations targeting how Dact3 proteins function within the hindbrain to integrate the Wnt and RA signaling networks.

Experimental procedures

Zebrafish husbandry and maintenance

Zebrafish (*Danio rerio*) were raised and maintained following standardized laboratory conditions (Westerfield, 2000). Embryos were collected from crosses between WT strains and raised at 28.5°C in an incubator before fixing at desired stages for analysis. Staging was done as reported previously (Kimmel et al., 1995).

Generation of *dact3a* and *dact3b* probes

629 bp of *dact3a* and 759 bp of *dact3b* sequence were amplified from mixed stage cDNA using the following primers: Forward *dact3a*: 5'-TGAAGGTGGACACAGAGAACAGC. Reverse *dact3a*: 5'- ATAGGTCGTGGGTCTTGCTTGG. Forward *dact3b*: 5'-ATTTTCTGCCCCATACCCCC Reverse *dact3b*: 5'-ACGCAACTGTCCTCTGAACGAG. The amplified products were cloned into pGemT-Easy (Promega), sequenced, and anti-sense probe was synthesized using standard methods. Probe used for *krox-20* was previously reported (ZDB-GENE-980526-283).

Phylogenetic analysis

Full-length sequence for all Dact members of human, mice and zebrafish was obtained from NCBI website. Sequence alignments were performed using Clustal W in MacVector.

RT-PCR analysis

30 embryos at the designated stages were homogenization in TRIzol reagent (Ambion). RNA was prepared using Pure link RNA Micro Kit (In Vitrogen). cDNA was synthesized using the ThermoScript Reverse Transcriptase kit (Invitrogen). RT-PCR was performed using the primers that were used to make probe for the respective genes. Control for expression is *solo* a ubiquitously expressed gene at the stages mentioned.

Wholemount *in situ* hybridization (WISH)

WISH was performed as described previously (Waxman et al., 2008). Images were taken using a Zeiss M2BioV12 stereomicroscope and Zeiss AxioImager 2. Pigmentation was prevented at later stages using 0.003% 1-phenyl-2-thiourea (PTU) treatment starting at 24 hpf.

Drug treatment

WT embryos were treated with 1 μ m RA and 1 μ m DEAB and placed on a Nutator at 28.5°C. Treatment was performed beginning at 16hpf and 24hpf followed by fixation at 24hpf and 30hpf, respectively.

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Highlights

- We investigated *dact3a* and *dact3b* expression during early zebrafish development.
- Sequence alignments show strongest conservation in the PDZ-binding domains.
- *dact3a* is expressed in the lateral plate mesoderm, somites, and neural crest.
- *dact3b* is expressed in the dorsal forerunner cells (DFC) and hindbrain.
- Retinoic acid signaling negatively regulates *dact3b* expression in the hindbrain.

Figure 1. Sequence alignments of vertebrate Dact3 proteins
A: Comparison of human (Hs), mouse (Mm), and zebrafish (Dr) Dact3 protein sequences revealed conservation of the N-terminal CD1 and LZD domains and the C-terminal PDZ-binding domain. Conserved domains are underlined in red. 6 conserved leucines of the LZD are indicated with red stars. **B:** Table with the amino acid identity (Y-axis) and similarity scores (X-axis) of Hs, Mm and Dr Dact3 homologs.

Figure 2. Sequence alignments of zebrafish Dact proteins
A: Comparison of zebrafish (Dr) Dact1, Dact2, and Dact3a and Dact3b protein sequences revealed greater conservation of the N-terminal CD1 and C-terminal PDZ-binding domains, with less conservation of the LZD domain. Conserved domains are underlined in red. 5 conserved leucines of the LZD are indicated with red stars. **B:** Table with the amino acid identity (Y-axis) and similarity scores (X-axis) of Dr Dact proteins.

Figure 3. RT-PCR analysis of *dact3a* and *dact3b* gene expression

Expression of *dact3a* and *dact3b* analyzed by RT-PCR at the indicated stages. *Solo* served as a positive control.

Figure 4. Zebrafish *dact3a* expression from 60% epiboly through 48hpf

A: Specific *dact3a* expression was not observed at 60% epiboly, even though it is detectable by RT-PCR (Fig. 3). **D:** *Dact3a* expression in the adaxial mesoderm at the TB stage (black arrow). **B,E:** *Dact3a* expression in the somites (black arrows) and anterior lateral plate mesoderm (white arrowhead) at the 8s stage. **C:** Expression at the telencephalon (white arrow), spinal cord neurons (black arrowhead), and the forming somites (black arrow) at the 18s stage. **F:** Mandibular (nc1), hyoid (nc2), and branchial (nc3) neural crest expression and medial eye expression (black arrowhead) at the 18s stage. **G–I:** Hindbrain expression at 24, 36 and 48hpf (black arrow). At 36 and 48 hpf, *dact3a* expression is visible in the pectoral fin bud (red arrow). **Inset:** Cartilage expression (black arrow) at 48hpf. A, C, G, H, I are lateral views. B, D, E, F are dorsal views. Anterior up in all the images.

Figure 5. *Dact3a* expression in the brain

A: *Dact3a* expression in the telencephalon (black arrowhead), medial eye (white arrow), neural crest (nc1, 2, 3) and hindbrain neurons (black arrows). **B:** Expression in the nV (trigeminal) and nVII (facial) cranial branchiomotor neurons (black arrows). **C:** Expression in the branchiomotor neurons (black arrows). **D:** Pectoral fin bud expression in two distinct domains (black arrowheads). All the images are dorsal with anterior right.

Figure 6. Zebrafish *dact3b* expression from dome through 48hpf

A–C, E–G: *Dact3b* expression in the dorsal blastoderm margin from dome through 60% epiboly stage (black arrow). **D:** Specific *dact3b* is not expressed at the 8s stage, even though we could detect it with RT-PCR (Fig. 3). **H,I:** *Dact3b* expression in the hindbrain starts at 18s and is maintained through 24 hpf embryos (arrow). **J,K:** *Dact3b* expression initiates in the 2nd pharyngeal arch (white arrowheads) and the dorsal region of the midbrain/tectum (black arrowheads) at 36hpf and is maintained through 48 hpf. *Dact3b* continues to be expressed in the hindbrain at 36 and 48hpf (black arrows). A–D and H–K are lateral views with dorsal rightward. E–G are dorsal views. In all images, anterior is up.

Figure 7. *Dact3b* expression in the hindbrain

A: Double WISH for *dact3b* and *krox-20* at 24 hpf. *Krox-20* (orange) is expressed in r3 and r5. *Dact3b* expression is from r2 through r6 (brackets). *dact3b* is expressed in the adjacent 2nd pharyngeal arch (black arrowhead). **B:** *Dact3b* expression extends more anteriorly past the midbrain-hindbrain boundary (black arrow) and is expressed in the tectum by 36 hpf (white arrowhead). 2nd pharyngeal arch expression is maintained (black arrowhead). **C:** By 48 hpf, *dact3b* expression is increased and is in stripes consistent with anterior branchiomotor neuron expression (black arrow) (Chandrasekhar et al., 1997). Embryos in images were flat mounted. Images are dorsal views with anterior to the left.

Figure 8. RA signaling negatively regulates *dact3b* expression

A, D: Control embryos **B, E:** Treatment with exogenous RA extinguishes *dact3b* expression in the hindbrain (black arrows) and 2nd pharyngeal arch (white arrow in E). **C, F:** Inhibition of RA signaling lead to an expansion of *dact3b* expression domain with the hindbrain (compare the length between arrows in D and F). Images are lateral views with anterior to upward.