

Synergistic regulation of vertebrate muscle development by *Dach2*, *Eya2*, and *Six1*, homologs of genes required for *Drosophila* eye formation

Tiffany A. Heanue,^{1,6} Ram Reshef,^{2,6,7} Richard J. Davis,³ Graeme Mardon,³ Guillermo Oliver,⁴ Stanislav Tomarev,⁵ Andrew B. Lassar,² and Clifford J. Tabin^{1,8}

¹Department of Genetics and ²Department of Biochemistry and Molecular Biology, Harvard Medical School, Boston, Massachusetts 02115 USA; ³Department of Pathology, Baylor College of Medicine, Houston, Texas 77030 USA; ⁴Department of Genetics, St. Jude's Children's Research Hospital, Memphis, Tennessee 38105 USA; ⁵National Eye Institute, National Institutes of Health (NIH), Bethesda, Maryland 20892 USA

We have identified a novel vertebrate homolog of the *Drosophila* gene *dachshund*, *Dachshund2* (*Dach2*). *Dach2* is expressed in the developing somite prior to any myogenic genes with an expression profile similar to *Pax3*, a gene previously shown to induce muscle differentiation. *Pax3* and *Dach2* participate in a positive regulatory feedback loop, analogous to a feedback loop that exists in *Drosophila* between the *Pax* gene *eyeless* (a *Pax6* homolog) and the *Drosophila dachshund* gene. Although *Dach2* alone is unable to induce myogenesis, *Dach2* can synergize with *Eya2* (a vertebrate homolog of the *Drosophila* gene *eyes absent*) to regulate myogenic differentiation. Moreover, *Eya2* can also synergize with *Six1* (a vertebrate homolog of the *Drosophila* gene *sine oculis*) to regulate myogenesis. This synergistic regulation of muscle development by *Dach2* with *Eya2* and *Eya2* with *Six1* parallels the synergistic regulation of *Drosophila* eye formation by *dachshund* with *eyes absent* and *eyes absent* with *sine oculis*. This synergistic regulation is explained by direct physical interactions between *Dach2* and *Eya2*, and *Eya2* and *Six1* proteins, analogous to interactions observed between the *Drosophila* proteins. This study reveals a new layer of regulation in the process of myogenic specification in the somites. Moreover, we show that the *Pax*, *Dach*, *Eya*, and *Six* genetic network has been conserved across species. However, this genetic network has been used in a novel developmental context, myogenesis rather than eye development, and has been expanded to include gene family members that are not directly homologous, for example *Pax3* instead of *Pax6*.

[Key Words: *Dach2*; *Eya2*; *Six1*; *Pax3*; myogenesis; *dachshund*; somite development]

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Somites are segmentally organized mesodermal structures that are the embryonic precursors of the axial skeleton and of all skeletal muscle (for review, see Christ and Ordahl 1995). Somites form by budding off from the anterior end of the presegmental mesoderm (PSM) to form epithelial balls of tissue. Patterning signals from surrounding tissues induce different regions of the somite to acquire distinct fates: The dorsal somite develops into the dermamyotome, the precursor to the dermis and to the muscles; and the ventral somite gives rise to the sclerotome, the precursor of the axial skeleton and ribs (Christ and Ordahl 1995). Subsequent inductive signal-

ing leads to further subdivision of cell fates within the somite.

The best studied aspect of this patterning and differentiation process is the specification of the myogenic cells. The establishment of muscle cell fate requires inductive signals both from axial tissues and from the dorsal ectoderm that overlays the somite (Christ and Ordahl 1995; Cossu et al. 1996). The progress of myogenic induction can be observed by following the expression of the *paired*-type transcription factor *Pax3*. In the chick embryo, *Pax3* is initially expressed throughout the PSM (Williams and Ordahl 1994). However, early inductive influences restrict this expression such that when the epithelial somite buds off from the PSM, *Pax3* expression is restricted to the dorsal aspect of the somite. Later in development, *Pax3* transcripts are confined to the dermamyotome (Williams and Ordahl 1994). The myogenic derivatives of the dermamyotome develop from two dis-

⁶These authors contributed equally to this study.

⁷Present address: Department of Zoology, Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv, Israel.

⁸Corresponding author.

E-MAIL tabin@rascal.med.harvard.edu; FAX (617) 432-7595.

tinct regions (Ordahl and Le Douarin 1992). The epaxial cells, which form the back and intercostal muscles, arise from the medial edge of the dermamyotome (Ordahl and Le Douarin 1992). These pass under the dermamyotome and then elongate to form a new ventral layer of differentiating and postmitotic cells, the myotome (Christ and Ordahl 1995; Denetclaw et al. 1997). Myotomal cells express *Myf-5* and *MyoD*, muscle-specific basic-helix-loop-helix (bHLH) transcription factors, and this expression marks the initiation of the myogenic differentiation program (Ott et al. 1991; Pownall and Emerson 1992). The hypaxial muscle precursors, which will form the limb and ventral body wall muscles, arise from the lateral portion of the dermamyotome and migrate ventrolaterally to populate their target structures (Ordahl and Le Douarin 1992). These migrating cells continue to express *Pax3* and only turn on *MyoD* and *Myf-5* after they have reached their destination (Williams and Ordahl 1994). The expression of *MyoD* and *Myf-5* in myogenic precursors is followed by the expression of *Myogenin* and *MRF-4*, which are downstream myogenic bHLH transcription factors in differentiating myoblasts, and by the expression of genes encoding sarcomeric proteins, such as *Myosin Heavy Chain (MHC)*, during the terminal differentiation phase (Molkentin and Olson 1996).

Pax3 and the myogenic bHLH genes are not only markers of myogenic fate but also play important roles in directing cells to form muscle. Transfection of 10T1/2 fibroblasts with any of the members of the myogenic bHLH family of transcription factors can drive these cells to adopt a muscle cell fate (for review, see Weintraub et al. 1991). *Pax3* also acts to induce the muscle differentiation program in vivo (Tajbakhsh et al. 1997) and when ectopically expressed in a variety of explanted embryonic tissues, including somites (Maroto et al. 1997), although transfection of 10T1/2 fibroblasts with *Pax3* is not sufficient to induce these cells to adopt a myogenic fate (Maroto et al. 1997). This suggests that *Pax3*-induced myogenesis requires additional factors that are not present in the 10T1/2 fibroblasts but are present in somites.

On the basis of their expression patterns, several genes are candidates to be acting with *Pax3* to direct muscle development. The putative transcriptional activators *Eya1*, *Eya2*, and *Eya4* are all expressed in the dorsal epithelial somite (Xu et al. 1997; Mishima and Tomarev 1998; Borsani et al. 1999). As the somites mature, they become restricted to the medial and lateral aspects of the dermamyotome and are subsequently seen in the myotome and limb muscle precursors. Similarly, the homeodomain-containing transcription factors *Six1* and *Six4* are also expressed in the dorsal region of the developing somite and subsequently in the myotome and limb muscle precursors (Oliver et al. 1995a; Esteve and Bovolenta 1999).

Eya1, *Eya2*, *Eya4*, and *Six1* and *Six4* are homologous to the *Drosophila* genes *eyes absent (eya)* and *sine oculis (so)*, respectively, and genetic studies in that organism suggest that they may function in common pathways. Both *so* and *eya* are expressed in the developing *Dro-*

sophila eye and are required for normal eye formation (Bonini et al. 1993; Cheyette et al. 1994). In addition, *eya* has the ability to induce ectopic eyes when misexpressed (Bonini et al. 1997). The ability to induce ectopic eye formation is shared with the *Drosophila* gene *eyeless (ey)*, which encodes a transcription factor of the *Pax* family, *Pax6* (Quiring et al. 1994; Halder et al. 1995). Recent studies have begun to analyze how the functions of these various gene products are integrated in normal *Drosophila* eye development. For instance, *eya* and *so* have been shown to act synergistically downstream of *ey* to regulate the formation of ectopic eyes, and it has been demonstrated that their protein products physically interact (Pignoni et al. 1997). In addition, there are indications that a positive feedback loop exists such that *eya* and *so* regulate the expression of *ey* (Pignoni et al. 1997; Halder et al. 1998).

The fact that members of the *Pax*, *Eya*, and *Six* gene families have overlapping expression patterns in the developing somite raises the intriguing possibility that the *Pax/Eya/Six* regulatory network first identified in the context of the *Drosophila* eye may play an important role in vertebrate somitogenesis as well. If true, however, it would mean that the regulatory relationships are not limited to the direct homologs of the specific family members implicated in *Drosophila* eye development but extend to more divergent members of these gene families. For example, the only paired domain containing proteins implicated in cooperating with *eya* and *so* in *Drosophila* are *ey* and *toy*, which are both orthologs of the vertebrate gene *Pax6* (Quiring et al. 1994; Halder et al. 1995; Bonini et al. 1997; Czerny et al. 1999). *Pax6* is not, however, expressed in the developing somite (Walther and Gruss 1991). Several more distantly related *Pax* genes are expressed in the somite, and of these, only *Pax3* and *Pax7* are specific to the dermamyotome (Goulding et al. 1994; Williams and Ordahl 1994). Therefore, if *Eya* and *Six* genes are working with a *Pax* gene in the context of muscle development, it must be with one more distantly related to *ey*, for example, *Pax3* or *Pax7*.

A fourth gene, *dachshund (dac)*, has been shown to participate in the pathway regulating *Drosophila* eye development. *dac* encodes a novel nuclear protein that functions as a putative transcriptional activator and is both required for normal eye development and capable of initiating ectopic eye formation when misexpressed (Mardon et al. 1994; Shen and Mardon 1997). Genetic experiments established that *dac* and *eya* function synergistically to induce ectopic eyes (Chen et al. 1997). Furthermore, biochemical experiments have shown that *Dac* and *Eya* proteins physically interact (Chen et al. 1997). No direct synergy or protein interactions have been observed between *Dac* and *So*. Epistasis experiments have demonstrated that, like *eya* and *so*, *dac* functions downstream of *ey* and regulates *ey* in a positive feedback loop (Shen and Mardon 1997). Thus, *Drosophila* eye development is governed by a complex, integrated, regulatory network in which *ey*, *eya*, *so*, and *dac* all play key roles. Because *Eya*, *Six*, and *Pax* genes are all expressed during somite development and poten-

tially play roles in vertebrate somitogenesis, we reasoned that vertebrate homologs of *dac* might also exist and participate in this same process.

Results

Cloning of Dach2

To identify vertebrate homologs of *Drosophila dachshund*, a chick library was screened with a human EST clone that showed homology to *dachshund*. Two distinct classes of chick *Dachshund* clones were isolated, *Dach1* (T. Heanue and C. Tabin, unpubl.) and *Dach2*. Independent studies identified a murine homolog of *dachshund*, referred to as *Dach* (Hammond et al. 1998; Caubit et al. 1999; Davis et al. 1999). Sequence comparison and expression analysis, which will be presented separately, suggest that *Dach1* is the chicken homolog of mouse *Dach* and the original human EST, whereas *Dach2* represents a novel vertebrate gene. Sequence analysis of *Dach2* indicates that the predicted coding region of *Dach2* is 1.8 kb, encoding 608 amino acids (Fig. 1A). Northern blot analysis and sequence analysis indicate that the *Dach2* transcript size is ~3.0 kb (Fig. 1B). Comparison of the predicted amino acid sequence of *Dach2* with *Drosophila* *Dac* (Mardon et al. 1994) (Fig. 1A) shows two regions of high sequence conservation. These regions correspond to domains previously identified in comparisons between mouse *Dach* and *Drosophila* *Dac*, and denoted DD1/Dachbox-N and DD2/Dachbox-C (Hammond et al. 1998; Davis et al. 1999). The similarity between *Dach2* and *Drosophila* *Dac* is 86% in the DD1/Dachbox-N domain and 70% in the DD2/Dachbox-C domain.

These high levels of sequence conservation suggested that these might be conserved functional domains. Functional conservation between *Dach2* and *Drosophila* *Dac* was tested by attempting to rescue *Drosophila dac* mutant phenotypes using the *GAL4-UAS* system (Brand and Perrimon 1993). To ensure that efficient translation of *Dach2* took place in vivo, we constructed a *Drosophila dac::Dach2* fusion transgene, encoding the first 31 amino acids of the *Drosophila* *Dac* protein fused to *Dach2*. We drove expression of the *Drosophila dac::Dach2* fusion transgene, or the first 31 amino acids of *Drosophila* *Dac* alone, using a *GAL4* driver that accurately reproduces the *dac* pattern of expression (*dac-GAL4*; G. Marden, unpubl.).

The normal fly eye consists of ~800-unit eyes or ommatidia that are arranged in a precise hexagonal array (Fig. 1B,i). The adult eye develops in the larva from an epithelial monolayer termed the eye-imaginal disc. Photoreceptor differentiation proceeds in a wave of development from the morphogenetic furrow (Wolff and Ready 1991). *decapentaplegic* (*dpp*) marks the position of the furrow (Fig. 1B,iv) as it moves across the eye disc (Blackman et al. 1991). *dac* mutant adults develop with no eyes because of a failure of furrow initiation during larval stages (Mardon et al. 1994). When *Dach2* is ectopically expressed in a *dac* null mutant background using the

Drosophila dac::Dach2 fusion transgene driven by *dac-GAL4*, the mutant eye phenotype is rescued, resulting in morphogenetic furrow initiation and progression and ommatidia formation in both larvae in adults (Fig. 1B,iii,vi). The first 31 amino acids of *Drosophila* *Dac* by itself had no effect (Fig. 1B,ii,v). Targeted *Drosophila dac::Dach2* expression driven by *dac-GAL4* in a wild-type (*dac*⁺) background had no discernible effect on fly development (data not shown). These experiments show that the chick *Dach2* gene encodes a functional protein capable of compensating for *Dac* function in the *Drosophila* eye. Therefore, because vertebrate *Dach2* can apparently interact productively with the presumed target proteins of *Drosophila* *Dac*, the interacting domains of the proteins must have been maintained during the divergence of these two evolutionarily distant organisms. This suggests that the interactions themselves may be conserved in vertebrates.

Dach2 is expressed dynamically during somite development

To determine whether *Dach2* is present in the developing somite, various stage chick embryos were analyzed by in situ hybridization (Fig. 2; data not shown). Expression profiles of *Dach2* are distinct from those of *Dach1* (data not shown), indicating that the RNA probes used are specific.

Somites develop in a rostral to caudal sequence; thus, the degree of maturation of a somite depends on both the age of the embryo and the location of the somite within the embryo (Christ and Ordahl 1995). Early somites are morphologically uniform and are unpatterned along the medial-lateral axis; however, some molecular differences are apparent along the dorsal-ventral axis (Williams and Ordahl 1994; Ebensperger et al. 1995). At this stage, *Dach2* is expressed throughout the medial-lateral extent of the somite but restricted to the dorsal region (Fig. 2A,B,F; see arrow to somite I in B). *Pax3* expression is similarly restricted to the dorsal region of these early somites, throughout their medial-lateral extent (Williams and Ordahl 1994).

As the somite matures, morphological changes in the dorsal region of the somite give rise to a visibly recognizable dermamyotome (Christ and Ordahl 1995). *Dach2* is expressed throughout the medial-lateral extent of the dorsal somite and at higher levels in the lateral regions (Fig. 2A,B; see arrows to somite VI). At this same somite level, myotomal precursor cells at the medial edge of the dermamyotome begin to express *MyoD* and down-regulate *Pax3*, resulting in a more lateral restriction of *Pax3* expression (Williams and Ordahl 1994).

After the somites become patterned along both the dorsal-ventral and medial-lateral axes, the migratory population of the lateral dermamyotome begins to invade the limb bud and body wall (Christ and Ordahl 1995). *Dach2* is expressed at high levels in the medial and lateral dermamyotome of somites at the limb level at this stage and in a punctate pattern in the proximal region of the emerging limb bud (Fig. 2C,G). The myo-

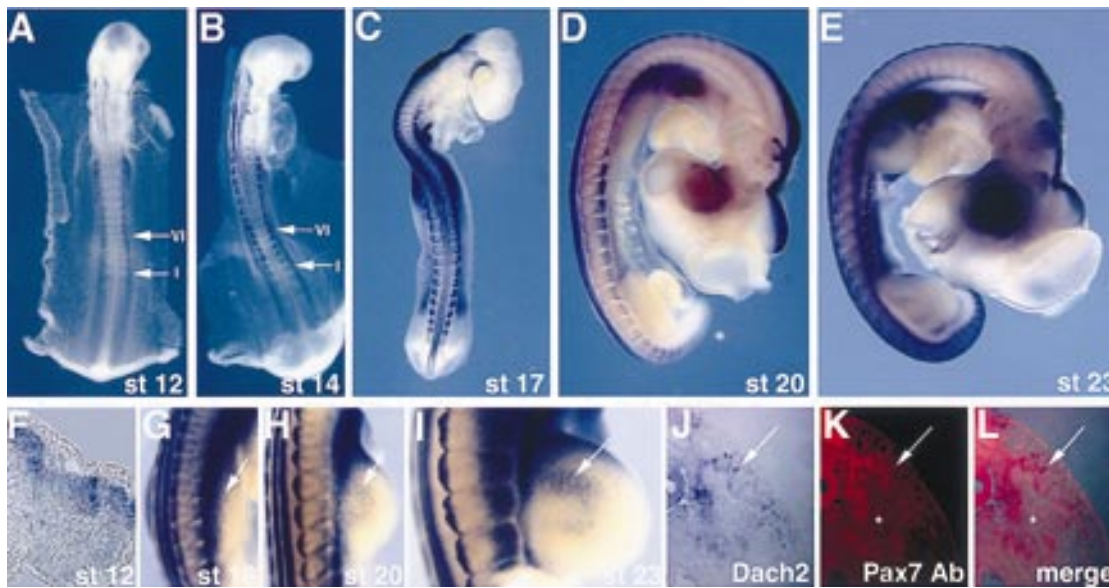


Figure 2. *Dach2* is expressed dynamically during somite development and in myoblast precursors migrating into the limb buds. (A–E, G–I) Whole-mount RNA in situ hybridization: (A) stage (st) 12 and (B) stage 14, arrows point to the newly formed somite I and the more mature somite VI; (C) stage 17; (D) stage 20; (E) stage 23; (G) stage 18 forelimb; (H) stage 20 forelimb; (I) stage 23 forelimb. Arrows in G–I point to myoblast precursors migrating into the forelimbs. (F) Section in situ hybridization on a transverse section of a stage 11 embryo at the level of somite I. (J–L) Transverse section through a stage 20 forelimb visualized for *Dach2* RNA distribution, seen as blue (J), and Pax7 protein distribution, seen as red (I). The two images are merged in K, and cells expressing *Dach2* and Pax7 overlap and are purple. Red blood cells autofluoresce, and some examples are indicated above and below the asterisk (*) in K and L.

Dach2 is regulated by signals from the ectoderm

The striking similarity in the expression patterns of *Pax3* and *Dach2* suggested that their expression might be regulated by the same signals. Dorsal somite fate and the expression of *Pax3* are dependent on signals from both the overlying ectoderm and the dorsal neural tube (Fan and Tessier-Lavigne 1994; Maroto et al. 1997; Reshef et al. 1998; Tajbakhsh et al. 1998). In the dorso-medial somite, expression of *Pax3* is regulated by the neural tube and is independent of the ectoderm (Dietrich et al. 1997). In contrast, in the dorsolateral somite, *Pax3* expression is dependent on the ectoderm. When a barrier is placed between the somite and the ectoderm, *Pax3* expression is down-regulated (Dietrich et al. 1997). To test whether *Dach2* expression in the dorsolateral somite is similarly regulated, we performed in ovo barrier experiments to separate somites from the influence of the overlying ectoderm. At stage 10–11, a barrier was placed under the ectoderm and over the PSM and somites I–III on one side of the embryo. After 24–36 hr, embryos were analyzed for gene expression. As previously reported, we observe down-regulation of lateral *Pax3* expression in the somites covered by the barrier (Fig. 4A,E) (Dietrich et al. 1997). Likewise, *Dach2* shows a dramatic down-regulation of dorsolateral expression when the somites are blocked from contacting the ectoderm (Fig. 4B,F). As previously noted, lateral *MyoD* expression is also down-regulated in the presence of such a barrier (Fig. 4C,G) (Dietrich et al. 1997). In contrast, expression of the control ventral somitic marker *Pax1* (Ebensperger et al. 1995) is expanded dorsally (Fig. 4D,H).

Moreover, analysis of these embryos in transverse sections shows a morphologically normal somite (Fig. 4E–H; data not shown).

Pax3 and *Dach2* positively regulate each other's expression

The similarities in *Pax3* and *Dach2* expression and their mutual dependence on ectodermal signals could reflect either independent regulation of the two genes or a more complex, integrated regulation. In *Drosophila* eye formation, *ey* (a *Drosophila Pax6* homolog; Quiring et al. 1994) acts in a positive feedback loop with *dac* (Shen and Marodon 1997). To test for a similar relationship between *Pax3* and *Dach2*, we used an in vitro somite culture system that we have previously shown faithfully recapitulates in vivo somite differentiation without the confounding influence of adjacent inductive tissues (Maroto et al. 1997). Somites were explanted into culture and infected with a retrovirus containing either *Pax3* or *Dach2*, and target gene expression was analyzed by RT-PCR after 5 days of culture.

As observed previously (Maroto et al. 1997), retroviral misexpression of *Pax3* in somites results in an induction of myogenic gene expression (Fig. 5A, lane 2), showing that *Pax3* regulates muscle differentiation in the somitic tissue. *Dach2* is expressed at a low level in uninfected somite cultures (Fig. 5A, lane 1); however, cultures exposed to a *Pax3* retrovirus show strong up-regulation of *Dach2* expression (Fig. 5A, lane 2). Thus, *Pax3* can positively regulate the expression of *Dach2*. In addition,

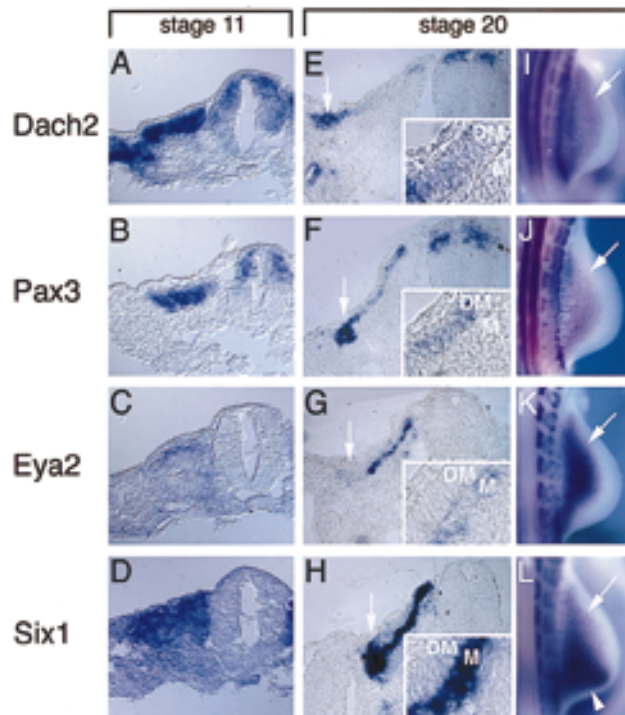


Figure 3. Expression patterns of *Dach2*, *Pax3*, *Eya2*, and *Six1* overlap in the developing somite and in limb muscle precursors. (A–H) Nonradioactive section in situ hybridizations on adjacent sections: (A–D) stage 11, adjacent transverse sections at the level of somite V; (E–H) stage 20, adjacent transverse sections at the anterior edge of the forelimb. Arrows in E–H point to the hypaxial myoblast precursors at the lateral edge of dermamyotome. (Insets) Magnifications of the dermamyotome (DM) and myotomal (M) layers of sections shown in E–H. (I–L) Whole-mount in situ hybridization of stage 20 embryos, hindlimbs. Arrows point to myoblast precursors migrating into the hindlimbs. (A,E,I) *Dach2* expression. (B,F,J) *Pax3* expression. (C,G,K) *Eya2* expression, *Eya2* probe gives weaker signals relative to other probes used. (D,H,L) *Six1* expression. Arrowhead in L points to an additional domain of *Six1* expression in the posterior limb mesenchyme.

Pax3 misexpression leads to weak *Eya2* up-regulation (Fig. 5A, lane 2), although *Six1* expression is detected at the same high level in the presence or absence of *Pax3* (Fig. 5, lanes 1,2).

To test for a possible reciprocal regulation of *Pax3* by *Dach2*, we infected similar somite cultures with a retrovirus containing *Dach2*. *Pax3* is not expressed at all in uninfected cultures. Retroviral *Dach2* misexpression in somites leads to a clear induction of *Pax3* (Fig. 5B, lane 2) when compared with the control culture (Fig. 5B, lane 1). In some experiments, weak up-regulation of myogenic genes was also seen (data not shown); however, these results were not consistently observed. These indicate that a positive regulatory feedback loop operates between *Pax3* and *Dach2* in the context of the developing somite.

Dach2, *Eya2*, and *Six1* synergistically regulate myogenic gene expression

In *Drosophila*, *dac*, *eya*, and *so* act synergistically to regulate eye development (Chen et al. 1997; Pignoni et al. 1997). Whereas *dac* and *eya* can each induce ectopic eye formation, the two genes together induce ectopic eyes at a frequency that is much greater than the sum of the rates of eye induction produced by the individual genes (Chen et al. 1997). Ectopic eyes are also induced at a higher rate when *eya* is coexpressed with *so* (Pignoni et al. 1997). Our results demonstrate that the vertebrate homologs of these genes are coexpressed in muscle precursor populations prior to muscle differentiation, suggesting that they may function together to regulate myogenesis. To test whether synergistic relationships, similar to those seen in *Drosophila* eye, exist between *Dach2*, *Eya2*, and *Six1* within the context of myogenesis, we misexpressed these genes in somite culture.

When *Dach2* is misexpressed in somite culture, low level *Pax3* expression is induced, but no myogenic gene expression is detected (Fig. 6A, lane 1; see also Fig. 5B, lane 2). Misexpression of *Eya2* in somites resulted in either trace or undetectable levels of *Pax3* and *MyoD* expression and no expression of *Myogenin* or *MHC* (Fig. 6A, lane 3, and Fig. 6B, lane 1). However, when *Dach2* and *Eya2* are misexpressed in combination, elevated levels of *Pax3* are detected, and *MyoD*, *Myogenin*, and *MHC* expression are induced (Fig. 6A, lanes 2,4). Like *Eya2*, *Six1* shows only weak *Pax3* and *MyoD* inducing ability and does not induce *Myogenin* or *MHC* (Fig. 6B, lane 3). In contrast, when *Eya2* and *Six1* are misexpressed together, a dramatic up-regulation of *Pax3*, *MyoD*, *Myogenin*, and *MHC* is seen (Fig. 6B, lanes 2,4). No synergistic up-regulation of *Pax3* or myogenic genes was observed when *Dach2* and *Six1* were misexpressed in combination (data not shown). These results demonstrate that *Dach2* and *Eya2*, as well as *Eya2* and *Six1*, act synergistically to regulate the expression of *Pax3* and the process of myogenic differentiation.

Dach2 and *Eya2*, and *Eya2* and *Six1*, physically interact

One possible mechanism for the synergistic action of *Dach2* with *Eya2* and *Eya2* with *Six1* is that the proteins function in a physical complex to regulate myogenesis. Consistent with this idea, the *Drosophila* homologs of these pairs of proteins have been shown to interact: *Dac* with *Eya* and *Eya* with *So* (Chen et al. 1997; Pignoni et al. 1997). To test whether similar physical interactions occur between the vertebrate proteins, we performed GST pull-down interaction assays.

GST-*Dach2* and GST-*Eya2* proteins were tested for their ability to interact with ³⁵S-labeled *Eya2* and *Six1* proteins, respectively. GST-*Dach2* fusion protein efficiently pulled down ³⁵S-labeled *Eya2* (Fig. 7A, lane 2), whereas control experiments showed that GST alone did not pull down any ³⁵S-labeled *Eya2* (Fig. 7A, lane 1) nor did GST fused to the first 170 amino acids of *Dach2* (Fig.

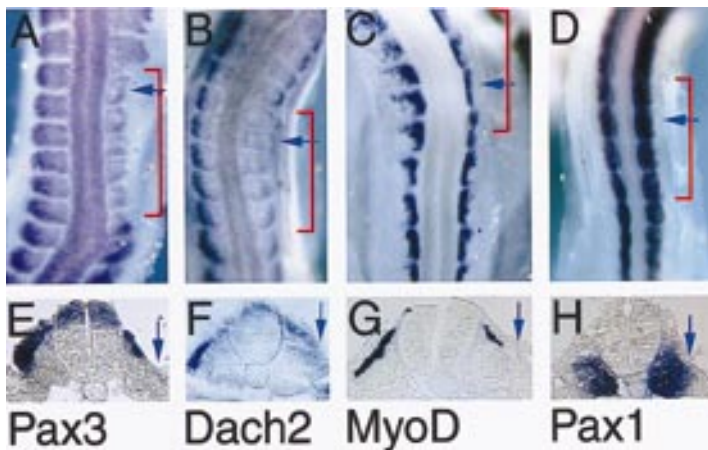


Figure 4. *Pax3*, *Dach2*, and *MyoD* are regulated by signals from the ectoderm. (A–D) Whole-mount in situ hybridizations of embryos 24–36 hr after barrier placement between the somite and the dorsal ectoderm. Embryos at stage 16–19. The region covered by the barrier is indicated with a red bracket. (E,F) Paraffin sagittal sections of the embryos shown in A–D at the position indicated by the blue arrows in A–D. (A,E) *Pax3*; (B,F) *Dach2*; (C,G) *MyoD*; (D,H) *Pax1*. Blue arrows in E–H point to the lateral edge of the somite where expression of *Pax3*, *Dach2*, and *MyoD* is lost and where *Pax1* expression is expanded.

7A, lane 3). This finding indicates that the amino 170 amino acids of *Dach2* are not sufficient to interact with *Eya2* and suggests that the interaction of *Dach2* with *Eya2* requires the carboxyl terminus of the *Dach2* protein. In a similar assay, GST–*Eya2* fusion protein was found to interact with ³⁵S-labeled *Six1* protein (Fig. 7A, lane 6), whereas the control GST alone showed no interaction with *Six1* protein (Fig. 7A, lane 5). The GST pull-down experiment was also used to assay potential *Dach2* and *Six1* interactions; however, no interactions were detected (data not shown). This finding is consistent with our failure to detect synergistic gene regulation by *Dach2* and *Six1* in somite cultures (data not shown).

A second method for determining the ability of proteins to physically interact is the GAL4 yeast two-hybrid system. In this system, “baits” are constructed as fu-

sions with a GAL4 DNA-binding domain capable of binding to a UAS target sequence upstream of a *lacZ* reporter. “Preys” are constructed as fusions with the GAL4 transcriptional activation domain. Thus, in vivo interactions between bait and prey will result in *lacZ* transcription.

We were unable to use this approach to verify that *Dach2* interacts with *Eya2* because both of these genes induce *lacZ* expression by themselves when expressed in a bait construct (data not shown). In contrast, we were able to use the GAL4 yeast two-hybrid system to confirm the *Eya2*/*Six1* interaction seen with GST pull-downs. Transformation of *Six1* bait construct (containing the *Six* domain of *Six1*; Oliver et al. 1995b) with either *Drosophila* *Eya* domain prey construct (Chen et al. 1997) or *Eya2* prey construct led to very strong *lacZ* expression, relative to the prey constructs transformed alone (Fig. 7B). To test the specificity of this interaction, we performed the same experiment with *Six3*. *Six3* is another member of the *Six* family (Oliver et al. 1995b), but it is the direct homolog of *Drosophila optix*, not of *so* (Toy et al. 1998). However, no activation of *lacZ* expression was seen when a *Six3* bait construct was transformed with the *Eya2* prey construct (Fig. 7B).

The results of our GST pull-down and yeast two-hybrid assays demonstrate conservation of protein interaction domains from flies to vertebrates and suggest that these conserved interactions might be responsible for their synergistic regulation of *Pax3* and myogenesis.

Discussion

Conserved domains in *Dach2*

The predicted *Dach2* amino acid sequence is highly similar to *Drosophila* *Dac* (Mardon et al. 1994) and to mouse *Dach* (Hammond et al. 1998; Caubit et al. 1999; Davis et al. 1999) in two domains denoted DD1/*Dachbox-N* and DD2/*Dachbox-C* (Hammond et al. 1998; Davis et al. 1999). These domains of the *Dach* proteins show some sequence similarities to domains in the proto-oncogene *c-ski* and the *c-ski*-like gene *sno* (Hammond et al. 1998; Davis et al. 1999). Mammalian *c-ski* is

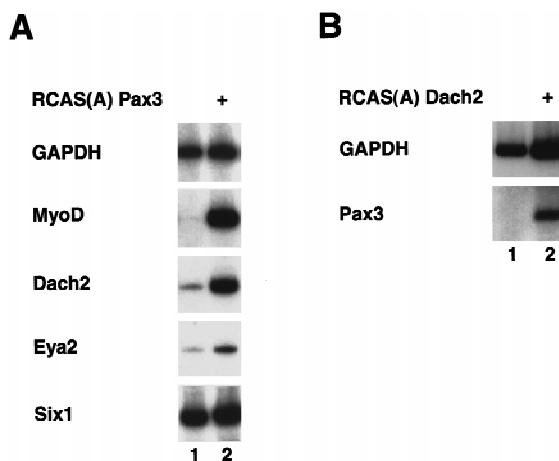
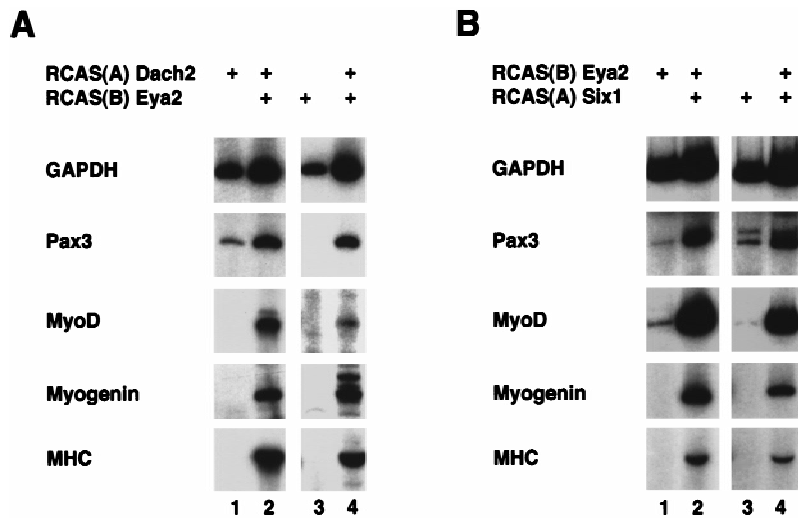


Figure 5. Misexpression of *Pax3* and *Dach2* in somite explant culture reveals a positive regulatory feedback loop between these two genes. (A) Misexpression of *Pax3*. (Lane 1) Somites cultured alone; (lane 2) somites cultured with *Pax3* retrovirus (+). Cultures analyzed by RT-PCR for *GAPDH*, *MyoD*, *Dach2*, *Eya2*, and *Six1*. (B) Misexpression of *Dach2* retrovirus. (Lane 1) Somites cultured alone; (lane 2) somites cultured with *Dach2* retrovirus (+). Cultures analyzed by RT-PCR for *GAPDH* and *Pax3*. (+) The addition of a retrovirus to the culture.

Figure 6. Misexpression of *Dach2* and *Eya2*, and *Eya2* and *Six1* in somite explant culture results in synergistic regulation of *Pax3* and myogenic marker genes. (A) Misexpression of *Dach2* and *Eya2* in somite explant culture. (Lane 1) Somites cultured with RCAS(A) *Dach2* alone; (lanes 2,4) somites cultured with RCAS(A) *Dach2* and RCAS(B) *Eya2*; (lane 3) somites cultured with RCAS(B) *Eya2* alone. Cultures analyzed by RT-PCR for *GAPDH*, *Pax3*, *MyoD*, *Myogenin*, and *MHC*. (B) Misexpression of *Eya2* and *Six1* in somite explant culture. (Lane 1) Somites cultured with RCAS(B) *Eya2* alone; (lanes 2,4) somites cultured with RCAS(B) *Eya2* and RCAS(A) *Six1*; (lane 3) somites cultured with RCAS(A) *Six1* alone. Cultures analyzed by RT-PCR for *GAPDH*, *Pax3*, *MyoD*, *Myogenin*, and *MHC*. (+) The addition of a retrovirus to the culture.



required for normal muscle development (Berk et al. 1997), and both *c-ski* and chicken *SnoN* have the ability to induce myogenic differentiation (Boyer et al. 1993; Zheng et al. 1997a). Ski and Sno are thought to function as dimers, and although they do not directly contact DNA, they are thought to act through alternate participation in either repressor or activator complexes (Nomura et al. 1999). The helical domain of Ski/Sno is required for dimerization, and is required for the complete activity of the *v-ski* oncogene to transform fibroblasts and induce myogenic differentiation (Nagase et al. 1993;

Zheng et al. 1997b). The predicted tertiary structure of the DD2 domain of Dach2 protein indicates that this domain could, like Ski and Sno, form an α -helical coiled-coil structure (data not shown). The sequence and protein structure similarities between Dach2 and Ski/Sno raises the intriguing possibility that these proteins are acting in similar ways to regulate myogenesis.

Cross-regulation between Pax3 and Dach2

The expression profile of *Dach2* is identical to that of

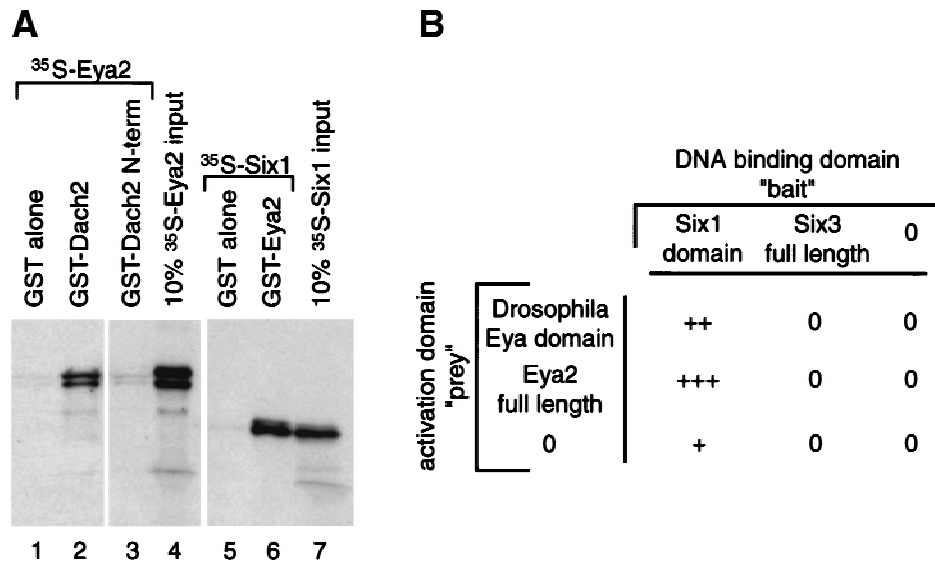


Figure 7. Interaction assays to reveal interactions between Dach2 and Eya2, and Eya2 and Six1 proteins. (A) GST pull-down interaction assays. (Lanes 1–4) Analysis of Dach2–Eya2 interactions. GST alone (lane 1), GST–Dach2 full-length protein (lane 2), and GST–Dach2 amino-terminal fragment (N-term) (lane 3) were tested for their ability to pull down radiolabeled Eya2 protein. (Lanes 5–7) Analysis of Eya2–Six1 interactions. GST alone (lane 5) and GST–Eya2 full-length protein (lane 6) were tested for their ability to pull down radiolabeled Six1 protein. Strength of the interactions can be assessed by comparing the amount of protein pulled down in the test lanes (lane 1–3 and 5–6, respectively) to the amount in the 10% input lane (lanes 4 and 7, respectively). (B) Yeast two-hybrid interaction assays. Cotransformants were analyzed for their ability to show *lacZ* color reaction. Strength of interactions were judged by the amount of time passed before a color reaction was observed: (0) no staining after 4 hr; (+++) staining in <60 min.

Pax3 during somitogenesis (Williams and Ordahl 1994). We have shown in barrier experiments that control of lateral *Dach2* expression is regulated by the dorsal ectoderm. Because *Dach2* transcripts are already present in the somites at the time of barrier placement, the experiments demonstrate a requirement for the ectoderm for maintenance of *Dach2* expression, perhaps mediated by *Pax3*. The dorsal ectoderm may also be responsible for the initial induction of *Dach2* expression.

Several secreted factors present in the dorsal ectoderm are candidates for the ectodermal signal that induces and maintains expression of *Pax3* and *Dach2*. Several *Wnt* genes, including *Wnt4* and *Wnt6* (Parr et al. 1993), are present at the correct time and place to be transmitting this signal. Moreover, these and other *Wnt* genes have been shown to induce *Pax3*, *MyoD*, and *Myf-5* expression in cultured somites (Munsterberg et al. 1995; Fan et al. 1997; Maroto et al. 1997; Tajbakhsh et al. 1998). It is interesting to note that in the *Drosophila* leg imaginal disc, *dac* gene expression is regulated, in part, by *wg* (the *Drosophila* homolog of vertebrate *Wnt* genes; Lecuit and Cohen 1997).

Not only are *Dach2* and *Pax3* under similar regulatory control by the ectoderm, but these two genes also participate in a positive regulatory feedback loop. Because *Pax3* is expressed in the PSM prior to *Dach2* expression (Williams and Ordahl 1994; data not shown), it is possible that the feedback loop is initiated by *Pax3*.

Our analysis of the cross-regulation between *Pax3* and *Dach2* was performed in vitro. We were unable to verify these results in vivo (data not shown), consistent with previous negative results with *Pax3* misexpression in vivo (A.B. Lassar, unpubl.). This is likely due to the buffering influence of other factors present in vivo. An alternative approach to working in vitro would be to remove some of the confounding influences in vivo. For example, the dorsal ectoderm can be physically separated from the underlying somite with a barrier before performing misexpression experiments, thus removing the normal regulatory influence of this tissue. Preliminary experiments, using such conditions, suggest that the regulatory interactions between *Pax3* and *Dach2* can indeed be observed in vivo (data not shown).

It is clear that additional regulators of *Dach2* expression must exist elsewhere in the embryo because *Dach2* is expressed in domains that do not overlap with *Pax3* expression, including the developing urogenital system. However, another *Pax* gene, *Pax2*, is expressed in the nephrogenic duct and is a plausible candidate for regulating *Dach2* expression (Dressler et al. 1990). Moreover, mouse *Dach2* (isolated in a screen using the chick *Dach2* probe; G. Mardon, unpubl.) is expressed normally in *Pax3* (*Splotch*; Epstein et al. 1991) mutant mouse embryos (T. Heanue and C. Tabin, unpubl.), indicating that *Pax3* is not the sole regulator of *Dach2*. However, it is possible that *Pax7*, which is up-regulated in the dermamyotome of *Splotch* mutants (Borycki et al. 1999), compensates for the loss of *Pax3* expression.

The regulatory feedback loop between *Pax3* and *Dach2* is analogous to the regulatory relationship seen

between *ey* and *dac* in *Drosophila*. However, *Pax3* is not the vertebrate ortholog of *ey* (Quiring et al. 1994). The fact that over evolutionary time, two *Pax* genes apparently substituted for one another in regulating a *Dach* gene could be based on recognition of a common *Pax* binding site upstream of the *Dach* gene.

Dach2 and Eya2, and Eya2 and Six1 act synergistically

In addition to *Dach2* and *Pax3*, we identified *Eya2* and *Six1* as important regulators of myogenesis. We have shown that *Pax3*, *Dach2*, *Eya2*, and *Six1* expression patterns overlap in the early dorsal somite and in limb muscle precursors. Both of these cell populations are composed of undifferentiated muscle precursor cells (Christ and Ordahl 1995), and neither population has begun to express markers of progressive muscle differentiation such as *MyoD*, *Myf-5*, *Myogenin*, or *MHC* (Christ and Ordahl 1995). *Pax3*, *Dach2*, *Eya2*, and *Six1* are expressed in a manner consistent with their working cooperatively, possibly setting the stage for myogenic differentiation. However, once further myogenic differentiation occurs (e.g., after myotome formation), the genes display different expression patterns and may function with other partners and on other targets.

Pax3 can induce myogenesis in cultured somites (Maroto et al. 1997). However, misexpression of *Dach2* on its own in somite cultures only rarely induced low level myogenic gene expression (data not shown). This result could mean that *Dach2* is a weak muscle inducer or that induction of myogenic genes was an indirect effect of inducing *Pax3*, which in turn induced *MyoD*. In either case, the lower level of induction of myogenic genes by *Dach2* relative to *Pax3* parallels the different potencies of the *Drosophila* homologs *dac* and *ey* to induce ectopic eyes: *ey* has a greater ability to induce ectopic eye formation than *dac* (Shen and Mardon 1997).

The ability of *dac* to induce ectopic eyes is potentiated when *dac* is misexpressed in combination with *eya* (Chen et al. 1997), and the ability of *eya* to induce ectopic eyes is also potentiated by *so* (Pignoni et al. 1997). We have found that the vertebrate homologs of *dac*, *eya*, and *so* have similar abilities to regulate myogenesis in a synergistic manner. For instance, *Dach2* and *Eya2* synergistically regulate both *Pax3* and myogenic gene expression, and indeed the proteins physically interact. However, because neither protein is capable of binding to DNA (Bonini et al. 1993; Mardon et al. 1994), this complex presumably interacts with a protein that binds to DNA. One possible candidate is *Six1*, which is present in explanted somites in the absence of any extrinsic factors (see Fig. 5A, lane 1). However, it has yet to be demonstrated that these three proteins are acting as a single protein complex, and they could alternatively have other partners.

Eya2 and *Six1* also synergize to regulate the expression of target genes and physically interact. Interaction between *Eya* and *Six* proteins has also been observed in recent independent studies (Ohto et al. 1999). Although *Six1* is normally present in somite cultures, endogenous

levels are apparently not high enough to induce *Pax3* or the myogenic genes when *Eya2* is misexpressed alone. However, when *Eya2* and *Six1* are ectopically expressed together, levels of *Six1* are considerably higher, allowing for induction of *Pax3* and myogenic genes.

Dach2, *Eya2*, *Six1* action in myogenesis

Dach2, *Eya2*, and *Six1* function in complexes to regulate *Pax3*, *MyoD*, *Myogenin*, and *MHC*. However it is still not known whether any of these genes are direct transcriptional targets of these complexes. Recent findings have shown that *Six1* and *Six4* are able to bind upstream of the *Myogenin* promoter, thereby regulating *Myogenin* gene expression (Spitz et al. 1998). More recently, it has been shown that *Six* and *Eya* proteins act synergistically to regulate this promoter (Ohto et al. 1999). Thus, at least one myogenic bHLH transcription factor is a direct target of *Six* and *Eya* proteins and is, therefore, a potential target of various *Eya*–*Six* or *Dach*–*Eya*–*Six* transcriptional complexes. Future experiments will determine the precise roles these proteins play in regulating the expression of the downstream myogenic regulatory genes.

Materials and methods

Cloning of *Dach2*

A human retinal cDNA (IMAGE Consortium cDNA clone, ID 381801; Lennon et al. 1996) was identified as sharing homology to *Drosophila dachshund* by a TBLASTN screen of the *Drosophila dachshund* sequence against the dbEST database and was obtained from Research Genetics, Inc. (Huntsville, AL). A stage 12–15 embryonic chick cDNA library cloned in λ ZAPII was screened with a 600-bp *SmaI*–*EcoRI* fragment of 381801. Filters were hybridized in 20% formamide, 10% dextran sulfate, 2 \times SSC, and 1% SDS at 42°C overnight and washed in 2 \times SSC, 1% SDS at 52°C. Positive clones were sequenced and fell into two classes, one more closely related to the EST clone representing *Dach1* (T. Heanue and C. Tabin, unpubl.) and a second representing *Dach2*. Several overlapping clones were used to construct a full-length *Dach2* clone, *Dach2-L*. *Dach2-L* was found to encode an ~1.8-kb open reading frame, 50 bp of 5' UTR, and 1.1 kb of 3' UTR. This sequence has been submitted to GenBank (accession no. AF198349). No upstream stops were identified 5' to the putative ATG; however, the nucleotide sequence surrounding the ATG is found to be a strong context Kozak sequence: GCCatgG (Kozak 1996). Northern blot analysis was performed using standard methods and using stage 22 embryo total RNA and a 800-bp 3' fragment of *Dach2-L* as a probe. This analysis revealed a *Dach2* transcript size of ~3 kb, further indicating that the complete coding region has been identified.

Fly genetics

Drosophila crosses were performed at 25°C on standard media. The *Drosophila dac::Dach-2* fusion transgene encodes the first 31 amino acids of *Drosophila* *Dac* and the last 556 amino acids of *Dach2* (named pUAS-DD31::CD556). This was constructed using a 700-bp *EcoRI*–*SacII* fragment from *Drosophila dac* cDNA p2-2 and a 1.9-kb *SacII* fragment from *Dach2* cDNA

p2c cloned into an *EcoRI*–*SacII* digested pUAST vector (Brand and Perrimon 1993). The 700-bp *EcoRI*–*SacII* fragment from *Drosophila dac*, which produces a truncated protein containing only the first 31 amino acids of *Drosophila* *Dac*, was used as a negative control (named pUAS-DD31). Flies were transformed using standard techniques (Spradling and Rubin 1982; Rubin and Spradling 1983). Five independent transformants for each construct were analyzed by driving expression using either *dpp-GAL4* (Staebling-Hampton and Hoffmann 1994) or *dac-GAL4* (to be described elsewhere). No ectopic expression phenotypes were observed for either construct when driven by *dac-GAL4*, and pUAS-DD31 had no phenotype when driven by *dpp-GAL4*. In contrast, *dpp-GAL4* driven pUAS-DD31::CD556 results in leg truncations reminiscent of that caused by misexpression of full-length *Drosophila dac* (Chen et al. 1997). Rescue of *dac* null mutant animals was performed as follows: *dac*³, *UAS-DD31::CD556/CyO*, *Kr-GFP;dpp-lacZ/+* flies were crossed to *dac-GAL4/CyO*, *Kr-GFP;dpp-lacZ/+* flies and *dac*³, *UAS-DD31::CD556/dac-GAL4;±dpp-lacZ* animals were selected as non-*GFP* larvae or non-*CyO* adults. Similar experiments were performed with the truncated form of the *Drosophila* *Dac* protein alone except that the transgene was located on the X chromosome.

Scanning electron microscopy and immunohistochemistry

Samples were prepared for scanning electron microscopy as described previously (Kimmel et al. 1990). Imaginal discs were dissected and stained with anti-ELAV (Robinow and White 1991) as described previously (Mardon et al. 1994). *dpp* expression was detected using a β -galactosidase reporter construct specific for imaginal discs (Blackman et al. 1991).

Chick embryos

Fertilized White Leghorn chicken embryos were obtained from SPAFAS (Norwich, CT). Embryos were staged according to Hamburger and Hamilton (1951). Somites were staged according to established nomenclature (Christ and Ordahl 1995).

Whole-mount and section RNA and Ab in situ hybridization

Whole-mount in situ hybridization and section in situ hybridization with nonradioactive and [³³P]UTP probes were performed as described previously (Riddle et al. 1993; Vortkamp et al. 1996; Bao and Cepko 1997). Probe templates were *Dach2* (*cd2c*, *SalI* digest, T3 polymerase), *Pax3* (CHPax3, *BamHI*, T3), *Eya2* (*cEya2*, *EcoRI*, T3), *Six1* (*chLZ54/x*, *SacI*, T3), *MyoD* (*pCMDmyoD*, *HindIII*, T7), and *Pax1* (QP1, *HindIII*, T7). For double labeling experiments with *Pax7*, embryos processed by whole-mount in situ hybridization were paraffin-sectioned. Slides were washed with PBS, blocked with 5% goat serum/PBS for 1 hr, and then incubated in mouse monoclonal *Pax7* antibody (Developmental Studies Hybridoma Bank), diluted 1:10, at 4°C overnight. After PBS washes, slides were incubated in Cy3 goat anti-mouse, diluted 1:200, for 1 hr at room temperature, and finally washed again in PBS.

Embryo surgery for barrier placement

Embryo surgeries were performed essentially as described (Dietrich et al. 1997). Fifteen-micron-thick cellophane barriers were inserted under the ectoderm overlapping the paraxial mesodermal tissues at the level of PSM or from somites number I–VI (Christ and Ordahl 1995) of stage 10–12 embryos. Embryos were incubated for an additional 24–36 hr, fixed in 4% para-

formaldehyde, and analyzed by whole-mount RNA in situ hybridization. After photographing, the embryos were paraffin sectioned (10 μ m thick) and rephotographed.

RCAS virus construction

Generation of viral constructs and production of high titer virus followed the protocols described previously (Logan and Tabin 1998). The *Pax3* viral construct was described previously (Maroto et al. 1997). cDNAs encoding the entire open reading frames of chick *Dach2*, chick *Eya2*, and mouse *Six1* were cloned in-frame with the initiator ATG of the pSLAX-13 shuttle vector, and transferred as *Clal* fragments to both the RCAS(A) and RCAS(B) retroviral vectors. Retroviral titers ranged from 4×10^8 to 1×10^9 CFU/ml.

Explant culture

Embryonic tissues were isolated and cultured as described (Munsterberg et al. 1995). Coculture of paraxial tissues with various RCAS constructs was performed as described (Maroto et al. 1997). When RCAS type B envelope was used, 8 μ g/ml of polybrene was added to the medium to increase infection. Medium (500 ml) was added to the collagen cultures after overnight incubation with 35 μ l of medium plus virus. The cultures were incubated for 5 days and analyzed by RT-PCR.

RT-PCR analysis

RT-PCR analysis was performed essentially as described (Munsterberg et al. 1995; Maroto et al. 1997). After production of cDNA by reverse transcriptase, single PCR reactions were performed with appropriate primer pairs for the designated genes. After individual PCR reactions were run, radiolabeled PCR transcripts were visualized by gel electrophoresis and autoradiography. Each PCR cycle was 93°C for 30 sec, 60°C for 35 sec, and 72°C for 1 min. *Pax3*, *Dach2*, and *Six1* were amplified in the presence of 5% formamide with an annealing temperature of 50°C for *Dach2*, 52°C for *Pax3*, and 55°C for *Six1*. Twenty-five cycles were used to assay GAPDH, and 30–33 cycles to assay other genes. The primers used for PCR amplification were as described (Munsterberg et al. 1995; Maroto et al. 1997) and as follows: *Dach2*, 5'-CGCCATTTCTTTTGCTGAT and 3'-CGCCTGTTCCACTTGTCTC (308 bp); *Eya2*, 5'-ACATAGAAGGCAACAGTAAAG and 3'-TGGGATGGCTGAAGGGCTGAT (497 bp); *Six1*, 5'-TTCCGCTTCACGCAGGAGCAG and 3'-CCTCCGCCGCCGGTCCCGCT (500 bp). The specificity of the PCR reactions was verified for these primers by restriction mapping of the PCR products.

GST pull-down

GST pull-down interaction assays were performed essentially as described (Pearse et al. 1999). Full-length *Dach2* and *Eya2* were cloned into the bacterial expression vector pGEX-KG vector to fuse GST to the *Dach2* and *Eya2* proteins. Also, a 5' fragment of *Dach2* corresponding to the first 170 amino acids was cloned into pGEX-KG. Recombinant proteins were purified from induced cultures and bound to glutathione resin as described. Full-length ³⁵S-labeled radioactive test proteins were generated using the TnT Rabbit Reticulocyte Lysate System (Promega) using T7 polymerase for the *Eya2* template pSlax-Eya2AS and using T3 polymerase for the *Six1* template pSlax-Six1. Proteins were analyzed on SDS-PAGE gels prior to performing interaction assays. Stringent conditions for interaction assays followed those used previously to test interactions between the homolo-

gous *Drosophila* proteins (Chen et al. 1997). ³⁵S-labeled *Eya2* and *Six1* proteins (50,000 TCA-precipitable cpm) were incubated with 50 μ l of a 50:50 slurry of glutathione resin containing bound GST, GST::Dach2, GST::Dach2-N-term, or GST::Eya2 in binding buffer [20 mM HEPES-KOH at pH 7.7, 150 mM NaCl, 0.1% NP-40, 10% glycerol, and 1 \times Complete protease inhibitors (Boehringer)] for 2 hr at 4°C. Resins were washed four times in 1 ml of binding buffer before elution by boiling in loading buffer and loading onto SDS-PAGE gels. After running, staining, and fixing, the gels were treated with Enlightening (NEN Life Sciences) to enhance the radioactive signal. Radioactive test proteins were visualized by autoradiography and were detectable after 6 hr. Intensity of the bands was compared with a 10% input lane.

Yeast two-hybrid

The MATCHMAKER Gal4 two-hybrid system (Clontech Laboratories, Inc.) was used for yeast interaction assays. Full-length chick *Eya2* and the *Drosophila Eya* interaction domain were cloned into the GAL4 activation domain vector (pACT2). The Six domain of mouse *Six1* (from the fourth amino acid of the protein to the second amino acid of the homeodomain) and full-length *Six3* were cloned into the GAL4 DNA-binding domain vector (pAS2-1). The Six domain has been shown to be the interaction domain of the *Drosophila* So protein (Pignoni et al. 1997). Small scale LiAc cotransformations of the plasmid DNAs into Y190 cells were performed as outlined in the Clontech protocols. β -Galactosidase colony-lift filter assays were performed on double transformants as described and incubated at 30°C. Strong positives were visible after 30–60 min, whereas weaker positives showed staining after 3 hr.

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