, 61, 4414-4417
Kubo A, Isumi Y, Ishizaka Y, Tomoda Y, Kangawa K, Dohi K, Matsuo H, Minamino N. C-type natriuretic peptide is synthesized and secreted from leukemia cell lines, peripheral blood cells, and peritoneal macrophages. Exp Hematol. 2001 May;29(5):609-15.

2. 学会発表
特にありません。

H. 知的財産権の出願。登録状況
1. 特許取得
特にありません。
2. 実用新案登録
特にありません。
3. その他
特にありません。

厚生科学研究費補助金（厚生科学特別研究事業）

分担研究報告書

# 細胞周期のチェックポイントに関係するホメオボックス遺伝子の各転移性腫瘍における意義 Six と HAT (Histone Acetyl Transferase)

分担研究者　川上　潔　自治医科大学　教授

転写因子SixファミリーのうちのひとつhSix1が、骨髄CD34陽性細胞ではGM-CSFによる分化をした方が、Epo, Tpoによる分化よりもより発現していた。また多方向に分化する株細胞 UT-7 を用いてノザン解析を行うと、GM-CSF によって刺激した場合により hSix1 が発現していた。

A. 研究目的

癌に対して多くの手術療法や化学療法、放射線療法が開発され、行われている。手術後や化学療法後に早期に再発する症例も依然として存在することも事実である。各種臓器の原発とするがんに対して特異的なマーカーである抗原や遺伝子の発見は、多くの期待を持って今後の癌の臨床経過や予後を推測させ、早期に対処できるようになることが期待される。しかし現実にはまだ多くの症例での検討や基礎的意味付けの必要な部分が多く残っている。そこで社会のニーズに答えるような臨床的検討から、微小残存病変や微小転移病変の有無の評価やその結果を生かして予後を推測することに生かせれば患者さんへの恩恵や社会への貢献度が高いと考えられる。そのための基礎的および臨床的研究を行う。

１．病変における細胞の特性の研究

B. 研究方法

増殖と分化に関係する転写因子 Six ファミリーのひとつ hSix1 について遺伝子の発現と分化の方向での検討を行った。ほ乳類細胞での検討の前に、zebrafish で検討した。

C. 結果

まず骨髄 CD34 陽性細胞では GM-CSF による分化をした方が、Epo, Tpo による分化よりもより発現していた。また多方向に分化する株細胞 UT-7 を用いてノザン解析を行うと、GM-CSFによって刺激した場合により hSix1 が発現していた。

D. 考察

増殖と分化に関係する転写因子 Six ファミリーのひとつhSix1について遺伝子の発現と分化の方向での検討を行った。ほ乳類細胞での検討の前に、zebrafish で検討した。分化の方向によっては発現が増強される遺伝子がある事がわかった。

E. 結論

転写因子 Six ファミリーのひとつ hSix1 について遺伝子の発現と分化の方向での検討を行った。特に GM-CSF による分化によって増強した。

F. 健康危険情報

特にありません。

G. 研究発表

１．論文発表

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

Ozaki, H., Yamada, K., Kobayashi, M., Asakawa S., Minoshima S., Shimizu N., Kajitani, M., and Kawakami, K. Structure and chromosomal mapping of human *SIX4* and mouse *Six4* genes. Cytogenet. Cell Genet. 87, 108-122. 1999

Muto, S., Nemoto, J., Okada, K., Miyata, Y., Kawakami, K., Saito, T., and Asano, Y. Intracellular $Na^+$ directly modulates $Na^+$,$K^+$-ATPase gene expression in normal rat kidney epithelial cells. Kidney Int., 57, 1617-1635. 2000

Kawakami, K., Sato, S., Ozaki, H. and Ikeda, K.*Six* family genes-Structure and function as transcription factors and their roles in development. BioEssays 22, 616-626. 2000
Kobayashi, M., Osanai, H., Kawakami, K., and Yamamoto, M. Expression of three zebrafish Six4 genes in the cranial sensory placodes and the developing somites. Mech. Dev. 98, 151-155. 2000
Kawakami, K. Transcriptional regulation of Na,K-ATPase alpha1 subunit gene. In: Control and diseases of sodium dependent transport proteins and ion channel, eds. Y. Suketa, E. Calafori, M. Lazdunski, K. Mikoshiba, Y. Okada, and E. M. Wright., pp. 27-30, 2000 Elsevier.
Ozaki, H., Watanabe, Y., Takahashi, K., Kitamura, K., Tanaka, A., Urase, K., Momoi, T., Sudo, K., Sakagami, J., Asano, M., Iwakura, Y. and Kawakami, K. Six4, a putative myogenin gene regulator, is not essential for mouse embryonal development. Mol. Cell. Biol. 21, 3343-3350.2001

2. 学会発表
特にありません。

H. 知的財産権の出願。登録状況
1. 特許取得
特にありません。
2. 実用新案登録
特にありません。
3. その他
特にありません。

20010157

以降 P.23－P.350は雑誌/図書等に掲載された論文となりますので下記の資料をご参照ください。

**【サイトカインに関する最近の進歩】 アポトーシスを誘導制御するサイトカイン** 畠 清彦　臨床免疫(0386-9695)36 巻 1 号 Page32-38(2001.07)

**【抗癌剤の作用機序と効果】 アミノペプチダーゼ阻害剤とアポトーシス**
畠 清彦, 三嶋 雄二　血液・腫瘍科(0915-8529)42 巻 5 号 Page443-448(2001.05)

**ベータ 2 ミクログロブリンの新しい細胞死への作用**
森 政樹, 畠 清彦　血液・腫瘍科(0915-8529)43 巻 1 号 Page75-82(2001.07)

**イダルビシンを用いて寛解導入療法を行った初発急性骨髄性白血病自験例 41 例の解析 特に t(8;21)を有する M2 の長期予後について**
松本 裕子, 森 政樹, 大月 哲也, 室井 一男, 畠 清彦, 小松 則夫, 小澤 敬也
臨床血液(0485-1439)42 巻 1 号 Page15-22(2001.01)

**CD13 is the resistance mechanism for endothelial interleukin-8 inducing apoptosis**
Yuji Mishima , Yasuhito Terui , Misa Katsuyama , Hiroshi Tomizuka , Masaki Mori , Masaya Uwai , Masuzu Ueda , Muneo Yamada , Hirotoshi Hayasawa , Keiya Ozawa , Noboru Horikoshi and Kiyohoko Hakake
Cell-Surface Aminopeptidases:Basic and Clinical Aspects 2001 p.177-184

**Antitumor effect of beta2-microglobulin in leukemic cell-bearing mice via apoptosis-inducing activity: activation of caspase-3 and nuclear factor-kappaB.** Mori M, Terui Y, Tanaka M, Tomizuka H, Mishima Y, Ikeda M, Kasahara T, Uwai M, Ueda M, Inoue R, Itoh T, Yamada M, Hayasawa H, Furukawa Y, Ishizaka Y, Ozawa K, Hatake K.
Cancer Res. 2001 Jun 1;61(11):4414-7.

20010157

Autologous stem cell transplantations for recurrent adult T cell leukaemia/lymphoma using highly purified CD34+ cells derived from cryopreserved peripheral blood stem cells.
Watanabe J, Kondo H, Hatake K.
Leuk Lymphoma. 2001 Sep-Oct;42(5):1115-7.

Detection of murine adult bone marrow stroma-initiating cells in Lin(-)c-fms(+)c-kit(low)VCAM-1(+) cells.
Tanaka-Douzono M, Suzu S, Yamada M, Wakimoto N, Hayasawa H, Hatake K, Motoyoshi K. J Cell Physiol. 2001 Oct;189(1):45-53.

EBM と新しい治療戦略】 再発乳癌化学療法 Evidence の解釈
伊藤 良則 乳癌の臨床(0911-2251)16 巻 3 号 Page220-223(2001.06)

【乳癌診断・治療の現状と展望】 化学ホルモン療法 現状と展望
伊藤 良則 日本医師会雑誌(0021-4493)125 巻 11 号 Page1713-1720(2001.06)

Dose-finding phase I study of simultaneous weekly infusion with doxorubicin and docetaxel in patients with advanced breast cancer. Ito Y, Aiba K, Horikoshi N, Saotome T, Irie T, Sugiyama K, Nakane M, Hashimoto D, Yoshida N, Mizunuma N, Takahashi S, Tanigawara Y. Int J Clin Oncol. 2001 Oct;6(5):242-7.

最新がん治療と看護 化学療法と看護ケア(後編)】 がん化学療法の臨床 乳がんに対する化学療法とその限界
伊藤 良則 がん看護(1342-0569)6 巻 1 号 Page16-19(2001.01)

特集:転移性肝癌治療ー最近の進歩 Ⅰ. 大腸癌肝転移 A. 肝切除術 4. 肝転移切除時の切除断端の意義と術式
國土 典宏 , 関 誠 , 多田 敬一郎 , 上野 雅資 , 畦倉 薫 , 太田 博俊 , 柳澤 昭夫 , 高橋 孝 , 中島 聰總 , 武藤 徹一郎
外科 62 巻 6 号 2000-6 p622-627

20010157

**Anatomical major resection versus nonanatomical limited resection for liver metastases from colorectal carcinoma.**
Kokudo N, Tada K, Seki M, Ohta H, Azekura K, Ueno M, Matsubara T, Takahashi T, Nakajima T, Muto T. Am J Surg. 2001 Feb;181(2):153-9.

**【癌治療のプロトコール 当施設はこうしている】 肝癌治療のプロトコール 癌研究会附属病院・外科** 國土 典宏, 関 誠, 猪狩 功遺, 松原 敏樹, 太田 博俊, 山口 俊晴, 高橋 孝, 中島 聰總, 武藤 徹一郎
臨床外科(0386-9857)55 巻 11 号 Page124-128(2000.10)

**Proliferative activity of intrahepatic colorectal metastases after preoperative hemihepatic portal vein embolization.**
Kokudo N, Tada K, Seki M, Ohta H, Azekura K, Ueno M, Ohta K, Yamaguchi T, Matsubara T, Takahashi T, Nakajima T, Muto T, Ikari T, Yanagisawa A, Kato Y. Hepatology. 2001 Aug;34(2):267-72.

**Hepatic parenchymal transection using ultrasonic coagulating shears: a preliminary report.** Kokudo N, Kimura H, Yamamoto H, Seki M, Ohta H, Matsubara T, Takahashi T.
J Hepatobiliary Pancreat Surg. 2000;7(3):295-8.

**特集:リンパ節転移ー微小転移, Sentinal Node の観点から**
**Ⅱ. 胃癌の Sentinal Node Navigation Surgery**
山口 俊晴 , 大山 繁和 , 太田 惠一朗 , 中島 聰總 , 武藤 徹一郎
外科 63 巻 7 号 2001-7 p.819-822

**【癌手術の手順と手技のポイント】 胃癌 標準的 D2 郭清手術**
山口 俊晴, 大山 繁和, 太田 惠一朗, 山本 順司, 松原 敏樹, 中島 聰總, 武藤徹一郎 外科(0016-593X)63 巻 10 号 Page1169-1173(2001.10)

20010157

**【直腸癌局所再発に対する治療戦略】 直腸癌局所再発に対する治療法の変遷** 上野 雅資, 武藤 徹一郎, 山口 俊晴, 畦倉 薫, 太田 博俊
外科治療(0433-2644)85 巻 6 号 Page613-617(2001.12)

**16 イレウス b. 老人の場合** 山口 俊晴 , 太田 惠一朗
消化器疾患最新の治療 2001-2002 p.238-239

**6. 幽門輪温存胃切除術の適応 8. 膵頭十二指腸切除術(PD)の適応**
山口 俊晴 消化器癌の外科治療 専門医にきく最新の臨床 2001 p.44-49

**【最新がん治療と看護 化学療法と看護ケア(後編)】 がん化学療法の臨床 消化器固形がんに対する化学療法とその限界**
山口 俊晴, 大山 繁和, 太田 惠一朗, 中島 聰總, 武藤 徹一郎
がん看護(1342-0569)6 巻 1 号 Page20-22(2001.01)

**【癌の抗体療法】 固形腫瘍に対する抗体療法 消化器癌**
山口 俊晴 癌治療と宿主(0915-4639)13 巻 1 号 Page61-65(2001.01)

**巻頭言 癌の転移征圧のための DDS -臨床が求めるもの, 基礎から提供できるもの-** 山口 俊晴 Drug Delivery System 16-2,2001

**【出血させない手術のコツとピットフォール】 胃癌/幽門側切除**
山口 俊晴, 太田 惠一朗, 大山 繁和, 中島 聰總, 武藤 徹一郎
手術(0037-4423)55 巻 9 号 Page1304-1308(2001.08)

**【胃癌治療のコンセンサス『胃癌治療ガイドライン』を踏まえて】 一般用ガイドライン解説 意義,内容紹介**
山口 俊晴, 大山 繁和, 太田 惠一朗, 山本 順司, 上野 雅資, 松原 敏樹, 太田 博俊, 武藤 徹一郎, 中島 聰總
消化器外科(0387-2645)24 巻 11 号 Page1643-1647(2001.10)

20010157

**Construction of preferential cDNA microarray specialized for human colorectal carcinoma: molecular sketch of colorectal cancer.** Takemasa I, Higuchi H, Yamamoto H, Sekimoto M, Tomita N, Nakamori S, Matoba R, Monden M, Matsubara K.
Biochem Biophys Res Commun. 2001 Aug 3;285(5):1244-9.

**Outcome of pancreatic cancer patients based on genetic lymph node staging.** Yamada T, Nakamori S, Ohzato H, Higaki N, Aoki T, Oshima S, Shiozaki K, Okami J, Hayashi N, Nagano H, Dono K, Umeshita K, Sakon M, Monden M. Int J Oncol. 2000 Jun;16(6):1165-71.

**Clinical application of quantitative analysis for detection of hematogenous spread of hepatocellular carcinoma by real-time PCR.** Miyamoto A, Nagano H, Sakon M, Fujiwara Y, Sugita Y, Eguchi H, Kondo M, Arai I, Morimoto O, Dono K, Umeshita K, Nakamori S, Monden M.
Int J Oncol. 2001 Mar;18(3):527-32.

**Genetic detection for micrometastasis in lymph node of biliary tract carcinoma.** Okami J, Dohno K, Sakon M, Iwao K, Yamada T, Yamamoto H, Fujiwara Y, Nagano H, Umeshita K, Matsuura N, Nakamori S, Monden M. Clin Cancer Res. 2000 Jun;6(6):2326-32.

**Assessment of Stanniocalcin-1 mRNA as a molecular marker for micrometastases of various human cancers.** Fujiwara Y, Sugita Y, Nakamori S, Miyamoto A, Shiozaki K, Nagano H, Sakon M, Monden M.
Int J Oncol. 2000 Apr;16(4):799-804.

**Hepatic resection of hepatocellular carcinomas based on tumor hemodynamics.** Sakon M, Nagano H, Shimizu J, Kondo M, Nakamori S, Dono K, Umeshita K, Nakamura H, Murakami T, Monden M.
J Surg Oncol. 2000 Mar;73(3):179-81. No abstract available.

20010157

**Expression of interferon alpha/beta receptor in human hepatocellular carcinoma.** Kondo M, Nagano H, Sakon M, Yamamoto H, Morimoto O, Arai I, Miyamoto A, Eguchi H, Dono K, Nakamori S, Umeshita K, Wakasa K, Ohmoto Y, Monden M.
Int J Oncol. 2000 Jul;17(1):83-8.

**Augmentation of antitumor activity of 5-fluorouracil by interferon alpha is associated with up-regulation of p27Kip1 in human hepatocellular carcinoma cells.** Eguchi H, Nagano H, Yamamoto H, Miyamoto A, Kondo M, Dono K, Nakamori S, Umeshita K, Sakon M, Monden M. Clin Cancer Res. 2000 Jul;6(7):2881-90.

**Role of the liver in alloimmune response following inoculation of donor spleen cells.** He L, Dono K, Gotoh M, Okumura M, Takeda Y, Shimizu J, Nagano H, Nakamori S, Umeshita K, Sakon M, Monden M.
Cell Transplant. 2000 Sep-Oct;9(5):725-8.

**Pancreatic mass due to chronic pancreatitis: correlation of CT and MR imaging features with pathologic findings.**
Kim T, Murakami T, Takamura M, Hori M, Takahashi S, Nakamori S, Sakon M, Tanji Y, Wakasa K, Nakamura H.
AJR Am J Roentgenol. 2001 Aug;177(2):367-71.

**Reciprocal functions of liver tumor cells and endothelial cells. Involvement of endothelial cell migration and tumor cell proliferation at a primary site in distant metastasis.**
Okamoto H, Ohigashi H, Nakamori S, Ishikawa O, Imaoka S, Mukai M, Kusama T, Fujii H, Matsumoto Y, Akedo H.
Eur Surg Res. 2000;32(6):374-9.

20010157

**Presence of active hepatitis associated with liver cirrhosis is a risk factor for mortality caused by posthepatectomy liver failure.**
Eguchi H, Umeshita K, Sakon M, Nagano H, Ito Y, Kishimoto SI, Dono K, Nakamori S, Takeda T, Gotoh M, Wakasa K, Matsuura N, Monden M.
Dig Dis Sci. 2000 Jul;45(7):1383-8.

**Prednisolone suppresses ischemia-reperfusion injury of the rat liver by reducing cytokine production and calpain mu activation.**
Wang M, Sakon M, Umeshita K, Okuyama M, Shiozaki K, Nagano H, Dohno K, Nakamori S, Monden M. J Hepatol. 2001 Feb;34(2):278-83.

**Involvement of calcium influx in hypoxia-induced bleb formation in human umbilical vein endothelial cells.**
Sakon M, Aono Y, Ariyoshi H, Ueda A, Tsuji Y, Umeshita K, Nagano H, Dono K, Nakamori S, Monden M.
Transplant Proc. 2001 Feb-Mar;33(1-2):898. No abstract available.

**Clinical significance of hepatic resection in hepatocellular carcinoma: analysis by disease-free survival curves.**
Sakon M, Umeshita K, Nagano H, Eguchi H, Kishimoto S, Miyamoto A, Ohshima S, Dono K, Nakamori S, Gotoh M, Monden M.
Arch Surg. 2000 Dec;135(12):1456-9.

**The inhibitory effect of prostaglandin E1 on oxidative stress-induced hepatocyte injury evaluated by calpain-mu activation.** Kishimoto S, Sakon M, Umeshita K, Miyoshi H, Taniguchi K, Meng W, Nagano H, Dono K, Ariyosi H, Nakamori S, Kawasaki T, Gotoh M, Monden M, Imajoh-Ohmi S. Transplantation. 2000 Jun 15;69(11):2314-9.

20010157

**Differential expression of cyclooxygenase-2 (COX-2) in human bile duct epithelial cells and bile duct neoplasm.**
Hayashi N, Yamamoto H, Hiraoka N, Dono K, Ito Y, Okami J, Kondo M, Nagano H, Umeshita K, Sakon M, Matsuura N, Nakamori S, Monden M.
Hepatology. 2001 Oct;34(4 Pt 1):638-50.

**膵癌の治療成績を向上させるために 膵癌診療の最近の進歩】 膵膵の診断 現況と新しい試み 膵癌の分子生物学的診断**
岡見 次郎, 中森 正二, 左近 賢人, 門田 守人
外科治療(0433-2644)84 巻 6 号 Page966-971(2001.06)

**Advanced hepatocellular carcinoma with distant metastases, successfully treated by a combination therapy of alpha-interferon and oral tegafur/uracil.** Miyamoto A, Umeshita K, Sakon M, Nagano H, Eguchi H, Kishimoto S, Dono K, Nakamori S, Gotoh M, Monden M.
J Gastroenterol Hepatol. 2000 Dec;15(12):1447-51.

**【高度進行大腸癌治療の最前線】 外科非切除例 分子生物学的手法を用いた診断と治療** 中森 正二, 竹政 伊知朗, 門田 守人
消化器外科(0387-2645)24 巻 9 号 Page1411-1419(2001.08)

**Six4, a putative myogenin gene regulator, is not essential for mouse embryonal development.** Ozaki H, Watanabe Y, Takahashi K, Kitamura K, Tanaka A, Urase K, Momoi T, Sudo K, Sakagami J, Asano M, Iwakura Y, Kawakami K. Mol Cell Biol. 2001 May;21(10):3343-50.

J. Hum. Genet in press 12/4



# Impaired interactions between mouse Eya1 harboring mutations found in patients with branchio-oto-renal syndrome and Six, Dach and G proteins

Hidenori Ozaki

Yoko Watanabe

Keiko Ikeda

Kiyoshi Kawakami

Department of Biology, Jichi Medical School,

Minamikawachi, Kawachi, Tochigi 329-0498, Japan

To whom correspondence should be addressed:

K. Kawakami

Department of Biology, Jichi Medical School,

Minamikawachi, Kawachi, Tochigi 329-0498, Japan

Fax: +81-285-44-5476;

Tel: +81-285-58-7311

e-mail: kkawakam@jichi.ac.jp

**Abstract** Mutations in *EYA1* gene are responsible for branchio-oto-renal (BOR) syndrome as well as for other ocular defects. Most of the mutations are located within or in the vicinity of the Eya domain, which is highly conserved among Eya protein family. The EYA domain is required for protein-protein interaction, which is important to the biological function of EYA proteins. To determine how *EYA1* mutations cause BOR syndrome and/or ocular defects, we tested the effects of Eya1 mutations on interactions with Six, Dach and G proteins by mammalian two-hybrid and GST-pulldown assays. Defective interactions were noted between BOR type mutations S486P and L504R of Eya1 and Dach1, G proteins and some of Six proteins. These mutations impaired the activation of transcription from a Six-responsive gene, *myogenin*, with Six5. S486P and L504R showed altered digestion pattern with trypsin and L504R also decreased the sensitivity to V8 protease digestion and produced a peptide fragment with a different Mr. Our results suggest that defective protein-protein interactions of the mutations in the Eya domain underlie BOR syndrome and that SIX, DACH and/or G proteins are possibly involved in the pathogenic processes.

**Key words** BOR syndrome, EYA1, EYA domain, Six, Dach, G protein, mammalian two-hybrid assay, GST-pulldown

## Introduction

*EYA* family genes are homologues of *Drosophila eyes absent* (*eya*) which is required for compound eye formation. A clue for molecular functions of *EYA* family gene products has been given by analyses using *Drosophila* system. Loss-of-function mutations of *eya*, as well as those of *sine oculis* (*so*, one of *Drosophila Six*) and *dachshund*, result in a reduction or a complete loss of compound eye development in the fly (Bonini et al. 1993; Cheyette et al. 1994; Mardon et al. 1994), while ectopic expression of *so* or *dachshund* with *eya* synergistically induced compound eye formation (Pignoni et al. 1997; Chen et al. 1997). These genetic interactions were shown to be mediated through physical interactions between their gene products (Pignoni et al. 1997; Chen et al. 1997).

*EYA1* was originally isolated as a gene responsible for branchio-oto-renal (BOR) syndrome, a human autosomal dominant disorder characterized by hearing impairment, branchial arch deformation and variable severity of renal anomaly (Abdelhak et al. 1997b). In a recent report, *EYA1* mutations were also found in certain ocular defects such as cataract and iris anomaly (Azuma et al. 2000). To date, more than 20 mutations of *EYA1* have been identified in patients with BOR syndrome or ocular defects, most of which are located in the EYA domain of the product (an amino acid substitution, a truncation or a frame-shift) (Abdelhak et al. 1997a,b; Azuma et al. 2000; Kumar et al. 1998).

The EYA domain is conserved among *EYA* family gene products and is required for protein-protein interaction. For example, in *Drosophila*, Eyes absent forms a

complex with So and/or Dachshund through the Eya domain synergistically to induce compound eye formation (Pignoni et al. 1997; Chen et al. 1997), and mouse Eya forms a complex with Six and synergistically activate the target gene promoter through nuclear translocation of Eya by Six, for which the Eya domain is indispensable (Ohto et al. 1999). These facts suggest that the protein-protein interaction mediated by EYA domain is important for functions of EYA proteins and for normal organogenesis.

To gain insight into the molecular basis of how *EYA1* mutations cause BOR syndrome, we analyzed the effects of *EYA1* mutations on such protein-protein interactions. For this purpose, we used the substitution and truncation mutations of *EYA1* identified in patients with BOR syndrome (R275X, L472R and R514G), ocular defects (E330K and S454P) or both (G393S) (Abdelhak et al. 1997b; Azuma et al. 2000) based on the following reasons. 1) Amino acid residues at these mutations are conserved (E330K, G393S and L472R) or homologous (R275X, S454P and R514G) among *EYA* family genes, suggesting that the molecular functions of these residues are common to *EYA* gene family. 2) In frame-shift mutations, sequences and lengths of thereafter amino acid stretches are not generally conserved and these stretches might have unexpected gain-of-function. The observations that human EYA1 and mouse Eya1 are highly homologous (99.6% identity in EYA domain, 98.7% identity in the whole molecule) and that mice carrying mutations in *Eya1* manifest BOR syndrome-like phenotype (Xu et al. 1999; Johnson et al. 1999), suggest that mouse Eya1 functions in the same molecular context as human *EYA1*. Thus we introduced the corresponding mutations found in human *EYA1* into mouse *Eya1* (R307X, S486P

and L504R for BOR type, E362K and R546G for ocular type and G425S for complex type) and performed molecular characterizations. We hypothesized that *Eya1* mutations impair molecular interactions with cofactors mediated by EYA domain and analyzed the interaction of these mutations with the known cofactors, Six, Dach and G proteins.

We tested these interactions by mammalian two-hybrid assays and/or GST-pulldown assays. Furthermore, to examine whether the impaired interaction influences the transactivation function of Eya1 with Six, we performed reporter gene assays using a promoter of a Six-responsive gene, *myogenin*. Structural analyses of Eya1 mutations were also performed by protease digestion.

## Materials and methods

### Construction of plasmids

pHM6Eya1R307X, pHM6Eya1E362K, pHM6Eya1G425S, pHM6Eya1L504R, pHM6Eya1R546G and pHM6Eya1S486P were constructed by introducing corresponding point mutations into pHM6Eya1 (Ohto et al. 1999) by cassette mutagenesis using the following sets of PCR primers: 5'-CGTGGCCGAGGCTGAAGAAACAATA-3' and 5'-TATTGTTTCTTCAGCCTCGGCCACG-3' (R307X); 5'-GGACTACGAATGAAAGAGATGATTT-3' and 5'-AAATCATCTCTTTCATTCGTAGTCC-3' (E362K); 5'-

ACTGGTGTCCGAAGTGGTGTGGACT-3' and 5'-AGTCCACACCACTTCGGACACCAGT-3' (G425S); 5'-CAAAGGTTTGGAGGGAAAGTGGTAT-3' and 5'-ATACCACTTTCCCTCCAAACCTTTG-3' (R546G); 5'-CTGAAGGCCCTCCCCCTCATCCACT-3' and 5'-AGTGGATGAGGGGGAGGGCCTTCAG-3' (S486P); and 5'-CAACTACGCAGCGCATCCCAGCATT-3' and 5'-AATGCTGGGATGCGCTGCGTAGTTG-3' (L504R). The point mutations introduced are underlined.

pMEya1 was constructed as follows. The initiation codon (154)-*Hin*dIII (654) fragment was amplified by PCR so as to generate an *Eco*RI site at 5'-terminus. The resulting *Eco*RI-*Hin*dIII fragment was inserted into the *Eco*RI/*Sal*I sites of pM (Clontech Laboratories, Palo Alto, CA) together with the *Hin*dIII (654)-*Xho*I (3'-terminus) fragment of pHM6Eya1.

pMEya1R307X was constructed by replacing the *Kpn*I (661)-*Pst*I (3'-terminus) fragment of pMEya1 with the *Kpn*I (661)-*Pst*I (3'-terminus) fragment of pHM6Eya1R307X. pMEya1E362K, pMEya1G425S, pMEya1L504R, pMEya1R546G and pMEya1S486P were constructed by replacing the *Bam*HI fragment (1199 to 3'-terminus) of pMEya1 with the *Bam*HI fragment (1199 to 3'-terminus) of pHM6Eya1E362K, pHM6Eya1G425S, pHM6Eya1L504R, pHM6Eya1R546G and pHM6Eya1S486P, respectively.

pfSix1 was constructed as follows. *Six1* cDNA was cloned from mouse mammary cancer cell SC-3 cDNA library and the coding region was amplified by

PCR using the following set of primers to generate a *Kpn*I site at 5'-terminus and an *Xba*I site at 3'-terminus: 5'-GGGTACCCATGTCGATGCTGCCGTCGT-3' and 5'-GCTCTAGATTAGGAACCCAAGTCCACCA-3'. The resulting PCR fragment was digested with *Kpn*I and *Xba*I and inserted into the *Kpn*I/*Xba*I sites of pFLAG-CMV-2 (Eastman Kodak, New Haven, Conn.).

pVP16Six1, pVP16Six2, pVP16Six4 and pVP16Six5 were constructed as follows. The full length of the insert of pfSix1 was excised as the *Bam*HI (blunt-ended)-*Xba*I fragment and inserted into the *Bam*HI (blunt-ended)/*Xba*I sites of pVP16 (Clontech) (pVP16Six1); the full length of the insert of pfSix2 (Ohto et al. 1999) was excised as the *Bss*HII (blunt-ended)-*Sma*I fragment and inserted into the *Sal*I (blunt-ended) site of pVP16 (pVP16Six2); the full length of the insert of pfSix4 (Ohto et al. 1999) was excised as the *Bgl*II (blunt-ended)-*Xba*I fragment and inserted into the *Bam*HI (blunt-ended)/*Xba*I sites of pVP16 (pVP16Six4); the full length of the insert of pfSix5 (Ohto et al. 1999) was excised as the *Xba*I (blunt-ended)-*Bgl*II (blunt-ended) fragment and inserted into the *Sal*I (blunt-ended) site of pVP16 (pVP16Six5).

pfDach1 was constructed as follows. pBSmdac (kindly provided by S. Krauss) was engineered to generate a *Hin*dIII site 5' adjacent to the initiation codon (266). The *Hin*dIII-*Ssp*I (2520) fragment was once cloned into the *Hin*dIII/*Eco*RV sites of pBluescript KS+ (Stratagene, La Jolla, CA). Then the insert was excised as the *Hin*dIII-*Xba*I fragment and inserted into the *Hin*dIII/*Xba*I sites of pFLAG-CMV-2.

pVP16Gz and pVP16GzQ205L were constructed as follows. The *Nco*I (13)-*Xba*I (3'-terminus) fragments from pCMV5Gz and pCMV5GzQ205L (Itoh et al. 1986) were excised, blunt-ended and inserted into the *Eco*RI (blunt-ended) site of

pVP16. pVP16Gi2 and pVP16Gi2Q205L were constructed as follows. The 262 bp *Hae*III fragments (34-295) from pCMV5Gi2 and pCMV5Gi2Q205L (Itoh et al. 1986) were inserted into the *Bam*HI (blunt-ended) site of pVP16. *Eco*RI (5'-terminus)-*Bst*XI (216) fragments of these plasmids and *Bst*XI (216)-*Xba*I (3'-terminus) fragments of pCMV5Gi2 and pCMV5Gi2Q205L were simultaneously ligated to *Eco*RI/*Xba*I sites of pVP16, respectively.

pGEXSix1 was constructed as follows. The full length of the insert of pfSix1 was excised as the *Eco*RV-*Xba*I (blunt-ended) fragment and inserted into the *Sma*I site of pGEX-6P-1 (Amersham Pharmacia Biotech, Buckinghamshire, UK).

pGL3MG-1.7 was constructed by inserting the *Hin*dIII fragment (5' upstream 1.7 kb region of *myogenin* gene promoter) of pMGNLacZ (Fujisawa-Sehara et al. 1993) into the *Hin*dIII site of pGL3-Basic (Promega, Madison, WI).

Cell culture and reporter gene assays

293 and NIH3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 4.5 g glucose/l, 10% fetal bovine serum with 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C under 5% $CO_2$. For mammalian two-hybrid assays, transfections into 293 cells were performed by the standard calcium phosphate method as described previously (Murakami et al. 1998) in 3.5 cm-diameter dishes. For coactivation activity of Eya1, transfections into NIH 3T3 cells were performed by SuperFect Transfection Reagent (QIAGEN, Hilden, Germany) in 24-well plates. Two days after the transfection, cells were collected and lysed for

luciferase assays. All values were normalized by the internal controls of β-galactosidase activities.

GST-pulldown assays

GST, GST-Six2, GST-Six4 and GST-Six5 fusion proteins were prepared as described previously (Kawakami et al. 1996a,b). GST-Six1 fusion protein was prepared using pGEXSix1 by the same procedure. pHM6Eya1 and its mutations were applied to TNT Quick Coupled Transcription/Translation Systems (Promega, Madison, Wis.) to obtain HA-tagged Eya1 and its mutation proteins labeled with [$^{35}$S]methionine (Amersham Pharmacia Biotech).

GST, GST-Six1, GST-Six2, GST-Six4 and GST-Six5 fusion proteins bound to Glutathione Sepharose beads in the binding buffer (50 mM K-$PO_4$, pH 7.5, 150 mM KCl, 1 mM $MgCl_2$, 10% glycerol, 1% Triton X-100) were incubated with $^{35}$S-labeled HA-Eya1 and its mutations at 4°C for 2 h with rotation. Supernatants were recovered as unbound fractions. Beads were washed five times with the binding buffer and dissolved in SDS sample buffer, followed by SDS-PAGE and fluorography.

Protease digestion

$^{35}$S-labeled Eya1 proteins and their mutations were incubated with V8 protease or trypsin (Sigma, St. Louis, MO.) in a digestion buffer (50 mM Tris-HCl, pH 7.6, 25mM NaCl, and 2 mM EDTA) at 4°C for 30 min. After digestion, samples were