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# Reciprocal signaling between the transcriptional co-factor Eya2 and specific members of the G $\alpha$ i family

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Reciprocal signaling between Eya2 and specific G $\alpha$ i proteins

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**ABBREVIATIONS:** G protein, guanine nucleotide binding regulatory protein; HEK, human embryonic kidney; MEF3, myocyte expression factor-3; Luc, luciferase; GST, glutathione S-transferase; DTT, dithiothreitol; PBS, Phosphate-buffered saline; GFP, green fluorescent protein; QL, glutamine-to-leucine substitution rendering the G protein GTPase deficient; GTP $\gamma$ S, guanosine 5'-3-O-(thio)-triphosphate; GAP, GTPase activating protein; pNPP, p-nitrophenyl phosphate; RGS, regulator of G protein signaling.

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## ABSTRACT

As part of a program to elucidate signaling processes controlled by the heterotrimeric G protein  $G\alpha_z$ , a human fetal brain cDNA library was screened for proteins that specifically interact with the activated form of  $G\alpha_z$ . One of the most-encountered molecules in this screen was Eya2, a member of the *eyes absent* family of proteins. Mammalian Eya proteins are predominantly cytosolic proteins that are known to interact with members of the *sine oculis* (Six) family of homeodomain transcription factors. This interaction facilitates the translocation of Eya into the nucleus, where the Eya/Six complex regulates transcription during critical stages of embryonic development. *In vitro* binding studies confirmed that  $G\alpha_z$  interacts with Eya2 in an activation-dependent fashion; furthermore, most other members of the  $G\alpha_i$  family including  $G\alpha_i1$ ,  $G\alpha_i2$ , and  $G\alpha_i3$  were found to interact with Eya2. Interestingly, one of the most abundant  $G\alpha_i$  proteins,  $G\alpha_o$ , did not interact with Eya2. Co-expression of the activated forms of  $G\alpha_i1$ ,  $G\alpha_i2$ , and  $G\alpha_i3$ , but not  $G\alpha_o$ , with Eya2 recruited Eya2 to the plasma membrane, prevented Eya2 translocation into the nucleus, and abrogated Eya2/Six4-mediated transcription. Additionally, Eya2 impinged on G protein-mediated signaling, as evidenced by its ability to relieve  $G\alpha_i2$ -mediated inhibition of adenylyl cyclase. These results demonstrate that the interaction between the  $G\alpha_i$  proteins and Eya2 may impact on seemingly disparate regulatory events involving both classes of proteins.

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## Introduction

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are crucial components of cellular communication pathways that mediate transmission of extracellular signals from cell surface receptors to a wide range of intracellular effectors (Gilman, 1987; Neer, 1995). G proteins are composed of two functional signaling units, an  $\alpha$  subunit that binds guanine nucleotides, and a  $\beta\gamma$  dimer that dissociates from the  $\alpha$  subunit upon G protein activation. The  $G\alpha_i$  family of heterotrimeric G proteins consists of five  $\alpha$  subunits expressed in a variety of tissues ( $G\alpha_i1$ ,  $G\alpha_i2$ ,  $G\alpha_i3$ ,  $G\alpha_z$ , and  $G\alpha_o$ ), and two  $\alpha$  subunits specifically expressed in sensory tissues ( $G\alpha_t$  (transducin), and  $G\alpha_{gust}$  (gustducin))(Simon et al., 1991). Of the former category,  $G\alpha_i1$ ,  $G\alpha_i2$ , and  $G\alpha_i3$  are expressed fairly ubiquitously,  $G\alpha_o$  is found predominantly in the nervous system, and  $G\alpha_z$  is primarily found in brain, platelets, and several neuroendocrine tissues (Offermanns, 2001).

While members of the  $G\alpha_i$  family are primarily thought to be mediators of adenylyl cyclase inhibition (Taussig et al., 1993), many studies suggest that signaling through  $G\alpha_i$  proteins can also regulate cell growth and differentiation. Mutations that lock  $G\alpha_i2$  in its GTP-bound active state have been identified in several endocrine tumors (Lyons et al., 1990), and ectopic expression of these  $G\alpha_i$  mutants can transform Rat1a fibroblasts (Pace et al., 1991).  $G\alpha_i$  proteins have also been shown to influence neuronal growth in Neuro2a cells through a direct interaction with the G protein-regulated inducers of neurite growth, GRIN1 and GRIN2 (Chen et al., 1999).

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In ongoing efforts to elucidate the signaling functions of the G $\alpha$ i family member, G $\alpha$ z, we (Glick et al., 1998) and others (Fan et al., 2000), have employed yeast two-hybrid technology to identify direct downstream effectors of mutationally-activated G $\alpha$ z (the Q205L variant). We report here that one of the predominant interactors identified in our screen is Eya2, a human orthologue of the *Drosophila eyes absent* gene (*eya*).

*Drosophila* Eya is a key component of a complex regulatory network that is best known for directing compound eye formation in flies (Bonini et al., 1997), however Eya clearly plays important roles in the development of other tissues. In mammals, there are four orthologues of *eya*, denoted Eya1-4. Mutations in the human EYA1 gene are responsible for a rare autosomal dominant disease, Branchio-Oto-Renal (BOR) Syndrome, in which patients suffer from varying degrees of deafness and kidney anomalies due to the improper formation of ear and kidney structures (Abdelhak et al., 1997). Consistent with an involvement in BOR syndrome, Eya1-deficient mice lack ears and kidneys altogether, have craniofacial abnormalities, and additionally lack thyroid, parathyroid, and thymus glands (Xu et al., 1999).

Although it is clear that Eya proteins are important for the correct development of many organs and tissues, the molecular mechanisms underlying these processes remain unclear. Studies in mammals and flies have shown that Eya proteins are transcriptional cofactors for members of the *Six/sine oculis* family of homeodomain transcription factors, and that they activate the expression of genes important in cell cycle progression and differentiation (Li et al., 2003; Ohto et al., 1999; Pignoni et al., 1997). Recently, three groups found that Eya can also act as a protein phosphatase and that this activity may be crucial for proper transcriptional activation and developmental regulation (Li et

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al., 2003; Rayapureddi et al., 2003; Tootle et al., 2003). In mammalian cells, ectopically expressed Eya2 exhibits a punctate cytosolic localization pattern (Fan et al., 2000; Ohto et al., 1999). However, when co-expressed with specific members of the Six family, Eya2 translocates into the nucleus where it can participate with Six proteins in regulating transcription of specific target genes (Ohto et al., 1999).

In the current study, we report that Eya2 specifically interacts with the activated GTP-bound forms of G $\alpha$ i1, G $\alpha$ i2, G $\alpha$ i3, and G $\alpha$ z, but interestingly not the closely related G $\alpha$ o. Introduction of activated forms of these G $\alpha$ i subunits into cells results in recruitment of Eya2 to the plasma membrane, which prevents its participation in Six4-mediated transcriptional activation of a myogenin reporter construct. Additionally, Eya2 blocks G $\alpha$ i2-mediated inhibition of adenylyl cyclase, raising the possibility that Eya2 may have alternate functions that include regulation of G $\alpha$ i signaling.

## Materials and Methods

**Materials.** Plasmids, strains, and instructions for performing the yeast two-hybrid screen were obtained from Stephen Elledge (Baylor College of Medicine). CosP and HEK293 cells were obtained from the American Type Culture Collection. The anti-G $\alpha$ z polyclonal antisera and the anti-G $\alpha$  antibody were described previously (Casey et al., 1990). The cDNAs for human G $\alpha$ z, G $\alpha$ i1, G $\alpha$ i2, G $\alpha$ i3, G $\alpha$ o, G $\alpha$ s, G $\alpha$ 12, and G $\alpha$ q were obtained from the Guthrie Research Center (Sayre, PA). The MEF3/TATA luciferase promoter pGL3-MEF3-Luc was a gift from Dave Manning (University of Pennsylvania).

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The cDNA for murine pSVSport-Six4 was obtained from the Riken DNA Bank (Japan) with permission from Kiyoshi Kawakami (Jichi Medical School, Japan). Purified recombinant RGS10 was a gift from Andy Nixon (Duke University).

**Plasmid Constructs.** A partial cDNA encoding amino acids 97-538 of human EYA2 was isolated from yeast two-hybrid positive colonies (see results). The EYA2 fragment was excised from pACT2 and ligated into pRSETB. The missing N-terminal sequence was constructed by PCR cloning using a fetal human brain library (Genetrapp<sup>TM</sup>, Invitrogen, Carlsbad, CA). The ~700 base pair PCR product was digested with EcoRI and EcoNI and ligated into pRSETB already containing the EYA2 fragment. The full cDNA was sequenced and determined to be identical to the complete coding sequence of EYA2 (Accession: Y10261). N-terminal-Flag (DYKDDDDK)-tagged EYA2 and N-terminal-Myc (EQKLISEEDL)-tagged Six4 were generated by PCR and subsequently inserted into pcDNA3.1(+) (Invitrogen). The glutathione-S-transferase (GST)-EYA2 (97-538) fusion construct was generated by excising the EYA2 fragment from pRSETB and subcloning it into pGEX5X-1. The green fluorescent protein (GFP)-tagged EYA2 was generated by subcloning full-length EYA2 into pEGFP-C2 (Clontech, Palo Alto, CA).

**Protein Production and Purification.** Recombinant G $\alpha$ <sub>z</sub>, G $\alpha$ <sub>i2</sub>, G $\alpha$ <sub>o</sub>, and G $\alpha$ <sub>s</sub> were purified from *E. coli* as described previously (Casey et al., 1990; Graziano et al., 1991; Linder et al., 1991). For production of GST proteins, plasmids encoding GST and the GST-Eya2 (97-538) fusion protein were transformed into the BL21 (DE3) strain of *E.*



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*coli*. Cultures were grown at 37 °C to an OD<sub>600nm</sub> of 0.8 and induced with 3 mM isopropyl β-D- thiogalactopyranoside (IPTG) (Teknova, Hollister, CA) for 3 h at 30 °C. Cells were harvested by centrifugation and lysed by french press in buffer A [50 mM Tris-HCl (pH 7.7), 0.4 M NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol, and complete Mini Protease Inhibitor Cocktail tablets (Roche, Indianapolis, IN)]. The lysate was centrifuged at 30,000 x g for 1 h at 4 °C and the resulting supernatant collected and incubated with glutathione-sepharose beads in buffer A for 2 h at 4 °C with continuous rocking. The beads were washed 3 times with buffer B [50 mM Tris-HCl (pH 7.7), 150 mM NaCl, 1 mM EDTA, 1 mM DTT] and stored in buffer B at -80°C. For use in GTPase assays, soluble GST-Eya2 was prepared by eluting GST-Eya2 from the beads with three equal volumes of buffer C [20 mM Tris (pH 7.7), 20 mM Glutathione, 1 mM EDTA] at 37°C for 10 min, concentrated using an Amicon YM30 (Millipore Corporation, Bedford, MA ), and exchanged into 50 mM Tris-HCl (pH 7.7-8), 1 mM EDTA, 1 mM DTT. Protein purity was assessed by SDS-PAGE analysis with Coomassie blue staining. RGS-10 used in GAP assays was purified as previously described (Hunt et al., 1996).

**Cell Culture and Transfection Conditions.** CosP and HEK293 cells were grown in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) with 10% fetal bovine serum (FBS). Transfections were performed using LipofectAMINE 2000™ (Invitrogen) or Polyfect® (Qiagen, Valencia, CA) in 6-well culture plates according to standard manufacturer protocols.

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**GST pull-down assays.** The assessment of specificity of G $\alpha$  interactions with Eya2 was conducted using G $\alpha$  subunits produced in a combined transcription/translation system. <sup>35</sup>S-labeled G $\alpha$  subunits were generated from cDNAs encoding the activated (QL variants) of G $\alpha$ <sub>z</sub>, G $\alpha$ <sub>i1</sub>, G $\alpha$ <sub>i2</sub>, G $\alpha$ <sub>i3</sub>, G $\alpha$ <sub>o</sub>, G $\alpha$ <sub>s</sub>, G $\alpha$ <sub>q</sub>, and G $\alpha$ <sub>12</sub> in pcDNA3.1(+) using the TNT<sup>®</sup> Rabbit Reticulocyte Lysate System (Promega, Madison, WI) according to manufacturer's protocol. To assess direct binding and G $\alpha$  specificity, 10  $\mu$ l of each reaction was incubated with 30  $\mu$ l of GST-Eya2 beads in buffer C [50 mM Hepes (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.1% Lubrol, 5 mM MgCl<sub>2</sub>, 10% casein acid hydrolysate (Sigma, St. Louis, MO)] for 2 h at 4°C while rocking. GST-Eya2 beads were washed 3 times with 1 ml buffer C, and assessed for binding to G $\alpha$  subunits by SDS-PAGE (12%) followed by autoradiography.

To assess the activation dependence of the interaction of Eya2 with G $\alpha$  proteins, selected purified recombinant G $\alpha$  proteins (20 pmol) were preloaded with either GDP or GTP $\gamma$ S in 50 mM HEPES, (pH 8.0), 1 mM DTT, 0.1% Lubrol, and 200  $\mu$ M GTP $\gamma$ S or GDP at 30°C for 90 min. The concentrations of EDTA and MgCl<sub>2</sub> varied depending on the G protein; G $\alpha$ <sub>z</sub> was loaded in the presence of 5mM EDTA, whereas G $\alpha$ <sub>i2</sub>, G $\alpha$ <sub>o</sub>, and G $\alpha$ <sub>s</sub> were loaded in the presence of 2.5 mM MgCl<sub>2</sub> and 0.5 mM EDTA. Binding studies were performed by incubating ~5 pmol of GTP $\gamma$ S or GDP-loaded G proteins with either GST-Eya2 (97-538) or GST bound to glutathione-sepharose beads (30  $\mu$ l suspension) in buffer C (250  $\mu$ l) for 2 hours at 4 °C with continuous rocking. GST-Eya2 beads were washed 3 times with 1 ml buffer C and processed by SDS-PAGE followed by immunoblot analysis.

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**Fluorescence microscopy.** For immunofluorescence experiments, CosP cells were seeded onto sterilized glass coverslips in 6-well plates and transfected with combinations of expression constructs containing Flag-EYA2, Myc-Six4, G $\alpha$ 1QL, G $\alpha$ 2QL, G $\alpha$ 3QL, G $\alpha$ oQL, and G $\alpha$ sQL. Following a 48 h incubation, cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde at room temperature for 10 min. The cells were washed with PBS, and then incubated in PBS containing 0.1% Triton X-100 and 10% calf serum for 15 min. Coverslips were incubated with mouse anti-Flag M2 monoclonal antibody (Sigma) for detection of Flag-Eya2, mouse anti-Myc monoclonal antibody (Zymed, San Francisco, CA) for detection of Myc-Six4 alone, or a combination of mouse anti-Flag M2 antibody and goat anti-Myc polyclonal antibody (Santa Cruz, Santa Cruz, CA) for colocalization of Flag-Eya2 and Myc-Six4. Antibody incubations were performed in PBS containing 10% calf serum for 1 h at dilutions of 1:10,000 for the anti-Flag antibody and 1:1000 for anti-Myc antibodies. The cells were washed three times with PBS containing 10% calf serum, then incubated for 1 h with either a 1:1000 dilution of donkey anti-mouse-Cy3 or a 1:100 dilution of donkey anti-goat-Cy2 (Jackson ImmunoResearch, Inc., West Grove, PA). Cells were washed again, as above, and mounted using Pro-long anti-fade kit (Molecular Probes, Inc., Eugene, OR) for viewing by fluorescence microscopy using a Nikon Eclipse TE300 inverted microscope at 600 x magnification (Melville, NY). Protein expression was verified in parallel experiments by SDS-PAGE followed by immunoblot analysis with the appropriate antisera. For fluorescence microscopy in live cells, CosP cells were seeded into coverglass dishes (MatTek, Ashland, MA) and transfected with 0.5  $\mu$ g of GFP-EYA2 alone or in the presence of expression constructs containing G $\alpha$ 1QL, G $\alpha$ 2QL,

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G $\alpha$ i3QL, G $\alpha$ oQL, and G $\alpha$ sQL. After 24 h, cells were washed with PBS and viewed using a Nikon Eclipse TE300 inverted microscope at 600 x magnification (Melville, NY).

**Transcriptional Reporter Assays.** HEK293 cells were seeded into 6-well plates and transfected with 0.3  $\mu$ g of pGL3-MEF3-Luc reporter construct, 0.01  $\mu$ g of pRL-TK renilla as an internal reporter control (Promega), and appropriate combinations of expression constructs containing 0.5  $\mu$ g of Flag-EYA2, 0.3  $\mu$ g of Myc-Six4, and 0.5  $\mu$ g of G protein as indicated. Forty-eight hours after transfection, cell lysates were prepared and luciferase activity was measured using the Dual Luciferase Assay kit (Promega) according to the manufacturer's protocol. Transcriptional activity was measured using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) and reported as a ratio of luciferase to renilla luminescence. Protein expression levels were determined by SDS-PAGE (12%) followed by immunoblot analysis with the appropriate antisera.

**GAP Assays.** Single-turnover GTPase experiments, commonly referred to as GAP assays, were performed as previously described (Hunt et al., 1996). Purified G $\alpha$ i2 was loaded with [ $\gamma$ -<sup>32</sup>P]-GTP (50,000 cpm. pmol<sup>-1</sup>) for 30 min at 30 °C. Magnesium was excluded from all loading reactions to slow hydrolysis. Free nucleotide was removed from the GTP-bound G protein by centrifugation at 200 x g for 3 min at 4 °C through 1 ml of G50 Sephadex (Sigma) that was equilibrated with 50mM HEPES (pH 8.0), 1 mM EDTA, and 1mM DTT. GTP hydrolysis of G $\alpha$ i2-GTP (13.3 nM) was determined by measuring the release of free phosphate after the addition of 5.0 mM MgCl<sub>2</sub> for 30 s at 4 °C in the presence or absence of purified recombinant GST-Eya2 (1 $\mu$ M) or RGS10 (50

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nM). Results are reported as a fold-change over the intrinsic rate of G $\alpha$ i2 GTP hydrolysis.

**Quantitation of cAMP.** CosP cells were seeded into 6-well plates and transfected with 1  $\mu$ g of empty vector (pcDNA3.1) or expression constructs encoding G $\alpha$ i2QL, Flag-EYA2, or both combined. Total DNA concentration was kept constant in each well by the addition of empty vector as required. After 24 h, cells were treated with 100  $\mu$ M isoproterenol for 5 min, lysed, and cAMP levels were measured using the cAMP Biotrak Enzymeimmunoassay System (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's protocol. Data are reported as the level of cAMP per mg protein. Statistical analysis of samples with G $\alpha$ i2QL and G $\alpha$ i2QL containing Eya2 following isoproterenol treatment was evaluated by 2-tailed paired t-test using GraphPad Prism (GraphPad Software Inc., San Diego, CA) 4.0a for Mac. A value of  $p < 0.05$  was considered significant.

## Results

**The activated forms of the G $\alpha$ i1, G $\alpha$ i2, G $\alpha$ i3, and G $\alpha$ z, but not G $\alpha$ o, interact with Eya2.** A yeast two-hybrid screen was conducted using a mutationally-activated form of G $\alpha$ z (the Q205L variant) as bait to screen a human brain cDNA library. We previously reported on two interactors identified in this screen, RGSZ (Glick et al., 1998), and Rap1GAP (Meng et al., 1999), both of which were further implicated in signaling processes specifically linked to G $\alpha$ z (Meng and Casey, 2002; Nixon et al., 2002). In

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addition to RGSZ and Rap1GAP, 26 of the 100 positive clones that emerged from the two-hybrid screen encoded Eya2, making it one of the predominant molecules identified in the screen. To confirm the interaction between Eya2 and G $\alpha$ <sub>z</sub>, and to assess the specificity of the interaction, we performed pull-down experiments using a GST-Eya2 fusion protein and <sup>35</sup>S-labeled G $\alpha$  subunits generated using an *in vitro* transcription/translation system. As shown in Fig. 1A, GST-Eya2 clearly bound not only the mutationally-active (QL variant) form of G $\alpha$ <sub>z</sub>, but also other G $\alpha$ <sub>i</sub> family members including G $\alpha$ <sub>i1</sub>, G $\alpha$ <sub>i2</sub>, G $\alpha$ <sub>i3</sub>. Eya2 showed no interaction with the closely related G $\alpha$ <sub>o</sub>, or other G $\alpha$  family members including G $\alpha$ <sub>s</sub>, G $\alpha$ <sub>q</sub>, or G $\alpha$ <sub>12</sub>. That Eya2 did not bind with G $\alpha$ <sub>o</sub> was surprising based on the high degree of sequence similarity between G $\alpha$ <sub>o</sub> and other G $\alpha$ <sub>i</sub> family members, as well as the fact that G $\alpha$ <sub>o</sub> couples to many of the same receptors as other G $\alpha$ <sub>i</sub> proteins indicating a distinct requirement for specific members of the G $\alpha$ <sub>i</sub> family in regulating Eya2-mediated signaling events.

To assess the activation dependence of the G $\alpha$ <sub>i</sub>-Eya2 interaction, recombinant G $\alpha$ <sub>z</sub>, G $\alpha$ <sub>i2</sub>, G $\alpha$ <sub>o</sub>, and G $\alpha$ <sub>s</sub> were loaded with either GDP or GTP $\gamma$ S and assessed for binding with GST-Eya2. In these experiments, only the active GTP $\gamma$ S forms of G $\alpha$ <sub>z</sub> and G $\alpha$ <sub>i2</sub> bound to GST-Eya2, while the active forms of G $\alpha$ <sub>o</sub> and G $\alpha$ <sub>s</sub> showed no binding to Eya2 (Fig. 1B). These results clearly demonstrate that G $\alpha$ <sub>i1</sub>, G $\alpha$ <sub>i2</sub>, G $\alpha$ <sub>i3</sub>, and G $\alpha$ <sub>z</sub>, but not G $\alpha$ <sub>o</sub>, or other G $\alpha$  families, bind with Eya2 in an activation dependent manner.

**Activated forms of G $\alpha$ <sub>i</sub> proteins recruit Eya2 to the plasma membrane.** To examine whether G $\alpha$ <sub>i</sub> proteins could interact with Eya2 in mammalian cells, we expressed a green

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fluorescent protein (GFP)-tagged Eya2 in CosP cells in the presence and absence of various mutationally-activated G $\alpha$  subunits and viewed by fluorescence microscopy. Consistent with previous findings (Fan et al., 2000; Ohto et al., 1999), GFP-Eya2 exhibited a punctate pattern within the cytosol (Fig. 2A). However, when co-expressed with the mutationally-activated forms of G $\alpha$ i1, G $\alpha$ i2, and G $\alpha$ i3, GFP-Eya2 translocated from the cytosol to the plasma membrane in greater than 80% of transfected cells (Fig. 2B-D). In agreement with the *in vitro* binding results in Fig. 1, expression of ectopic G $\alpha$ o, the most abundant G $\alpha$ i family member in vertebrates, did not alter the cellular localization of GFP-Eya2 (Fig 2E). The altered localization of GFP-Eya2 was also not observed when co-expressed with G $\alpha$ s, even though protein expression was similar for all G $\alpha$  subunits (Fig. 2F).

**G $\alpha$ i proteins attenuate the interaction of Eya2 with Six4 and block Eya2-mediated transcriptional activation.** Eya2 is known to activate transcription via an interaction with the Six family of homeodomain transcription factors, and this property is dependent on the cellular localization of Eya2 (Ohto et al., 1999). Previous studies have shown that, when exogenously expressed in cells, members of the Six family localize to the nucleus, while Eya2 resides predominantly in the cytosol. However, co-expression of Eya2 with Six proteins results in a translocation of Eya2 into the nucleus where the complex activates transcription of specific target genes important in embryonic development (Fan et al., 2000; Ohto et al., 1999). To investigate whether activated G $\alpha$ i proteins are able to influence the ability of Six to recruit Eya2 into the nucleus, we assessed the localization pattern of Eya2 and Six4 in the presence and absence of G $\alpha$ i expression. Consistent with

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previous results, when Flag-tagged Eya2 was introduced into CosP cells, it localized predominantly in the cytosol (Fig. 3A), while Myc-tagged Six4 localized exclusively to the nucleus (data not shown). Moreover, when Flag-Eya2 was co-expressed with Myc-Six4, Eya2 was now found exclusively in the nucleus together with Six4 (Fig. 3B). Introduction of the activated forms of  $G\alpha i1$ ,  $G\alpha i2$ ,  $G\alpha i3$ , but again not  $G\alpha o$  or  $G\alpha s$ , prevented the nuclear localization of Eya2 (Fig. 3C-F), even though Six4 was still found in the nucleus by colocalization studies (data not shown). Greater than 80% of transfected cells expressing Flag-Eya2 exhibit the expression patterns presented in Figure 3. When co-expressed with Myc-Six4, Flag-Eya2 localized exclusively to the nucleus in 295 of 300 (98%) cells counted, however when Myc-Six4 and Flag-Eya2 were co-expressed with  $G\alpha i2QL$ , for example, 243 of 300 cells counted (81%) exhibited a membrane/cytosolic pattern similar to that seen in Figure 3. In the majority of the remaining 57 of 300 cells, Flag-Eya2 was distributed in both membrane/cytosolic and nuclear pools within each cell, suggesting that  $G\alpha i$  proteins may not always entirely exclude Eya2 from the nucleus, but may help regulate the level of Eya2 nuclear accumulation. Results were similar for  $G\alpha i1QL$  and  $G\alpha i3QL$ , while in almost all of the cells co-expressing Flag-Eya2, Myc-Six4, and  $G\alpha oQL$  or  $G\alpha sQL$  together, Flag-Eya2 was localized in the nucleus, consistent with the finding that Eya2 does not interact with  $G\alpha o$  or  $G\alpha s$ . The results of Fig. 2 and 3 suggest that, by recruiting Eya2 to the plasma membrane,  $G\alpha i1$ ,  $G\alpha i2$ , and  $G\alpha i3$ , are able to prevent the nuclear localization of Eya2 triggered by the expression of Six4 while  $G\alpha o$  cannot.

Nuclear Eya2 significantly enhances the Six-mediated transcription of a myogenin luciferase reporter construct (Fan et al., 2000; Ohto et al., 1999). Based on the *in vitro*



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binding data (Fig. 1) and cellular localization studies of Eya2 (Fig. 2 and 3), it seemed likely that activated G $\alpha$ i proteins would attenuate the ability of Eya2 to enhance the transcriptional activity of Six4. We used a luciferase reporter construct fused to the MEF3 region of the myogenin promoter (pGL3-MEF3-Luc) (Fan et al., 2000) to further characterize other members of the G $\alpha$ i family including G $\alpha$ i1, G $\alpha$ i3, and G $\alpha$ o. We chose the Six4 homologue to examine Eya2-mediated co-activation of transcription of this reporter because previous studies indicated that of the characterized Six family members, Eya2 has the strongest co-activation response with Six4 (Ohto et al., 1999). As expected, co-expression of Six4 and Eya2 strongly activates the transcription of the MEF3 reporter (Fig. 4). This synergistic activity of Eya2 was significantly inhibited when the active forms of G $\alpha$ i1, G $\alpha$ i2, and G $\alpha$ i3 were introduced into the cells. Consistent with the *in vitro* and cellular translocation studies, expression of activated G $\alpha$ o had no effect on transcriptional co-activation activity of Eya2. These results confirm that G $\alpha$ i1, G $\alpha$ i2, and G $\alpha$ i3 are able to attenuate Eya2/Six4-mediated transcription of a MEF3/myogenin promoter element and provide the final piece of evidence that the closely-related G $\alpha$ o does not share this property.

**Eya2 can relieve G $\alpha$ i2-mediated inhibition of adenylyl cyclase.** The data summarized above, and other currently available data on G $\alpha$ i-Eya2 interactions (Fan et al., 2000), indicate that activated G $\alpha$ i proteins can prevent the nuclear localization and function of Eya2. While one functional consequence of the G $\alpha$ i-Eya2 interaction is an attenuation of Eya2-mediated transcriptional co-activation, the possibility that Eya2 could impinge on aspects of G $\alpha$ i signaling has not been addressed. Since Eya2 shares a property of RGS

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proteins in that it selectively interacts with the activated GTP-liganded form of the  $G\alpha$  protein, we examined whether Eya2 binding might enhance the intrinsic GTPase activity of  $G\alpha i$  proteins. However, even using concentrations of Eya2 that were 20-fold greater than those of RGS10, no effect on the GTPase activity of  $G\alpha i2$  was observed (Figure 5A).

We then hypothesized that, since Eya2 binds to the active form of  $G\alpha i$  subunits, it may block the ability of activated  $G\alpha i$  to inhibit adenylyl cyclase, which is a well-characterized effector function of  $G\alpha i$  proteins. To test this possibility, we assessed  $G\alpha i$ -mediated inhibition of adenylyl cyclase in CosP cells. Addition of isoproterenol to CosP cells markedly increased intracellular levels of cAMP, and this stimulation was substantially attenuated by expression of  $G\alpha i2QL$  (Fig. 5B). However, co-expression of Eya2 completely relieved the  $G\alpha i$ -mediated cyclase inhibition. The effect of Eya2 expression on cAMP levels was not due to an alteration in expression levels of activated  $G\alpha i2$ , as the levels of this protein were the same in the presence and absence of Eya2 (data not shown). These results indicate that binding of Eya2 to activated  $G\alpha i2$  can attenuate G protein signaling and suggest that reciprocal regulation of Eya2 and  $G\alpha i$  signaling functions may be important in biological response pathways coupled to these distinct proteins.

## Discussion

Primarily studied as components in processes involving receptor-mediated inhibition of adenylyl cyclase, members of the  $G\alpha i$  family have been implicated in the

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modulation of mitogenic pathways and transcriptional regulation, although G $\alpha$ i involvement in most of these processes is not well understood and often involves  $\beta\gamma$  signaling (Gutkind, 1998). In an effort to identify signaling pathways directly mediated by the G $\alpha$ i family member G $\alpha$ z, we used yeast two-hybrid technology to screen for molecules that interact directly with the activated form of G $\alpha$ z. One of the predominant interacting proteins identified in this screen was Eya2, the human orthologue of a *Drosophila* gene product important in embryonic development. In this study, we report that the interaction of Eya2 with several, but not all, members of the G $\alpha$ i family of proteins has a profound impact on both Eya2 and G $\alpha$ i functions in cells.

Several studies hint at G $\alpha$ i family member involvement in aspects of development. Mice with tissue-specific expression of antisense RNA directed toward G $\alpha$ i2 display a runted phenotype and abnormal development in targeted organs (Moxham et al., 1993). Subsequently, G $\alpha$ i2-deficient mice generated by homologous recombination were found to grow more slowly than wild-type mice, die prematurely, and develop adenocarcinoma of the colon (Rudolph et al., 1995). G $\alpha$ o-deficient mice are also smaller and weaker than littermates, have much lower survival rates, and have CNS defects (Jiang et al., 1998; Valenzuela et al., 1997).

To date, no obvious phenotype has been reported for mice with homozygous inactivation of G $\alpha$ i1 and G $\alpha$ i3 (Offermanns, 2001), while the only phenotypes reported for G $\alpha$ z-deficient mice are altered responses to psychoactive drugs and mild defects in platelet activation (Hendry et al., 2000; Yang et al., 2000). While it is somewhat surprising that targeted inactivation of G $\alpha$ i family proteins does not produce more striking phenotypes, functional redundancy among the family members is the likely

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explanation. G $\alpha$ i proteins are widely expressed, have high degrees of sequence similarity, and couple to many of the same receptors. In this regard, a recent study in zebrafish has provided strong evidence for significance of G $\alpha$ i family proteins in development (Hammerschmidt and McMahon, 1998). In this study, zebrafish embryos were treated with pertussis toxin, a bacterial toxin that inactivates G $\alpha$ i signaling by catalyzing the ADP-ribosylation of a C-terminal cysteine of all G $\alpha$ i family members except G $\alpha$ z. Embryos thus treated display abnormal head morphologies, fused eyes, and have other brain and somite patterning defects, indicating that broad removal of G $\alpha$ i function has profound consequences on development (Hammerschmidt and McMahon, 1998).

While it is difficult to ascribe a direct biological role for the binding of G $\alpha$ i proteins and Eya2, the data presented in this paper suggest several possible consequences of the interaction. The selective binding of Eya2 to active GTP-bound forms of G $\alpha$ i proteins suggests that this is a biologically-relevant interaction that is dependent on signaling events initiated through G $\alpha$ i-coupled receptors. The interaction is likely important during embryonic or postnatal development since the highest levels of Eya2 expression and signaling appear to be during time periods critical in the correct patterning of organs and tissues (Fougerousse et al., 2002; Xu et al., 1997). While there is limited data on G $\alpha$ i protein expression during development, several studies have shown that members of the G $\alpha$ i subfamily are specifically upregulated in distinct patterns in the developing brain (Schuller et al., 2001). Interestingly, the *Drosophila* G $\alpha$ i homologue plays a critical role in asymmetric cell division in the developing neuroblast and sensory organ precursor cells (Schaefer et al., 2001). Further, the only identified Regulator of G

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protein signaling (RGS) in *Drosophila, loco*, is required for the correct development of glial cells (Granderath et al., 1999). Additionally, the regulatory proteins Rap1GAP and GRIN1/2 have recently been implicated in neuronal growth and differentiation through interactions with members of the G $\alpha$ i family (Chen et al., 1999; Meng and Casey, 2002).

Based on the high degree of sequence similarity between G $\alpha$ i family members, we were somewhat surprised that, while G $\alpha$ i1, G $\alpha$ i2, and G $\alpha$ i3, and G $\alpha$ z all interacted with Eya2, G $\alpha$ o showed no such interaction. While G $\alpha$ o is highly expressed in the brain, the signaling functions of G $\alpha$ o have been elusive (Offermanns, 2001). If the interaction between Eya2 and G $\alpha$ i proteins is important in brain growth, differentiation, or function, the fact that Eya2 does not interact with G $\alpha$ o may be a means of ensuring that other G $\alpha$ i signaling events are not overwhelmed by the presence of the highly-abundant G $\alpha$ o. Alternatively, the inability of Eya2 to interact with G $\alpha$ o could also suggest that the interaction between Eya2 and G $\alpha$ i proteins is primarily important in areas peripheral tissues, since G $\alpha$ i1, G $\alpha$ i2, and G $\alpha$ i3 are nearly ubiquitously expressed.

The data in this report and in an earlier study (Fan et al., 2000), clearly demonstrate that activated G $\alpha$ i proteins can inhibit the ability of Eya2 to localize to the nucleus with Six proteins. In HEK293 cells transcriptional reporter assays using the MEF3 binding element of the myogenin promoter showed that the activated forms of G $\alpha$ i proteins attenuate Eya2/Six4-mediated transcriptional responses. Although the activation of heterotrimeric G proteins is a fairly short-lived event, it is possible that transcription of Eya2 target genes is highly dependent upon strict concentration gradients within the cell, and requires tight regulation during critical developmental time periods. In this situation, it is possible that activation of G $\alpha$ i proteins may serve to limit the amount of Eya2

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entering the nucleus at a given time. A second potential consequence of the interaction between Eya2 and G $\alpha$ i family members is that Eya2 impinges on certain aspects of G $\alpha$ i signaling. Support for this hypothesis comes from our finding that Eya2 can relieve G $\alpha$ i2-mediated inhibition of adenylyl cyclase (Fig. 5). There are many examples that illustrate important roles for cAMP in both developmental and post-developmental signaling processes (Defer et al., 2000).

The importance and diversity of signaling events mediated by members of the heterotrimeric G $\alpha$ i family have been well documented, and recent studies are beginning to provide insight into the many critical roles that Eya proteins play in embryonic development. Due to the highly conserved nature of Eya proteins, it is likely that G $\alpha$ i proteins interact with other members of the Eya family as well as Eya2. In fact, Kawakami *et al.* (Ozaki et al., 2002) recently showed that mutations in Eya1 that are associated with Branchio-Oto-Renal syndrome interfere with the ability of Eya1 to bind not only to its associated transcription factors but also the activated forms of G $\alpha$ z and G $\alpha$ i2. Based on the many possible combinations of interaction between G $\alpha$ i and Eya proteins, it appears that the interaction of these two families may have multiple consequences in developmental signaling. It will also be interesting to see whether targeted disruption of multiple G $\alpha$ i family members can provide further insight into the functional consequence of the interaction between G $\alpha$ I and Eya proteins during development.

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## Footnotes

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## Figure legends

**Figure 1.** The activated forms of  $G\alpha_z$ ,  $G\alpha_i1$ ,  $G\alpha_i2$ , and  $G\alpha_i3$  interact with Eya2 *in vitro*.

A, Mutationally-active [ $^{35}\text{S}$ ]-methionine-labeled  $G\alpha\text{QL}$  subunits were generated by *in vitro* transcription/translation and then incubated with GST-Eya2 (97-538) attached to glutathione-sepharose beads as described in “Materials and Methods.” GST-Eya2 beads were washed, and protein interactions were evaluated by SDS-PAGE followed by autoradiography. An autoradiograph of mutationally-active subunits that precipitated with GST-Eya2 is shown in the top panel, and the corresponding  $G\alpha\text{QL}$  subunits generated by *in vitro* transcription/translation used in the experiment are shown below (load). The data presented are representative of three separate experiments. B, Purified recombinant  $G\alpha$  subunits were loaded with either  $\text{GTP}\gamma\text{S}$  or  $\text{GDP}$  as described in “Experimental Procedures” and then incubated with GST-Eya2 beads. GST-Eya2 beads were washed and bound proteins were separated by SDS-PAGE and evaluated by Western blotting with anti- $G\alpha_z$  antisera (P961) or a pan- $G\alpha$  antisera (P960). The analysis of  $G\alpha_z$ ,  $G\alpha_i2$ ,  $G\alpha_o$ , and  $G\alpha_s$  precipitated by GST-Eya2 is shown in the top panel, and the corresponding recombinant  $G\alpha$  subunits input into the experiment, designated as load, are shown below. Data shown are from two separate experiments that were each repeated three separate times.

**Figure 2.**  $G\alpha_z$ ,  $G\alpha_i1$ ,  $G\alpha_i2$ , and  $G\alpha_i3$  recruit GFP-Eya2 to the plasma membrane.

CosP cells were seeded into coverglass dishes and transfected with 0.5  $\mu\text{g}$  of expression plasmids encoding GFP-EYA2 alone, or in the presence of mutationally-active forms of

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G $\alpha$  subunits as described under “Materials and Methods.” Cellular localization of GFP-Eya2 expressed alone (A), GFP-Eya2 co-expressed with G $\alpha$ i1QL (B), GFP-Eya2 co-expressed with G $\alpha$ i2QL (C), GFP-Eya2 co-expressed with G $\alpha$ i3QL (D), GFP-Eya2 co-expressed with G $\alpha$ oQL (E), and GFP-Eya2 co-expressed with G $\alpha$ sQL (F). Imaging of GFP-Eya2 in live cells was performed using a Nikon Eclipse TE300 inverted microscope at 600  $\times$  magnification. Data are representative of four separate experiments.

**Figure 3.** G $\alpha$ z, G $\alpha$ i1, G $\alpha$ i2, and G $\alpha$ i3 inhibit the Six4-mediated nuclear translocation of Eya2. Combinations of plasmids encoding Flag-Eya2, Myc-Six4, and activated G $\alpha$  subunits were introduced into CosP cells by transient transfection. After forty-eight hours, cells were fixed in 4% paraformaldehyde and stained for Flag-Eya2 with a mouse monoclonal anti-Flag antibody followed by Cy3-conjugated donkey anti-mouse as described under “Materials and Methods.” Cellular localization of Flag-Eya2 expressed alone (A), Flag-Eya2 co-expressed with Myc-Six4 (B), Flag-Eya2 co-expressed with Myc-Six4 and G $\alpha$ i1QL (C), Flag-Eya2 co-expressed with Myc-Six4 and G $\alpha$ i2QL (D), Flag-Eya2 co-expressed with Myc-Six4 and G $\alpha$ i3QL (E), and Flag-Eya2 co-expressed with Myc-Six4 and G $\alpha$ oQL (F). Flag-Eya2 localization was performed using a Nikon Elipse TE300 inverted microscope at 600  $\times$  magnification. Data are representative of four separate experiments.



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**Figure 4.**  $G\alpha_z$ ,  $G\alpha_i1$ ,  $G\alpha_i2$ , and  $G\alpha_i3$  inhibit Eya2/Six4-mediated transcription.

HEK293 cells were transiently transfected with pGL3-MEF3-Luc reporter, pRL-TK, and combinations of plasmids encoding Flag-Eya2, Myc-Six4, and mutationally-activated  $G\alpha$  subunits as described under “Materials and Methods.” Empty vector (pcDNA3.1+) was used to adjust samples to an equal amount of total DNA for all transfections. After 48 hours, cell lysates were analyzed for firefly luciferase (pGL3-MEF3-Luc) activity and *Renilla* luciferase (pRL-TK) activity as an internal standard for transfection efficiency using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). The results of this experiment are reported as a ratio of Firefly luciferase reporter activity to control *Renilla* luciferase activity. The data presented are the mean and standard error of quadruplicate measurements from a single experiment, and are representative of data from three separate experiments.

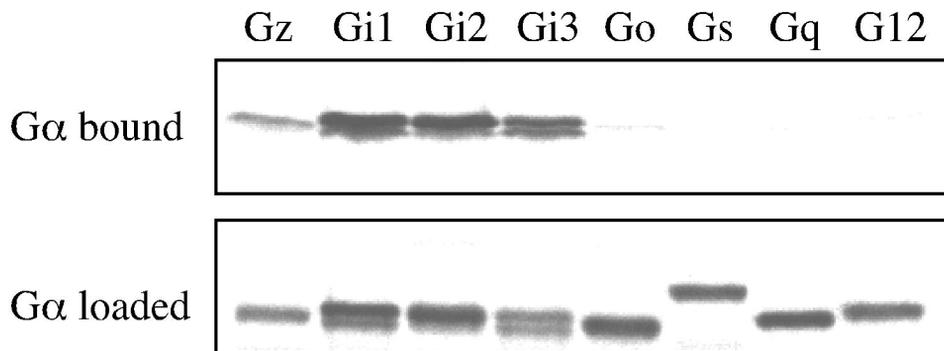
**Figure 5.** Effect of Eya2 on  $G\alpha_i2$  biochemistry and signaling. *A*, GTP hydrolysis by  $G\alpha_i2$  was determined as described under “Materials and Methods.” Purified  $G\alpha_i2$  (13.3 nM) was loaded with [ $\gamma$ - $^{32}$ P]GTP and incubated with GST-Eya2 (1  $\mu$ M) or RGS10 (50 nM) at 4 °C for 30 sec. The data presented are the mean and standard error of quadruplicate measurements from a single experiment, and are representative of data from three separate experiments. *B*, CosP cells were transfected with combinations of pcDNA3.1(+),  $G\alpha_i2QL$ , and Flag-Eya2. Empty vector (pcDNA3.1(+)) was used to adjust samples to an equal amount of total DNA for all transfections. After twenty-four hours, cells were either left untreated, or treated with 100  $\mu$ M isoproteranol for six minutes.

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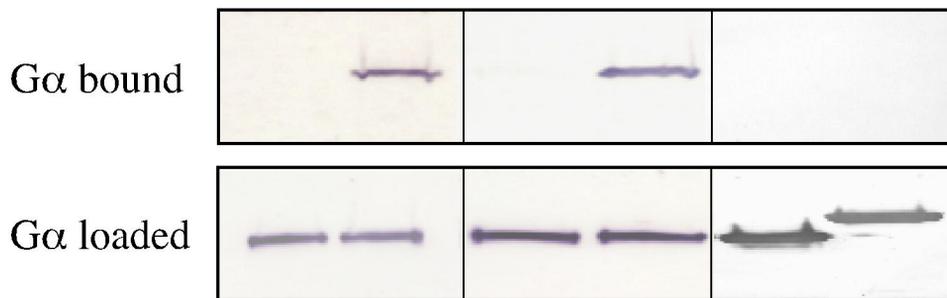
Cells were lysed and intracellular cyclic AMP levels were determined using a competitive enzyme-immunoassay as described under “Materials and Methods.” The data presented are the mean and standard error of quadruplicate measurements from a single experiment, and are representative of data from three separate experiments. The cAMP level in unstimulated cells was 18.67 +/-1.02 pmol/mg protein. The means of the values of samples containing G $\alpha$ i2QL and G $\alpha$ i2QL with Eya2 were evaluated by 2-tailed paired t-test. \*  $p < 0.05$ .

# Figure 1

A



B



Gα form	Gz		Gi2		Go		Gs
	GDP	GTPγS	GDP	GTPγS	GTPγS		GTPγS
Gα	Gz		Gi2		Go		Gs

Figure 2

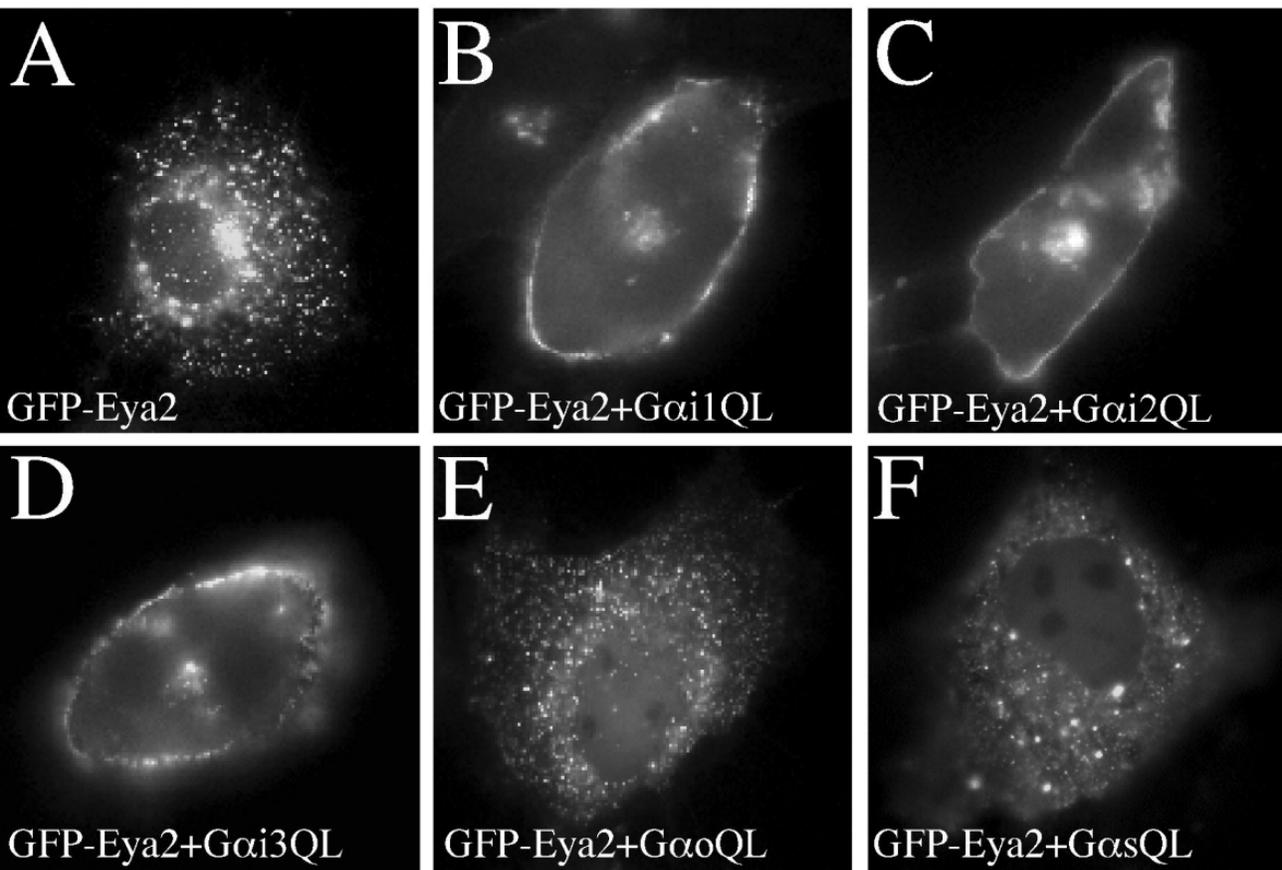


Figure 3

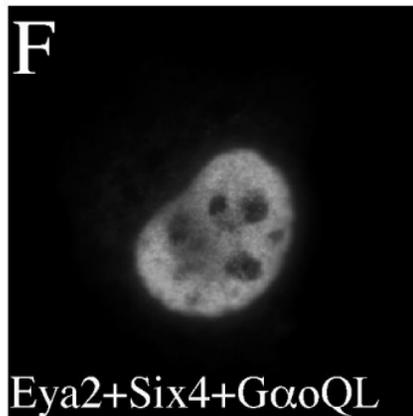
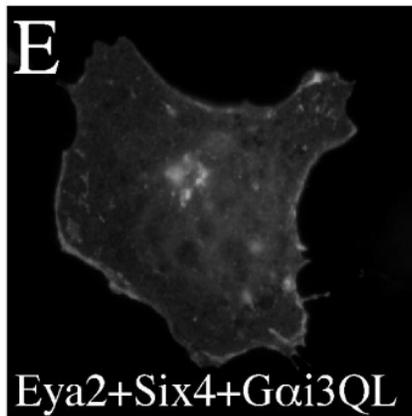
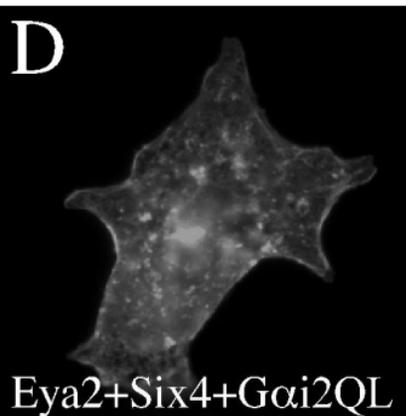
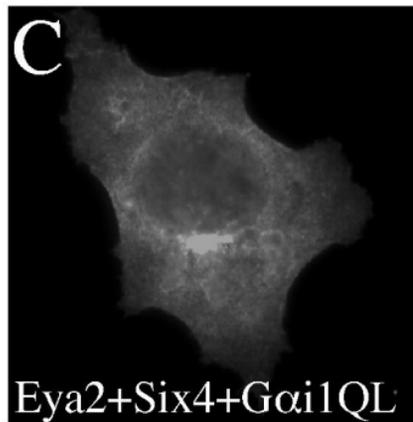
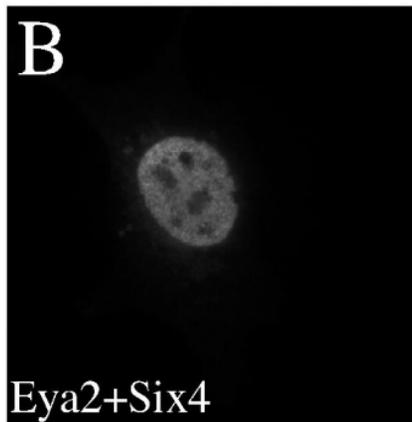
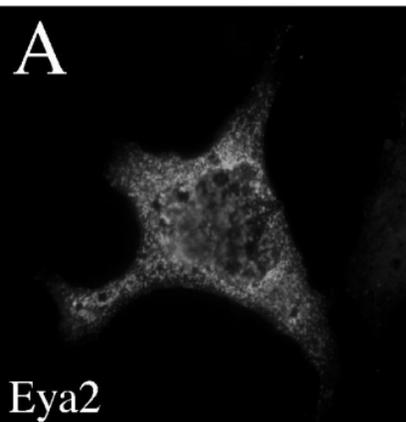


Figure 4

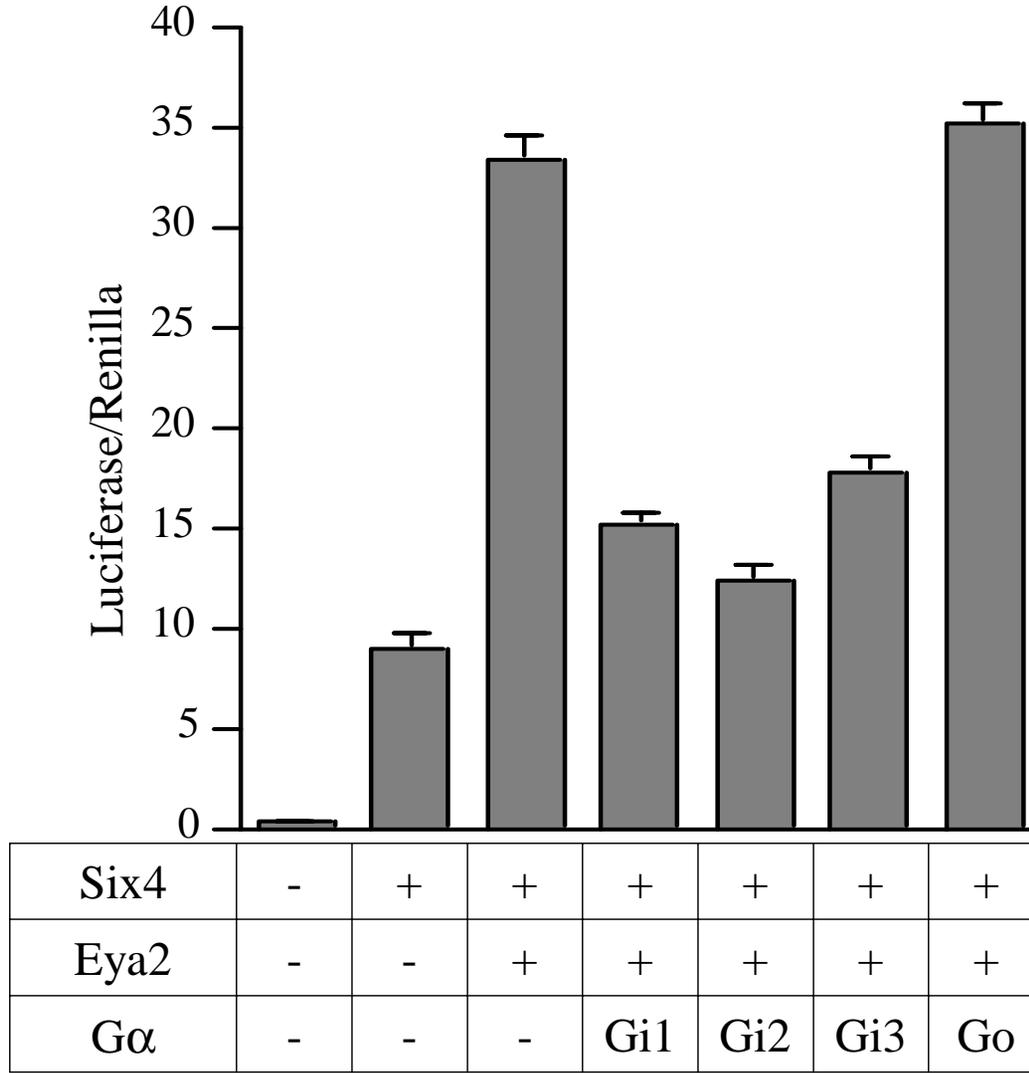


Figure 5

