added with SDS-sample buffer, heated at 95°C for 5 min, and applied to SDS-PAGE, followed by fluorography.

Results

BOR type mutations abolish the interaction between Dach1 and Eya1

Interactions between Dachshund and Eyes absent proteins in *Drosophila* were identified by yeast two-hybrid assays and GST-pulldown analyses (Chen et al. 1997). The interaction is mediated through the Eya domain and Dachshund domain 2 (DD2), both of which are conserved among various species (Chen et al. 1997; Xu et al. 1997; Caubit et al. 1999). We assessed the interaction between mouse Dach1 and mouse Eya1 by mammalian two-hybrid assay. A plasmid expressing Eya1 or Eya1 mutations fused with GAL4 DNA binding domain (pMEya1 and its mutation derivatives) was used as a bait (Table 1). FLAG-tagged Dach1 expressing plasmid, pfDach1, as a prey and a luciferase reporter plasmid consisting of five Gal4 DNA binding sites upstream of the synthetic core promoter, pGL-MRG5 (Ikeda et al. in press), were co-transfected into 293 cells. FLAG-tagged Dach1 was used because Dach1 itself shows a potent transactivation activity and that its activity is reduced in the context of Dach1-VP16 fusion protein (Kawakami, K., unpublished observation). As shown in Fig. 1, pfDach1 stimulated the transcription of the reporter 12- to 36-fold in the presence of pMEya1 in a dose dependent manner, indicating that Dach1 interacts with the wild type Eya1.

We tested three BOR type mutations, pMEya1R307X, pMEya1S486P and pMEya1L504R, as well as ocular type of mutations, pMEya1E362K and pMEya1R546G, as baits. The former three BOR type mutations marginally activated the transcription of the reporter gene (< 3-fold), whereas the latter two ocular type mutations enhanced the transcription (11- to 37-fold), similar to the wild type. Interestingly, a complex type mutation pMEya1G425S, which had been described in a patient with both BOR syndrome and cataract (Azuma et al. 2000), also enhanced the transcription (9- to 26-fold) similar to the wild type. These results indicate that the three BOR type mutations abolished the interaction with Dach1, while the ocular and complex type mutations retained the interaction with Dach1.

BOR type mutations abolish the interaction between G proteins and Eya1

Human EYA2, another member of the *EYA* family gene products, was found capable of binding to constitutively active forms of a subset of Gα proteins, Gαz and Gαi2, through EYA domain, suggesting that EYA2 is one of the effector molecules of these G proteins (Fan et al. 2000). This prompted us to assess the interaction between Eya1 and these Gα proteins. In the following studies, we used the constitutive active mutations of rat GαzQ205L and Gαi2Q205L, which mimic the phosphorylated form of each G protein (Fan et al. 2000). Mammalian two-hybrid assays were performed using pMEya1 as a bait and plasmids expressing VP16-Gα fusion proteins, pVP16Gαz, pVP16GαzQ205L, pVP16Gαi2 or pVP16Gαi2Q205L as a prey (Fig. 2a). pVP16Gαz and pVP16Gαi2 showed no activation of the reporter pGL-MRG5,

whereas each constitutive active mutation, pVP16GαzQ205L and pVP16Gαi2Q205L activated the transcription of the reporter from 33- to 79- and from 42- to 132-fold in a dose dependent manner, respectively. These results indicate that GαzQ205L and Gαi2Q205L interact with Eya1.

Next, we examined the effect of Eya1 mutations on the interaction between Eya1 and GαzQ205L or Gαi2Q205L (Figs. 2b, c). Three BOR type mutations pMEya1R307X, pMEya1S486P and pMEya1L504R showed no activation of the reporter. On the other hand, an ocular type mutation pMEya1E362K enhanced the transcription of the reporter 52- to 103-fold (pVP16GαzQ205L) or 69- to 116-fold (pVP16Gαi2Q205L). This enhancement was slightly higher than that of the wild type pMEya1 (23- to 52-fold for pVP16GαzQ205L and 62- to 89-fold for pVP16Gαi2Q205L). The other ocular type mutation pMEya1R546G enhanced 39- to 77-fold (pVP16GαzQ205L) or 56- to 75-fold (pVP16Gαi2Q205L), similar to wild type pMEya1. The complex type mutation pMEya1G425S showed a comparable level of activation (57- to 58-fold for pVP16GαzQ205L and 57- to 96-fold for pVP16Gαi2Q205L) of the reporter gene transcription to the pMEya1. The results indicate that three BOR type mutations, R307X, S486P and L504R failed to interact with the constitutive active mutations of Gαz and Gαi2, and that other Eya1 mutations retained their abilities to interact with these G proteins.

BOR type mutations weaken the interaction between Six and Eya1

The functional synergy between Six2, Six4 or Six5, and Eya1 has been reported with regard to transcription from their target gene (*myogenin*) promoter (Ohto et al. 1999). To examine whether the above Eya1 mutations influence the interaction with various Six, we performed mammalian two-hybrid assays (Fig. 3). A plasmid pVP16Six1, pVP16Six2, pVP16Six4 or pVP16Six5 expressing VP16-Six1, -Six2, -Six4 or -Six5 fusion proteins, respectively, was used as a prey. These Six proteins are expected to be involved in BOR syndrome based on their expression in branchial arch, otic vesicle and/or nephrogenic cord during embryogenesis. pMEya1 and its mutation derivatives were used as a bait (Table 1). Various combinations of bait-prey were co-transfected into 293 cells with the reporter plasmid pGL-MRG5.

For pVP16Six1, wild type pMEya1 showed 14- to 15-fold activation of the reporter. pMEya1E362K (19- to 30-fold), pMEya1G425S (16- to 19-fold), pMEya1R546G (18- to 28-fold) and pMEya1S486P (13- to 15-fold) showed comparable or even higher activation of the reporter, relative to pMEya1. In contrast, pMEya1R307X and pMEya1L504R showed little activation up to 1- and 3-fold, respectively (Fig. 3a).

For pVP16Six2, wild type pMEya1 showed 4.6- to 4.9-fold activation of the reporter. pMEya1E362K (4.4- to 5.9-fold), pMEya1G425S (5.3-fold), pMEya1R546G (4.8- to 5.6-fold) and pMEya1S486P (4.7- to 5.1-fold) showed comparable or a slightly higher activation of the reporter, relative to pMEya1. In contrast, pMEya1R307X showed little activation (< 1.5-fold) and pMEya1L504R up to 2.3- to 2.7-fold activation (Fig. 3b).

For pVP16Six4, wild type pMEya1 showed 6.7- to 11-fold activation of the reporter. pMEya1E362K (4.7- to 10-fold), pMEya1G425S (5.2- to 10-fold) and pMEya1R546G (5.5- to 13-fold) showed comparable activation of the reporter, relative to pMEya1. In contrast, pMEya1R307X showed little activation (< 1.1-fold) and S486P and L504R showed 5.5- to 7.7- and 2.3- to 3.3-fold activation, respectively (Fig. 3c).

In case of pVP16Six5, wild type pMEya1 showed 13- to 24-fold activation of the reporter. pMEya1E362K (13- to 35-fold), pMEya1G425S (22- to 30-fold) and pMEya1R546G (28- to 42-fold) showed comparable or higher activation of the reporter than pMEya1. In contrast, pMEya1R307X showed little activation (< 1.1-fold), while S486P and L504R showed 10- to 11- and 3.2- to 4.2-fold activation, respectively (Fig. 3d).

These results indicate that the ocular type and the complex type mutations E362K, G425S and R546G showed comparable or stronger interaction with any Six compared with wild type Eya1, but that R307X abolishes the interaction with either Six. In contrast, S486P exhibits comparable or slightly weaker interaction with Six1, Six2 and Six4, but significantly weaker interaction with Six5, and L504R shows marginal interactions with Six1 and Six5, although it retains significant interactions with Six2 and Six4 (summarized in Table 1).

To address whether the defects in interactions observed in mammalian two-hybrid assays are due to lack of the direct interaction between Eya1 and Six proteins, GST-pulldown assays were performed (Fig. 4). *In vitro* translated ^{35}S-labeled Eya1 or its mutation proteins were incubated with Glutathione Sepharose beads bound to

GST-Six fusion proteins. Bound and unbound fractions (denoted as B and S, respectively in Fig. 4) were analyzed by SDS-PAGE, followed by fluorography. Wild-type Eya1 (WT) was observed in the bound fraction of any GST-Six fusion proteins, but not in the bound fraction of GST alone, and observed in smaller quantity in unbound fraction of any GST-Six fusion proteins than in unbound fraction of GST alone. Likewise, E362K, G425S and R546G were observed in larger quantities in the bound fraction of any GST-Six fusion proteins than in the bound fraction of GST alone. In contrast, R307X, S486P and L504R were mostly observed in unbound fraction of all GST-Six and GST alone, and little or no amount of these proteins were observed in bound fraction of any GST-Six. These results indicate that the wild type Eya1 efficiently bound to all of the GST-Six fusion proteins, and that E362K, G425S and R546G interact with any GST-Six fusion proteins at significant, but varying levels. In contrast, BOR type mutations, R307X, S486P and L504R did not show significant binding to any GST-Six proteins.

BOR type mutations perturb the transcription from *myogenin* promoter

As a coactivator, Eya1 synergistically activates *myogenin* gene transcription with Six2, Six4 and Six5 that bind to its promoter region (Ohto et al. 1999). We checked whether mutation versions of Eya1 could activate the *myogenin* gene transcription with Six5 as well as wild type Eya1. Wild-type pHM6Eya1 activated the transcription from *myogenin* promoter with Six5 about 4.6-fold. In contrast, pHM6Eya1S486P and pHM6Eya1L504R showed little or no activation of 1.0- and 0.6-fold, respectively.

When we used mutations of pHM6Eya1E362K and pHM6Eya1G425S, comparable levels of activation (5.4- and 5.0-fold) were observed respectively. pHM6Eya1R546G showed slightly lower level of around 3.2-fold activation (Fig. 5). These transactivation of the *myogenin* promoter by wild type and mutations of Eya1 exhibited a good correlation with the two-hybrid interactions depicted in Fig. 3. This indicates that the defective coactivator function of Eya1 could be attributed to the reduced physical interactions between Eya1 and Six in transactivation of the *myogenin* promoter.

BOR type mutations alter sensitivity to V8 protease and trypsin digestion

The fact that two BOR type substitution mutations, S486P and L504R, simultaneously lost the interaction with Dach1 and G proteins, strongly suggests a gross conformational change in these mutations. To address this possibility, we performed V8 protease mapping. In the case of S486P, we did not observe any difference in sensitivity and in digestion pattern compared with those of the wild type (Fig. 6a, lanes 1-6 and 7-12). In contrast, we detected an alteration of digestion pattern by V8 protease in L504R (Fig. 6a, lanes 13-18). Two- to 3-fold higher amount of the undigested polypeptide (denoted as A) remained in L504R than that of wild type when we added 200 ng to 2 μg of V8 protease (compare lanes 16-18 with lanes 4-6), while the patterns of fragments D, E and F in L504R digestion did not change, relative to that in wild type digestion (compare lanes 15-18 with lanes 3-6). Fragment C was detected at higher levels in L504R than the wild type with the addition of 650

ng and 2 μg of V8 protease (lanes 17 and 18, compare with lanes 5 and 6). Furthermore, fragment B was hardly detected and fragments with slightly different mobility from that of fragment B appeared (Fig. 6a, lanes 14-18). In addition, fragment B' was present at higher level at 2 μg of V8 protease in L504R than in wild type (discerned by short exposure, data not shown). These results indicate that L504R is more resistant to V8 protease digestion and the digestion pattern is distinct from those of wild type and S486P, suggesting a different conformation of L504R.

We also performed protease mapping using trypsin and detected alterations of digestion pattern in S486P and L504R (Fig. 6b). Far more amount of undigested polypeptide (denoted as A) remained in both mutations than in wild type when we added 12 ng of trypsin (compare lanes 11 and 17 with lane 5). Fragment B was hardly detected at 4 ng of trypsin (Fig. 6b, lanes 10 and 16). Fragments D, E, and H were missing or detected only at lower levels in these mutations than in the wild type with the addition of 40 ng of trypsin, while fragment I was detected at higher levels (lanes 12 and 18, compare with lane 6). The patterns of the other proteolytic fragments, C, F, G and I in S486P and L504R digestion were unchanged to those in the wild type digestion (Fig. 6b, compare lanes 12 and 18 with lane 6). These results indicate that S486P and L504R are more resistant to trypsin digestion and the digestion patterns of these mutations are distinct from that of the wild type, suggesting the altered conformation of S486P and L504R.

Discussion

In this study, we found that two BOR type Eya1 substitution mutations, S486P and L504R, and a truncation mutation R307X were defective in protein-protein interaction mediated by EYA domain. Our results also suggest the possible involvement of Six, Dach1, and G proteins in the pathogenicity of BOR syndrome.

S486P and L504R mutations are located in the central region of Eya domain corresponding to the subregion of Eya domain of *Drosophila* Eyes absent (EF2), which is required for the association with *Drosophila* Dachshund (Bui et al. 2000). Dachshund and Eyes absent form a complex and synergistically induce compound eye formation in *Drosophila* (Chen et al. 1997). Likewise in chicken, Dach2 and Eya2 physically interact and synergistically induce myogenic gene expression (Heanue et al. 1999). Thus, it is possible that the interaction between DACH1 and EYA1 is necessary for the development of certain organs. Actually, both Eya1 and Dach1 are expressed in otic vesicles and branchial arches in mouse (Xu et al. 1997; Caubit et al. 1999). The abrogation of such interaction by these BOR type mutations may perturb the normal organogenesis, leading to BOR syndrome.

We also found that Eya1 interacted with the active form of the two types of G proteins, Gαz and Gαi2 (Fig. 2a). These G proteins have been reported to bind to EYA2 and negatively regulate its coactivator function by inhibiting the nuclear translocation of EYA2 mediated by Six1 and Six4 (Fan et al. 2000). We propose that the G proteins also modulate the distribution of Eya1 proteins and regulate the Eya1 functions in organogenesis, and that impaired interaction of Eya1 mutations with these G proteins may result in their aberrant subcellular distribution, which might be involved in the onset of BOR syndrome.

Mostly consistent results were noted in the mammalian two-hybrid and GST-pulldown assays as for the interaction of Eya1 mutations with four types of Six proteins (Figs. 3 and 4). The BOR type truncation mutation, R307X, and substitution mutations, S486P and L504R, showed little interaction with Six1, Six2, Six4 or Six5 by GST-pulldown assays (Fig. 4). R307X showed little or no interactions with any Six by mammalian two-hybrid assays, whereas L504R showed weak but significant two-hybrid interaction with any Six and S486P reduced two-hybrid interaction only with Six4 and Six5 (Fig. 3). The difference between the results of the two assays may be due to their different sensitivity or may be explained by the involvement of a third factor(s) in the living cells, which bridge or stabilize the interaction between Eya1 and Six proteins. Impaired interaction of Six4 and Six5 with all three BOR type Eya1 mutations suggests the involvement of these Six proteins in the pathogenicity of BOR syndrome. In fact, Six4 as well as Six1 is expressed in branchial arches, otic vesicles and nephrogenic tissues (Oliver et al. 1995; Ohto et al. 1998; Ozaki et al. 2001), and Six5 as well as Six2 are expressed in branchial arches and nephrogenic tissues (Oliver et al. 1995; Ohto et al. 1998; Klesert et al. 2000). Thus it is possible that BOR type mutations reduce the interaction with these Six proteins to perturb the expression of the Six-responsive genes, leading to BOR syndrome.

Contrary to the BOR type mutations, the ocular and complex type mutations E362K, G425S and R546G did not show any defects in two-hybrid, GST-pulldown and transactivation assays. This finding suggests that unidentified factors other than Six, Dach or G proteins, are probably involved in cascades that lead to ocular defects. Alternatively, a possible impairment of transactivation of these Eya1 mutations might

not be the main pathway to cause ocular defects. However, the fact that *Six5*-deficient mice developed cataract (Klesert et al. 2000; Sarkar et al. 2000) suggests the involvement of Six5 in ocular developmental defects. Furthermore, Six5 and Eya1 showed high synergistic activation (Fig. 5). In this context, it is plausible that defects of transactivation of these mutations of Eya1 are detected if we use the promoter of the relevant target genes of Six5 which operate in the lens.

The BOR type substitution mutations, S486P and L504R, were associated with the disappearance or weakening the interaction with structurally unrelated groups of proteins (Figs. 1-4). In *Drosophila* Eyes absent, the regions that interact with So and Dachshund is reported to be distinct (EF1 and EF2 subdomain of Eya domain, respectively) (Bui et al. 2000). S486P and L504R are located within the region corresponding to EF2, not to EF1. Nevertheless these two mutations were not only associated with the loss of interaction with Dach1, but also weakening of the interaction with Six5. Considering that serine to proline substitution in S486P changes the structure of the backbone and that leucine to arginine substitution in L504R changes the charge and the size of the amino acid side chains, these results suggest a gross conformational change in these mutations. In fact, S486P and L504R showed a low sensitivity to and distinct pattern of trypsin digestion from those of wild type Eya1 (Fig. 6b). L504R also showed a low sensitivity to and distinct pattern of V8 protease digestion (Fig. 6a). These results suggest that S486P and L504R mutations cause the conformational changes which disturb the protein-protein interactions mediated by EYA domain.

Contrary to our expectation, the complex type mutation G425S which had been identified in a patient with combined BOR syndrome and ocular defects (Azuma et al. 2000) interacted with Dach, the two G proteins and the four Six proteins and held the transactivation activity at levels similar to wild type Eya1. Patients carrying this mutation may need further evaluation including search for additional mutation(s) outside the exons of *EYA1* loci, for example in its promoter region. However, the possibility is not excluded that G425S mutation, as well as S486P and L504R, may disturb the interaction of Eya1 with unidentified cofactor(s), which is essential for normal organogenesis.

Structural studies including determination of the three dimensional structure of EYA1, combined with biochemical analyses including the search for other factors binding to EYA1 and the target genes of SIX, should further enhance our understanding of the function of EYA1 and the pathogenic mechanisms of BOR syndrome.

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References

Abdelhak S, Kalatzis V, Heilig R, Compain S, Samson D, Vincent C, Levi-Acobas F, Cruaud C, Le Merrer M, Mathieu M, König R, Vigneron J, Weissenbach J, Petit C, Weil D (1997a) Clustering of mutations responsible for branchio-oto-renal (BOR) syndrome in the eyes absent homologous region (eyaHR) of EYA1. Hum Mol Genet 6:2247-2255

Abdelhak S, Kalatzis V, Heilig R, Compain S, Samson D, Vincent C, Weil D, Cruaud C, Sahly I, Leibovici M, Bitner-Glindzicz M, Francis M, Lacombe D, Vigneron J, Charachon R, Boven K, Bedbeder P, Van Regemorter N, Weissenbach J, Petit, C (1997b) A human homologue of the Drosophila eyes absent gene underlies branchio-oto-renal (BOR) syndrome and identifies a novel gene family. Nature Genet 15:157-164

Azuma N, Hirakiyama A, Inoue T, Asaka A, Yamada M (2000) Mutations of a human homologue of the Drosophila eyes absent gene (EYA1) detected in patients with congenital cataracts and ocular anterior segment anomalies. Hum Mol Genet 9:363-366

Bonini NM, Leiserson WM, Benzer S (1993) The eyes absent gene: genetic control of cell survival and differentiation in the developing Drosophila eye. Cell 72:379-395

Bui QT, Zimmerman JE, Liu H, Bonini NM (2000) Molecular analysis of Drosophila eyes absent mutants reveals features of the conserved Eya domain. Genetics 155:709-720

Caubit X, Thangarajah R, Theil T, Wirth J, Nothwang HG, Rüther U, Krauss S (1999)

Mouse Dac, a novel nuclear factor with homology to Drosophila dachshund shows a dynamic expression in the neural crest, the eye, the neocortex, and the limb bud. Dev Dyn 214:66-80

Chen R, Amoui M, Zhang Z, Mardon G (1997) Dachshund and eyes absent proteins form a complex and function synergistically to induce ectopic eye development in Drosophila. Cell 91:893-903

Cheyette BNR, Green PJ, Martin K, Garren H, Hartenstein V, Zipursky SL (1994) The Drosophila sine oculis locus encodes a homeodomain-containing protein required for the development of the entire visual system. Neuron 12:977-996

Fan X, Brass LF, Poncz M, Spitz F, Maire P, Manning DR (2000) The α subunits of Gz and Gi interact with the eyes absent transcription cofactor Eya2, preventing its interaction with the six class of homeodomain-containing proteins. J Biol Chem 275:32129-32134

Fujisawa-Sehara A, Hanaoka K, Hayasaka M, Hiromasa-Yagami T, Nabeshima Y (1993) Upstream region of the myogenin gene confers transcriptional activation in muscle cell lineages during mouse embryogenesis. Biochem Biophys Res Commun 191:351-356

Heanue TA, Reshef R, Davis RJ, Mardon G, Oliver G, Tomarev S, Lassar AB, Tabin CJ (1999) Synergistic regulation of vertebrate muscle development by Dach2, Eya2, and Six1, homologs of genes required for Drosophila eye formation. Genes Dev 13:3231-3243

Ikeda K, Stuehler T, Meisterernst M (2002) The H1 and H2 regions of the activation domain of herpes simplex virion protein 16 stimulate transcription through distinct

molecular mechanisms. Genes Cells (in press)

Itoh H, Kozasa T, Nagata S, Nakamura S, Katada T, Ui M, Iwai S, Ohtsuka E, Kawasaki H, Suzuki K, Kaziro Y (1986) Molecular cloning and sequence determination of cDNAs for α subunits of the guanine nucleotide-binding proteins Gs, Gi, and Go from rat brain. Proc Natl Acad Sci USA 83:3776-3780

Johnson KR, Cook SA, Erway LC, Matthews AN, Sanford LP, Paradies NE, Friedman RA (1999) Inner ear and kidney anomalies caused by IAP insertion in an intron of the Eya1 gene in a mouse model of BOR syndrome. Hum Mol Genet 8:645-653

Kawakami K, Ohto H, Ikeda K, Roeder RG (1996a) Structure, function and expression of a murine homeobox protein AREC3, a homologue of Drosophila sine oculis gene product, and implication in development. Nucleic Acids Res 24:303-310

Kawakami K, Ohto H, Takizawa T, Saito T (1996b) Identification and expression of six family genes in mouse retina. FEBS Lett 393:259-263

Klesert TR, Cho DH, Clark JI, Maylie J, Adelman J, Snider L, Yuen EC, Soriano P, Tapscott SJ (2000) Mice deficient in Six5 develop cataracts: implications for myotonic dystrophy. Nature Genet 25:105-109

Kumar S, Deffenbacher K, Cremers CW, Van Camp G, Kimberling WJ (1998) Branchio-oto-renal syndrome: identification of novel mutations, molecular characterization, mutation distribution, and prospects for genetic testing. Genet Test 1:243-251

Mardon G, Solomon NM, Rubin GM (1994) dachshund encodes a nuclear protein

required for normal eye and leg development in Drosophila. Development 120:3473-3486

Murakami Y, Ohto H, Ikeda U, Shimada K, Momoi T, Kawakami K (1998) Promoter of mDMAHP/Six5: differential utilization of multiple transcription initiation sites and positive/negative regulatory elements. Hum Mol Genet 7:2103-2112

Ohto H, Takizawa T, Saito T, Kobayashi M, Ikeda K, Kawakami K (1998) Tissue and developmental distribution of Six family gene products. Int J Dev Biol 42:141-148

Ohto H, Kamada S, Tago K, Tominaga SI, Ozaki H, Sato S, Kawakami K (1999) Cooperation of Six and Eya in activation of their target genes through nuclear translocation of Eya. Mol Cell Biol 19:6815-6824

Oliver G, Wehr R, Jenkins NA, Copeland NG, Cheyette BNR, Hartenstein V, Zipursky SL, Gruss P (1995) Homeobox genes and connective tissue patterning. Development 121:693-705

Ozaki H, Watanabe Y, Takahashi K, Kitamura K, Tanaka A, Urase K, Momoi T, Sudo K, Sakagami J, Asano M. Iwakura Y, Kawakami K (2001) Six4, a putative myogenin gene regulator, is not essential for mouse embryonal development. Mol Cell Biol 21:3343-3350

Pignoni F, Hu B, Zavitz KH, Xiao J, Garrity PA, Zipursky SL (1997) The eye-specification proteins So and Eya form a complex and regulate multiple steps in Drosophila eye development. Cell 91:881-891

Sarkar PS, Appukuttan B, Han J, Ito Y, Ai C, Tsai W, Chai Y, Stout JT, Reddy S (2000) Heterozygous loss of Six5 in mice is sufficient to cause ocular cataracts. Nature Genet 25:110-114

Xu PX, Woo I, Her H, Beier DR, Maas RL (1997) Mouse Eya homologues of the Drosophila eyes absent gene require Pax6 for expression in lens and nasal placode. Development 124:219-231

Xu PX, Adams J, Peters H, Brown MC, Heaney S, Maas R (1999) Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia. Nature Genet 23:113-117

Figure legends

Fig. 1. Interaction between Eya1 and Dach1 in 293 cells. Increasing amounts (0.25 or 0.50 μg) of pMEya1 wild type (WT) or the indicated Eya1 mutations were cotransfected with 0.3 μg of pfDach1. 0.5 μg of pGL-MRG5 was cotransfected as a reporter gene. Luciferase activity in the cell lysate was normalized with β-galactosidase activity of pEFBOSβ-gal as an internal control. The activity of each datum point is relative to that obtained by the control vector pM (-). Each experiment was performed in triplicates and the mean fold activation is shown with the standard deviation. Similar results were obtained at least three independent experiments

Fig. 2. Interaction between Eya1 and G proteins in 293 cells. a Increasing amounts (0.05 and 0.1 μg) of pVP16Gαz, pVP16GαzQ205L, pVP16Gαi2 or pVP16Gαi2Q205L were cotransfected with 0.5 μg of pMEya1. b,c Increasing amounts (0.25 and 0.5 μg) of pMEya1 (WT) or the indicated mutations were cotransfected with 0.05 μg of pVP16GαzQ205L (b) or pVP16Gαi2Q205L (c). 0.5 μg of pGL-MRG5 was cotransfected as a reporter gene. Luciferase activity in the cell lysate was normalized with β-galactosidase activity of pEFBOSβ-gal as an internal control. The activity of each datum point is relative to that obtained by the control vector pM (-). Each experiment was performed in triplicates and the mean fold activation is shown with the standard deviation. Similar results were obtained at least three independent experiments

Fig. 3. Interaction between Eya1 mutations and Six proteins in 293 cells. 0.5 μg of pVP16Six1 (a), pVP16Six2 (b), pVP16Six4 (c) or pVP16Six5 (d) was cotransfected with increasing amounts (0.25 or 0.5 μg) of pMEya1 (WT) or the indicated mutations. 0.5 μg of pGL-MRG5 was cotransfected as a reporter gene. Luciferase activity in the cell lysate was normalized with β-galactosidase activity of pEFBOSβ-gal as an internal control. The activity of each datum point is relative to that obtained by the control vector pM (-). Each experiment was performed in triplicates and the mean fold activation is shown with the standard deviation. Similar results were obtained at least two independent experiments

Fig. 4. Direct interaction between Eya1 and Six proteins *in vitro*. One microgram of GST-Six1, 0.5 μg of GST-Six2, 1 μg of GST-Six4 or 5 μg of GST-Six5 fusion protein bound to Glutathione Sepharose beads was incubated with *in vitro* translated, ^{35}S-labeled Eya1 (WT) or the indicated mutations at 4°C for 2 h. Ten percent of input, a half of bound (B) and 17% of supernatant (S) were electrophoresed on a 12% polyacrylamide SDS gel followed by fluorography

Fig. 5. Effects of Eya1 mutations on *myogenin* promoter transcription in NIH 3T3 cells. One hundred nanogram of pfSix5 was cotrancfected with 300 ng of pHM6Eya1 or its mutations. Two microgram of pGL3MG-1.7 was cotransfected as a reporter gene. Luciferase activity in the cell lysate was normalized with β-galactosidase activity of pEFBOSβ-gal as an internal control. The activity of each datum point is relative to that obtained by the control vector pHM6 (-). Each experiment was

performed in triplicates and the mean fold induction is shown with the standard deviation. Similar results were obtained at least two independent experiments

Fig. 6. Protease digestion profiles of Eya1 wild type and its mutations by V8 protease (a) and trypsin (b). ^{35}S-labeled Eya1 wild type or its mutations translated *in vitro* in rabbit reticulocyte lysate was digested without (panel a, lanes 1, 7 and 13) or with 20 ng (panel a, lanes 2, 8 and 14), 65 ng (panel a, lanes 3, 9 and 15), 200 ng (panel a, lanes 4, 10 and 16), 650 ng (panel a, lanes 5, 11 and 17) and 2 μg (panel a, lanes 6, 12 and 18) of V8 protease or without (panel b, lanes 1, 7 and 13) or with 0.4 ng (panel b, lanes 2, 8 and 14), 1.2 ng (panel b, lanes 3, 9 and 15), 4 ng (panel b, lanes 4, 10 and 16), 12 ng (panel b, lanes 5, 11 and 17) and 40 ng (panel b, lanes 6, 12 and 18) of trypsin for 30 min at 4 °C and analyzed by SDS-PAGE followed by fluorography. Undigested full-length polypeptides are denoted as A. The major proteolytic fragments are designated B to F (panel a) and B to H (panel b) according to their electrophoretic mobilities