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Three Dact Gene Family Members are Expressed During Embryonic Development and in the Adult Brains of Mice

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Abstract

Members of the Dact protein family were initially identified through binding to Dishevelled (Dvl), a cytoplasmic protein central to Wnt signaling. During mouse development, *Dact1* is detected in the presomitic mesoderm and somites during segmentation, in the limb bud mesenchyme and other mesoderm-derived tissues, and in the central nervous system (CNS). *Dact2* expression is most prominent during organogenesis of the thymus, kidneys, and salivary glands, with much lower levels in the somites and in the developing CNS. *Dact3*, not previously described in any organism, is expressed in the ventral region of maturing somites, limb bud and branchial arch mesenchyme, and in the embryonic CNS; of the three paralogs it is the most highly expressed in the adult cerebral cortex. These data are consistent with studies in other vertebrates showing that *Dact* paralogs have distinct signaling and developmental roles, and suggest they may differentially contribute to postnatal brain physiology.

Introduction

Signaling downstream of secreted Wnt ligands is a conserved process in multicellular animals that plays important roles during development and, when misregulated, contributes to cancer and other diseases (Polakis, 2000; Moon et al., 2002). In mammals many Wnt signaling components are expressed in the postnatal brain, where manipulations of their activity can lead to effects on behavior (Madsen et al., 2003; Beaulieu et al., 2004; Kaidanovich-Beilin et al., 2004; O'Brien et al., 2004; Shimogori et al., 2004). Although more than one molecular cascade has been identified downstream of Wnt receptors, all such cascades involve a cytoplasmic scaffold protein called Dishevelled (Dvl in mammals) (Veeman et al., 2003; Wharton, Jr., 2003). Because of its central role in Wnt signal transduction, efforts have been made to identify the direct binding partners of Dvl. One such protein, which binds to the Dvl PDZ domain via a conserved C-terminal PDZ-binding motif (Cheyette et al., 2002), has alternately been named Dapper (Dpr), Frodo/Frd, THYEX3, HNG3, MTNG3, and Dact in various organisms (Cheyette et al., 2002; Gloy et al., 2002; Gillhouse et al., 2004; Yau et al., 2004; Zhang et al., 2004; Hunter et al., 2005; Katoh, 2005). For simplicity, hereafter we use the symbol "*Dact"* assigned by the Human Genome Organization Nomenclature Committee and the Mouse Genome Informatics website for all members of this gene family. Interestingly, despite the importance of Wnt signaling during invertebrate development, we have been unable to identify *Dact* orthologs in the completely sequenced genomes of the invertebrates *Drosophila melanogaster* and *Caenorhabditis elegans,* nor in that of the simple chordate, *Ciona intestinalis* (data not shown).

The function of Dact proteins in signal regulation remains ambiguous, with some studies indicating they act positively in Wnt signal transduction (Gloy et al., 2002; Waxman et al., 2004), and others indicating an inhibitory function (Cheyette et al., 2002; Wong et al., 2003; Yau et al., 2004; Brott and Sokol, 2005a; Zhang et al., 2006). Previous research in zebrafish has shown that two members of the Dact family have distinct effects on Wnt signaling, with Dact1 having a greater impact on ß-catenin-dependent signaling, and Dact2 having a greater impact on a ß-catenin-independent process called planar cell polarity/convergent-extension signaling (Waxman et al., 2004). Furthermore, in zebrafish and when overexpressed in mammalian cells, Dact2 but not Dact1 can inhibit Nodal signaling by promoting the endocytic degradation of Type I TGFß receptors (Zhang et al., 2004). Taken together, the evidence suggests that different Dact paralogs have distinct signaling activities, and that even a single Dact protein may have more than one role that can vary under changing cellular conditions (Hikasa and Sokol, 2004; Brott and Sokol, 2005b).

Because of this gene family's manifold yet conserved functions in vertebrate signal transduction, we have cloned cDNAs corresponding to the full-length coding regions of all three mouse *Dact* homologs, and have characterized their developmental and adult expression patterns.

Results and Discussion

Identification of a Three-Member *Dact* Gene Family

Using the previously described *Dact* sequences from frogs and fish, we scanned the mouse genome and expressed sequence tag (EST) databases for similar sequences,

and then cloned full-length cDNAs by RT-PCR (Fig. 1A). Based on sequence similarity, *Dact1,* which maps to mouse chromosome 12D1, is the closest mammalian homolog to the *Dpr* and *Frodo* sequences identified in *Xenopus*, and corresponds to the mammalian *Dpr1* and *Frd1* genes reported in the literature (Cheyette et al., 2002; Katoh and Katoh, 2003; Yau et al., 2004; Zhang et al., 2004; Brott and Sokol, 2005; Hunter et al., 2005). *Dact2*, which is most closely related to the *frd2/dpr2* sequences identified in zebrafish (Gillhouse et al., 2004; Waxman et al., 2004), maps to mouse chromosome 17A2. *Dact3*, which has not previously been described, maps to mouse chromosome 7A2.

Dact3 is a bone fide member of the *Dact* gene family (Fig.1A-C). Upon alignment at the amino acid sequence level, mouse Dact3 is approximately 27% similar to Dact1 and 24% similar to Dact2 (compared to 26% similarity between Dact1 and Dact2, Fig. 1B). In and around a conserved leucine zipper domain, Dact1 and Dact2 are more closely similar to each other than to Dact3. However, at the C-terminus Dact1 and Dact3 are more closely related (compare PDZ-binding domains in Fig. 1A).

The predicted amino acid sequence for the Dact3 protein is approximately 20% shorter than either Dact1 or Dact2 (610 amino acids for Dact3 *vs.* 778 amino acids for Dact1 and 757 amino acids for Dact2). There is an open reading frame that continues upstream to an ATG located at position -156 in the genomic locus of *Dact3*, which if transcribed and translated could therefore theoretically add 52 amino acids to the amino terminus of the Dact3 polypeptide. We have excluded this upstream sequence as a part of the *Dact3* transcript produced in newborn forebrain by using 5' RACE to determine the start of transcription (see Methods for details). A highly conserved ortholog of *Dact3* is identifiable in the human genome and EST databases. The human *DACT3* gene maps to

chromosome 19q13.32 and is predicted to encode a protein 85% identical to mouse Dact3 (Fig. 1C, Supplemental Fig. S1). A similar *Dact3* gene distinct from *Dact1* and *Dact2* is also identifiable in the zebrafish and pufferfish genomic and EST public sequence databases (data not shown).

The 5' region of each of the mouse *Dact* genes is extremely GC-rich, and the intron-exon structure is also conserved, consisting of three small 5' coding exons which together encode a short 5' UTR and the amino-terminus of the polypeptide, and a larger fourth exon containing approximately 2/3 of the translated sequence plus a longer 3' UTR (data not shown). To summarize, we have identified three paralogous *Dact* genes in mouse, one of which (*Dact3*) is entirely novel. Overall homology relationships between the principal members of the proposed *Dact* gene family are diagrammed schematically in Fig. 1C.

Developmental and Tissue-Specific Expression

Using Northern blots we have profiled the expression of each *Dact* gene across embryonic stages and adult tissues. This has been complemented by Quantitative PCR (Q-PCR) to more accurately compare relative mRNA levels between the three genes. Over the course of embryogenesis, the *Dact* genes have quite different temporal patterns of expression. From embryonic day (E) 4.5-8.5 there is only weak expression of these genes, some of which may occur in maternal and extra-embryonic tissues (Fig. 1D). In the embryo proper, *Dact1* expression increases dramatically from E9.5-10.5, peaks between E11.5-13.5, then diminishes slowly thereafter. In contrast, *Dact2* expression is very low overall for most of embryogenesis (Fig. 1D middle blot). *Dact3* expression is

initially low, peaks at E10.5, then declines again (Fig. 1D bottom blot). Because both *Dact1* and *Dact3* levels decline while *Dact2* remains relatively constant overall, all three genes are expressed at roughly comparable levels at E18.5, three days prior to birth (Fig. 1D, G).

Using similar methodology, the three *Dact* genes show quite different adult tissue expression patterns. In the adult, *Dact1* is present primarily in the brain, lung, and uterus, with significantly weaker expression in other tissues (Fig. 1E). *Dact2* is present in the brain and uterus, but is also quite notable in the kidneys, small intestines, thymus, and testes (Fig. 1E, H, I). The adult distribution of *Dact3* is most restricted: it is present in the uterus (Fig. 1E, I), and is the principally-expressed Dact family member in the adult brain (Fig. 1E, H).

In summary, the three *Dact* genes are broadly expressed during mouse embryogenesis and in adult tissues, and yet have distinct temporal and tissue-specific signatures. This is consistent with these molecules playing separable roles during development and in adult tissue physiology. To further clarify these differences, we have performed mRNA in situ hybridization analysis in whole mounts (WISH) and sections (ISH) during embryonic development and in the adult brain.

Embryonic Expression of the *Dact* gene family through Segmentation Stages

As expression of all three family members is low at early embryonic stages, and as the expression of *Dact1* has been described up to E8.5-9, we have focused our attention primarily on later developmental stages. Consistent with a prior report (Hunter et al., 2005), at E7.5 our *Dact1*-specific probe detects expression primarily in the

mesoderm and at very low levels in the neurectoderm (Dr. Uta Grieshammer and BNRC, data not shown). In the E9.0 embryo, *Dact1* expression is highest in the septum transversum, cranial mesenchyme, the caudal presomitic mesoderm (PSM), and in the somites that derive from it (Hunter et al., 2005), as well as in the wall of Rathke's pouch, the dorsal aorta, the aortic sac, and the branchial arch arteries (Fig. 2A). Within the PSM, a band of low *Dact1* is apparent between high expressing zones in the caudal PSM and the newly forming somite at the rostral edge (Fig. 2A). *Dact1* exhibits a strong caudal to rostral gradient that inversely correlates with the developmental age of somites: highest expression in the most recently formed (*i.e.* caudal) somite, and diminishing expression in more mature (*i.e.* rostral) somites. Within individual somites *Dact1* shows a progressively restricted spatial pattern. In younger (caudal) somites, *Dact1* is preferentially expressed ventromedially along the rostral-caudal extent and along both the rostral and caudal somite walls (Fig. 2A, I). As the somite matures, *Dact1* expression decreases rostrally, such that its localization becomes progressively restricted to the ventromedial and caudal domains (Fig. 2A inset). By section ISH, *Dact1* expression is also prominent in the nephrogenic cords, the ventral mesentery and the mesenchymal outer walls of the foregut, the dorsal aorta, and its main branches (Fig. 2G).

Dact2 is detectable only at very low levels in the E9.0 embryo by mRNA in situ techniques (Fig. 2B), though it is fairly widely distributed. At this stage, low *Dact2* expression is appreciable in the retina, otic vesicle, ventral mesentery of the foregut, the umbilical veins, dorsal neural tube, and in a gradient in the somites with highest levels in the caudal (youngest) somites much like *Dact1* (Figure 2B and insets). Unlike *Dact1*, *Dact2* is not detected within the caudal PSM at this stage (Fig. 2B). Compared to *Dact1*,

Dact2 also shows a significantly different domain of expression within each somite. Whereas *Dact1* is most highly expressed ventromedially, *Dact2* is more highly expressed dorsolaterally. Furthermore, unlike *Dact1* which becomes more *caudally* restricted, *Dact2* becomes progressively more *rostrally* restricted (Fig. 2B left inset). As a consequence, the two paralogs occupy complementary intrasomitic distributions as somites mature.

At E9.0, *Dact3* mRNA is found in a tissue distribution distinct from the other two *Dact* genes (Fig. 2C). Like *Dact1*, *Dact3* is expressed in craniofacial mesenchyme, but it is more prominent in the branchial arch mesenchyme, the aortic sac, and the aortic arches (Fig. 2C, P) where *Dact1* expression is comparatively lower. Also different from *Dact1*, *Dact3* is not expressed in the PSM, nor is it present in a caudal-rostral gradient among developing somites like both *Dact1* and *Dact2*. Instead, *Dact3* is expressed in the ventral domain of more mature somites, located centrally along the rostral-caudal axis (Fig. 2C, Q).

At E10.5, high *Dact1* expression continues in the PSM and caudal somites (Fig. 2D, L, compare to 2A). *Dact1* is also present at low levels in other tailbud tissues, such as the ventral mesoderm of the tail bud (Fig. 2D). More anteriorly, *Dact1* at this stage is present in the forelimb and hindlimb buds, where it is expressed in mesoderm in a proximal (low)-apical (high) gradient (Fig. 2D, J). It continues to be expressed significantly in mesenchyme surrounding foregut derivatives such as the left and right main bronchi, as well as in the sclerotome derived from the ventral somite (Fig. 2J), but comparatively is only weakly detectable in the branchial arch mesenchyme (data not

shown). At this stage, it first starts to be expressed in post-mitotic neurons, chiefly evident in the differentiating motor pools of the ventral spinal cord (Fig. 2J).

Consistent with the Northern and Q-PCR data, at E10.5 *Dact2* is only weakly detectable by mRNA in situ hybridization and appears to be more restricted in its tissue distribution than at E9.0. The main loci of expression at this time are in the otic vesicle and in the caudal-most somites where the strongest signal is in the most-recently formed somite and a diminishing signal is in the next two youngest somites (Fig. 2E, inset E). In contrast, *Dact3* at this stage is very prominent throughout the branchial arch mesenchyme, limb bud mesenchyme, as well as continued expression in maturing somites (Fig. 2F, Q, R, S).

Taken together, the expression of *Dact* family members at embryonic stages through E10.5 suggests overlapping roles especially during mesoderm and neural crest development. At E9.0 *Dact1* and *Dact3* overlap in the facial mesoderm and septum transversum, where they may play either complementary or redundant roles. The exclusive expression of *Dact1* in the PSM suggests a more unique function in that tissue during segmentation. The robust expression of *Dact3* in the branchial arches, facial mesenchyme, and ventral somites is consistent with this gene being important in the migration or differentiation of neural crest cells and of mesoderm-derived mesenchyme. The expression pattern of *Dact1* at early stages has been proposed to indicate a role in mesenchymal to epithelial transitions (Hunter et al., 2005). A comprehensive view based on the embryonic expression patterns of all three *Dact* genes suggests involvement in a subset of signaling events, including those that control morphogenesis but extending to the regulation of cellular differentiation and tissue patterning.

The distinct domains of *Dact* gene expression within developing somites correlates with domains of signaling activity that pattern this tissue. Sonic hedgehog (Shh) secreted from the notochord and floor plate is an important ventromedial somite patterning signal, whereas TGFßs and Wnts play a similar role dorsolaterally (Lee et al., 2000; Christ et al., 2004). Given that *Dact1* and *Dact3* are primarily restricted to the ventromedial domain, and that *Dact2* is concentrated dorsally and laterally, these signaling cascades could differentially regulate *Dact* expression. Simultaneously, based on their known functions Dact proteins are likely to be involved in the intracellular modulation of the signaling cascades that pattern these tissues. The ventromedial expression of *Dact1* and *Dact3* is consistent with a role in signaling within the presumptive sclerotome, which produces the cartilage and vertebral bodies making up the axial skeleton (Christ et al., 2004). The complementary expression of *Dact2* dorsolaterally is consistent with a signaling role in the presumptive dermomyotome, which at later stages gives rise to the dermis as well as the deep back and intercostal musculature (Borycki et al., 1999; Wagner et al., 2000).

Prenatal Expression of Dact genes in the Developing Central Nervous System

After E10.5, expression of *Dact1* and *Dact3* becomes concentrated in the developing CNS. In situ hybridization of sagittally-sectioned embryos at E14.5 shows that *Dact1* and *Dact3* RNA are broadly expressed in the brain and spinal cord, (Fig. 3A, C). By contrast, *Dact2*, although also expressed in the developing CNS, is clearly present at higher levels in several non-neuronal tissues, particularly the developing kidneys, salivary glands, and thymus (Fig. 3B, K-N).

In the developing brain, *Dact1* is expressed in some progenitor zones. In the ventricular zone of the cerebral cortex at E14.5, it is expressed in a ventral (high)-dorsal (low) gradient (Fig. 3D). There is high expression in the ventricular zone of the basal ganglia anlagen (lateral and medial ganglionic eminences) (Fig. 3D); here, *Dact1* expression labels radially aligned clusters of cells (Fig. 3D, G). *Dact1* also shows differential regional expression in postmitotic neurons. For instance, within the cerebral cortex (Fig. 3D), *Dact1* is expressed in the cortical plate in a rostroventral (low)-caudodorsal (high) gradient, which is complementary to the gradient in the underlying ventricular zone. *Dact3* is also concentrated in the cortical plate zone at this stage (Fig. 3F). By contrast, using a carefully-validated probe to avoid cross-detection of the two more heavily expressed paralogs (see Methods), *Dact2* message is detectable only very weakly in either the proliferative zones or post-mitotic domains of the forebrain (Fig. 3E).

Since *Dact1* is regionally expressed in the CNS at this stage of development, we have conducted a more thorough analysis of its distribution in developing nervous tissue. At the level of the developing midbrain (Fig. 3H), *Dact1* message is notable dorsally in the tectum, in postmitotic neurons of the ventral midbrain, as well as in some nuclei of the developing hypothalamus. Moving more caudally within the CNS, *Dact1* is also found in cerebellar precursors near the midbrain-hindbrain junction, as well as in the rhombic lip region and in the pons (Fig. 3A, I). In the spinal cord, *Dact1* is detected in primary sensory neurons of the developing dorsal horns, and in neurons of the motor pools located ventrally (Fig. 3J).

Postnatal Expression of the *Dact* genes in the Central Nervous System

In sharp contrast to the embryonic period, *Dact1* is the most weakly expressed of the gene family in the adult brain (*cf.* Fig. 1E, H). Nonetheless, *Dact1* message can be detected in many postnatal neuronal populations, and it is differentially expressed in neuronal sub-types (Fig. 3O). For example in the adult cerebellum, although *Dact1* is present at relatively high levels in the granule cell layer, it is not detectable in most Purkinje cells (Fig. 3O, inset). This pattern of expression in the adult cerebellum is complementary to *Dact2* and *Dact3*, both of which are detected more strongly in the Purkinje cell layer (Fig. 3P, Q insets). Elsewhere in the brain, all three *Dact* genes are co-expressed in the hippocampus. In the dorsal forebrain *Dact1* and *Dact3* are expressed throughout all layers of the cerebral cortex, *Dact2* is preferentially expressed in more superficial layers (Fig. 3P, compare to Fig. 3O, Q). Given prior studies showing that changes in Wnt signaling components can alter complex behaviors (Madsen et al., 2003; Beaulieu et al., 2004; Long et al., 2004; Kaidanovich-Beilin et al., 2004; O'Brien et al., 2004), the regional adult brain expression patterns of *Dact* family members suggest different roles in brain function.

Implications for signaling

Sequence similarities and differences among the three mouse *Dact* genes, together with prior studies focused on *Dact1* and *Dact2* in other organisms (Waxman et al., 2004; Zhang et al., 2004; Zhang et al., 2006), suggest that each *Dact* paralog has both conserved and divergent functions in signal transduction. The tissue distribution of the three murine genes during embryogenesis is consistent with roles in a subset of

developmental events downstream of Wnt signaling, as well as perhaps in other types of signaling as has been suggested for *Dact2* in modulating TGFß receptors (Zhang et al., 2004). The postnatal expression of the mouse *Dact* genes points to important functions in several adult organs including the CNS, uterus, testes, thymus, and kidneys. Ongoing work in our lab will explore the molecular and cellular roles of these signal scaffold molecules during development, and especially in the postnatal CNS.

Methods

5' RACE to determine transcriptional start of *Dact3*.

5'RACE was carried out using an RNA ligation-mediated protocol to ensure capture of the 5'end of the mRNA. FirstChoice RLM-RACE kit (Ambion, Austin TX) was used according to the manufacturer's protocol with the addition of Thermo-X RT polymerase (Invitrogen, Carlsbad, CA). The RT reaction was performed at 62ºC for 2 h with reverse primer: 5'CAGGCGTCCATAGGAGCCAGATCCGGAG3' on total RNA extracted from mouse strain C57Bl/6 neonatal forebrain. Dissected brain was frozen on dry ice and RNA isolated with RNeasy kit (Invitrogen) using the manufacturer's animal tissue protocol. RT products were amplified with the 5'RACE outer primer provided by the manufacturer and gene-specific reverse primer: 5'GTGGTGAATCTGGGCCTCCAGTAGAACTG3' using Pfx DNA polymerase (Invitrogen, Carlsbad, CA). Purified PCR products were treated with Taq polymerase in the presence of 2mM dATP and cloned into pCR-4 TOPO vector (Invitrogen Carlsbad, CA). 12 RACE clones were sequenced to determine the mRNA start site. Relative to the

proposed translational start, the distribution of transcriptional start sites was: 1 clone of each: positions –120, -111, -104, -77, -47, +11; 2 clones of each: -115, -110, -56.

Consistent with the proposed translational start site for *Dact3* based on 5'RACE, the sequence surrounding this codon corresponds to the Kozak consensus site (Kozak, 1987) at 8 out of 10 residues (gccgcagccATGa). This methionine is also conserved in the predicted sequence of human *DACT3*, and aligns well with the starts of the two other Dact family members (Fig. 1A).

Cloning of Mouse *Dact* Genes

The cloning of mouse *Dact1* (*Dpr1*) has previously been described (Cheyette et al., 2002). The full-length clones of mouse *Dact2* and *Dact3* were obtained by RT-PCR from adult cerebral cortex and neonatal forebrain respectively (see above for mRNA extraction). RT reactions were performed using Thermoscript (*Dact2*) and Thermo-X RT (*Dact3*) polymerases (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions for GC-rich templates, and the following gene-specific primers:

gene	RT primer
Dact2	5'AGCGCAATAGCAAGGTTGATAC3'
Dact3	5'ATTAACTGCAGTGAAGTTCAAGCCCATCCCGCCCCAAC3'

RT product was amplified by PCR with Pfu (Stratagene, La Jolla, CA) and Pfx (Invitrogen, Carlsbad, CA) polymerases for *Dact2* and *Dact3* respectively, using a forward primer specific for the 5'UTR of each gene and a reverse primer specific for the 3'UTR internal to the first-strand synthesis primer. Amplified cDNAs were isolated and subcloned using standard molecular biology techniques, and confirmed by sequencing with both vector-based and gene-specific primers.

Accession Numbers and Sequence Comparisons

The GenBank accession number for mouse *Dact1* (*Dpr1*) has previously been reported (Cheyette et al., 2002) and is AF488775. For the mouse *Dact2* (*Dpr2*) and *Dact3* sequences whose cloning is described here, accession numbers are AY297430 and DQ832319 respectively. Chromosomal positions were determined using the June 2006 update of the Ensembl Genome Browser (v 39).

Protein sequences were compared with VectorNTI Advance v. 9.1 (Invitrogen, Carlsbad, CA) AlignX software using an amino acid identity matrix. In the phylogenetic tree shown (Fig. 1C), distance from a node along the horizontal axis indicates sequence divergence. Distance from a node along the vertical axis is arbitrary and has been manually enhanced to emphasize family subgroupings (*i.e. Dact1 vs. Dact2 vs*. *Dact3* subfamilies).

Northern Blotting

DNA probes were labeled by incorporation of ^{32}P-labeled dCTP. Mouse embryonic multi-stage and postnatal multi-tissue Northern blots (Seegene, Seoul S. Korea) containing 20 μg total RNA per lane were hybridized according to the manufacturer's instructions and the following stringencies and times: hybridization overnight at 55^{O}C in Ultrahyb buffer (Ambion, Austin TX), wash 3 X 15 minutes in 0.2x SSC, 0.5% SDS at 60 ^{O}C. Exposure to film was overnight (15 hours) at -80 ^{O}C with two intensification screens. Two different probes were used to validate each gene pattern, and a pair of fresh blots (1 embryonic and 1 adult multi-tissue) was used for the initial

characterization of each gene. Probes used for data shown (numbers relative to translational start): *Dact1* 613-1377; *Dact2* 586-1769; *Dact3* 853-1673.

Quantitative Reverse-Transcriptase PCR (Q-PCR)

For preparation of template, 2 μg total RNA was isolated from the experimental tissue indicated, taken from CD1 outbred mice (Charles River Laboratories, Wilmington MA), DNaseI-treated (Roche, Indianapolis IN), and reverse-transcribed (25°C x 10 min, 42°C x 50 min, 72°C x 10 min) using random primers and Superscript II (Invitrogen, Carlsbad, CA). Q-PCR primers for *Dact1*, *Dact2*, and *Dact3* have been designed using PrimerExpress (Applied Biosystems, Foster City, CA) and validated to ensure: 1) amplification of a single product and 2) appropriate efficiency of amplification. The linear plot of cycle number determined at threshold (C_T) *vs.* cDNA concentration (log ng) gives a linear slope of -3.3±0.1 for the housekeeping gene (mouse cyclophilin) and for *Dact1, Dact2*, and *Dact3*. Furthermore, a no-template control was conducted in each trial to ensure that the primers did not dimerize, and that amplified DNA is not the result of contamination. Steady-state mRNA was measured using an ABI 7300 quantitative real time PCR thermal cycler and standard conditions [1 cycle x (2 min @ 50°C, 10 min @ 95°C), then 40 cycles x (15 sec @ 95°C, 1 min @ 60°C)]. Sybr green (Applied Biosystems, Foster City, CA) was utilized to detect the PCR product in real-time, and a standard dissociation curve was generated. Mouse cyclophilin (NM 011149) was employed as an internal control for standardizing the measurements between reactions. Experimental PCR products were subcloned and sequenced to verify their identity. Data from each experiment (n=3 for 2 independent tissue samples in each case) was calculated

using the ΔΔCt method (Livak and Schmittgen, 2001). The following primer pairs were used:

gene	*forward*	*reverse*
Cyclophilin	5'TGGAGAGCACCAAGACAGACA3'	5'TGCCGGAGTCGACAATGAT3'
Dact1	5'TCAGGGTTTTATGAGCTGAGT3'	5'GAACACGGAGTTGGAGGAGTTA3'
Dact2	5'GGCTGACGGGCATGTTC3'	5'CCCCACGTCAGCTGGAA3'
Dact3	5'AGGCTTCTATGAAGACCCCAGTT3'	5'AGATCCGGAGAAGCCACTGT3'

Probes for mRNA in situ Hybridization

Riboprobes were labeled by incorporation of digoxigenin-labeled UTP (DIG RNA Labeling Mix, Roche Applied Science, Indianapolis IN). Sense controls were performed in parallel and compared in each case to confirm the specificity of the expression patterns shown (Supplemental Fig. S3). The validity of tissue expression observed for each gene was further confirmed by the observation of identical expression patterns using multiple non-overlapping antisense probes derived from the same cDNA. Probes used (nt numbers relative to translational start):

gene	probe for data shown (nt)	pattern validated with probe (nt)
Dact1	1250-1601	316-692
Dact2	1639-1963	610-1080
Dact3	239-607	813-1153, 1162-1641, 1643-1910

mRNA in situ Hybridization: Tissue Preparation

Embryos were fixed by immersion, neonatal and postnatal animals by perfusion, with 4% paraformaldehyde in PBS. Embryos or tissue (*e.g.* brains) were dehydrated in sequential concentrations of ethanol and stored in 100% ethanol at -20°C. Prior to experimental use, tissue was rehydrated sequentially from ethanol into PBS containing 0.1% Tween-20.

mRNA in situ Hybridization: Thick sections

Embryos/tissues were embedded in gelatin/albumin gel polymerized with glutaraldehyde. Gel solution was 30% ovalbumin (w/v), 0.5% gelatin in 0.1M sodium acetate, pH 6.5, filtered. Gel was polymerized by addition of 2.5% glutaraldehyde, and solidified by storing overnight with embedded tissue at 4°C. Embedded tissue was sectioned to 100 μm thickness on a Leica VT1000S fluid immersion vibratome.

mRNA in situ Hybridization: Hybridization and Development

Embryos or thick sections were collected in PBT (PBS containing 0.1% Tween-20), and treated with 3% H_2O_2 (for whole mounts) or 6% H_2O_2 (for sections, including adult brain sections) in PBT for 1 h. Embryos/sections were washed sequentially in 3 X 5 min PBT, 5 min 10ug/mL proteinase K in PBT, 5 min 2mg/mL glycine in PBT, 2 X 5 min PBT, 20 min 4% paraformaldehyde, 0.2% glutaraldehyde in PBT, 3 X5 min PBT. Tissue was prehybridized for 2 h in hybridization solution at 70°C, followed by hybridization overnight in fresh hybridization solution containing 0.5 μg/ml digoxigenin labeled RNA probe. Hybridization solution was 50% formamide, 5X SSC pH4.5 (pH 7.0 for adult brain sections), 1% SDS, 50ug/mL yeast tRNA, 50ug/mL heparin. Stringency washes were used to remove unbound probe. These consisted of 2 X 30 min in 50% formamide, 4X SSC, 1% SDS at 70ºC, followed by 2 X 30 min in 50% formamide, 2X SSC, 1% SDS at 70ºC.

Following hybridization, tissue was washed with MABT (0.1M maleic acid buffer pH 7.5 with 0.1% Tween-20) for 2 X 10 min at room temperature. Tissue was labeled with 1:4000 anti-digoxigenin (Roche, Indianapolis, IN) overnight at 4°C. Blocking for 2

h and immunolabeling were performed in 10% heat inactivated sheep serum, 2% BM blocking reagent (Roche, Indianapolis, IN), in MABT. Following antibody incubation, tissues were washed 5 X 30 min with MABT at room temperature.

For development reactions, tissue was washed 3 X 10 min in NTMT (0.1 M Tris pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$, 0.1% Tween-20), and incubated in dark in NBT/BCIP (Bio-Rad) in NTMT. Incubation times were variable depending on when clear development was visible, but usually 4-6 h at room temperature for embryos, and 10-14 h for adult brain sections.

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Figure Legends

Fig. 1. Dact gene family molecular data. **A.** Alignment of primary protein sequences, mouse Dact1, Dact2, Dact3. Chromosomal positions are shown in the first line. Black blocks indicate identity; grey indicates similarity. Blocks of four or more amino acids conserved in all three paralogs are underlined in black. The coiled-coil domain (with four absolutely conserved leucines indicated) and the Dvl/PDZ-binding domain (Cheyette et al, 2002) are underlined in grey. **B.** Table showing percentage conservation in the primary protein sequences, aligned as in **A**. Numbers to the top right indicate % identity; numbers (in parentheses) to the bottom left indicate % identity plus highly similar residues. **C.** Deduced phylogenetic relationships between the Dact proteins described in this paper and those previously described. The three mouse cDNAs whose coding sequences have been cloned in their entirety are boxed. The originally-described *Dpr* and *Frodo* genes are both homologs of *Dact1*, corresponding to a recent duplication event in the *Xenopus* lineage (not shown). The human DACT3 sequence is a predicted cDNA based on public database information (see text and also Fig. S1). **D, E.** Northern blots. *Note:* Different blots were probed for each gene, loading controls are provided in Fig. S2. **D.** Embryonic stages. Samples from the first three days post-coitus (E4.5-6.5) include both extra-embryonic and maternal uterine tissue, while the next three (E7.5-9.5) are the embryo plus extra-embryonic membranes. E10.5-18.5 correspond to embryonic tissues only. **E.** Adult tissues: (br) brain, (hrt) heart, (lng) lung, (lvr) liver, (spl) spleen, (kd) kidney, (stm) stomach, (si) small intestine, (ml) striated muscle, (thy) thymus, (ts) testis, (ut) non-pregnant uterus, (plc) placenta. **F-I.** Q-PCR showing relative expression of *Dact1 vs. Dact 2 vs. Dact3* at selected developmental stages and adult tissues. **F.** E10.5 **G.** E18.5 **H.** adult

(8 week postnatal) brain, **I**. adult (8 week postnatal; non-pregnant) uterus. *Note:* Y-axis scale changes from F-I; units denote *relative* expression within each sample, absolute levels are not measured by this technique.

Fig. 2. Developmental expression of *Dact* genes. **A-C.** WISH at E9.0. **A**. *Dact1*-specific probe. Expression is most prominent in presomitic mesoderm (psm), somites (so), septum transversum (st), craniofacial mesenchyme (cfm), and the ganglion of cranial nerve V (V), and is also present in the retina (r), around Rathke's pouch (Rp), the otic vesicle epithelium (ov), the mandibular arch (md), aortic sac (as), aortic arches (aa), and dorsal aorta (da), and in the forelimb bud (fl). Inset (lateral aspect): *Dact1* becomes polarized ventromedially (v/m) and caudally (c) as somites mature. **B.** *Dact2*-specific probe. Only weak expression is detectable: in caudal somites (so), umbilical veins (uv), ventral mesentery of the foregut (vm), otic vescicle (ov), mandibular arch (md), and retina (r). Left inset (lateral aspect): *Dact2* becomes polarized dorsolaterally (d/l) and rostrally (r) in the caudal somites. Right inset (dorsal aspect): *Dact2* is expressed in the dorsal neural tube caudally. **C.** *Dact3*-specific probe. Expression in the craniofacial mesenchyme (cfm), mandibular arch mesenchyme (md), aortic sac (as), aortic arches (aa), and dorsal aorta (especially caudally; da), umbilical artery (ua), vitelline artery (va), ventral mesentery of the foregut (vm), forelimb bud (fl), and ventrally in mature somites (so). **D-F.** WISH at E10.5. **D.** *Dact1*-specific probe. Expression in the presomitic mesoderm (PSM), caudal somites (so), more weakly in the ventral mesoderm of the tail bud (tb; vtm), limb buds (fl, hl), as well as the ventral spinal cord (sc). **E.** *Dact2*-specific probe. Expression is detected in the otic vesicle plus the rostral portion of the most recently formed somites (so). Inset: another example of caudal somite expression plus no expression detected in the forebrain (fb) or mandibular arch (md; obscured in **E)**. **F.** *Dact3*-specific probe. Expression in facial mesenchyme (fm), branchial arch mesenchyme: (mx) maxillary, (md) mandibular, (hy) hyoid, limb buds (fl, hl), as well as ventral somites (so). **G-S**. Dact family member ISH on representative

sections from E9-E10.5. **G.** At E9.0 *Dact1* is detected in the ventral somite (vs), nephrogenic cords (nc), septum transversum and hepatic primordium (st/hp), and the gut wall mesenchyme (gwm). **H.** Schematic showing approximate level and orientation of sections in **G**, **I.** **I.** At E9.0 *Dact1* is expressed in the PSM, in the ventral domains of somites, and along the rostral and caudal somite walls. **J.** At E10.5 *Dact1* is detected in the limb bud mesoderm (lb), outer walls of the right and left main bronchi (rb, lb), sclerotome (scl), and the motor pools of the spinal cord (mp, sc). **K.** Schematic showing approximate level and orientation of sections in **J**, **L.** **L.** At E10.5 and earlier *Dact1* is highly expressed in the PSM and caudal somites. **M.** At E9.0 *Dact2* is detected in the otic vesicle (ov), ventral mesentery of the foregut (vm), and umbilical veins (uv). **N.** Schematic showing approximate levels and orientation of sections in **M** and **O**. **O.** *Dact2* is detected in the umbilical veins (uv) and within somites (so) rostrally and dorsally. **P.** At E9.0 *Dact3* is detected throughout mesenchyme, including that of the limb buds such as the forelimb (fl), as well as the hyoid (hy) and mandibular (md) branchial arches, in a section corresponding to the plane shown in the schema at right. **Q-S.** E10.5 ISH on sections corresponding to the planes shown in the accompanying diagram. **Q.** Expression ventrally in maturing somites. **R.** Expression in forelimb mesoderm (fl). **S.** Expression in branchial arch mesenchyme: (mx) maxillary, (md) mandibular, (hy) hyoid, (III) third. Other abbreviations: (lda) left dorsal aorta, (bl) blood. Scale bars: **A-C** 0.5 mm; **D-F** 1.0 mm.

Fig. 3. Expression of *Dact* genes at E14.5 and in adult brain. **A-C.** Sagittal sections of whole embryos at E14.5 stained by in situ hybridization with probes for *Dact1* (**A**), *Dact2* (**B**), and *Dact3* (**C**). *Dact1* and *Dact3* are most prominent in the developing CNS, *Dact2* has domains of higher expression in the developing salivary glands (sal) and kidneys (kd). **D-F.** Horizontal sections of forebrain at E14.5 stained with probes for *Dact1* (**D**), *Dact2* (**E**), and *Dact3* (**F**). **G.** Close-up of horizontal section through the ventricular proliferative zone at the level of the lateral ganglionic eminence, showing *Dact1* expression in radially-arranged cell clusters (arrows). **H-J.** Horizontal sections at progressively more caudal planes of the E14.5 CNS showing *Dact1* expression in many populations of differentiating neurons. **H.** Midbrain. **I.** Midbrain-hindbrain junction and pons. **J.** Spinal cord. Abbreviations: (tc) tectum/dorsal midbrain, (vm) ventral midbrain, (mn) migrating neurons of the ventral midbrain, (hy) hypothalamic nuclei, (pt) pontine tegmentum, (rl) rhombic lip of the fourth ventricle, (cpr) cerebellar precursors, (dh) developing dorsal horn of the spinal cord. Arrows: neurons in the motor pools of the ventral spinal cord (vsc). **K-N.** Sections showing *Dact2* expression in E14.5 tissues. **K**. Horizontal section through spinal cord (sc) and sympathetic ganglia (sg). Compare to higher level of expression in the nearby edge of the developing thymus (thy). **L**. Section of developing kidney showing high levels of expression in the collecting system and renal pelvis (rp). **M**. Section through the main lobe of the thymus (thy). **N**. Section of the oral cavity showing expression in the developing salivary glands (sal) as well as weaker expression in the toothbuds (thb). **O-Q.** Sagittal sections of adult brains stained with in situ hybridization probes for *Dact1* (**N**), *Dact2* (**O**), and *Dact3* (**P**). All three genes are expressed in the hippocampus (hc), in different patterns in the cerebral cortex (Ccx) and other structures of the forebrain (see text). Insets:

Expression in the adult cerebellum. *Dact1* (inset O) is specifically expressed in the granule cell layer (gcl), whereas both *Dact2* (inset P) and *Dact3* (inset Q) are expressed significantly in the Purkinje cell layer (Pcl). Sense and no probe controls provided in Fig. S3. Other abbreviations: (fb) forebrain, (mb) midbrain, (hb) hindbrain, (thal) adult thalamus, (str) striatum, (t) thalamic eminence, (p) pons, (lvr) liver, (hl) hindlimb, (cp) cortical plate zone, (ne) neuroepithelium, (lge) lateral ganglionic eminence, (mge) medial ganglionic eminence, (ml) molecular layer of the cerebellum, (bl) extravasated blood, (*) folded tissue. Scale bars: A-C 1 mm; D-N 0.2 mm; O-Q 2 mm.

Supplemental Figure Legends

Fig. S1. Predicted human DACT3 sequence (*H.s.* Dact3) compared to translation of cloned mouse *Dact3* cDNA (*M.m.* Dact3). Chromosomal positions are shown in the first line. Black blocks indicate identity; grey indicates similarity. The human sequence is based on publicly-available human cDNA fragments (*e.g.* GenBank CV029753, BG715516, BF515069, BF115250, BM468105, etc), previously identified 5'truncated cDNAs (*e.g.* BC016161), and human genomic sequence corresponding to chromosome 19q13.32. Genscan also identifies this as a transcribed locus based on genomic sequence-level criteria.

Fig. S2. Ethidium bromide stained gels corresponding to all Northern blots shown in Fig. 1D, E, demonstrating similar levels of 18S and 28S ribosomal RNA in each lane as a loading control. A total of 6 (3 pairs of embryonic (**A**) and adult (**B**) blots) were used to generate data in Fig 1: one pair of fresh blots for each *Dact* gene. Expression data was

cross-validated by sequentially stripping and re-testing each blot pair with the other 2 probes.

Fig. S3. In situ hybridization controls using reverse complementary (sense) probes corresponding to the cDNAs, hybridization, and development conditions used in Figs. 2 and 3. **A-C**.WISH at E 9.0: (**A**) *Dact1*. (**B**) *Dact2*. (**C**) *Dact3*. Aside from very weak staining of the retina and otic vesicle with the sense probe for *Dact1*, essentially no signal comparable to the antisense staining is seen with any of the sense controls. Note the absence of PSM, somite, septum transversum, and major arterial staining with the *Dact1* sense probe. *Abbreviations:* (r) retina, (ov) otic vesicle, (psm) presomitic mesoderm. **D-F.** ISH on representative sections at E9.0 and E10.5 using sense probes: (**D**) *Dact1*. (**E**) *Dact2*. (**F**) *Dact3*. No specific signal is detected. **G-I.** ISH on adult brain sections, forebrain and cerebellum (insets): (**G**) *Dact1*. (**H**) *Dact2*. (**I**) *Dact3*. No signals comparable to the cortical, hippocampal, or cerebellar staining in Fig. 3**O-Q** is observed. **J.** No-probe control for adult brain section ISH, showing background levels of endogenous peroxidase activity under conditions used to generate adult brain expression data shown in Fig. 3**O-Q** and Fig. S3**G-I** . Note absence of staining in cortical structures including the cerebellum (Clm), hippocampus (hc), and cerebral cortex (Ccx). Brownish peroxidase stain is apparent in the striatum (str) and thalamus (thal).

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