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4 The *Pax/Six/Eya/Dach* Network in Development and Evolution

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Introduction

An emerging theme in developmental and evolutionary biology is the conservation of networks of regulatory genes working together during the development of a wide range of metazoan taxa. In many cases, these networks of genes, often referred to as regulatory cassettes, are used in conserved processes for the development of homologous structures. However, these cassettes are often deployed in different temporal and spatial developmental contexts and expanded to include different gene family members and downstream targets. In fact, such modification of regulatory cassettes may be an important mechanism for generating evolutionary novelty.

In this chapter, we examine one evolutionarily conserved cassette of transcriptional regulators. The network of *eyeless* (*ey*), *sine oculis* (*so*), *eyes absent* (*eya*), and *dachshund* (*dac*) was first identified in *Drosophila* as a critical regulator of eye development (reviewed in Wawersik and Maas 2000). Their respective homologues, the *Pax, Six, Eya,* and *Dach* genes, have also been found in vertebrates (reviewed in Wawersik and Maas 2000) and are coexpressed in a variety of developmental contexts, including the developing eye. The expression patterns of these genes often overlap in a manner suggesting that these genes may indeed be functioning as a network and implying that this network has acquired new functions in vertebrate development. Recently, Heanue and colleagues (Heanue et al. 1999) have demonstrated that the *Pax/Six/Eya/Dach* network plays a critical role in myogenesis. Data from several labs also indicate that this network is important for eye and ear development (Torres et al. 1996; Xu et al. 1999; reviewed in Wawersik and Maas 2000). Comparison of the networks used during vertebrate myogenesis, eye and ear development reveals that different mem-

bers of the *Pax, Six, Eya,* and *Dach* gene families have been employed and that they are differently regulated. The evolutionary expansion and inclusion of other members of these four gene families appears to have been critical for the deployment of the cassette in novel developmental processes. Not only are the new family members expressed in different temporal and spatial contexts, but also they interact with different partner proteins and activate different downstream targets.

The *Pax, Six, Eya,* and *Dach* Gene Families

The *Pax/Six/Eya/Dach* network is composed of interactions between four different families of genes. Two of the families, the *Pax* and *Six* genes, encode DNA-binding transcription factors, while the other two families, the *Eya* and *Dach* genes, encode transcriptional coactivators. With the evolution of vertebrates, all four gene families have expanded, and new family members have been co-opted into the *Pax/Six/Eya/Dach* network.

The *Pax* genes constitute a large and relatively diverse family of transcription factors (reviewed by Miller et al. 2000). *Pax* genes are defined by the presence of a paired domain, a 128-amino-acid DNA-binding domain. In addition, some *Pax* genes contain a complete or partial homeodomain and/or a distinctive octapeptide motif. Based on a comparison of domain structure and sequence, *Pax* genes are divided into four classes, *PaxA–PaxD*. In *Drosophila,* there are single members of the *A* and *B* classes, but two members of the *C* and three members of the *D* family (fig. 4.1). In vertebrates, no members of the *A* class exist. However, there has been an expansion of the other classes: there are three members of the *B* class, two members of the *C* class, and four members of the *D* class (Miller et al. 2000; fig. 4.1).

The *Six* genes comprise a family of transcription factors that contain a homeodomain and a Six domain (reviewed by Kawakami et al. 2000). The homeodomain is unique because it lacks two highly conserved amino acid residues typical of most homeodomains. Both the Six domain and the homeodomain are necessary for specific DNA binding. In addition to its DNA-binding role, the Six domain is essential for binding to Eya proteins (Pignoni et al. 1997). Members of the *Six* family have been divided into three subfamilies based on the lengths of the region C-terminal to the homeodomain (Kawakami et al. 2000). In *Drosophila,* each of the subfamilies contains one family member. In vertebrates, the *Drosophila sine oculis, optix,* and *six4* subfamilies have been expanded to include two orthologues each (Kawakami et al. 2000; fig. 4.1).

The *Eya* genes constitute a family of transcriptional coactivators, each of which contains an Eya domain. The Eya domain is a highly

Drosophila	Vertebrate
PaxA: pox neuro	
PaxB: sparkling	*Pax 2,5,8*
PaxC: twin of eyeless	*Pax 4,6*
eyeless	
PaxD: pox meso	*Pax 1,9*
gooseberry	
gooseberry neuro	*Pax 3,7*
paired	
sine oculis	*Six 1,2*
optix	*Six 3, Optx 2*
six 4	*Six 4,5*
eyes absent	*Eya 1,2,3,4*
dachshund	*Dach 1,2*

Fig. 4.1.—Comparison of the members of the *Pax, Eya, Six,* and *Dach* genes found in *Drosophila* and vertebrates. The *Pax* and *Six* families contain several subfamilies. The *Pax* family is divided into *PaxA, PaxB, PaxC,* and *PaxD* subfamilies. The *Six* family is divided into the *Six1, Six2, Six3,Optx2,* and *Six4,5* subfamilies. See references in text.

conserved region at the C-terminus of the Eya proteins (Xu et al. 1997b). This domain has been shown to be the site of protein-protein interactions between the *Drosophila* Eya and So proteins and between Eya and Dac proteins (Pignoni et al. 1997; Chen et al. 1997). At the N-terminus of Eya proteins is a nonconserved proline-, serine-, threonine-rich region capable of functioning as a transcriptional activator (Xu et al. 1997a). Eya proteins do not contain known DNA-binding motifs, suggesting that Eya must act in concert with DNA-binding proteins to regulate transcription (Wawersik and Maas 2000). In *Drosophila,* there is a single *eyes absent* gene, and in vertebrates, the family has expanded to include four members (Bonini et al. 1993; Borsani et al. 1999; Xu et al. 1997a; fig. 4.1).

The other family of transcriptional coactivators in the network is the *Dach* genes. Two regions of high similarity, an N-terminal region termed DD1/Dachbox-N and a C-terminal region termed DD2/Dachbox-C, have been identified in all known members of the Dach

family (Hammond et al. 1998; Davis et al. 1999). The N-terminal domain has been shown to be necessary for transcriptional activation in yeast (Chen et al. 1997), while the C-terminus has been demonstrated to be critical for binding to Eya proteins in both *Drosophila* and chick (Chen et al. 1997; Heanue et al. 1999). Because there is no known DNA-binding domain in Dach proteins, the transcriptional activation function of Dach must be mediated by interactions with DNA-binding proteins. In *Drosophila,* there is a single *dachshund* gene, and in vertebrates, the family has expanded to two members (Mardon et al. 1994; Hammond et al. 1998; Davis et al. 1999; Heanue et al. 1999; Kozmik et al. 1999; Caubit et al. 1999; fig. 4.1).

Drosophila Eye Development

The *Drosophila* eye consists of a hexagonal array of approximately 750 ommatidia, each containing photoreceptor and accessory cells (reviewed in Wolff and Readt 1993). The eye develops from a small number of cells set aside in the eye imaginal disc (Younoussi-Hartenstein et al. 1993). During the third instar of larval development, ommatidia are generated as a wave of differentiation, known as the morphogenetic furrow, moves from posterior to anterior across the eye disc (Tomlinson and Ready 1987). Anterior to the furrow, cells are undifferentiated, whereas posterior to it, cells are recruited into ommatidia and differentiate into photoreceptors. The initiation and propagation of the morphogenetic furrow is necessary for the proper formation of ommatidia, and the *eyeless/sine oculis/eyes absent/dachshund* network is essential for this process.

The importance of the *ey, so, eya,* and *dac* genes for *Drosophila* eye development was revealed through both loss- and gain-of-function studies. In *ey, so, eya,* and *dac* mutants, the eye anlagen initially form normally. However, during the third instar the eyes fail to develop in all four mutant backgrounds because of lack of morphogenetic furrow initiation and massive cell death in the eye disc (Quiring et al. 1994; Halder et al. 1998; Cheyette et al. 1994; Bonini et al. 1993; Mardon et al. 1994). Thus, each of these four genes is necessary for proper eye development. Conversely, gain-of-function studies have shown that these genes are also sufficient to initiate eye development. In particular, targeted misexpression of *ey, eya,* or *dac* in the antennal imaginal disc induces the formation of ectopic eyes (Halder et al. 1995; Bonini et al. 1997; Shen and Mardon 1997). Interestingly, these genes are not equally potent inducers of ectopic eyes: *ey* is capable of inducing large ectopic eyes with high frequency, while *eya* and *dac* induce smaller eyes and at a much lower frequency.

Consistent with their role in eye development, all four genes are expressed in the developing eye. *ey* is expressed first in the earliest eye anlagen and subsequently becomes restricted to the cells anterior to the morphogenetic furrow (Quiring et al. 1994). *so* and *eya* have similar patterns of expression (Cheyette et al. 1994; Bonini et al. 1993). Prior to morphogenetic furrow formation, both are expressed along the posterior and lateral edges of the eye disc with decreasing levels towards the central region. After morphogenetic furrow propagation, the two genes are expressed anterior to, within, and posterior to the furrow. Prior to morphogenetic furrow formation, *dac* is expressed at the posterior margin of the eye, and subsequently, during furrow propagation, it becomes restricted to cells anterior to the furrow (Mardon et al. 1994).

The initial expression and regulation of *ey, so, eya,* and *dac* is primarily linear (fig. 4.2, *A*). *ey* is the earliest expressed component of the

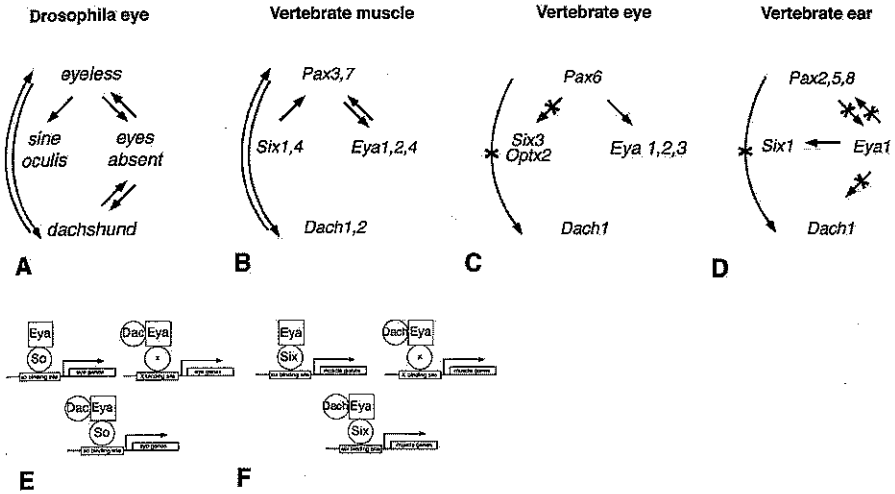

Fig. 4.2.—*A–D,* Regulatory relationships between members of the *Pax/Six/Eya/Dach* network acting during the development of the *Drosophila* eye, vertebrate muscle, eye, and ear. *A,* The known sufficient interactions between *ey, so, eya,* and *dac* in the *Drosophila* eye as established by experiments ectopically misexpressing these genes. Not shown are the necessary interactions between these genes as established by analysis of mutants. Analysis of mutants has determined that *so, eya,* and *dac* are not necessary for *ey* expression and that *dac* is not necessary for *eya* expression. In addition, mutant analysis has determined that *ey* is necessary for *so* and *eya* expression, *eya* is necessary for *so* and *dac* expression, and *so* is necessary for *eya* expression. *B,* The known sufficient interactions between members of the network in vertebrate muscle as determined by ectopic misexpression studies in the chick using *Pax3, Six1, Eya2,* and *Dach2.* Not indicated is the fact that *Pax3* is not necessary for *Dach2* expression, as revealed by normal *Dach2* expression in *Splotch* mice. *C,* Known necessary interactions in the vertebrate eye between members of the network as determined by analysis *Six3, Eya1,* and *Dach1* expression in mice with mutations in *Pax6. D,* Known necessary interactions in the vertebrate ear between members of the network as determined by analysis of *Pax2* and *8, Six1,* and *Dach1* expression in *Eya1* mutant mice and analysis of *Eya1* and *Dach1* expression in *Pax2* mutant mice. *E* and *F,* Proposed interactions between Six, Eya, and Dach proteins functioning in the development of the *Drosophila* eye (*E*) and vertebrate muscle (*F*). See references in text.

ey/so/eya/dac network (Quiring et al. 1994) and is the most potent inducer of ectopic eyes (Halder et al. 1995). In addition, *ey* is expressed in the absence of *so, eya,* or *dac* (Halder et al. 1998; Bonini et al. 1997; Shen and Mardon 1997). *ey,* in turn, is required for the expression of *eya* and *so* and is sufficient to induce *so* and *eya* (Chen et al. 1997; Halder et al. 1998). Recently, Niimi and colleagues have shown that *ey* regulation of *so* is direct, as the Ey protein binds and activates *so* through an eye-specific enhancer (Niimi et al. 1999). *eya* and *so* regulate each other's expression; in the absence of *eya,* no *so* is expressed, and in the absence of *so, eya* is downregulated (Halder et al. 1998). Finally, *dac* is downstream of *ey, so,* and *eya.* For instance, ectopic *ey* induces *dac,* and *ey* is expressed in the absence of *dac* (Shen and Mardon 1997). Also, in the absence of *eya,* no *dac* is expressed, but in the absence of *dac, eya* is expressed normally (Chen et al. 1997).

Although *ey, so, eya,* and *dac* appear initially to be regulated in a linear manner, these genes ultimately function in a nonlinear network, and all four are required for eye development (fig. 4.2, *A*). For instance, gain-of-function studies demonstrate that both *eya* and *dac* are able to induce expression of genes initially upstream in the network. In particular, ectopic *eya* induces *ey* expression, and ectopic *dac* induces both *eya* and *ey* expression (Bonini et al. 1997; Chen et al. 1997; Shen and Mardon 1997). Also, experiments combining loss- and gain-of-function approaches show that the ability of *so, eya,* or *dac* to induce ectopic eyes requires the function of the initially upstream *ey.* In *ey* mutants, ectopic *so, eya,* and *dac* are unable to induce ectopic eyes either singly or in combination (Bonini et al. 1998; Chen et al. 1997; Pignoni et al. 1997). Conversely, *so, eya,* and *dac* are as critical for eye development as *ey.* In *eya, so,* or *dac* mutants ectopic *ey* is unable to induce ectopic eyes (Chen et al. 1997; Halder et al. 1998).

The network is further complicated by the synergistic relationship between *so* and *eya* and between *eya* and *dac.* As mentioned previously, ectopic expression of *eya* or *dac* is able to induce ectopic eyes, albeit small ones and at a low frequency. However, ectopic expression of *so* and *eya* or *eya* and *dac* is able to induce larger ectopic eyes and at a higher frequency (Pignoni et al. 1997; Chen et al. 1997). This synergistic relationship between *so* and *eya* and between *eya* and *dac* is underlain by interactions between the proteins they encode. In particular, GST pull-down and yeast two-hybrid assays establish that So and Eya proteins and Eya and Dac proteins physically interact. Several models may explain the relationship between So, Eya, and Dac proteins and their downstream target genes (fig. 4.2, *E*). The transcriptional coactivator Eya may bind to the transcription factor So, which in turn binds to *so* binding sites upstream of target genes. The transcriptional coactivators Dac and Eya may bind to a third unidentified transcription factor which binds upstream of target genes. Alternatively, a third, as yet undemonstrated, possibility is that Dac, Eya, and So together form a transcriptional complex which activates downstream targets.

Recently, a second *Pax* family member, *twin of eyeless* (*toy*), has been found to play an important role in *Drosophila* eye development (Czerny et al. 1999). Like *ey, toy* is a member of the *PaxC* family and is more likely orthologous to *Pax6* than *ey. toy* is expressed earlier in development than *ey* in the developing head ectoderm. Later, *toy* is found in the cells anterior to the morphogenetic furrow within the eye disc. *toy* appears to act upstream of the entire network, and of *ey* in particular. Ectopic Toy induces both ectopic eyes and *ey* expression. *toy* may in fact directly regulate *ey* expression; several *toy*-binding sites essential for eye-specific expression have been identified in the *ey* enhancer (Hauck et al. 1999). This expansion of the *ey/so/eya/dac* network to include a second member of the *Pax* family appears to be unique to *Drosophila.*

Interestingly, a second *Six* gene, *optix,* has been identified in *Drosophila* and appears to be involved in eye morphogenesis by an *ey*-independent mechanism (Seimiya and Gehring 2000). *optix* is a member of the *Six3* subfamily and is expressed in a pattern different from *so* in the developing eye. Early in eye development, *optix* is expressed throughout the eye disc and later becomes restricted to the region anterior to the morphogenetic furrow (more like the expression pattern of *ey* or *toy*). Unlike *so,* ectopic *optix* can induce ectopic eyes. In addition, unlike *so, optix* does not function synergistically with *eya* in eye development; ectopic *optix* with ectopic *eya* does not induce ectopic eyes at a higher frequency. Moreover, unlike *eya* or *dac,* ectopic *optix* can induce ectopic eyes in an *ey* mutant background. In total, these results suggest that in the context of ectopic eye formation, *optix* acts in a partially different pathway from the one regulated by *ey.* However, since no loss-of-function mutants for *optix* are currently available, the functional role of *optix* in the eye disc remains uncertain.

Vertebrate Myogenesis

In vertebrates, somites are segmentally organized mesodermal structures that are the embryonic precursors of all skeletal muscle (reviewed in Christ and Ordahl 1995). Somites initially form as epithelial balls and, in response to patterning signals from surrounding tissues, acquire distinct fates. The dorsal region of the somite forms the dermamyotome, which gives rise to the dermatome and myotome. The myotome, in turn, gives rise to the epaxial (deep back) muscles that differentiate in situ and to the hypaxial muscles that form from cells that migrate away from the somites and differentiate into body wall and limb

muscles. In both epaxial and hypaxial muscle cells the expression of the muscle-specific helix-loop-helix transcription factors, *Myf5* and *MyoD,* marks the initiation of the myogenic differentiation program.

The role of the *Pax/Six/Eya/Dach* network in myogenesis was suggested by the expression of several gene family members in the somite. In particular, *Pax3* and *Pax7; Six1* and *Six4; Eya1, Eya2,* and *Eya4;* and *Dach1* and *Dach2* are all expressed in the dorsal somite prior to expression of the myogenic genes (Williams and Ordahl 1994; Jostes et al. 1990; Oliver et al. 1995b; Esteve and Bovolenta 1999; Borsani et al. 1999; Mishima and Tomarev 1998; Xu et al. 1997b; Heanue et al. 1999; Heanue et al. 2002). In addition, some of these genes are also expressed in the undifferentiated myogenic precursors migrating into the limbs. These genes therefore appear to be good candidates for genes acting upstream of the myogenic regulatory factors.

Recent studies in the chick have revealed some of the regulatory relationships between *Pax3, Six1, Eya2,* and *Dach2* within the somite and their role during myogenesis. Previous somite culture experiments and analysis of mouse splotch (*Pax3*) mutants had firmly established the importance of *Pax3* for induction of myogenesis (Maroto et al. 1997; Tajbakhsh and Buckingham 1999). However, the regulatory relationships of *Six, Eya,* and *Dach* to *Pax* and the roles of these three gene families in myogenesis were unknown. Somite culture and in vivo misexpression experiments in chick now have established that *Pax3* positively regulates *Dach2* and *Eya2* expression (Heanue et al. 1999; Kardon et al. 2002; fig. 4.2, *B*). Interestingly, analysis of *Dach2* expression in splotch mutants reveals that at least in the mouse, *Pax3* is not necessary for *Dach2* expression (Davis et al. 2001). Conversely, *Six1, Eya2,* and *Dach2* are able to weakly induce *Pax3*. However, misexpression of *Dach2* with *Eya2* or *Eya2* with *Six1* in somite culture is able to strongly upregulate *Pax3*. Thus, *Dach2* and *Eya2* or *Eya2* with *Six1* synergistically regulate *Pax3* expression. In addition, these culture experiments also have established that these pairs of genes synergistically regulate myogenesis. In particular, while misexpression of *Dach2, Eya2,* or *Six1* alone was unable to induce *MyoD* and *Myosin heavy chain,* misexpression of *Dach2* with *Eya2* or *Eya2* with *Six1* was able to induce these myogenic genes.

The synergistic regulation of myogenesis by *Dach2* with *Eya2* and by *Eya2* with *Six1* is underlain by specific protein-protein interactions (fig. 4.2, *F*). GST pull-down and yeast two-hybrid assays demonstrate that Dach2 and Eya2 proteins physically interact and also Eya2 and Six1 proteins interact (Heanue et al. 1999). The protein-protein interactions appear to be specific for particular family members. In particular, while Eya2 and Six1 proteins strongly interact, Eya2 does not appear to interact with Six3 (Heanue et al. 1999). The importance of

Eya/Six function in regulating the myogenic regulatory gene *myogenin* has been demonstrated in several studies. Gel mobility shift assays have demonstrated that Six1 and Six4 proteins bind to the MEF3 binding site of *myogenin* (Spitz et al. 1998). More recently, Ohto and colleagues (Ohto et al. 1999) have shown that Six and Eya proteins synergistically activate *myogenin.* Furthermore, the magnitude of cooperative activation of *myogenin* transcription depends on particular combinations of Six and Eya proteins. Although not yet tested, it is possible that Dach2 participates directly in this Six-Eya transcriptional complex.

Many aspects of the *Pax/Six/Eya/Dach* network functioning in *Drosophila* eye development are strikingly conserved in the *Pax/Six/Eya/Dach* network functioning in vertebrate myogenesis. In both networks, *Pax* and *Dach* act in a positive feedback loop, and *Pax* positively regulates *Eya.* Similar to the synergistic regulation of eye development by *dac* and *eya* and by *eya* and *so,* myogenesis is regulated synergistically by *Dach* and *Eya* and by *Eya* and *Six.* Moreover, in both *Drosophila* and chick, this synergism is underlain by interactions between Dach and Eya proteins and between Eya and Six proteins. There is also evidence that the interaction domains of these proteins have been conserved between *Drosophila* and chick. When chick *Dach2* is ectopically expressed in *Drosophila dac* null mutants, the mutant eye phenotype is rescued. This suggests that chick *Dach2* can functionally interact with *Drosophila eya* and that the interaction domain of the Dac and Dach2 proteins is similar (Heanue et al. 1999). In addition, yeast two-hybrid assays have directly shown that chick Six1 can physically interact with *Drosophila* Eya demonstrating that the interaction domains of Six1 and So are conserved (Heanue et al. 1999).

One notable dissimilarity between the fly and the chick *Pax/Six/Eya/Dach* networks is the employment of *Pax3,* and potentially *Six4,* in vertebrate myogenesis. *Pax3* is a member of the *PaxD* subfamily and therefore is not orthologous to *ey,* a *PaxC* subfamily gene (Miller et al. 2000), and likewise *Six4* is not orthologous to *so* (Kawakami et al. 2000). Thus, it appears that during vertebrate evolution, different members of the *Pax* and *Six* families have been substituted in the network.

Vertebrate Eye Development

The morphological development of the vertebrate eye differs dramatically from the development of the fly eye (reviewed in Grainger 1992). Vertebrate eye development begins with the outpouching of the diencephalic portion of the neural tube. This outpouching, the optic vesicle, subsequently contacts the head ectoderm and interacts with the overlying ectoderm as it thickens into the lens placode. The lens pla-

code then invaginates, detaches from the adjacent ectoderm, forms a lens vesicle, and eventually lengthens to form the lens of the eye. Concurrently, the optic vesicle folds inward on itself and surrounds the developing lens. The cells of this optic cup proliferate and differentiate into the neural and pigmented layers of the adult retina.

Pax6, Six3, Optx2, Eya1 and *Eya2,* and *Dach1* are all expressed in the developing eye. *Pax6* is initially expressed in the optic vesicle and in the head surface ectoderm in both the lens and otic regions prior to placode formation. As development proceeds, *Pax6* becomes localized to the lens placode and the neural retina (Grindley et al. 1995; Li et al. 1994; Walther and Gruss 1991). *Six3* is also expressed in the developing optic vesicle and later in both the neural retina and the lens (Ohto et al. 1998; Oliver et al. 1995a). *Optx2* has a slightly different expression pattern: it is found in the developing optic vesicle and later in the neural retina but appears to be absent in the developing lens (Jean et al. 1999; Lopez-Rios et al. 1999; Ohto et al. 1998; Toy and Sundin 1999; Toy et al. 1998). Two members of the *Eya* family, *Eya1* and *Eya2,* are expressed in complementary patterns in the developing eye (Xu et al. 1997b). Early in development, *Eya1* is expressed in the lens vesicle and the peripheral region of the optic vesicle, and it later is localized to the anterior epithelium of the lens and the retinal pigmented epithelium. In contrast, *Eya2* is never found in the lens or pigmented epithelium, but instead is expressed in the neural retina. Finally, *Dach1* is expressed in the developing optic vesicle and later in the neural retina (Hammond et al. 1998; Kozmik et al. 1999; Heanue et al. 2002).

The critical role of the *Pax, Six,* and *Eya* genes in vertebrate eye development has been revealed primarily by analysis of mouse and human mutations (reviewed in Wawersik and Maas 2000). Mutations in *Pax6, Six3,* and *Optx2* all lead to severe eye defects. Mutations in mouse *Pax6* cause the *Small eye* (*Sey*) phenotype (Hill et al. 1991; Hogan et al. 1986). *Sey/+* heterozygotes have lens and cornea abnormalities, while *Sey/Sey* homozygotes lack eyes altogether. Similarly, in humans haploinsufficiency for *Pax6* leads to aniridia, and homozygous *Pax6* mutations lead to anophthalmia (Glaser et al. 1994a, 1994b; Ton et al. 1991). In addition, mutations in human *Six3* cause microphthalmia, while mutations in *Optx2* result in anophthalmia (Gallardo et al. 1999; Wallis et al. 1999). Analysis of mice with null mutations in *Eya1* has not revealed any major defects in eye development (Xu et al. 1999). However, a subset of human *Eya1* mutations does result in cataracts and anterior segment abnormalities (Azuma et al. 2000).

Gain-of-function studies also confirm the importance of the *Pax* and *Six* genes for vertebrate eye development. Ectopic expression, via RNA injection, of *Pax6* in *Xenopus* or *Six3* in the teleost medaka results in ectopic retina and lenslike structures (Altmann et al. 1997; Chow et al. 1999; Loosli et al. 1999; Oliver et al. 1996). In addition, misexpression of *Optx2* in chick retinal pigmented epithelium induced cells to express neural-retina-specific markers (Toy et al. 1998). Overexpression of *Optx2* in *Xenopus* induced proliferation of retinal cells (Zuber et al. 1999).

At present, little is known about the regulatory relationships between the *Pax, Six, Eya,* and *Dach* genes functioning during eye development. However, some data on the relationship of *Pax6* to *Six, Eya,* and *Dach* genes have been gathered from analysis of early *Sey* embryos. In the *Sey/Sey* mice, *Eya1* is downregulated in the developing lens in the absence of functional *Pax6* (Xu et al. 1997a). The expression of *Six3* and *Dach1* in the optic vesicle and neural retina is unaffected in the *Sey/Sey* mice (Oliver et al. 1995a; Heanue et al. 2002). Therefore, in the developing eye *Eya1, Six3,* and *Dach1* are differentially regulated by *Pax6.* Understanding of the regulatory relationships among the *Eya, Six,* and *Dach* genes awaits the further analysis of mouse single and double knockouts.

The *Pax/Six/Eya/Dach* network appears to be critical for eye development in both vertebrates and *Drosophila,* despite the radically different structure and morphogenesis of their eyes. In *Drosophila* these genes are important for the initiation and propagation of the morphogenetic furrow, while in vertebrates these genes are required for the development of the lens and the retina. Members of the *Pax, Six, Eya,* and *Dach* gene families are all expressed in both vertebrate and *Drosophila* eyes, but there are some interesting differences in the particular genes expressed and their regulation. *ey* in *Drosophila* and its orthologue, *Pax6,* in vertebrates are key regulators of eye development. However, while *so* is critical for *Drosophila* eye development, there is no evidence that its orthologues, *Six1* and *Six2,* are used in vertebrate eyes. Instead, members of the *optix/Six3* subfamily, *Six3* and *Optx2,* are important for vertebrate eye development. Comparison of the regulation of these genes in fly and vertebrate eyes reveals that some, but not all, aspects of the regulatory network are conserved. In both the vertebrate and *Drosophila* eye, *Eya* gene expression is dependent on *Pax* genes. Interestingly, both *Six3* expression is independent of *Pax6* in the vertebrate eye and *optix* expression is independent of *ey* in the *Drosophila* eye. The regulation of *Dach* genes appears to differ between the two systems; in vertebrates expression of *Dach1* is independent of *Pax6,* while in *Drosophila dac* expression is dependent on *ey.*

Vertebrate Ear Development

The vertebrate inner ear derives from a thickened area of ectoderm, the otic placode, localized close to the hindbrain (reviewed in Torres and

Giraldez 1998). The otic placode invaginates to give rise successively to the otic cup and then to the otic vesicle. From the early otic cup, cells delaminate to give rise to the cochlear and vestibular neurons. The other components of the inner ear derive from the otic vesicle. The vesicle undergoes intense proliferative growth and differentiates into the endolymphatic duct, semicircular canals, vestibule, and cochlea. The expression of *Pax, Six, Eya,* and *Dach* genes in the developing otic cup and vesicle, and the ear defects resulting from mutations in some of these genes, indicate that the *Pax/Six/Eya/Dach* network is also critical for vertebrate ear development.

Pax2, Pax5, and *Pax8, Six1, Eya1,* and *Dach1* are expressed in various temporal and spatial patterns in the developing inner ear. *Pax8* is the earliest *Pax* gene to be expressed in the developing otic region. *Pax8* is expressed in the prospective otic placode and in the developing otic vesicle but is downregulated as the vesicle differentiates (Heller and Brändli 1999; Plachov et al. 1990). *Pax2* is associated with the auditory region of the inner ear. It begins to be expressed in the otic cup and is restricted to the ventral half of the otic vesicle that will give rise to the cochlea and adjacent sacculus (Nornes et al. 1990). In addition in *Xenopus, Pax5* is transiently expressed in the invaginating otic vesicle (Heller and Brändli 1999). *Six1* is found in the otic placode, vesicle, and facioacoustic ganglion (Oliver et al. 1995b). Finally, both *Eya1* and *Dach1* are expressed in the otic vesicle. *Eya1* is initially expressed in the ventromedial wall of the otic vesicle, which is the site of the future sensory epithelia of the cochlea (Xu et al. 1997b; Kalatzis et al. 1998). *Dach1* is also expressed in the ventromedial wall of the otic vesicle and in the vestigial ganglia (Heanue et al. 2002).

Loss-of-function studies have demonstrated that at least *Pax2* and *Eya1* are required for normal ear development. Analysis of *Pax2* mutant mice shows that *Pax2* is necessary for differentiation of the auditory regions of the inner ear. In these mutants, the otic vesicle invaginates and the cochlear neurons segregate normally from the vesicle. However, in the subsequent morphogenesis of the otic vesicle, neither the cochlea nor the cochlear ganglion differentiates. *Eya1* mutant mice have an even more dramatic ear phenotype (Xu et al. 1999). These mice have defects in their inner, middle, and outer ears. With regard to the inner ear, the otic vesicle forms but fails to develop further, and no inner structures form. The critical role of *Eya1* in ear development is also found in humans. Haploinsufficiency in human *Eya1* results in branchio-oto-renal syndrome (Abdelhak et al. 1997a, 1997b; Kumar et al. 1998), which is characterized by hearing loss. Mice with null mutations in another gene, *Pax8,* expressed in the developing ear have also been generated (Mansouri et al. 1998). However, these mutant mice do not have ear phenotypes. It is possible, although not yet tested, that upregulation of *Pax2* and/or *Pax5* compensates for the loss of functional *Pax8.*

Analysis of *Eya1* and *Pax2* mutant mice reveals some of the regulatory relationships between *Pax2* and *Pax8, Six1, Eya1,* and *Dach1.* In mice lacking functional *Eya1, Six1* expression is lost (Xu et al. 1999). This demonstrates that *Six1* expression is regulated by *Eya1.* In contrast, *Pax2, Pax8,* and *Dach1* expression is unaffected in the *Eya1* mutant, indicating that these genes are regulated independently of *Eya1* (Heanue et al. 2002; Xu et al. 1999). In *Pax2* mutant mice expression of *Eya1* and *Dach1* is unaffected, suggesting that their expression is regulated independently of *Pax2.* However, their expression may be regulated by *Pax5* and/or *Pax8* (Heanue et al. 2002).

In summary, the coexpression of *Pax, Six, Eya,* and *Dach* genes in the developing ear together with the ear phenotypes in mice mutant for *Pax2* and *Eya1* strongly suggests that the *Pax/Six/Eya/Dach* network is functionally important in vertebrate ear development. In the employment of this network for ear development the *sine oculis* orthologue, *Six1,* has been used, but the *eyeless PaxC* orthologues have not. Instead, *PaxB* subfamily members *Pax2, Pax5,* and *Pax8* have been utilized. Although there has been little analysis of the regulatory relationships between the genes, some aspects of the regulation found in the *Drosophila* eye have been conserved in the vertebrate ear, while others have not. For instance, as in *Drosophila, Six1* expression is dependent on *Eya1.* However, unlike the fly eye, *Dach1* expression appears to be independent of *Eya1.*

Comparison of the *Pax/Six/Eya/Dach* Networks Employed in *Drosophila* and Vertebrate Development

The *Pax/Six/Eya/Dach* network has been employed in *Drosophila* and vertebrates in a variety of different developmental contexts (summarized in fig. 4.2). In *Drosophila* the network in the eye primarily consists of *eyeless, sine oculis, eyes absent,* and *dachshund.* Although not explicitly tested, it is possible that the network is employed in *Drosophila* in other developmental contexts, perhaps using other members of the *Pax* and *Six* families. For example, in the *Drosophila* larval eye (Bolwig's organ) both *sine oculis* and *eyes absent* are required for its proper development, but *eyeless* and *twin of eyeless* are not (Suzuki and Saigo 2000). Potentially, another *Pax* family member is important in this developmental context. In the future it will be interesting to see whether in other parts of the *Drosophila* embryo the network is found to function with members of the *Pax A, B,* and *D* subfamilies or with *optix* or *six4.*

With the evolution of vertebrates, each of the gene families has un-

dergone duplications, and the network has been employed in several different developmental contexts. As might be expected, *ey* and *so* orthologues have been used in the vertebrate *Pax/Six/Eya/Dach* networks. *ey* and *toy* and their orthologue, *Pax6,* have been used in *Drosophila* and vertebrate eye development, respectively. Similarly, *so* and its orthologue, *Six1,* have been employed in *Drosophila* eye development and in vertebrate muscle and ear development. However, the vertebrate *Pax* and *Six* families are complex and include multiple subfamilies. Nonorthologous members of the *Pax* and *Six* families have been employed in the *Pax/Six/Eya/Dach* network. Members of the *PaxB* (*Pax2/Pax5/Pax8*) and *D* (*Pax3* and *Pax7*) subfamilies have been used in vertebrate ear and muscle development, respectively. In vertebrate eye development, *Six3* and *Optx2* and *not* the *so* orthologues *Six1* or *Six2* are used. In addition, all known members of the *Eya* and *Dach* families appear to have been employed in the network in different developmental contexts. Comparison of the particular members of the *Pax, Six, Eya,* and *Dach* families used suggests that there is no necessary relationship between which particular family members must be used together in concert. For example, *Six1* can work in a network with either *Eya1* or *Eya2,* and *Eya1* can work in a network with either *Six3* (*Optx2*) or *Six1.*

Many aspects of the regulatory relationships between *Pax, Six, Eya,* and *Dach* genes have been conserved between *Drosophila* and vertebrates. Initially *Pax* genes are most upstream, followed by *Six, Eya,* and *Dach* genes. Subsequently, positive regulatory loops are established between the components to form a complex network. How have these tight regulatory relationships been maintained? In *Drosophila,* part of this regulation is direct; the Ey protein binds to an eye-specific enhancer of *so* and activates *so* (Niimi et al. 1999). Although it has not yet been demonstrated, *Pax* genes may bind to *Six* upstream regions. Another possibility is that the *Six* transcription factors may directly bind to and transactivate *Pax, Eya,* and *Dach* genes. The tight regulatory relationships between *Six* and *Eya* and between *Eya* and *Dach* may be indirect yet made necessary by the physical interactions between the proteins they encode. A third possibility is that the entire network of genes is maintained by common regulatory regions upstream of the *Pax, Six, Eya,* and *Dach* genes. The upstream regions may be important for restricting the temporal and spatial distribution of these genes.

Although many of the regulatory relationships have been conserved in the *Pax/Six/Eya/Dach* network, there are significant instances of nonconservation. Within vertebrates, some genes have been decoupled from the tight network of internal regulation. For instance, within the vertebrate eye, *Dach1* expression is independent of *Pax6* (Heanue et al. 2002). In fact, there are multiple documented cases where members of the *Pax, Six, Eya,* and *Dach* families are operating entirely independently of the network. In the *Drosophila* wing and vertebrate limb *dac* and *Dach1* (Mardon et al. 1994; Hammond et al. 1998; Kozmik et al. 1999; Davis et al. 1999; Heanue et al. 1999), respectively, are clearly functioning independently of the network, as no *Pax, Six,* or *Eya* genes are coexpressed in these regions (LeClair et al. 1999; Xu et al. 1997a, 1997b; Oliver et al. 1995a, 1995b). In vertebrates *Pax1* is strongly expressed in the sclerotomal region of the somites, but no *Six, Eya,* or *Dach* genes are expressed in this region (Hammond et al. 1998; Kozmik et al. 1999; Davis et al. 1999; Heanue et al. 1999). Another interesting evolutionary novelty has been the expansion of the network in *Drosophila* to include both *ey* and *toy* (Czerny et al. 1999; Hauck et al. 1999). So far, no such similar expansion has been discovered in vertebrates.

Evolution of the *Pax/Six/Eya/Dach* Regulatory Network

The evolution of the *Pax, Six, Eya,* and *Dach* genes is characterized by the expansion of each of these gene families (fig. 4.3). In the lower Metazoa, the only gene family that has been currently identified is the *Pax* family. In cnidarians, four *Pax* genes, *A–D,* are present (Miller et al. 2000). The identification of cnidarian *Six, Eya,* and *Dach* genes awaits further research. On the basis of the distribution of genes in *Drosophila* and vertebrates, it appears that members of all four families are present before the protostome-deuterostome split. At this node,

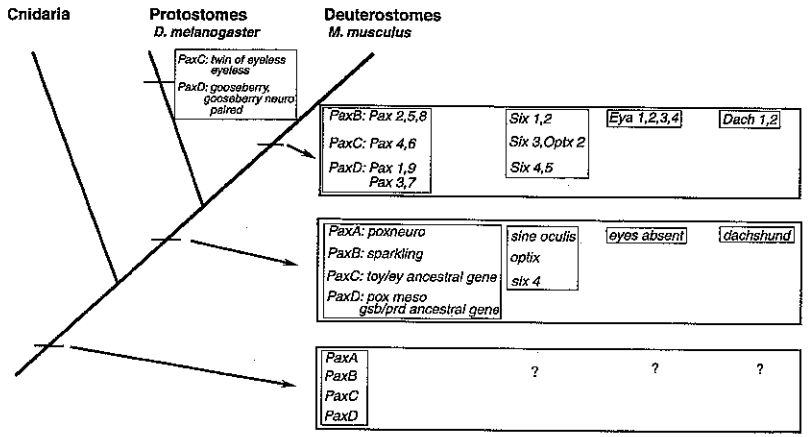

Fig. 4.3.—Evolutionary history of the *Pax, Six, Eya,* and *Dach* gene families as deduced from the distribution of genes in cnidarians, *Drosophila,* and mouse (Miller et al. 2000; Kawakami et al. 2000).

the *Pax* family had undergone duplication and contained at least five members. Also, three *Six* family genes, one *Eya,* and one *Dach* gene were present. With the evolution of protostomes, there were further duplications in the *Pax* family, so that *Drosophila* has eight *Pax* genes. Within the evolution of deuterostomes, there has been an expansion of all four gene families. Vertebrates have at least nine *Pax* genes, six *Six* genes, four *Eya* genes, and two *Dach* genes.

The phylogenetic distribution and the developmental expression of the *Pax, Six, Eya,* and *Dach* genes provide some insights into the origin of the *Pax/Six/Eya/Dach* network. The presence of all four gene families in the protostome/deuterostome common ancestor suggests that the network may have been present and functional very early in animal phylogeny. Moreover, if cnidarians are found to have *Six, Eya,* and *Dach* genes, the network may have originated even earlier. The ancestral developmental function of *Pax* genes, perhaps in the context of the *Pax/Six/Eya/Dach* network, may have been in the development of the nervous system. Although *Pax* genes are expressed in a variety of tissues in higher animals, most are expressed in the nervous system during development (Miller et al. 2000). Intriguingly, the one *Pax* gene, *Pax-Cam,* examined in the anthozoan cnidarian *Acoropora* is found in the presumptive developing neurons (Miller et al. 2000). It will be interesting to see whether *Six, Eya,* and *Dach* genes are coexpressed in the developing neurons and whether all four genes function in a regulatory network important for nervous system development.

With the evolution of protostomes and deuterostomes, the *Pax/Six/Eya/Dach* network has acquired a diversity of functions in the developing embryo. This diversification of function has been accompanied by the expansion and use of different members of the *Pax, Six, Eya,* and *Dach* families. In fact, it could be argued that it is the deployment of other gene family members that has allowed the *Pax/Six/Eya/Dach* network to be successfully used and functional in so many developmental contexts. The use of different family members may allow for diversification of function via three different mechanisms. First, the use of genes with different temporal and spatial patterns of expression may permit the network to operate in novel developmental contexts. Second, the differences in the DNA-binding specificities of different Pax and Six proteins may allow activation of different downstream targets. Finally, variation in protein-protein interactions between Six and Eya proteins and between Eya and Dach proteins may allow activation of different target genes. Such protein-protein specificity and its importance for target gene activation have been clearly demonstrated with the Six/Eya activation of *myogenin* (Ohto et al. 1999; Spitz et al. 1998). Overall, the important and diverse functions of the *Pax/Six/Eya/Dach* network in the developing embryo are striking and may have served a critical role in the evolution of both protostomes and deuterostomes. The expansion and use of different members of the *Pax, Six, Eya,* and *Dach* families in the network has allowed for developmental modification of the *Pax/Six/Eya/Dach* network. Such modification of developmental cassettes may be an important mechanism for generating evolutionary novelty in the animal lineage.

Evolution of Regulatory Cassettes

In this chapter we have examined one example of a regulatory cassette, a network of genes working together in many different developmental contexts. In general, the continued maintenance of regulatory cassettes in different developmental contexts in a wide variety of taxa suggests that there is some developmental and evolutionary utility to these cassettes. Here we have examined in detail the *Pax/Six/Eya/Dach* network. Another example of such a cassette is the *tinman/dmef2/pannier* and *Nkx/Mef2c/Gata4* networks important for heart development in both *Drosophila* and vertebrates, respectively (reviewed in Harvey and Rosenthal 1999). In the case of the *Pax/Six/Eya/Dach* network, the tight regulatory relationships between *Six* and *Eya* and between *Eya* and *Dach* probably originated and were maintained by selection for the necessary physical interactions between the proteins these genes encode. The origin and maintenance of the relationship between *Pax* and the other three genes is less clear. In *Drosophila, ey* directly regulates *so.* It is possible that *Pax* genes, in general, directly regulate *Six* (excluding members of the *optix* subfamily) and perhaps also *Eya* and *Dach* genes. Potentially, transcriptional regulation by *Pax* genes of *Six, Eya,* and *Dach* genes has allowed *Six, Eya,* and *Dach* genes to be coselected as a gene network. Over the course of evolution, regulatory cassettes have proved to be extremely versatile. In the case of the *Pax/Six/Eya/Dach* network, each of the gene families has expanded, and different members of these families have been used in the network. The use of different family members may have allowed the *Pax/Six/Eya/Dach* network to be used in different spatial and temporal developmental contexts and to activate different downstream targets. In addition, in some developmental contexts the internal regulatory relationships within the *Pax/Six/Eya/Dach* network have been modified and may have made the network more versatile. Both the developmental utility and the evolutionary flexibility of regulatory cassettes may make these cassettes central to animal development and evolution. Future examination of a broad array of animal taxa will reveal how universally these regulatory cassettes have been used.

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5 The Notch Signaling Module

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In the context of developmental biology, cell-to-cell communication includes the mechanisms by which cells interchange molecular signals that affect and/or direct the acquisition of particular cell fates. Cell signaling influences many aspects of cell behavior, such as cell division, growth, and polarity. In many instances the immediate consequences of signaling are changes in gene expression triggered by the transcriptional regulators that constitute the final elements of each signaling pathway. Signaling pathways consist of a number of proteins that are functionally related in a hierarchical manner. Each pathway generally includes extracellular ligands, membrane receptors, and a chain of intracellular transducers that modify the activity of a transcription factor. The interactions between members of a given pathway are determined by molecular recognition and therefore are context independent. This makes each signaling pathway a "module" of interacting proteins that contributes to the regulation of gene expression.

The Notch signaling pathway influences many cell fate choices during the development of multicellular organisms (Artavanis-Tsakonas et al. 1999). The elements that constitute the pathway are conserved in vertebrates and invertebrates. Furthermore, Notch affects similar developmental operations in all organisms where its functional requirements have been analyzed, including lateral inhibition during cell fate choice and local induction in the establishment of developmental boundaries (Artavanis-Tsakonas et al. 1999). For these reasons, the Notch pathway can be considered a signaling module that regulates gene expression. According to this view, the elements of the pathway constitute a conserved set of interacting proteins that modify the activity of a transcriptional regulator. There are two fundamental aspects