“Identification and characterization of Eya1-interacting proteins”

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ZUSAMMENFASSUNG

Eya1 ist als Komponente des gene-regulatorischen *Pax-Eya-Six-Dach*-Netzwerkes essentiell für die Entwicklung unterschiedlichster Organe in Wirbeltieren. Mutationen im menschlichen *EYA1*-Gen sind eng verknüpft mit erblich bedingten Entwicklungsstörungen des Menschen, wie zum Beispiel BOR (Branchio-Oto-Renales)- und BO (Branchio-Oto)-Syndrom. BOR-Patienten leiden unter Fehlentwicklungen des Ohres, der Kiemenbögen und der Niere, während bei BO-Patienten die Niere nicht betroffen ist. Die Mechanismen, durch welche *EYA1*-Mutationen zu diesen Krankheiten führen, sind bisher nur schlecht verstanden.

In dieser Arbeit wurden mehrere krankheitsassoziierte *EYA1*-Mutationen im Hinblick auf ihren Effekt auf die Funktion des Proteins analysiert. Dabei wurde gezeigt, dass einige der Mutationen zu einem beschleunigten proteasomalen Abbau des Proteins in Säugerzellen führen. Der beschleunigte Abbau des Eya1-Proteins und der damit verbundene Verlust an Proteinmenge und -aktivität könnten einen neuen Mechanismus für die Entstehung von *EYA1*-bedingten Krankheitsbildern darstellen. Weitere Analysen ergaben, dass die Ubiquitinierung im C-Terminus von Eya1 erfolgt und durch die Interaktion mit Six1 inhibiert wird. Dies deutet darauf hin, dass Six1 die Stabilität des Eya1-Proteins reguliert.

Ein Hauptziel dieser Arbeit war die Identifizierung von neuen Eya1-interagierenden Proteinen. Mit Hilfe des Hefe-Zwei-Hybrid-Systems wurden Sipl1 und Rbck1 als Interaktionspartner identifiziert und *in vitro* bzw. durch Ko-Immunopräzipitation aus Säugerzellen verifiziert. Als Bindestellen wurden der C-terminus von Eya1 und die Ubl-Domäne von Sipl1 bzw. Rbck1 bestimmt. Im Zebrafisch konnten Orthologe von *Sipl1* und *Rbck1* identifiziert werden. Es wurde gezeigt, dass *Sipl1* und *Rbck1* in verschiedenen embryonalen Geweben der Maus und des Zebrafisches mit *Eya1* koexprimiert sind. Interessanterweise führte der Knockdown eines *Sipl1*-Orthologs zu einem BOR-Syndrom-ähnlichen Phänotyp des Zebrafisches. Diese Ergebnisse deuten auf eine physiologische Relevanz der Eya1-Sipl1/Rbck1-Interaktion während der Organogenese hin und wurden durch die Identifizierung von *SIPL1*- und *RBCK1*-Mutationen in BOR-Patienten unterstrichen. Eine erste mechanistische Grundlage lieferten Transaktivierungsstudien, die zeigten, dass Sipl1 und Rbck1 die Funktion von Eya-Proteinen als Kofaktoren der Six-Transkriptionsfaktoren verstärken.

ABSTRACT

Eya1 is a component of the gene-regulatory *Pax-Eya-Six-Dach* network and essential for the development of various organs in vertebrates. Mutations in the human *EYA1* gene are associated with several congenital disorders, as for example BOR (branchio-oto-renal) and BO (branchio-oto) syndrome. BOR patients suffer from severe malformations of the ear, the branchial arches and the kidneys, while in BO patients the kidney is not affected. The mechanisms by which *EYA1* mutations cause human disease are only poorly understood.

Several disease-associated *EYA1* mutations were characterized in this work regarding their effect on Eya1 protein function. It was shown that some of the mutations lead to enhanced proteasomal degradation of the protein in mammalian cells. Loss of Eya1 activity due to loss of Eya1 protein might represent a so far unknown mechanism for the onset of *EYA1*-associated diseases. Further analyses revealed that ubiquitination occurs in the C-terminus of Eya1 and is inhibited by the interaction with Six1. These findings indicate that Six1 is involved in the regulation of Eya1 protein stability.

A central aim of this work was the identification of novel Eya1-interacting proteins. Using yeast two-hybrid analysis two novel interaction partners were identified: Sipl1 and Rbck1. The interactions were confirmed *in vitro* and by co-immunoprecipitation from mammalian cells. Binding studies demonstrated that the interaction is mediated via the C-terminal part of Eya1 and the Ubl domain of Sipl1 or Rbck1, respectively. Furthermore, orthologs of *Sipl1* and *Rbck1* were identified in zebrafish. It was shown that *Sipl1* and *Rbck1* are co-expressed with *Eya1* in several organs during embryogenesis of both mouse and zebrafish. Interestingly, the knockdown of one *Sipl1* ortholog in zebrafish led to a BOR syndrome-like phenotype. The results of expression studies and knockdown analyses indicate that, indeed, the Eya1-Sipl1/Rbck1 interaction is of physiological relevance in the context of organ development. This hypothesis was further underlined by the identification of *SIPL1* and *RBCK1* mutations in patients suffering from BOR syndrome. A first mechanistic basis was provided by results from transactivation studies which demonstrated that Sipl1 and Rbck1 enhance the function of Eya proteins to act as co-activators for the Six transcription factors.

ABBREVIATIONS

A	Adenine
A	Alanine
aa	Amino acid
Amp^r	Ampicillin resistance marker
β-Gal	β-galactosidase
bp	Base pairs
C	Cysteine
C	Cytosine
cDNA	copy DNA
CMV	Cytomegalo virus
C-Terminus	Carboxy-terminus
D	Aspartate
Dach	Dachshund
DIG	Digoxigenin
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
E	Glutamate
EDTA	Ethylenediaminetetraacetic acid
Eya	Eyes absent
EGFP	Enhanced green fluorescent protein
Fig.	Figure
G	Glycine
G	Guanine
Gal4-BD	Gal4 DNA-binding domain
Gal4-AD	Gal4 activation domain
GST	Glutathione-S-transferase
H	Histidine
HA	Hemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IB	Immunoblot
IP	Immunoprecipitation
IPTG	Isopropyl-thio-galactoside
K	Lysine
Kan^r	Kanamycin resistance marker
L	Leucine
LB	Luria Bertani
mRNA	messenger RNA
N-Terminus	Amino-terminus
OD	optical density
ONPG	o-Nitrophenyl-beta-galactopyranoside
ORF	Open reading frame
P	Proline
PFA	Paraformaldehyde
PCR	Polymerase chain reaction
Q	Glutamine
R	Arginine
RFP	Red fluorescent protein
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Reverse transcriptase
S	Serine
SDS	Sodium dodecylsulfate
siRNA	Short interfering RNA
Six	Sine oculis homeobox homolog
NP-40	Nonidet P-40
Pax	Paired box
PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethanesulphonylfluoride
T	Thymine
Taq	*Thermus aquaticus*
Tris	Tris-hydroxymethyl-aminomethane
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1 INTRODUCTION

Development of a multicellular organism from a single fertilized egg is a fascinating process: One single cell gives rise to hundreds of different cell types which are precisely organized into complex tissue and organ structures. In order to gain a deeper insight into the associated processes, developmental biology focuses on deciphering the underlying mechanisms. Nowadays, it is known that development of the body plan of a multicellular organism is controlled by large gene regulatory networks. During the evolution of body plans, the architecture of these gene-regulatory networks has changed, while the basic principles have been conserved (Davidson and Erwin, 2006). One of these regulatory networks is the *Pax-Eya-Six-Dach* network, which has been first identified in the context of *Drosophila* compound eye development. During the recent years, ample evidence accumulated that this network is conserved throughout evolution and re-employed in the development of various organs in different organisms.

1.1 THE *PAX-EYA-SIX-DACH* NETWORK

Development of the compound eye in *Drosophila* is mediated by several genes, such as *twin of eyeless* (*toy*), *eyeless* (*ey*), *sine oculis* (*so*), *eyes absent* (*eya*), and *dachshund* (*dach*). *Toy*, *ey* and *so* encode transcription factors, while *eya* and *dach* encode transcriptional co-factors. The genes act together in a regulatory gene network in which *toy* controls *ey*, *ey* activates *eya* and *so*, which in turn regulate *ey* and activate *dach* (Halder *et al.*, 1995; Chen *et al.*, 1997; Wawersik and Maas, 2000). It has been shown that mutation in any of these genes leads to malformation or even complete loss of the *Drosophila* eye. Furthermore, ectopic expression of *toy* and *ey* leads to the formation of ectopic eyes (Halder *et al.*, 1995; Czerny *et al.*, 1999). Misexpression of *eya* or *dach* induces ectopic eye formation of smaller size especially in the head region of the fly, which is markedly enhanced by ectopic co-expression of *eya* and *so* or *eya* and *dach*. The reason for this is the synergistic action of the respective proteins, which have been shown to physically interact with each other in yeast two-hybrid system (Bonini *et al.*, 1997; Chen *et al.*, 1997; Pignoni *et al.*, 1997). Vertebrate homologs of *toy* and *ey* (*Pax6*), *so* (*Six1-6*), *eya* (*Eya1-4*), and *dach* (*Dach1-2*) have been identified (reviewed in

Kawakami *et al*., 2000). Interestingly, the vertebrate homologs *Pax6*, *Six3*, *Optix2*/*Six6*, *Eya1* and *Eya2* are co-expressed during eye development in mice, suggesting that the gene network operating in *Drosophila* is also conserved in vertebrate eye development, despite the differences in eye morphology and the mode of development between these two species (Halder *et al.*, 1995; Chow and Lang, 2001; Lagutin *et al.*, 2003). In addition to that, members of the *Pax*, *Eya*, *Six* and *Dach* gene families show overlapping expression patterns in several other developing organs of vertebrate embryos, implicating that the *Pax-Eya-Six-Dach* network plays important roles also in other developmental contexts (Fig. 1.1). One of the best studied examples is vertebrate muscle development, which is regulated by the concerted action of *Pax3* (a *Pax6* homolog), *Eya2*, *Six1*, and *Dach2* in chick myogenesis. All genes are co-expressed in the developing somites, which are precursors of the axial skeleton and all skeletal muscles (Williams and Ordahl, 1994; Oliver *et al.*, 1995; Xu *et al.*, 1997; Mishima and Tomarev, 1998; Heanue *et al.*, 1999). Similar to the *Drosophila* homologs, *Eya2* and *Six1*, as well as *Eya2* and *Dach2*, act synergistically to regulate expression of *Pax3* and the process of myogenic differentiation, which is mediated by direct interactions between the respective proteins.

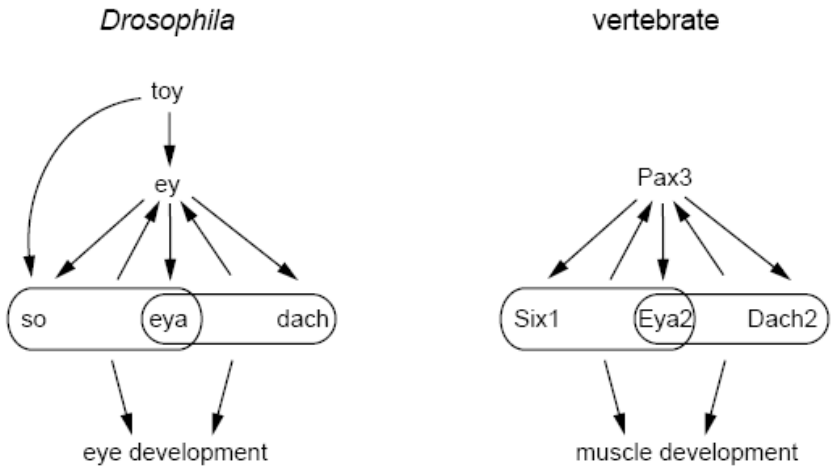

Fig. 1.1. The *Pax-Eya-Six-Dach* network is conserved throughout evolution in different developmental contexts. Comparison of *Pax-Eya-Six-Dach* networks involved in *Drosophila* eye development (left panel) and vertebrate muscle development (right panel). Arrows indicate induction pathways. Six and Eya or Eya and Dach homologs form a complex, and show synergistic action (modified from Kawakami *et al*., 2000)).

To clarify the molecular mechanisms by which members the *Pax-Eya-Six-Dach* network regulate organogenesis, it is important to understand the functions of the respective proteins. Activity of a protein can be regulated by post-translational

modifications or by interactions with other proteins. In this regard, especially studies on Eya proteins revealed surprising features concerning their function and mode of regulation, as described in the following section.

1.2 EYA DOMAIN STRUCTURE AND FUNCTION

Homologs of *Drosophila eya* have been found in several invertebrate and vertebrate species, including *C. elegans*, *Xenopus*, chick, mouse, and human, as well as in higher plants as *Oryza sativa* and *Arabidopsis thaliana*. All of them have been shown to share a common protein structure consisting of two domains: an N-terminal domain and the C-terminally located Eya domain (Fig. 1.2 A).

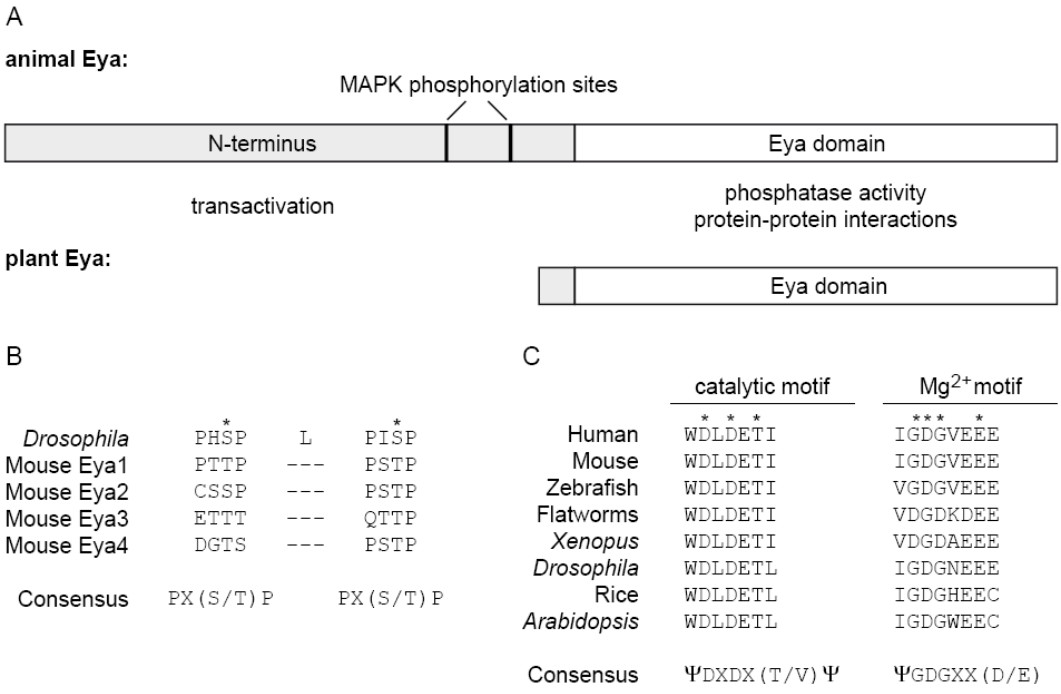

Fig. 1.2. Eya protein domain structure and function. (A) Eya protein domain structure and function compared between animals and higher plants. (B) Conservation of MAPK phosphorylation sites between *Drosophila* and mouse Eya homologs. Asterisks indicate targets for phosphorylation (modified from Hsiao *et al*., 2001). (C) Eya proteins of different species share a conserved catalytic motif and a metal-binding motif similar to that of phosphatases of the HAD (haloacid dehalogenase) family. Asterisks indicate conserved residues (modified from Li *et al*., 2003). Details are described in the text.

In contrast to the plant Eya homologs, which possess only a very short N-terminal domain of about 20 amino acids, animal Eya homologs have an N-terminus of 240-490 amino acids length, suggesting that the protein function located within this region was gained later during evolution (Xu *et al.*, 1997; Takeda *et al.*, 1999; Silver *et al.*, 2003). The N-terminal domains of animal Eya homologs are highly divergent. They consist of 35-40% proline, serine and threonine residues and

resemble the proline-serine-threonine (PST) transactivation domains found in other transcription factors (Mermod *et al.*, 1989; Theill *et al.*, 1989). Eya proteins do not possess a DNA binding domain. The Eya domain is highly conserved in both length (271-274 amino acids) and sequence between all Eya family members. All described interaction partners of Eya bind to this domain. Using yeast two-hybrid and GST pulldown analyses, *Drosophila* Eya has been shown to physically interact with So and Dach. It was proposed that complex formation mediates the synergistic action of the respective proteins during *Drosophila* eye development (Bonini *et al.*, 1997; Chen *et al.*, 1997; Pignoni *et al.*, 1997). The Eya-So interaction also occurs between the respective mouse homologs, as shown by *in vitro* analysis. In contrast to *Drosophila* Eya, which is a nuclear protein, vertebrate homologs are localized in the cytoplasm. Interaction with Six leads to their translocation into the nucleus, where Six mediates DNA-binding and the two proteins synergize in activation of gene expression (Ohto *et al.*, 1999). Natural target genes of the vertebrate Eya-Six complex are for example *Six2*, *Sall1*, and *Myogenin*. Activation of *Six2* and *Sall1* expression have been shown to be essential for proper kidney development in mouse, whereas activation of *Myogenin* is required for muscle development (Spitz *et al.*, 1998; Brodbeck, 2003; Chai *et al.*, 2006). Very recent results showed that expression of *Six2* during kidney development is also activated by complex formation of Hox11, Eya1 and Pax2 (Gong *et al.*, 2007). In contrast to *Drosophila* Eya and Dach, the interaction between the respective mouse homologs could be detected in mammalian two-hybrid assays, but not in GST pulldown experiments, leading to the conclusion that the interaction is not direct, but rather mediated through other proteins, such as the co-activator CBP (CREB binding protein) (Ikeda *et al.*, 2002). Furthermore, the interactions of mammalian Eya with two inhibitory Gα subunits, Gαi and Gαz, are quite poorly understood. Eya2 has been isolated as an interaction partner of Gαz in a yeast two-hybrid screen using constitutively active Gαz as bait. The interaction was confirmed *in vitro*, and further analysis implicated that binding to Gα subunits prevents Six-mediated translocation of Eya2 into the nucleus, and hence, Six-Eya2-mediated transactivation (Fan *et al.*, 2000). Conversely, interaction with Eya2 affects Gαi function *in vitro* by relieving Gαi2 mediated inhibition of adenylyl cyclase. The physiological relevance of this effect remains to be determined (Embry *et al.*, 2004).

Several studies indicated that Eya proteins directly link signal transduction events and regulation of gene expression. In *Drosophila*, Eya function is positively regulated via phosphorylation at two MAPK (mitogen-activated protein kinase) phosphorylation sites located within in the N-terminal domain. *In vivo*, Eya phosphorylation by ERK (extracellular signal-regulated kinase) significantly enhanced its ability to induce ectopic eyes. A possible mechanism was provided by studies in cell culture showing that MAPK activation can potentiate Eya-mediated transactivation (Hsiao *et al.*, 2001; Silver *et al.*, 2003). Examination of mammalian Eya protein sequences revealed similarly located MAPK phosphorylation sites in mouse and human Eya1, Eya2, and Eya4, but not in Eya3 (Fig. 1.2 B). However, phosphorylation of these sites, and associated regulation of mammalian Eya, still needs to be shown. Moreover, in 2003, three independent groups discovered the function of Eya proteins as phosphatases in addition to their function as a co-activator of transcription, which was a unique combination of activities at this time point (Li *et al.*, 2003; Rayapureddi *et al.*, 2003; Tootle *et al.*, 2003). All groups performed sequence analyses showing that a consensus of two sequence motifs corresponding to the haloacid dehalogenases (HAD) family of phosphohydrolases is conserved in all Eya family members (Fig. 1.2 C). However, results regarding the specificity of Eya phosphatases differed between the three groups. Rayapureddi *et al*. and Tootle *et al*. demonstrated specificity of Eya towards phosphotyrosine peptides, whereas Li *et al.* claimed that it has dual specificity dephosphorylating both phosphotyrosine and phosphoserine/threonine peptides. Two potential substrates of Eya phosphatase activity have been identified *in vitro*: Eya itself and RNA polymerase II (Li *et al.*, 2003; Tootle *et al.*, 2003). The physiological importance of this novel function of Eya proteins is not fully understood. It has been shown that the phosphatase activity of Eya is required to promote normal eye development in *Drosophila in vivo* (Rayapureddi *et al.*, 2003). Furthermore, some *EYA1* mutations, which are associated with human disease, result in loss of phosphatase activity (Mutsuddi *et al.*, 2005; Rayapureddi and Hegde, 2006). However, *in vivo* substrates have not been identified so far.

The following sections are focusing on mammalian *Eya1*, which is one homolog of *Drosophila eya*. The facts that *Eya1* knockout mice show a severe phenotype affecting the development of several organs, and mutations in human *EYA1* are

associated with several congenital disorders point to an essential role for this gene and the respective protein during mammalian organogenesis.

1.3 ROLE OF *EYA1* DURING MAMMALIAN ORGAN DEVELOPMENT

Eya1 has been shown to be essential for the development of various organs in mammals, as for example the kidney. Early kidney development in mammals serves as a classical model of organogenesis and is described in the following section.

1.3.1 Mammalian kidney development

Development of the kidney in mammals proceeds in three successive steps from the initial pronephros via the mesonephros to the metanephros which is the adult kidney (Fig. 1.3).

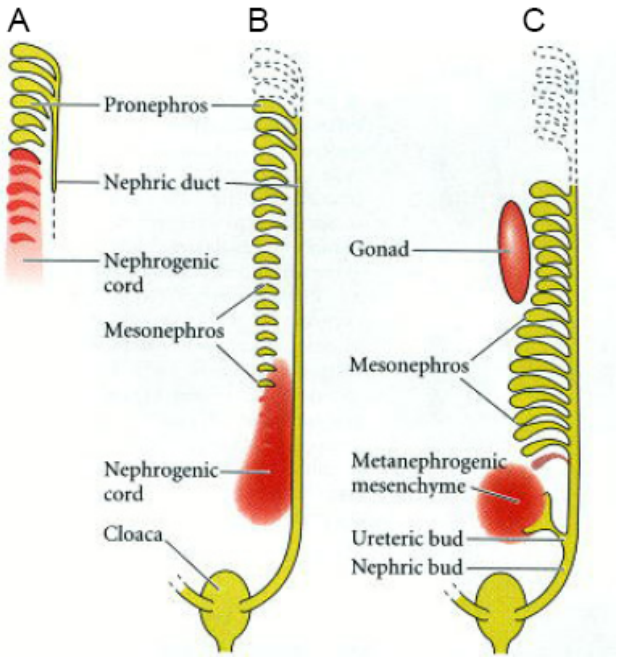

Fig. 1.3. Scheme of kidney development in mammals. (A) Pronephros and the caudally migrating Wolffian (or nephric) duct. (B) Degeneration of the pronephros and formation of the mesonephric tubules. (C) The outgrowth of the ureteric bud into the metanephric mesenchyme induces formation of the metanephros (modified from Gilbert and Singer, 2006).

All three stages are characterized by mesenchymal-to-epithelial transformation of cells that are derived from the intermediate mesoderm. Bouchard *et al*. demonstrated that formation of both the pronephros and the mesonephros

depends on the expression of *Pax2* and *Pax8*. In mouse embryos lacking both *Pax2* and *Pax8*, the intermediate mesoderm is unable to undergo the initial mesenchymal-to-epithelial transitions which are required for the formation of the nephric duct (Bouchard *et al.*, 2002). The expression of both *Pax* genes is induced by yet unknown signals from the adjacent paraxial mesoderm (Mauch *et al.*, 2000). Development of the adult metanephros depends on the reciprocal inductive interactions of two tissues, the metanephric mesenchyme and the nephric (or Wolffian) duct. The metanephric mesenchyme arises from the posterior part of the intermediate mesoderm. The Wolffian duct arises initially as the pronephric duct in the intermediate mesoderm early in development (day 8 in mouse). In mice, development of the metanephros starts at embryonic day 10.5 by secretion of Gdnf (glial cell line-derived neurotrophic factor) from the metanephric mesenchyme. Gdnf is a member of the TGFβ (transforming growth factor β) family and acts on the receptor tyrosine kinase c-Ret which is presented by the epithelial cells of the Wolffian duct (Trupp *et al.*, 1996). Activation of the receptor by binding of Gdnf induces outgrowth of the ureteric bud into the metanephric mesenchyme, which subsequently induces condensation of the metanephric mesenchyme and differentiation into the nephrons of the mammalian kidney. In *Gdnf* null mice, the initial interaction between the metanephric mesenchyme and the Wolffian duct does not occur and the mesenchymal cells undergo apoptosis (Moore *et al.*, 1996; Pichel *et al.*, 1996; Sanchez *et al.*, 1996). Gene knockout experiments have identified a number of factors that regulate *Gdnf* expression in the kidney mesenchyme. Most of these regulators are transcription factors, although deletion of genes coding for signaling molecules, such as *Gdf1* (*growth differentiation factor 1*), can also result in loss of *Gdnf* expression (Esquela and Lee, 2003). Recent studies suggested that the transcriptional co-activator Eya1 acts as key regulator specifically for the determination of the metanephric mesenchyme and ureteric bud growth by modulating the levels of *Gdnf* expression (Sajithlal *et al.*, 2005). *Eya1* knockout mice show a similar phenotype as *Gdnf* knockout animals with failure of ureteric bud outgrowth and apoptosis of the metanephric mesenchyme (Xu *et al.*, 1999). *Gdnf* expression, as well as *Six1* and *Six2* expression, is lost. Several independent studies indicated that *Eya1* is directly involved in the activation of *Gdnf*. Brodbeck *et al*. demonstrated that the Eya1-Six2 complex directly regulates the *Gdnf* promoter (Brodbeck, 2003). In contrast to this,

a recent study by Self *et al*. demonstrated that in *Six2* null mice *Gdnf* expression is retained. *Six2*-deficient mice show premature and ectopic differentiation of mesenchymal cells into epithelial cells which results in severe kidney hypoplasia. Based on these observations Self *et al*. claimed that *Six2* is required for maintaining the mesenchymal progenitor population in an undifferentiated state, which allows continuous growth of the kidney (Self *et al.*, 2006). However, Eya1 has also been described to directly activate *Gdnf* expression after complex formation with the transcription factors Pax2 and Hox11 (Gong *et al.*, 2007). In line with these data, inactivation of *Pax2*, as well as inactivation of all three *Hox11* paralogs *Hoxa11*, *Hoxc11* and *Hoxd11*, leads to loss of *Gdnf* expression (Brophy *et al.*, 2001; Wellik *et al.*, 2002). In addition to that, in *Hox11* null mice, also *Six2* expression is reduced, whereas *Pax2*, *Wt1* (*Wilms' tumor suppressor*) and *Eya1* expression are unaffected (Wellik *et al.*, 2002).

The role of several other genes is not completely understood. *Wt1*-, *Sall1*- and *Six1*- deficient mice all exhibit a phenotype similar to that of the *Gdnf* knockout mice, even though *Gdnf* expression is still detectable (Kreidberg *et al.*, 1993; Nishinakamura *et al.*, 2001; Xu *et al.*, 2003). An explanation for this might be that *Gdnf* expression is present but reduced to a level which is not sufficient for metanephric development. In line with this assumption, it has been described for *Six1* and *Sall1* null mice that the metanephric mesenchyme and, thus, the expression domain of *Gdnf* is reduced in size (Nishinakamura *et al.*, 2001; Xu *et al.*, 2003). Further examination of *Six1* null mice revealed that expression of *Pax2*, *Six2* and *Sall1* is markedly reduced, whereas *Eya1* expression is normal (Xu *et al.*, 2003). Recently, Chai *et al*. demonstrated that Six1 directly activates the *Sall1* promoter and this activation is significantly enhanced by interaction with Eya1 (Chai *et al.*, 2006). Interestingly, in *Six1*/*Six4* double mutant animals, *Gdnf*, as well as *Pax2* and *Pax8* expression are completely absent. The phenotype of *Six1*/*Six4* knockout animals is more severe than the phenotype of each of the single knockouts, indicating that *Six1* and *Six4* have redundant roles in kidney development (Kobayashi *et al.*, 2007).

Furthermore, Kume *et al*. demonstrated that *Foxc1* (*forkhead box C1*) is essential for the positioning of the ureteric bud (Kume *et al.*, 2000). During embryogenesis of *Foxc1* knockout mice, ectopic mesonephric tubules and ectopic ureteric buds are formed more anteriorly which results in duplex kidneys and double ureters.

Kume *et al*. suggested that this phenotype is due to abnormal expression of *Gdnf* and *Eya1*, since also the region of *Eya1* and *Gdnf* expression is extended anteriorly in *Foxc1*-deficient mice. They hypothesized that Foxc1 acts as a repressor of *Eya1* and *Gdnf* (Kume *et al.*, 2000).

To further complicate the issue, some of the identified factors seem to regulate expression at the post-transcriptional level. In *Wt1* knockout mice for example, *Pax2* expression can be detected at the mRNA level but not at the protein level (Kreidberg *et al.*, 1993; Donovan *et al.*, 1999). It was suggested that Wt1 acts as a splicing factor regulating *Pax2*, and perhaps also *Gdnf* expression (Englert, 1998).

An overview of the gene-regulatory network which mediates the development of the adult metanephros in mammals is given in Fig. 1.4. The model is based on the described results from knockout analysis and is, presumably, not complete yet.

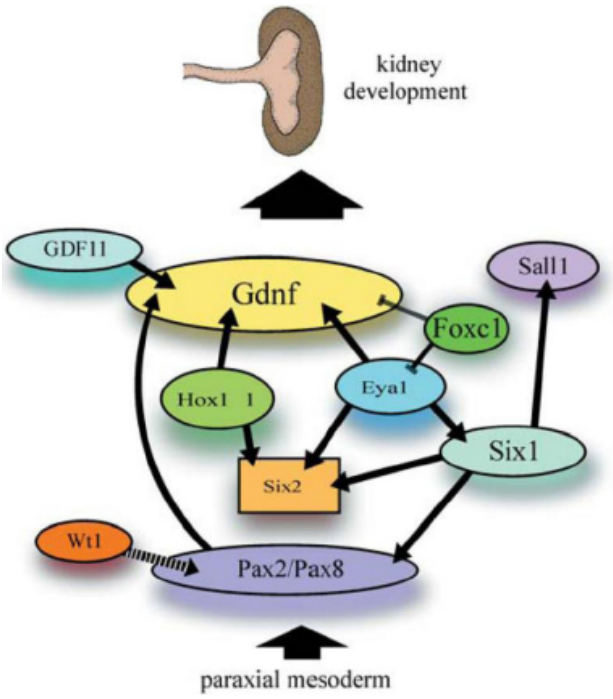

Fig. 1.4. A complex genetic network regulates initiation of mammalian kidney development in the metanephric mesenchyme. The model is based on knockout studies in mice. Arrows represent loss of gene expression in the respective knockout mouse, dashed arrow represents possible post-transcriptional effect, and inhibitory lines indicate repression. (Brodbeck and Englert, 2004)

Taken together, the expression of members of the *Pax-Eya-Six-Dach* network is the basis for development of the adult kidney. However, in contrast to *Drosophila* eye development the network has been expanded by additional factors to allow a tight regulation of nephrogenesis.

1.3.2 *Eya1*-knockout studies in mice

Analysis of an *Eya1*-specific knockout mouse by Xu *et al.* revealed an essential role for *Eya1* not only in the development of the kidney, but also in the development of several different organs, as ear, thymus, thyroid gland, parathyroid gland, and skeleton (Xu *et al.*, 1999; Xu *et al.*, 2002). The phenotype of *Eya1*-heterozygous mice resembled the symptoms of human patients suffering from BOR syndrome. *Eya1* heterozygotes showed renal abnormalities, including renal hypoplasia and unilateral agenesis, at a low penetrance. Furthermore, *Eya1* heterozygosity led to conductive hearing loss due to abnormal sound conduction through the middle ear. Additionally, inner ear abnormalities were detected in some heterozygous mice. In contrast, all *Eya1* homozygous mice died at birth showing severe craniofacial and skeletal defects, and absence of thymus, parathyroid glands, ears and kidneys. A more detailed investigation of these defects revealed that the otic anomalies involved structures of the inner, middle and outer ear. In the inner ear, the otic vesicle forms but fails to develop further due to abnormal induction of apoptosis. Analysis of the molecular effects of the *Eya1* mutations regarding the expression of *Pax* and *Six* family genes demonstrated that expression of the corresponding *Pax* gene (*Pax2* and *Pax8* in the ear) was not affected, whereas expression of the respective *Six* gene (*Six1* in the ear) could not be detected. Conclusively, as described for kidney development, also the formation of the ear seems to involve a *Pax-Eya-Six-Dach* network similar to that of *Drosophila* eye development. In both kidney and ear, *Dach* homologs were identified but there is no data on a possible role of the homologs in the development of these organs. This suggests that other genes might be involved (Xu *et al.*, 1999). Similar results, as described for ear and kidney, were obtained for the role of *Eya1* during organogenesis of thymus, parathyroid and thyroid, implicating that *Eya1* is also critical for the regulation of early inductive events involved in the morphogenesis of these organs (Xu *et al.*, 2002). The importance of *Eya1* in mammalian organogenesis was further underlined by the identification of disease-associated *EYA1* mutations in humans.

1.4 ASSOCIATION OF *EYA1* WITH HUMAN DISEASE

Mutations in the human *EYA1* gene have been associated with several human diseases including branchio-oto (BO) and branchio-oto-renal (BOR) syndrome, as well as congenital cataracts and ocular anterior segment anomalies. BOR syndrome is an autosomal-dominant disorder characterized by branchial arch anomalies, hearing loss and kidney defects (Melnick *et al.*, 1975; Melnick *et al.*, 1976; Fraser *et al.*, 1978). BO syndrome is a related disorder without renal anomalies. BOR/BO syndrome exhibits low penetrance and variable expressivity. It occurs with a prevalence of 1:40000 in the general population and is responsible for 2% of profound deafness in children (Fraser *et al.*, 1980). Up to now more than 50 different mutations of *EYA1* have been associated with BOR syndrome including frame shift or nonsense, missense, splice site, and complex mutations involving large deletions or chromosomal rearrangements. There is no single common mutation. Analysis of the molecular mechanisms, by which mutations in *EYA1* lead to the disease, showed that several different aspects of Eya1 protein function can be affected, as for example the phosphatase activity, the interactions of Eya1 with Six, Dach, and Gα subunits, or both. Some mutations result in an altered protein conformation thereby disturbing interactions mediated via the Eya domain (Ozaki *et al.*, 2002; Mutsuddi *et al.*, 2005; Rayapureddi and Hegde, 2006). However, many of the BOR-associated *EYA1* mutations do not affect any of these functions, suggesting that yet unknown functions or interactions of the Eya1 protein might be involved.

Interestingly, mutations in two additional genes, *SIX1* and *SIX5*, which both code for interaction partners of Eya1 have been shown to be associated with BOR syndrome as well (Ruf *et al.*, 2004; Hoskins *et al.*, 2007). The fact that most of the BOR-associated mutations in these genes abolish the interaction of the corresponding protein with Eya1 further underlines the central role of Eya1 or the Pax-Eya-Six-Dach network in organogenesis.

1.5 AIM OF THIS WORK

A central aspect of this work was to achieve a more detailed insight into the mechanisms by which Eya1 regulates mammalian organogenesis. At the beginning of this study, no antibodies for detection of endogenous Eya1 protein were available. In the course of this work, two Eya1-specific antibodies, which were generated in our lab, had to be further characterized regarding specificity and detection of endogenous Eya1 using different cell lines.

Another part of this work focused on the analysis of several BOR-associated mutations regarding their effect on Eya1 protein function.

Furthermore, Eya1 is essential for the development of several different mammalian organs, as for example ears and kidneys. The development of these organs is affected in human BOR syndrome which is mainly caused by mutations in *EYA1*. It is known that Eya1 acts as a co-factor for the Six transcription factors. Also mutations in *SIX1* lead to BOR syndrome due to loss of interaction with Eya1. In mice, *Eya1* and *Six1* are co-expressed in the developing ears and kidneys, and both *Eya1* and *Six1* null mice fail to form these organs. Conclusively, these results suggest that the complex formation of Eya1 and Six1 is crucial for the development of both ears and kidneys (Xu *et al.*, 1999; Laclef *et al.*, 2003). It is therefore predictable that additional factors bind to the complex, presumably to Eya1, and mediate the specification of organ identity (Fig. 1.5).

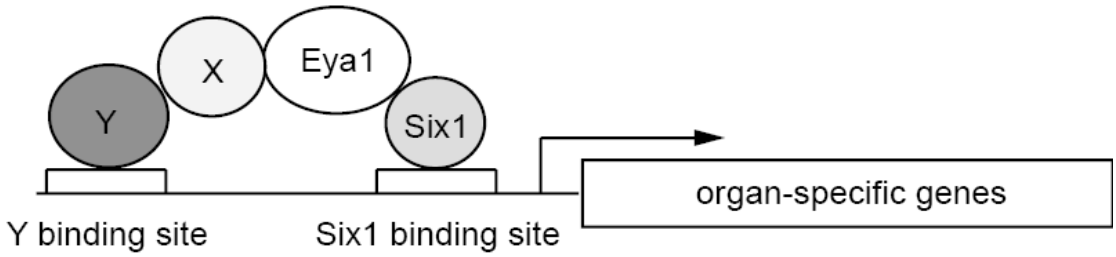

Fig. 1.5. Model for Eya1-Six1 transactivation complex involved in mammalian ear and kidney development. Eya1 directly interacts with Six1 which mediates binding to the promoter region of the respective genes. An unknown factor X mediates specific activation of gene expression by binding to the Eya1-Six1 complex potentially interacting with another adjacent transcription factor/complex Y (modified from Relaix and Buckingham, 1999).

Hence, the main focus of this work was on the identification of those so far unknown interaction partners of Eya1 using a yeast two-hybrid approach. Subsequently, novel interaction partners should be characterized regarding their roles in organogenesis with relation to Eya1.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Bacterial strains

Escherichia coli strains used for plasmid propagation and heterologous gene expression are listed in table 2.1.

Table 2.1 Genotype of *Escherichia coli* strains used in this work

Strain	Purpose	Genotype
DH5α	plasmid propagation	F$^-$ φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ$^-$
BL21	heterologous gene expression	F$^-$ ompT hsdS_B (r_B^- m_B^-) dcm gal (DE3) pLysS (Cam^R)
TOP10F' (Invitrogen)	plasmid propagation	F´c *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 n*up*G
HB101	plasmid propagation	F$^-$ *mcr*B *mrr* *hsd*S20(r_B^-, m_B^-) *rec*A13 *leu* *ara*-14 *pro*A2*lac*Y1 *gal*K2 *xyl*-5 *mtl*-1 *rps*L20(Sm^r) *sup*E44 λ$^-$

2.1.2 Yeast strains

The following *Saccharomyces cerevisiae* strains were used for transformation and mating:

Table 2.2 Genotype of *Saccharomyces cerevisiae* strains used in this work

Strain	Purpose	Genotype
Y187 (Harper *et al.*, 1993)	mating	*MATα*, *ura3-52*, *his3-200*, *ade2-101*, *trp1-901*, *leu2-3*, *112*, *gal4Δ*, *gal80Δ*, *met*$^-$, *URA3::GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*LacZ*, *MEL1*
KFY1 (Kristina Fahr, PhD thesis, 2001)	transformation, mating	*MATa*, *ura3-52*, *his3-200*, *lys2-801*, t*rp1-901*, *leu2-3*, *112*, *gal4Δ*, *gal80Δ*, *cyh*^{r}*2*, *LYS2::GAL1*$_{UAS}$-*HIS3*$_{TATA}$-*HIS3*, *MEL1*, *URA3:: GAL1*$_{UAS}$-*GAL1*$_{TATA}$- *LacZ*

2.1.3 cDNA library for yeast two-hybrid screening

The Pretransformed Mouse 11-day Embryo MATCHMAKER cDNA library (in *S. cerevisiae* Y187) used for the yeast two-hybrid screening procedure was purchased from Clontech.

2.1.4 Eukaryotic cell lines

The following cell lines were used in this work:

Cos-7: African green monkey kidney cell line (DSMZ)

mK3: clonal cell line representing early metanephric mesenchyme (Valerius *et al.*, 2002)

mK4: clonal cell line representing induced metanephric mesenchyme undergoing epithelial conversion (Valerius *et al.*, 2002)

Cells were cultured in 10 cm-cell culture dishes in Dulbecco's modified Eagle medium (DMEM; Invitrogen) containing 10% fetal bovine serum at 37°C, 5% CO_2 and 95% humidity.

2.1.5 Antibodies

Anti-β-actin (ab8224): monoclonal mouse antibody directed against conserved region of human β-actin (Abcam);

Anti-c-Myc (9B11): monoclonal mouse antibody directed against the c-Myc-epitope tag (Cell Signaling);

Anti-digoxigenin: polyclonal sheep antibody directed against digoxigenin, Fab fragments, alkaline-phosphatase conjugated (Roche);

Anti-Eya1.1: polyclonal rabbit antibody directed against mouse Eya1 (Jürgen Tomasch, diploma thesis, 2007)

Anti-Eya1.2: polyclonal rabbit antibody directed against mouse Eya1 (Jürgen Tomasch, diploma thesis, 2007)

Anti-Flag (M2): monoclonal mouse antibody directed against the Flag-epitope tag (Sigma-Aldrich);

Anti-HA (12CA5): monoclonal mouse antibody directed against the HA-epitope tag (Harlow lab, Boston, USA);

Anti-HA (6E2): monoclonal mouse antibody directed against the HA-epitope tag (Cell Signaling);

Anti-HA (Y11): polyclonal rabbit antibody directed against the HA-epitope tag (Santa Cruz Biotechnology);

Anti-LDH: polyclonal goat antibody directed against rabbit Lactate Dehydrogenase (Chemicon);

Goat anti-mouse: polyclonal goat antibody directed against mouse immunoglobulins, horse radish-peroxidase conjugated (DakoCytomation);

Goat anti-rabbit: polyclonal goat antibody directed against rabbit immunoglobulins, horse radish-peroxidase conjugated (DakoCytomation);

Rabbit anti-goat: polyclonal rabbit antibody directed against goat immunoglobulins, horse radish-peroxidase conjugated (DakoCytomation);

2.1.6 Culture media

2.1.6.1 Culture media for *E.coli*

LB medium: 10 g/L Bacto-tryptone, 5 g/L yeast extract, 5 g/L NaCl

LB agar: LB medium, 1.5% agar

M9 agar: 1.5% agar, 64 g/L Na_2HPO_4, 15 g/L KH_2PO_4, 2.5 g/L NaCl, 5 g/L NH_4Cl, 4 g/L glucose, 40 µg/ml proline, 340 µg/ml thiamine hydrochloride, 1x Dropout solution (-Leu/Trp/Ade/His, Clontech), 20 µg/ml adenine, 20 µg/ml histidine, 20 µg/ml tryptophan

Ampicillin (100 µg/ml) or Kanamycin (40 µg/ml) were added to liquid medium or agar to select for growth of colonies containing the plasmid of interest.

2.1.6.2 Culture media for *S. cerevisiae*

YPD medium: 20 g/L Bacto-peptone, 10 g/L yeast extract, 20 g/L glucose

YPD agar: YPD medium, 1.5% agar

selection medium 6.7 g/L Yeast Nitrogen Base, 20 g/L glucose
selection agar selection medium, 1.5% agar

Amino acids (see 2.1.7) were added to selection medium or agar according to the auxotrophy markers of the transformed plasmids.

2.1.7 Amino acids

Amino acids used for cultivation of *S. cerevisiae* on agar plates or in liquid medium are listed in table 2.3.

Table 2.3 Amino acids for cultivation of *S. cerevisiae*

Amino acid	Final concentration in medium
L-Adenine, hemisulfate salt	20 µg/ml
L-Histidine HCl monohydrate	20 µg/ml
L-Leucine	30 µg/ml
L-Lysine HCl	30 µg/ml
L-Methionine	20 µg/ml
L-Tryptophan	20 µg/ml

2.1.8 Plasmids

Plasmids used in this work are listed in table 2.4.

Table 2.4 Plasmids used in this work

Name	Description	Source	Reference
pGADT7	Yeast two-hybrid vector; full-length *S. cerevisiae ADH1* promoter 5' of Gal4-AD coding sequence followed by a multiple cloning site (MCS); HA-epitope tag, *LEU2* marker, Amp^r	Clontech	
pGBKT7	Yeast two-hybrid vector; *S. cerevisiae ADH1* promoter fragment 5' of Gal4-BD coding sequence followed by a multiple cloning site (MCS); c-Myc-epitope tag, *TRP1* marker, Kan^r	Clontech	
pGBT9	Yeast two-hybrid vector; *S. cerevisiae ADH1* promoter fragment 5' of Gal4-BD coding	Clontech	

	sequence followed by MCS; *TRP1* marker; Amp^r		
pCRII-TOPO	TA-cloning vector; Amp^r; Kan^r	Invitrogen	
pRcCMV	Eukaryotic expression vector; CMV promoter; Amp^r	Invitrogen	
pcDNA3.1-Flag	Eukaryotic expression vector containing Flag-epitope tag; CMV promoter; Amp^r	our lab	
pCMV-Sport6-Sipl1	IMAGE clone containing mouse Sipl1 coding sequence; ID: 4527839; Amp^r	ATCC	
pCMV-Sport6-Rbck1	IMAGE clone containing mouse Rbck1 coding sequence; ID: 4192310; Amp^r	ATCC	
pHM6-Eya1 (-Eya2, -Eya3)	Eukaryotic expression vector for N-terminal HA-tagged mouse Eya1 (Eya2, Eya3); Amp^r	Kawakami lab, Japan	Ohto *et al*., 1999
pGBKT7-E4HR19 (-E4HR20)	Yeast two-hybrid vector; Gal4-BD fused to coding sequence of the mouse Eya4 homology region containing exon 19 (exon 20); Kan^r	Smith lab, Iowa, USA	Zhang *et al*., 2004
ED-CHD	Eukaryotic expression vector for the N-terminal HA-tagged C-terminus of mouse Eya1; Amp^r	our lab	
pcDNA3.2-hSix1	Eukaryotic expression vector for N-terminal Flag-tagged human SIX1; Amp^r	Edgar Otto, Ann Arbor, USA	
pFlagFull	pcDNA3.1-Flag containing mouse Six2 coding sequence	our lab	Brodbeck *et al*., 2003
pCR3-Six4	Eukaryotic expression vector for mouse Six4; Amp^r	Maire lab, Paris, France	Spitz *et al*., 1998
pGEX-KG	Bacterial expression vector; IPTG-inducible bacterial promoter 5' of GST coding sequence followed by MCS; Amp^r		Guan and Dixon, 1991
pEGFP-C2	Eukaryotic expression vector; CMV promoter 5' of EGFP coding sequence followed by MCS; Kan^r	Clontech	
pEGFP-Eya1	pEGFP-C2 containing mouse Eya1 coding	our lab	

	sequence		
pRFP-C2	analogous to pEGFP-C2; Eukaryotic expression vector; CMV promoter 5' of RFP coding sequence followed by MCS; Kan^r	C. Hoischen, Diekmann lab, Jena,	
pMT107	Eukaryotic expression vector for His-ubiquitin; CMV promoter; Amp^r	Treier lab, Heidelberg, Germany	Treier *et al*., 1994
pMT123	Eukaryotic expression vector for HA-ubiquitin; CMV promoter; Amp^r	Treier lab, Heidelberg, Germany	Treier *et al*., 1994
pGL3-TATA	Promoter-less reporter-vector, containing MCS 5' of a TATA box followed by *Photinus pyralis* (Firefly) luciferase cDNA; Amp^r	Maire lab, Paris, France	Fan *et al*., 2000
pGL3-MEF3/TATA	pGL3-TATA containing 6 copies of the MEF3 element upstream of the TATA box	Maire lab, Paris, France	Fan *et al*., 2000
pGL4.74 (*hRluc*/TK)	Eukaryotic expression vector containing *Renilla reniformis* luciferase coding sequence under the control of HSV-TK-promoter; Amp^r	Promega	

2.1.9 Oligonucleotides

Oligonucleotides used in this work are listed in the appendix. All oligonucleotides were synthesized by Metabion (Matinsried) or MWG (Ebersberg).

2.1.10 Morpholinos

Morpholinos used for knockdown of gene expression in zebrafish were synthesized by Gene Tools (see table 2.5).

Table 2.5 Morpholinos used in this work

Target gene	Morpholino	Sequence (5'-3')	Target site
sipl1	sipl1-4-4	AGGCCCTATGATATACCTGATGTCT	exon4-intron4
sipl1-rbck1	sipl1-rbck1-1-1	CAAGTTGGACATTTACTCACCACAC	exon1-intron1
	sipl1-rbck1-2-2	GCAGAAGAAATGCAAACCTCTGTGT	exon2-intron2

2.1.11 siRNAs

siRNAs were obtained from Qiagen or Dharmacon as shown in table 2.6.

Table 2.6 siRNAs used in this work

siRNA	Target sequence (5'-3')	Target	Source
Eya1_1	CAGGATTATATTCAGGAAATA	mouse Eya1	Qiagen
Eya1_2	CCGAGGCAGAAGAAACAATAA	mouse Eya1	Qiagen
Luciferase	TAAGGCTATGAAGAGATACTT	luciferase; non-targeting in mouse and human	Dharmacon

2.2 METHODS

2.2.1 Standard techniques

Molecular biology standard techniques, including plasmid transformation and propagation, small-scale purification of plasmid-DNA from *E. coli*, enzymatic manipulation of DNA and RNA, polymerase chain reaction, and analysis of proteins by SDS-PAGE and immunoblot were performed according to Sambrook *et al*. (Sambrook and Russell, 2001) or Ausubel *et al*. (Ausubel, 2002).

2.2.2 Yeast two-hybrid system

2.2.2.1 Preparation of competent yeast cells (KFY1)

For preparation of competent yeast cells, 10 ml YPD medium were inoculated with cells of *S. cerevisiae* KFY1 and incubated overnight in a shaker at 30°C and 200 rpm. Next morning, 5 ml of the overnight culture were transferred into 100 ml YPD medium and shaken at 30°C for 3 h until cells were in mid-log phase. After that, cells were collected by centrifugation (3,000 rpm, 10 min, 4°C). The pellet was resuspended in 10 ml solution A, centrifuged again (3,000 rpm, 10 min, 4°C), dried, and dissolved in 1 ml solution A. After addition of 55 µl of DMSO, cells were aliquoted into 200 µl per tube and stored at -80°C until usage.

solution A: 0.5 M ethylene glycol, 10 mM bicin, 1 M D-sorbit

2.2.2.2 Transformation of competent yeast cells (Klebe *et al.*, 1983)

For transformation of *S. cerevisiae* KFY1, 3 µg plasmid-DNA were added to one aliquot of frozen competent cells, which was directly followed by a heat-shock for 5 min at 37°C and 800 rpm using a thermomixer. After addition of 1 ml solution B, cells were incubated for 1 h at 30°C and 600 rpm. Cells were collected by centrifugation (3,000 rpm, 3 min), washed with 1 ml solution C, and plated onto appropriate 10 cm-selection agar plates. Plates were incubated at 30°C for about 3 days until colonies were clearly visible.

solution B: 0.4 M PEG 1000, 200 mM bicine

solution C: 150 mM NaCl, 10 mM bicine

2.2.2.3 Screening of the cDNA library

To identify interaction partners of Eya1, the MATCHMAKER Two-Hybrid system (Clontech) was used according to the manufacturer's protocol. The method is based on the mating of two yeast strains of different mating types containing bait or prey plasmid, respectively. Yeast two-hybrid plasmids used for the screening procedure are shown in Fig. 2.1.

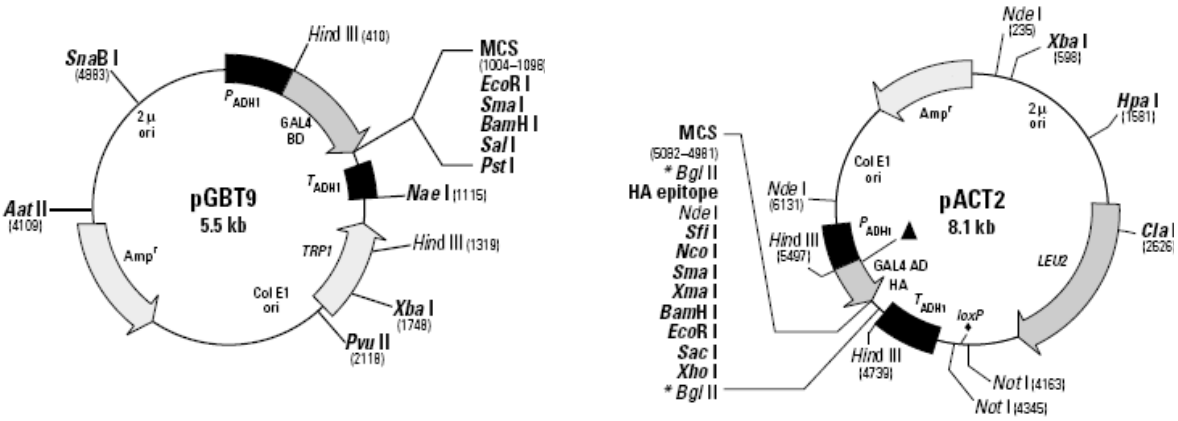

Fig. 2.1. Schematic overview about yeast two-hybrid vectors used in this work. pGBT9 (left) contains the coding region of the Gal4-BD and a *TRP1* marker, and was used for construction of the bait plasmid. pACT2 (right) contains the coding sequence of the Gal4-AD and a *LEU2* marker, and was used for construction of the prey constructs each containing a cDNA fragment of a mouse embryonic cDNA library. The auxotrophy markers *TRP1* and *LEU2* allow for selection of successfully mated colonies by growth on selection agar lacking tryptophan and leucine. (from „MATCHMAKER GAL4 Two-Hybrid Vectors Handbook", Clontech)

Briefly, three aliquots of the MATa-yeast strain KFY1 were transformed with the bait plasmid, pGBT9-mEya1-C, as described above, plated onto one 15 cm-selection agar plate and grown for 3 days at 30°C. The day before mating, an aliquot (100 µl) of the pretransformed mouse embryonic cDNA library (in MATα strain Y187) was plated onto a 15 cm-selection agar plate lacking histidine, adenine, tryptophane, leucine and methionine to select for clones containing the library plasmid pACT2. On the day of mating, colonies were rinsed off the plates with 15 ml YPD medium each. Cells were collected by centrifugation (3,000 rpm, 10 min, 4°C) and each pellet was resuspended in 300 µl YPD medium. Bait- and prey-containing suspensions were pooled and plated onto YPD agar where mating occurred. After 6 h of incubation at 30°C, YPD agar was rinsed with selection medium. Cells were pelleted by centrifugation (3,000 rpm, 10 min, 4°C), washed in selection medium, and resuspended in 4 ml selection medium. The cell suspension was evenly spread onto 20 selection agar plates and one control plate

(15 cm-format). The control plate contained histidine, but lacked tryptophane and leucine, which allows for selection of successfully mated clones. Colonies grown on the control plate after 3 days of incubation at 30°C were counted and mating efficiency was determined. Selection agar plates contained 5 mM 3-amino-1,2,4-triazole, but lacked histidine, tryptophane and leucine, which allows for selection of colonies containing interacting Gal4-BD- and Gal4-AD-fusion proteins. 3-amino-1,2,4-triazole is a competitive inhibitor of the HIS3 gene product and leads to reduction of background due to leaky *HIS3* expression. Selection agar plates were incubated at 30°C for 14 days, and colonies grown on these plates were subsequently analyzed for *β-gal* expression by performing a colony-lift filter assay as described below. Plasmid-DNA was extracted from β-gal positive clones as described in section 2.2.2.6, and used for re-transformation together with the bait plasmid, pGBT9-Eya1-C, followed by colony-lift filter assay to verify the results. If re-transformation assay was positive, plasmid-DNA was sequenced.

2.2.2.4 Qualitative colony-lift filter assay

HIS3-expressing yeast colonies were checked for β-gal activity by performing a colony-lift filter assay (Breeden and Nasmyth, 1985) as described by Clontech. Briefly, a sterile Whatman filter (7 cm or 12.5 cm, Sigma-Aldrich) was directly attached to the plate of colonies to be assayed and transferred into a pool of liquid nitrogen. After the filter had frozen completely, it was placed, colony side-up, on a second Whatman filter pre-soaked in Z/X-buffer in a clean petri dish. The filters were incubated at 30°C until a blue colour developed or for maximum 24 h. β-gal-producing colonies were identified by aligning the filter to the agar plate and subjected to further analysis.

Z/X buffer: Z buffer (see 2.2.2.5), 0.8 mM X-gal, 35 mM β-mercaptoethanol

2.2.2.5 Quantitative β-galactosidase liquid assay

To assess the strength of a protein-protein interaction, a β-gal liquid assay (Breeden and Nasmyth, 1987) was carried out according to the Clontech protocol. The method is based on the assumption that the strength of an interaction correlates with the level of *lacZ* expression which can be determined by measuring the enzymatic activity of its gene product β-gal.

Briefly, colonies were grown in liquid selection medium overnight at 30°C with shaking at 200 rpm. Next day, 2 ml of the overnight culture were transferred to 8 ml YPD medium. The fresh culture was incubated at 30°C and 200 rpm for 3-5 h until cells were in mid-log phase (OD_{600} of 1 ml = 0.5-0.8). For later calculation, the exact OD_{600} was recorded when cells were harvested. For harvesting, 1.5 ml of culture were placed into each of three 1.5-ml microcentrifuge tubes. After centrifugation (13,200 rpm, 30 sec), the pellet was washed in 1.5 ml Z buffer, centrifuged again and resuspended in 100 µl Z buffer. Tubes were placed in liquid nitrogen until the cells were frozen (1 min). Frozen tubes were then put into a 37°C water bath for 1 min to thaw. This freeze/thaw cycle was repeated two more times to ensure that the cells have broken open. 700 µl Z-buffer containing 35 mM β-mercaptoethanol were added to each tube and a blank (100 µl Z buffer) before starting the reaction with 160 µl ONPG in Z buffer (4 mg/ml). Tubes were incubated at 30°C until a yellow colour developed or for maximum 24 h. Reaction was stopped by the addition of 400 µl of 1 M Na_2CO_3 and elapsed time was recorded. After centrifugation (13,200 rpm, 10 min) OD_{420} of the supernatant was determined relative to the blank. The amount of β-gal units was calculated according to the following formula:

$$\text{β-gal units} = 1000 \times OD_{420}/(t \times V \times OD_{600})$$

where: t = time of incubation in min
V = volume of culture used for measurement

Thus, 1 unit of β-gal is defined as the amount which hydrolyzes 1 µmol of ONPG to *o*-nitrophenol and D-galactose per min per cell.

Z buffer: 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 0.1 mM $MgSO_4$

2.2.2.6 Plasmid extraction from yeast cells

For isolation of plasmid-DNA from yeast, a single colony was inoculated in 10 ml selection medium and grown at 30°C and 200 rpm. After 48 h, 1.5 ml of the culture were transferred into each of three microcentrifuge tubes and centrifuged at 13,200 rpm for 1 min. The cell pellet was resuspended in 200 µl yeast lysis buffer. 0.3 g glass beads and 200 µl phenol/chloroform were added. Samples were vortexed for 2 min and subsequently centrifuged for 5 min at 13,200 rpm. DNA-containing upper phases were pooled and 300 µl chloroform/isoamylalcohol were

added. After centrifugation (13,200 rpm, 5 min) the upper phase was transferred into a new tube, DNA was precipitated by addition of 50 µl 5 M NaCl and 550 µl isopropanol and spun down for 10 min at 13,200 rpm. The DNA pellet was washed in 70% ethanol and finally resuspended in 20 µl of ddH_20.
3 µl of the DNA solution were used for electroporation of *E. coli* HB101. Colonies containing the prey plasmid were selected on M9-agar plates lacking leucine. Plasmid-DNA was purified using standard techniques and subjected to retransformation into yeast, or sequencing.

yeast lysis buffer: 10 mM Tris pH 8.0, 1 mM EDTA, 100 mM NaCl, 2% Triton X-100, 1% SDS

2.2.2.7 Protein extraction from yeast cells

Protein extraction from yeast was basically performed as described by Lussier *et al*. (Lussier *et al.*, 2005). Briefly, co-transformed yeast cells were grown in appropriate selection medium for 24 h at 30°C and 200 rpm. An aliquot (1.5 ml) of the overnight culture was centrifuged for 5 min at 13,200 rpm and 4°C. The cell pellet was weighed and resuspended in 1 ml of 0.25 N NaOH containing 1% β-mercaptoethanol. After incubation on ice for 10 min, 160 µl of 50% trichloroacetic acid were added. The mixture was incubated on ice for 10 min again, and centrifuged for 10 min at 13,200 rpm and 4°C. The pellet was washed with 1 ml of ice-cold acetone, dried, and dissolved in 100 µl 2x Laemmli loading buffer per 15 mg of cell pellet. Samples were boiled for 5 min, resolved by SDS-PAGE and subsequently analyzed by immunoblotting.

2x Laemmli loading buffer: 120 mM Tris pH 6.8, 20% glycerol, 4% SDS, 8% β-mercaptoethanol, bromphenol blue

2.2.3 GST pulldown system

2.2.3.1 Purification of GST fusion proteins from *E. coli* BL21

The plasmid harbouring the cDNA of the respective GST fusion construct was transformed into *E. coli* BL21. 5 ml LB medium were inoculated with a single colony and grown overnight at 37°C and 180 rpm. Next morning, 1 ml of the overnight culture was transferred into 20 ml LB medium and incubated for 1.5 h at 37°C and 180 rpm. Induction of expression occurred by addition of 3.5 µl of 1 M

IPTG followed by a further incubation step (37°C, 180 rpm, 2 h). Then, bacterial cells were pelleted by centrifugation (5,000 rpm, 5 min, 4°C) and resuspended in 1 ml PBS containing 1 mM PMSF and 10 mM DTT. Cells were disrupted by sonication. Afterwards, the cell lysate was incubated for 30 min at 4°C in presence of 1% Triton X-100, and cleared by centrifugation (13,200 rpm, 15 min, 4°C). In parallel, 250 µl of a glutathione agarose slurry (3.5 % in ddH_2O, Sigma-Aldrich) were washed in 1 ml PBS. The cleared cell lysate was added to the pre-washed glutathione agarose beads and incubated for 1 h at 4°C on a rotator. Beads were collected by centrifugation (3,000 rpm, 5 min, 4°C), washed 4 times in PBS, and used for *in vitro* interaction assay. 10 µl of beads were analyzed by SDS-PAGE followed by coomassie staining of the gel to control efficient purification of GST fusion protein.

PBS: 140 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4

2.2.3.2 *In vitro* interaction assay

For *in vitro* transcription and translation, the TNT T7 Coupled Reticulocyte Lysate System (Promega) was used according to the manufacturer's protocol. GST fusion proteins coupled to agarose beads (see 2.2.3.1) were washed in 250 µl HBB containing 0.5% NP-40 (HBB-NP-40) and 0.5 mM DTT. Beads were resuspended in 50 µl HBB plus 50 µl HBB-NP-40, and 30 µl of *in vitro* synthesized protein were added. The mixture was incubated overnight at 4°C while rotating. Next day, beads were washed 6 times in 300 µl HBB-NP-40, resuspended in 25 µl of 2x Laemmli loading buffer and analyzed by SDS-PAGE and immunoblotting.

HBB: 20 mM HEPES pH 7.8, 100 mM KCl, 5 mM $MgCl_2$

2x Laemmli loading buffer: see 2.2.2.7

2.2.4 RNA isolation

Total RNA from embryonic mouse tissues was isolated using Absolutely RNA Microprep Kit (Stratagene) and eluted in 30 µl pre-warmed elution buffer.

Total RNA from zebrafish embryos or eukaryotic cell lines was isolated using RNeasy Mini Kit (Qiagen) after homogenization with QIAshredder spin columns (Qiagen) and eluted in a total volume of 30-50 µl RNase free water.

RNA concentration was determined by measuring the absorbance at 260 nm (A260) and calculated according to the following formula:

$$c = (A260 * e)/b \qquad \text{(modified Beer-Lambert equation)}$$

where: c = concentration in µg/µl
e = extinction coefficient in ng-cm/µl (RNA: 40 ng-cm/µl)
b = path length in cm

2.2.5 RT-PCR analysis

2.2.5.1 cDNA synthesis

An aliquot of 10 µl of RNA (100-500 ng) was reverse transcribed using SuperScript II RNase H^- Reverse Transcriptase (Invitrogen) and oligo(dT) primers (Roche) in a total volume of 20 µl. For control of specificity, a parallel reaction without reverse transcriptase was performed in each case. 1 µl of cDNA was used for subsequent PCR analysis.

2.2.5.2 Polymerase Chain Reaction (PCR)

For a standard PCR, an appropriate amount of DNA-template was used with 2.5 µl 10x PCR-Puffer (200 mM Tris pH 8.4, 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM Primers and 0.5 U Taq-Polymerase in a final volume of 25 µl. The cycling program was started with denaturation at 94°C for 1 min followed by 35 cycles (40 cycles for real-time RT-PCR) of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 30 s.

2.2.5.3 Quantitative Real-Time RT-PCR (qRT-PCR)

qRT-PCR analysis was performed in 96-well format. For comparison of gene expression by qRT-PCR, the standard RT-PCR mix plus 0.75 µl of a 1/2000 dilution of SYBR Green I nucleic acid gel stain (BioWhittaker Molecular Applications) and 0.25 µl of 1 µM Fluorescein Calibration Dye (BioRad) was used. The factor difference in expression of a gene of interesting in different samples was calculated according to the double delta method (Livak and Schmittgen, 2001) including normalization to expression of the housekeeping gene *β-actin*.

2.2.6 RNA *in situ* hybridization

2.2.6.1 Generation of digoxigenin-labeled riboprobes

For generation of digoxigenin labeled riboprobes, plasmids containing cDNA fragments of the respective gene were linearized by restriction enzyme digestion and purified using the QiaExII Gel Extraction kit from Qiagen. 1-4 µg purified plasmid-DNA were used for transcription with T3, T7 or Sp6 RNA-Polymerase (Roche) in presence of DIG RNA Labeling Mix (Roche) and rRNasin (Promega).

2.2.6.2 RNA *in situ* hybridization on paraffin sections

In situ hybridizations on paraffin sections were performed according to Leimeister et al. (Leimeister *et al.*, 1998) with some modifications. Briefly, embryos were fixed in 4% PFA/PBS, washed in PBS, dehydrated in a graded isopropanol or ethanol series, infiltrated with chloroform or xylol and embedded in paraffin. Sections of 10 µm were mounted on poly-lysine coated slides (Menzel), dried on a heating plate at 45°C and stored at 4°C.

Sections were dewaxed in chloroform, rehydrated in a graded ethanol series, washed in PBS, re-fixed for 30 min in 4% PFA/PBS, digested with proteinase K (10 µg/ml in Proteinase K buffer) for 10 min, washed, and fixed again. After washing in PBS, 2x SSC, and Tris/glycine buffer, sections were hybridized with 0.1 µg/ml digoxigenin-labeled probes in hybridization buffer for 16-20 h at 70°C in a 5x SSC humified chamber. Following hybridization, sections were washed three times for 20 min in 5x SSC at room temperature, 1 h in 0.5x SSC/20% formamide at 60°C, 15 min at 37°C in RNase buffer, and treated with 10 µg/ml RNase A for 30 min. After washes in RNase buffer for 15 min at 37°C, in 0.5x SSC/20% formamide for 30 min at 60°C and in 2x SSC for 30 min at room temperature, sections were blocked with 1% blocking reagent (Roche) in MABT and incubated with anti-digoxigenin antibodies (Roche) at a dilution of 1:5000 for 16-20 h at 4°C. Sections were washed five times for 10 min in TBS-T, once for 10 min in NTMT, 10 min in NTMT containing 2 mM levamisol and then developed for 1-5 days in BM-purple substrate (Roche) containing 2 mM levamisol and 0.1% Tween20. After staining, sections were washed twice in NTMT for 15 min and 10 min in PBS. Slides were mounted in Kaiser's glycerol gelatine (Merck).

PBS:	see 2.2.3.1
4% PFA/PBS:	4% paraformaldehyde in PBS
Proteinase K buffer:	20 mM Tris pH 7.5, 1 mM EDTA
1x SSC:	150 mM NaCl, 15 mM tri-sodium citrate; pH 5
Tris/glycine buffer:	100 mM Tris, 100 mM glycine
Hybridization buffer:	50% formamide, 1.3x SSC pH5, 0.5% CHAPS, 5 mM EDTA, 0.2% Tween20, 100 µg/ml heparin, 100 µg/ml torula yeast RNA (Sigma-Aldrich)
RNase buffer:	0.5 M NaCl, 10 mM Tris pH 7.5, 5 mM EDTA
MABT:	100 mM maleic acid, 150 mM NaCl, 0.1% Tween20; pH 7.5
TBS-T:	25 mM Tris pH 7.5, 140 mM NaCl, 2.7 mM KCl, 0.1% Tween20
NTMT:	100 mM Tris pH 9.5, 10 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween20

2.2.6.3 Whole mount *in situ* hybridization (WISH) of zebrafish embryos

To get an idea about the expression of *Sipl1/Rbck1* orthologous genes in zebrafish embryos, the method of whole mount *in situ* hybridization was employed. Therefore, zebrafish embryos were collected at different stages, dechorionated, and fixed in 4% PFA/PBS overnight at 4°C. Then, embryos were washed four times in PBS-T, resuspended in methanol, and stored at -20°C until further usage. Before hybridization, embryos were re-hydrated by incubation in a graded methanol series, washed in PBS-T, treated with proteinase K (5 µg/ml) in PBS-T for 1-15 min depending on the stage of the embryo, washed twice in PBS-T plus glycine (2 mg/ml), and then re-fixed for exactly 20 min in 4% PFA/PBS-T. After repeated washing of the embryos in PBS-T, embryos were pre-incubated in hybridization buffer for 1 h at 65°C. Afterwards, embryos were incubated with digoxigenin-labeled RNA probe in hybridization buffer (0.5 µg/ml) overnight at 65°C. Following hybridization, embryos were washed twice in 2x SSC containing 50% formamide at 65°C, once in 1x SSC at 65°C, twice in 0.2x SSC at 65°C, and once in PBS-T at room temperature. Embryos were blocked for 1-8 h in PBS-T plus 5% sheep serum (Roche) and incubated with anti-digoxigenin antibodies (Roche) in PBS-T (1:2000) overnight at 4°C, followed by several washing steps in PBS-T for 20 min each. After washing in SB buffer twice, embryos were stained in SS buffer in the dark until a blue colour developed. After this, embryos were washed three times in PBS-T, and finally fixed in 4% PFA/PBS for 30 min. Fixed embryos were stored in PBS-T at 4°C.

4% PFA/PBS:	see 2.2.6.2

PBS-T:	PBS (see 2.2.3.1) containing 0.1% Tween20
hybridization buffer:	5x SSC, 50% formamide, 0.1% Tween20, 50 µg/ml heparin, 5 mg/ml yeast torula RNA (Sigma-Aldrich)
SB buffer:	100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween20
SS buffer:	100 mM NaCl, 0.1% Tween20, NPT/BCIP (1 tablet/10 ml)

2.2.7 RNA interference

2 x 10^6 mK4 cells were seeded into 10 cm-dishes and transfected 24 h later applying 15-25 nM siRNA and siLentFect Lipid Reagent (Bio-Rad) according to the manufacturer. 72 h post-transfection, cells were harvested and subjected to RNA isolation for confirmation of knockdown efficiency or protein analysis.

2.2.8 Luciferase reporter assay

For luciferase reporter assay, 1.5 x 10^5 Cos-7 cells were seeded into 6-well format and transfected 24 h later with a total of 4 µg of plasmid-DNA, including a luciferase reporter construct, appropriate expression constructs and an internal control plasmid, using Lipofectamin (Invitrogen). 48 h post-transfection, cells were harvested and luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega).

2.2.9 Lysis of eukaryotic cells for direct Immunoblotting

1.5 x 10^5 Cos-7 cells were seeded in 6-well cell culture dishes and transfected with 2-3 µg of the appropriate expression vectors using Superfect transfection reagent (Qiagen) according to the manufacturer's instruction. 48 h post-transfection, cells were harvested in 1 ml PBS by scraping. After centrifugation (3,000 rpm, 5 min, 4°C), cells were lysed in 150 µl RIPA lysis buffer and the lysate was passed through a QIAshredder spin column (Qiagen) by centrifugation (13,200 rpm, 2 min). Protein concentration was determined by measuring absorbance at 280 nm (A280). 20-40 µg of protein were subsequently analyzed by SDS-PAGE and immunoblotting.

PBS:	see 2.2.3.1
RIPA lysis buffer:	50 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS

2.2.10 Immunoprecipitation

48 h after transfection with siRNAs or expression constructs, cells were scraped in 1 ml PBS, collected by centrifugation (3,000 rpm, 5 min, 4°C), and lysed in 500 µl HEPES lysis buffer for 1 h on ice. The lysate was cleared by centrifugation (10,000 rpm, 5 min, 4°C) and added to the appropriate rabbit or mouse antibody which had been pre-incubated with protein A or G agarose (Calbiochem), respectively. The mixture was incubated on a rotator in the cold room overnight to allow binding of antigen to the antibody. After centrifugation (1,000 rpm, 2 min, 4°C), removal of the supernatant, and washing in lysis buffer four times, immunocomplexes were eluted by boiling in 25 µl 2x Laemmli loading buffer. Samples were analyzed by SDS-PAGE and immunoblotting using appropriate antibodies.

PBS:	see 2.2.3.1
HEPES lysis buffer:	25 mM HEPES pH 7.9, 0.5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1x cømplete proteinase inhibitor cocktail (Roche)

2.2.11 *In vivo* ubiquitination assay

For *in vivo* ubiquitination assay, 1 x 10^6 Cos-7 cells were seeded into a 10 cm-cell culture dish. 24 h after seeding, cells were co-transfected with 10-15 µg of appropriate expression constructs using Superfect transfection reagent (Qiagen) according to the manufacturer's protocol. Again 24 h later, cells were treated with either 10 µM MG132 (Sigma-Aldrich) or DMSO as a control. 48 h post-transfection, cells were washed in PBS, and then harvested by scraping in 1 ml PBS. 100 µl of the cell suspension were transferred into a new microcentrifuge tube, centrifuged (3,000 rpm, 5 min 4°C) to pellet the cells, and the supernatant was removed. Cells were resuspended in 100 µl 2x Laemmli loading buffer, sonicated, and stored at –20°C for input control. The rest of the cell suspension was centrifuged (3,000 rpm, 5 min, 4°C), supernatant was removed, and the pellet was lysed in 1 ml buffer A. To reduce viscosity, the lysate was passed through a QIAshredder spin column (Qiagen) by centrifugation for 2 min at 13,200 rpm. After addition of 75 µl Ni-NTA agarose (Qiagen), the mixture was incubated on a rotator in the cold room overnight. Then, agarose beads were transferred to a Micro Bio-Spin chromatography column (Bio-Rad) and supernatant was passed through by centrifugation at 3,000 rpm for 30 sec. Beads were washed sequentially in 750 µl buffer A, 750 µl buffer B, and 750 µl buffer C before elution in 75 µl Elution buffer.

Both the eluate and the stored input control were boiled for 5 min and subsequently analyzed by SDS-PAGE and immunoblotting.

PBS:	see 2.2.3.1
2x Laemmli loading buffer:	see 2.2.2.7
buffer A:	6 M guanidium hydrochloride, 10 mM β-mercaptoethanol, 100 mM NaH_2PO_4, 10 mM Tris, 5 mM imidazol; pH 8.0
buffer B:	8 M urea, 10 mM β-mercaptoethanol, 100 mM NaH_2PO_4, 10 mM Tris pH 8.0
buffer C:	8 M urea, 10 mM β-mercaptoethanol, 100 mM NaH_2PO_4, 10 mM Tris; pH 6.3
Elution buffer:	150 mM Tris pH 6.8, 30% glycerol, 5% SDS, 200 mM imidazol, 720 mM β-mercaptoethanol

2.2.12 Fluorescence microscopy

2 x 10^5 Cos-7 cells were seeded onto cover slips in 6-well cell culture dishes and transfected with 3 µg of appropriate RFP- or EGFP-expression constructs using Superfect (Qiagen) according to the manufacturer's instructions. 40 h post-transfection, cells were washed in PBS, and fixed in 4% PFA/PBS for 20 min. After washing in PBS again, cell nuclei were stained with Hoechst 33258 (Sigma-Aldrich-Aldrich) in PBS (1:500) for 1min. Coverslips were mounted onto microscope slides using Glycergel (DakoCytomation) and analyzed by microscopy using an Axiovert 135 TV microscope (Zeiss).

PBS:	see 2.2.3.1

3 RESULTS

At the beginning of this study, no Eya1-specific antibodies were available. Because of this, in the first part of this work two Eya1-specific antibodies generated in our lab were analyzed regarding their ability to specifically detect the endogenous Eya1 protein.

3.1 CHARACTERIZATION OF EYA1-SPECIFIC ANTIBODIES

An important tool for analyzing the function of a protein of interest *in vivo* is the use of specific antibodies, which enable you to analyze protein modifications or protein-protein interactions. Jürgen Tomasch, a former diploma student in our lab, generated two polyclonal rabbit antibodies, anti-Eya1.1 and anti-Eya1.2, directed against fragments located within the N-terminus of mouse Eya1, which is, in contrast to the Eya domain, poorly conserved between Eya family members. He characterized both antibodies in immunoblot, immunoprecipitation and immunofluorescence using cell lines overexpressing *Eya1*. He could show that both antibodies worked in immunoprecipitation with anti-Eya1.1 being more efficient than anti-Eya1.2, whereas only anti-Eya1.2 detected Eya1 in immunoblot (Jürgen Tomasch, diploma thesis, 2007). According to his results, I have used anti-Eya1.1 for immunoprecipitation and anti-Eya1.2 for immunoblot analyses. However, a point still to be investigated was whether the antibodies are specific for mouse Eya1 or can also recognize the family members Eya2, Eya3 and Eya4. To verify specificity of anti-Eya1.2 in immunoblot, all four proteins were synthesized *in vitro*, loaded on a 10% SDS-PAA gel and detected by immunoblotting using anti-Eya1.2. Since Eya1, Eya2 and Eya3 carried HA-epitope tags at their N-terminus, the membrane was stripped and re-probed using anti-HA antibody to control the input. Input of Eya4 could not be determined because it was not fused to an HA-tag. In a second experiment, the antibody anti-Eya1.1 was tested for its ability to specifically pull down Eya1 in immunoprecipitation. Eya1-4 were synthesized *in vitro* in presence of S^{35}-labeled methionine. Part of the radioactively labeled protein was stored as input control, the rest was diluted in HEPES lysis buffer and immunoprecipitation was carried out as described. Precipitates and input controls were resolved on a 10% SDS-PAA gel and analyzed by autoradiography.

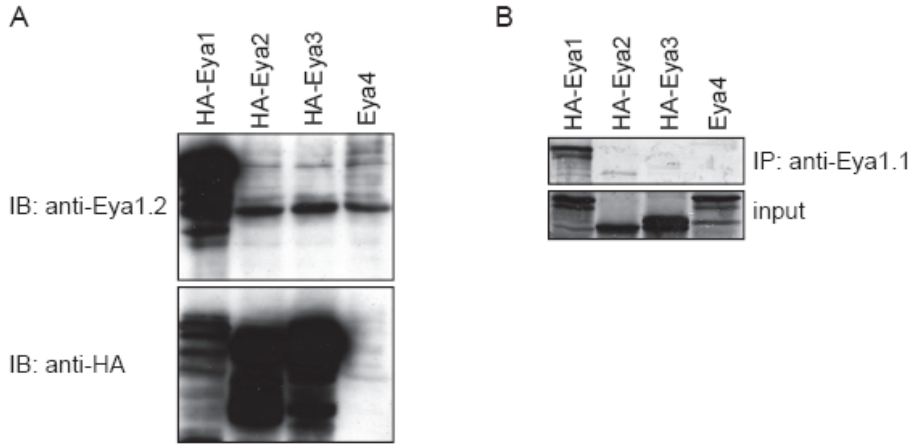

Fig. 3.1. The Eya1-specific antibodies detect Eya1, but not Eya2, Eya3 and Eya4 in immunoblot and immunoprecipitation. (A) HA-Eya1-3 and Eya4 were synthesized *in vitro*, resolved by 10%-SDS-PAGE and analyzed by immunoblotting using anti-Eya1.2 (upper panel). Membrane was stripped and re-detected using anti-HA for control of input (lower panel). (B) HA-Eya1-3 and Eya4 were synthesized and S^{35}-labeled *in vitro*, diluted in HEPES lysis buffer, and subjected to immunoprecipitation using anti-Eya1.1. Immunocomplexes and input control were analyzed by 10%-SDS-PAGE and autoradiography.

Figure 3.1 A shows that the antibody anti-Eya1.2 specifically detected Eya1 in immunoblot analysis, even though the input level of Eya1 was lower compared to that of Eya2 and Eya3 as shown by detection with anti-HA. Weaker background signals were detected in all lanes. Moreover, immunoprecipitation using anti-Eya1.1 and radioactively labeled Eya1-4 was also specific for Eya1 as seen in Fig. 3.1 B. The amounts of protein used for immunoprecipitation were comparable between all four mouse Eya homologs.

Having shown that both antibodies specifically recognize Eya1, but not Eya2, Eya3 and Eya4, the next step was to identify a cell line which is suited for the detection of endogenous Eya1. Jürgen Tomasch tried to detect endogenous Eya1 protein in several cell lines which were positive for *Eya1* expression in RT-PCR, as for example TM4 (mouse Sertoli) cells. But, probably due to very low Eya1 protein levels within these cell lines, he was not able to detect a clear signal for endogenous Eya1 after immunoprecipitation and immunoblot (Jürgen Tomasch, diploma thesis, 2007). Valerius *et al*. created four kidney cell lines, mK1-4, with mK1-3 cells representing stages of the early uninduced metanephric mesenchyme and mK4 cells representing a later induced stage of the metanephric mesenchyme (Valerius *et al*., 2002). Since it is known from literature that *Eya1* is expressed in both uninduced and induced metanephric mesenchyme during mouse embryonic kidney development (Xu *et al*., 1999), mK3 and mK4 cells were checked for *Eya1* expression by RT-PCR using gene-specific primers.

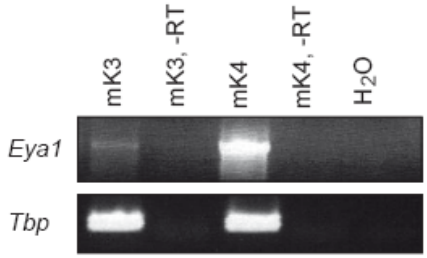

Fig. 3.2. *Eya1* is expressed in mK3 and mK4 cells. RNA isolated from mK3 and mK4 cells was reverse-transcribed into cDNA and analyzed for expression of *Eya1* by PCR using gene-specific primers (upper panel). Expression of *Tbp* (*TATA-box binding protein*) was determined from the same samples to control input (lower panel). For verification of specificity samples without reverse transcriptase (-RT) and a water control were included.

Using RT-PCR analysis *Eya1* expression was detected at low levels in mK3 and at high levels in mK4 cells (Fig. 3.2). To check for endogenous Eya1 protein in those cell lines, immunoprecipitation was performed.

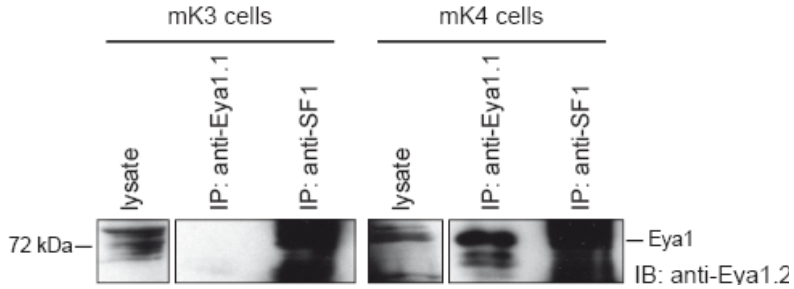

Fig. 3.3. Endogenous Eya1 protein is detected in mK4, but not in mK3 cells. Cells were grown to about 90% confluency in 10 cm-cell culture dishes and lysed in HEPES lysis buffer. The cell lysate was subjected to immunoprecipitation using anti-Eya1.1 or anti-SF1 (steroidogenic factor 1) antibody. An aliquot of the lysate before immunoprecipitation and the immunoprecipitate were loaded on a 10% SDS-PAA gel followed by immunoblotting using anti-Eya1.2 antibody.

Detection of a specific Eya1 signal in the cell lysate was difficult because of the presence of several signals in both mK3 and mK4 cells. After immunoprecipitation a specific signal of about 80 kDa was detectable in mK4 but not in mK3 cells (Fig. 3.3). This signal is likely to represent endogenous Eya1 protein taking into account that Eya1 protein appears at a similar size upon overexpression, and RT-PCR results show higher expression of *Eya1* in mK4 cells compared to mK3.

To verify that the signal which is detected in mK4 cell extracts after precipitation and detection with Eya1-specific antibodies indeed represents endogenous Eya1 protein, an RNA interference approach was employed. mK4 cells were transfected with specific siRNAs directed against mouse *Eya1* or a non-targeting control-siRNA directed against *luciferase*. 72 h post-transfection, cells were harvested and subjected to immunoprecipitation using anti-Eya1.1 or a rabbit control-antibody. In parallel, part of the cells was used for RNA isolation and cDNA synthesis followed

by quantitative RT-PCR (qRT-PCR) *Eya1*-specific primers to determine the knockdown efficiency.

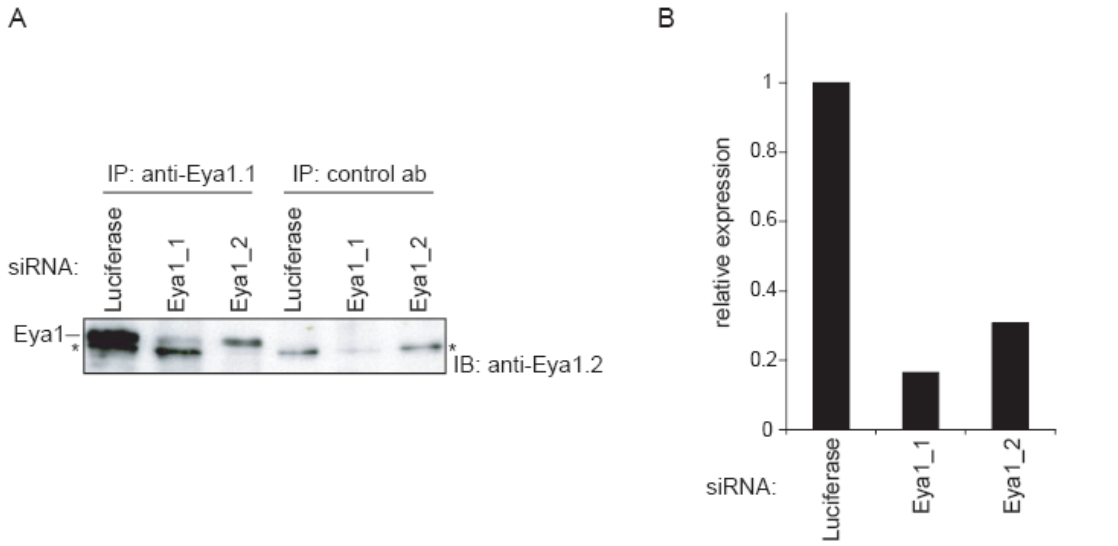

Fig. 3.4. The protein level of endogenous Eya1 is reduced after siRNA-mediated knockdown of *Eya1* expression in mK4 cells. mK4 cells were transfected with siRNAs directed against *Eya1* or a control siRNA directed against *luciferase*. 72 h post-transfection, cells were harvested. (A) siRNA-transfected cells were lysed in HEPES lysis buffer followed by immunoprecipitation using anti-Eya1.1 or rabbit control antibody (anti-HA, Y11) and immunoblot analysis using anti-Eya1.2 antibody. The asterisks indicate unspecific signals. (B) Part of the cells was used for RNA isolation. Efficiency of *Eya1*-specific knockdown was determined by qRT-PCR. Relative *Eya1* expression compared to cells transfected with control siRNA (luciferase) and normalized to *β-actin* expression was calculated using the double delta method.

After transfection of a control-siRNA into mK4 cells, a specific signal was detected after immunoprecipitation with anti-Eya1.1 and detection with anti-Eya1.2 antibody (Fig. 3.4 A). This signal was not present after precipitation with a control antibody suggesting that the detected signal represents endogenous Eya1 protein. Indeed, after knockdown using each of two *Eya1*-specific siRNAs, the respective signal was weakened, whereby Eya1_1 siRNA seemed to have a stronger effect compared to Eya1_2. This is in line with results obtained by qRT-PCR, which was performed from the same cells to control knockdown efficiency on the mRNA level (Fig. 3.4 B). Both *Eya1*-specific siRNAs mediated efficient knockdown of *Eya1* expression compared to the *luciferase*-specific control-siRNA with Eya1_1 being more efficient than Eya1_2.

In summary, it was shown that both Eya1-specific antibodies generated in our lab specifically recognize Eya1 in immunoprecipitation and immunoblot, respectively. Furthermore, using those antibodies endogenous Eya1 protein could be detected in mK4 cells, providing a starting point for analysis of Eya1 protein function at the physiological level.

3.2 BIOCHEMICAL CHARACTERIZATION OF EYA1

Eya1 is a multifunctional protein essential for the development of various organs as for example kidney, ears and thymus (Xu *et al*., 1999; Xu *et al*., 2002). It is known that mutations in the human *EYA1* gene are associated with several human diseases, as branchio-oto-renal (BOR) or branchio-oto (BO) syndrome as well as ocular defects. However, the molecular mechanisms by which these mutations lead to human disease are not understood. To gain a deeper insight into the underlying mechanisms, seven disease-associated Eya1 mutants, all single amino acid substitutions clustering within the conserved Eya domain (Table 3.1), were characterized concerning their influence on known protein-protein interactions of Eya1. All mutations were analyzed in the background of mouse Eya1 which has been shown to be highly conserved to human Eya1 in both protein sequence and function.

Table 3.1 Disease-associated Eya1 mutants analyzed in this work

Eya1 mutant (human)	associated disease	corresponding mouse Eya1 mutant	Reference
G393S	BOR	G425S	Azuma *et al*., 2000
D396G	BO	D428G	Namba *et al*., 2001
R407Q	BO	R439Q	Kumar *et al*., 1997
S454P	BOR	S486P	Abdelhak *et al*., 1997
L472R	BOR	L504R	Abdelhak *et al*., 1997
R514G	ocular defects	R546G	Azuma *et al*., 2000
L550P	BOR	L582P	Rickard *et al*., 2000

All mutations were introduced into the yeast two-hybrid bait construct which encodes the conserved C-terminus of mouse Eya1 using site-directed mutagenesis and primers designed by Amna Musharraf, a PhD student in our lab. In order to test the effect of the mutations on the interaction of Eya1 with two already known interaction partners of Eya1, Six1 as a representative for the Six protein family, and the inhibitory Gα subunit Gαi2 (Buller *et al*., 2001; Ozaki *et al*., 2002), β-gal liquid assays were performed. Therefore, human Six1 (hSix1) was cloned into the yeast two-hybrid prey vector pGADT7. The coding sequence of the Gαi2 subunit was obtained from cDNA of mouse lung tissue by RT-PCR using

gene-specific primers and also cloned into pGADT7. Since it is known that Eya proteins interact only with active Gαi2 (Fan *et al.*, 2000), an activating mutation (Q205L) was introduced by site-directed mutagenesis. *S. cerevisiae* KFY1 cells were co-transformed with respective bait and prey plasmids and β-gal liquid assay was performed to determine the interaction strength.

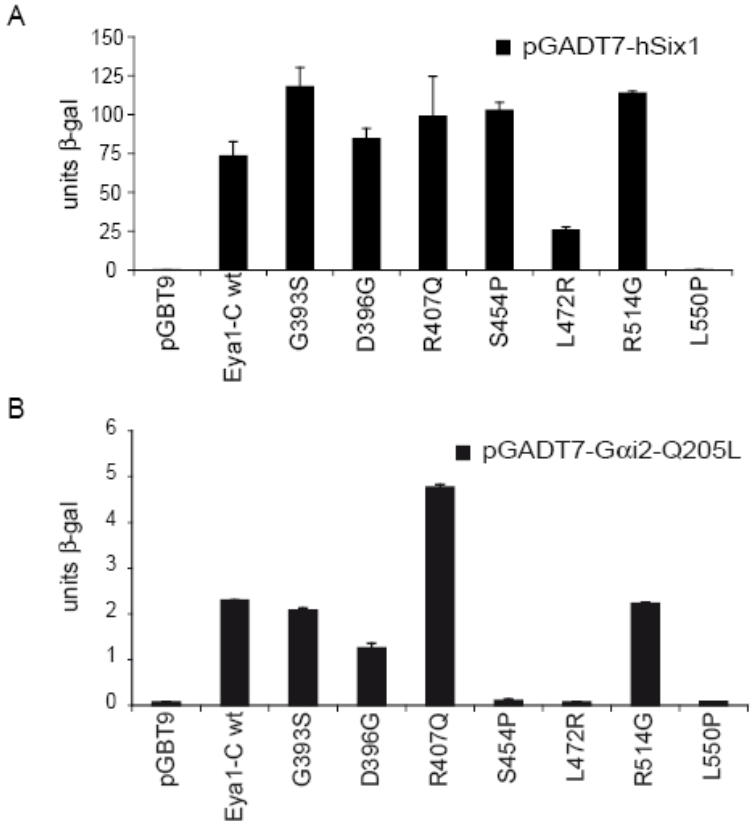

Fig. 3.5. Some of the disease-associated *Eya1* mutations affect the interaction with Six1 and Gαi2. Each mutation was introduced into the yeast vector pGBT9-Eya1-C. Mutant or wild type pGBT9-Eya1-C or empty vector pGBT9 were introduced into yeast KFY1 together with pGADT7-hSix1 (A) or pGADT7-Gαi2-Q205L (B). After 3 days of growth interaction strength was determined from 3 pooled colonies by β-gal liquid assay. Each measurement was performed in triplicates. Error bars represent standard deviation.

β-gal liquid assay revealed clear differences in the interaction profile of the tested mutant proteins (Fig. 3.5). All Eya1 mutants interacted with hSix1 comparable to the wild type protein with two exceptions: L472R and L550P. The substitution of the leucine residue at position 472 to arginine (L472R) led to weakening of the interaction to about 30%. Substitution of the leucine at position 550 to proline (L550P) abolished the interaction completely. The two mutations L472R and L550P, which inhibited interaction with Six1, had a similar effect on the interaction with Gαi2 which was not surprising since it is has been published by Fan *et al*. that binding of Six1 and Gαi2 is mutually exclusive indicating that the two proteins bind

to an overlapping region of the Eya domain (Fan *et al*., 2000). In contradiction to this, there was one mutation, S454P, which led to complete loss of interaction with Gαi2, but did not affect the interaction with Six1. Furthermore, introduction of the mutation D396G led to weakening of the interaction with Gαi2 to about 50%, whereas the mutation R407Q stabilized the interaction to about 2 fold compared to the wild type. Similar results were obtained by Ozaki *et al*. who performed mammalian two-hybrid assay and showed that the Eya1 mutants G393S and R514G still interact with Gαi2, while S454P and L472R completely abolish the interaction. The other mutants have not been tested in their approach (Ozaki *et al.*, 2002). In summary, most of the Eya1 mutants analyzed in this work still interact with Six1 and Gαi2. Some, however, loose the interaction.

To confirm that the differences in the interaction profiles of the Eya1 mutants were not due to alterations in the amount of protein produced within yeast, I performed immunoblot analysis. The bait vector pGBT9 which was used for the interaction studies is a so-called “low-expression” vector. Due to this low expression, it was not possible to directly detect the respective Gal4-BD fusion proteins in yeast cell lysates. Thus, Eya1-C and its mutant variants were introduced into the yeast two-hybrid bait vector pGBKT7 which is, in contrast to pGBT9, a “high-expression” vector. Furthermore, pGBKT7 includes a c-Myc epitope tag allowing the detection of the Gal4-BD fusion proteins in immunoblot by using an anti-c-Myc antibody.

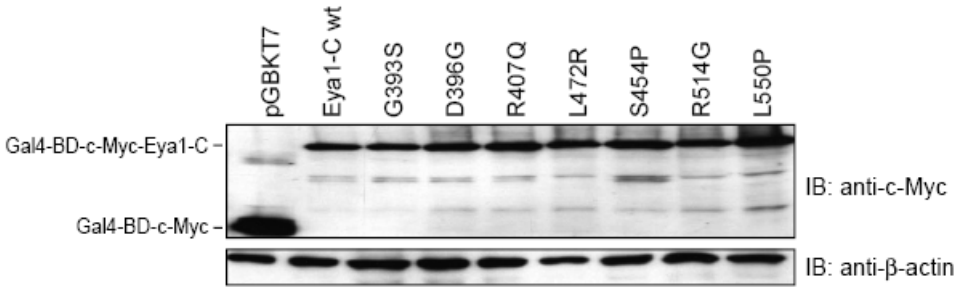

Fig. 3.6. Changes in the interaction profiles of the Eya1 mutants are not due to differences in protein levels. *S.cerevisiae* KFY1 were transformed with Gal4-BD-c-Myc expression constructs encoding wild type or mutant Eya1-C. After 3 days of growth, proteins were extracted and equal amounts of extracts were analyzed by 10% SDS-PAGE and immunoblotting using anti-c-Myc antibody (upper panel). Equal loading was confirmed by detection of β-actin (lower panel).

Fig. 3.6 shows that in yeast, all Eya1 mutants are present at a similar amount as the wild type. Thus, the possibility that the differences in the interaction profiles observed in yeast binding studies are caused by differences in expression levels can be excluded.

The next step was to establish a system which allows the analysis of the effect of the disease-associated *Eya1* mutations on Eya1 function in mammalian cells. Therefore, all mutations were introduced into the mammalian expression vector pHM6-Eya1 and checked for their expression in Cos-7 cells.

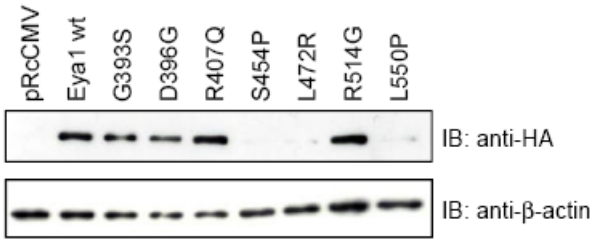

Fig. 3.7. The protein levels of the BOR-associated Eya1 mutants S454P, L472R, and L550P are dramatically decreased in Cos-7 cells. Each *Eya1* mutation was introduced into the eukaryotic expression vector pHM6-Eya1. Cos-7 cells were transfected with mutant pHM6-Eya1, wild type pHM6-Eya1 or empty vector pRcCMV as a control. 48 h post-transfection cells were lysed in RIPA lysis buffer and equal amounts of lysate were analyzed by 10% SDS-PAGE and immunoblotting using anti-HA (6E2) antibody (upper panel). Equal loading was confirmed by detection of β-actin (lower panel).

Surprisingly, three of the mutant Eya1 proteins (S454P, L472R, and L550P) were almost undetectable upon overexpression in Cos-7 cells (Fig. 3.7). To examine whether this fact was due to enhanced degradation of the mutant proteins via the proteasomal pathway, the experiment was repeated in presence or absence of the proteasome inhibitor lactacystin. In case Eya1 is degraded via the proteasomal pathway, one would expect accumulation of the protein in presence of lactacystin.

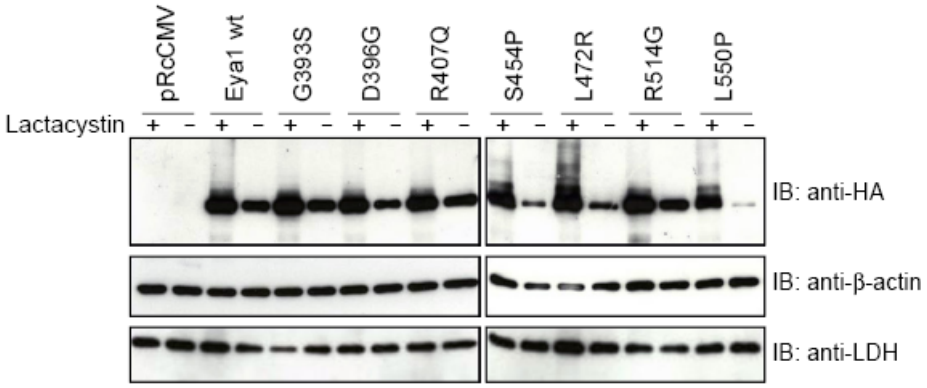

Fig. 3.8. Eya1 and its mutants accumulate in presence of lactacystin. Cos-7 cells were transfected with mutant pHM6-Eya1, wild type pHM6-Eya1 or empty vector pRcCMV as a control. 24 h post-transfection cells were treated with lactacystin (1 µM) or left untreated for another 24 h. Cells were lysed in RIPA lysis buffer and equal amounts of lysate were analyzed by 10% SDS-PAGE and immunoblotting using anti-HA (6E2) antibody (upper panel). Equal loading was confirmed by detection of β-actin (middle panel) and lactate dehydrogenase (LDH; lower panel).

Lactacystin treatment of Cos-7 cells overexpressing *Eya1* led to accumulation of the protein indicating that Eya1 is degraded via the proteasome (Fig. 3.8).

Moreover, the three BOR-associated Eya1 mutants S454P, L472R and L550P which are present at lower levels in untreated cells accumulated to a similar amount as the wild type protein in lactacystin-treated cells. This suggests that introduction of each of those three mutations leads to instability of the protein due to enhanced proteasomal degradation.

All proteins which are degraded via the proteasomal pathway are labeled for their degradation by covalent attachment of ubiquitin molecules to exposed lysine residues. A method to show that a protein indeed is ubiquitinated is the so-called *in vivo* ubiquitination assay which was established by Treier *et al*. (Treier *et al*., 1994). To show that Eya1 is ubiquitinated, *in vivo* ubiquitination assay was performed. Therefore, Cos-7 cells were co-transfected with expression constructs for Eya1 and His-ubiquitin or HA-ubiquitin. As a control, Cos-7 cells transfected with the empty eukaryotic expression vector pRcCMV were used. Cells were treated with the proteasome inhibitor MG132 (10 µM) or vehicle 24 h post-transfection, and harvested 24 h later. His-ubiquitin-labeled proteins were purified using Ni-NTA agarose and subsequently analyzed by SDS-PAGE and immunoblotting. Eya1 was detected using the anti-Eya1.2 antibody.

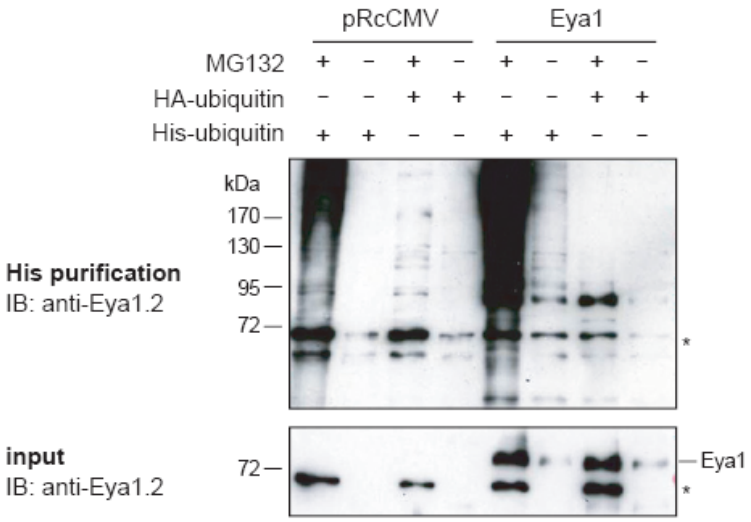

Fig. 3.9. Eya1 ubiquitination can be detected in Cos-7 cells by *in vivo* ubiquitination assay. Cos-7 cells were co-transfected with pHM6-Eya1 or empty vector pRcCMV and expression construct for HA-ubiquitin or His-ubiquitin. 24 h later, cells were treated with MG132 (10 µM) or DMSO for 24 h. His-ubiquitin-labeled proteins were purified from cell lysates and analyzed by 8% SDS-PAGE and immunoblotting with anti-Eya1.2 antibody. Asterisk indicates unspecific signal.

Similarly to treatment with lactacystin (see Fig. 3.8), presence of the proteasomal inhibitor MG132 led to accumulation of Eya1 protein indicating that Eya1 is a target of the proteasomal pathway (Fig. 3.9). After His-purification from cells

overexpressing *Eya1*, detection of higher molecular weight proteins was enhanced in presence of His-ubiquitin compared to HA-ubiquitin. This finding suggests that the higher molecular weight signals detected in presence of His-ubiquitin represent purified His-ubiquitin-labeled Eya1. In line with this assumption, treatment with the proteasomal inhibitor MG132 drastically increased the accumulation of these proteins. Note that, higher molecular weight proteins were also detected at a lower level after His-purification from cells co-transfected with empty vector and His-ubiquitin and treated with MG132. A reason for this might be that the endogenous Eya1 protein of Cos-7 cells is His-ubiquitin-labeled, purified by Ni-NTA agarose and subsequently detected by the anti-Eya1.2 antibody in immunoblot. Cos-7 cells are green monkey kidney cells and express *Eya1* as revealed by RT-PCR analysis (Jürgen Tomasch, diploma thesis, 2007). Taken together, it could be shown that Eya1 indeed is ubiquitinated, thus underlining earlier results regarding Eya1 as a target of the proteasomal pathway.

To determine in which part of the protein ubiquitination takes place, *in vivo* ubiquitination assay was performed using N- or C-terminal fragments of Eya1 (see 3.11 C for scheme of structure).

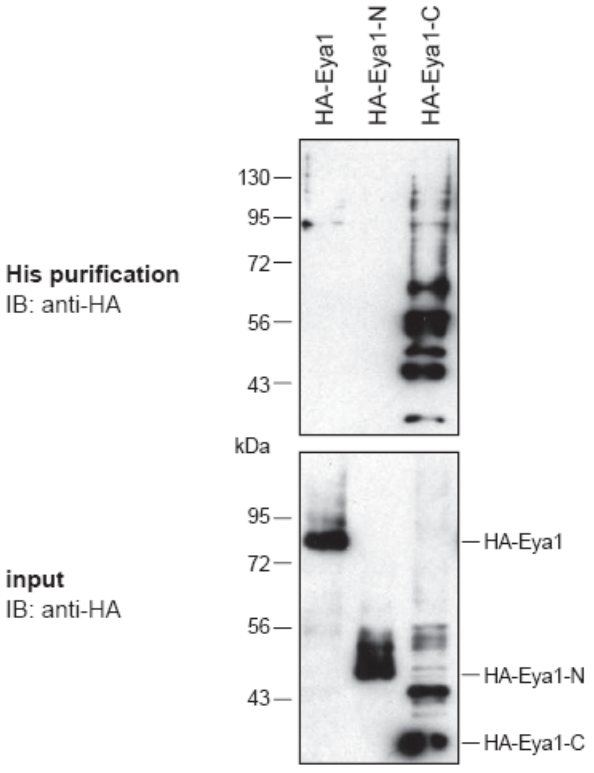

Figure 3.10. Eya1 ubiquitination is mediated via the conserved C-terminus of the protein. Cos-7 cells were transfected with expression constructs for HA-fusions of full length Eya1 (HA-Eya1), N-terminus (HA-Eya1-N) or C-terminus (HA-Eya1-C) together with His-ubiquitin. Cells were treated with MG132 (10 µM) for 24 h, lysed and subjected to His-purification. An aliquot of input (lower panel) and purified proteins (upper panel) were loaded on a 10% SDS-PAA gel and detected by immunoblotting using anti-HA (6E2) antibody.

His-ubiquitin-labeled proteins could only be purified from extracts of cells overexpressing full-length HA-Eya1 or its C-terminal part, but not of cells

overexpressing the HA-tagged N-terminal part of Eya1 (Fig.3.10), indicating that ubiquitination of Eya1 occurs in the conserved C-terminal part of the protein.

The expression construct for the C-terminus of Eya1 which was used for the *in vivo* ubiquitination assay contains 13 lysine residues which can be targets for ubiquitination. To further narrow down the ubiquitination site, different mutants were created using site-directed mutagenesis: a substitution of the most N-terminally located lysine residue to alanine (K301A), a deletion of overall 66 amino acids including a cluster of 6 lysine residues in the middle part of the Eya1 C-terminus (Δ426-491), and a deletion of the C-terminally located 103 amino acids including 6 lysine residues (H489stop). Each mutation was introduced into expression constructs for full-length Eya1 (HA-Eya1) or its C-terminal part (HA-Eya1-C). All mutants were tested for ubiquitination by *in vivo* ubiquitination assay after transfection of Cos-7 cells together with His-ubiquitin expression construct. An overview of the mutants used in this experiment and the results of the *in vivo* ubiquitination assay are given in Fig. 3.11 C. It has to be mentioned that ubiquitination of the lysine residue at position 301 could be excluded because this residue is also present in the N-terminal fragment of Eya1 (Eya1-N) which did not show ubiquitination in earlier experiments (see Fig. 3.10). Nevertheless, it was included in this study since the mutants had been generated before this finding.

All mutants tested were still ubiquitinated indicating that ubiquitination occurs in two regions of the protein, one located in the central part and the other located in the C-terminal part of the Eya domain (Fig. 3.11).

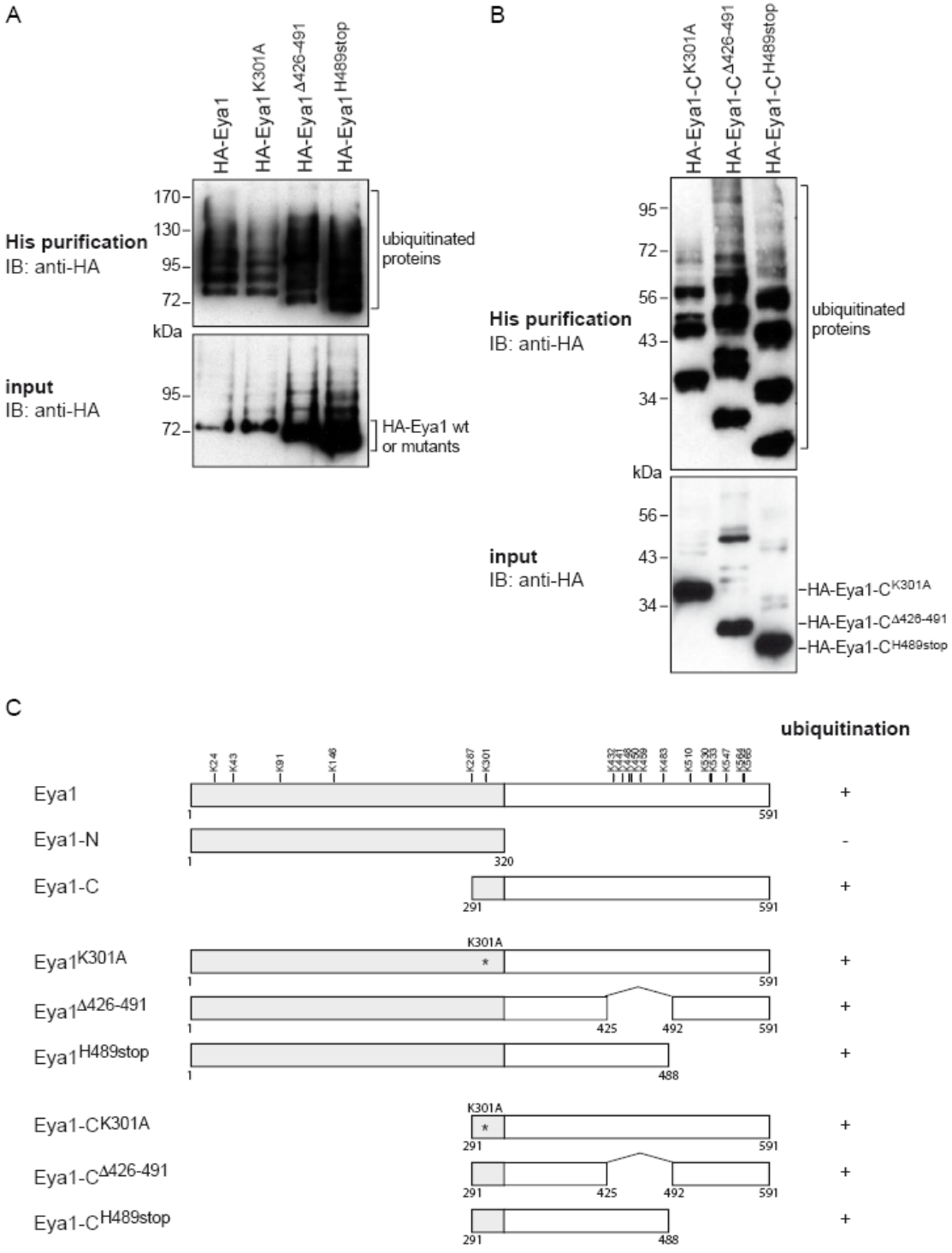

Figure 3.11. Eya1 ubiquitination occurs in two distinct regions of the Eya domain. Cos-7 cells were co-transfected with His-ubiquitin and the indicated expression construct for Eya1 or the respective mutant form, and treated with MG132 (10 µM) 24 h post-transfection. 24 h later, cells were lysed and *in vivo* ubiquitination assay was performed as described. (A) Ubiquitination pattern of Eya1 mutants in the full length background compared to the wild type. (B) Ubiquitination pattern of Eya1 mutants in the background of the Eya1-C-terminus. (C) Schematic overview depicting mutants analyzed in A and B (left) and respective results of *in vivo* ubiquitination assay (right). Position of lysine residues is indicated in the scheme of full-length Eya1. Asterisk indicates point mutation.

Data from Amna Musharraf provides evidence that Eya1 protein is stabilized in presence of Six proteins, as for example Six1 (Amna Musharraf, PhD thesis). To determine the possibility that Six1 might inhibit polyubiquitination of Eya1 and therefore prevent its degradation, *in vivo* Eya1 ubiquitination assays were performed in presence or absence of Six1.

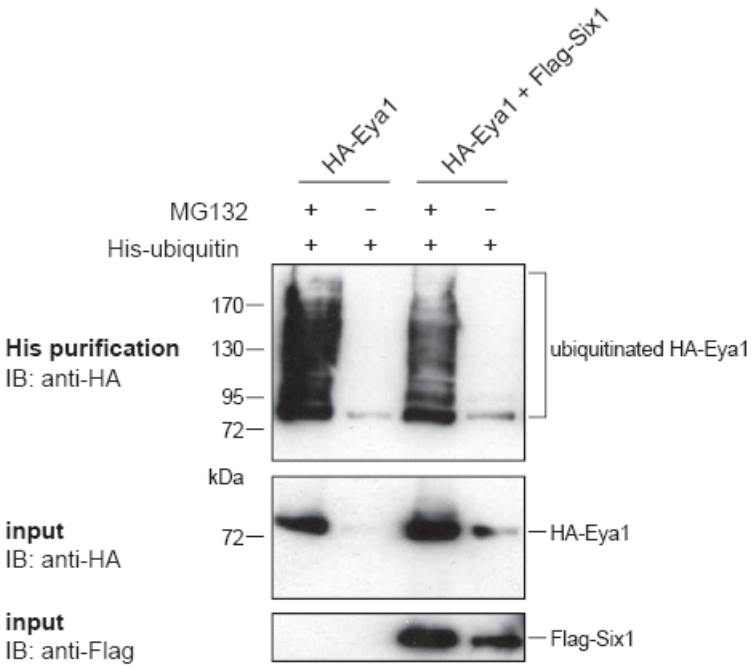

Figure 3.12. Interaction with Six1 inhibits ubiquitination of Eya1. Cos-7 cells were co-transfected with pHM6-Eya1 and His-ubiquitin in presence of either pcDNA-Flag-Six1 or empty vector. 24 h post-transfection cells were treated with MG132 (10 µM) or DMSO for another 24 h. His-ubiquitin-labeled proteins were purified from cell lysates and analyzed by 8% SDS-PAGE and immunoblotting using anti-HA (6E2) antibody for detection of HA-Eya1 and anti-Flag antibody for detection of Flag-Six1. An aliquot of the cell lysate before His-purification was analyzed in parallel for control of input.

As shown in Fig. 3.12, polyubiquitination of Eya1 was tuned down in presence of Six1, which was not due to alteration of Eya1 protein levels in the input. Total Eya1 protein level was even increased in presence of Six1 indicating that Eya1 is stabilized by interaction with Six1, which is in line with the data from Amna Musharraf.

Conclusively, the facts that Eya1 is stabilized in presence of proteasomal inhibitors and the detection of Eya1 ubiquitination within the conserved Eya domain provide evidence for the degradation of Eya1 via the proteasomal pathway. Interestingly, ubiquitination and, thus, degradation of Eya1 is inhibited by interaction with Six1. Furthermore, some BOR associated Eya1 mutations lead to enhanced protein degradation providing a potential mechanism for the cause of BOR syndrome.

3.3 IDENTIFICATION OF EYA1-INTERACTING PROTEINS

One aim of this work was to identify novel interaction partners of Eya1 in order to gain a deeper insight into the molecular mechanisms by which Eya1 is involved in the specific development of several organs. Method of choice was the classic Gal4-based yeast two-hybrid system, which is an efficient approach for the identification of interaction partners *in vivo.* The method is based on the ability of an interacting protein pair to bring together the DNA-binding domain and the transactivation domain of the transcription factor Gal4 to produce a functional transcriptional activator which activates the expression of reporter genes. The protein of interest, also called “bait”, is fused to the Gal4-binding domain (Gal4-BD) and used for screening of a “prey” library where cDNAs have been fused to the Gal4-activation domain (Gal4-AD).

3.3.1 Yeast two-hybrid analysis

A pre-requisite for a successful yeast two-hybrid screening procedure is the use of a bait construct, which does not lead to activation of reporter gene expression itself. Transcription factors often lead to so-called “autoactivation” due to the presence of a transcriptional activation domain within their structure. Initially, I wanted to screen full length Eya1 for so far unknown interaction partners. For this purpose three bait constructs (Eya1-fl, Eya1-N, and Eya1-C) were generated and cloned into the Gal4-BD-encoding vector pGBT9 via a PCR-based strategy. Each was checked for autoactivation by transformation of the *S. cerevisiae* strain KFY1 followed by colony-lift filter assay to detect expression of the reporter gene *lacZ*.

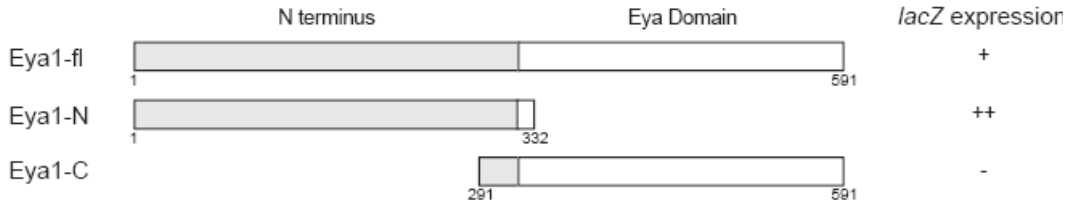

Fig 3.13. Full-length Eya1 as well as the N-terminus of Eya1 mediate autoactivation of reporter gene expression. Protein domain structure of Eya1 bait constructs (left) and results of autoactivation assay (right). The respective expression constructs were used for transformation of *S. cerevisiae* KFY1. Expression of *lacZ* by the transformants was determined by colony-lift filter assay and graded on a scale from no expression (-) to high expression (++).

Full-length Eya1, as well as the N-terminus of Eya1 led to autoactivation of the reporter gene *lacZ* when fused to the Gal4-BD (Fig. 3.13). The reason for this is probably the presence of a transactivation activity located within the N-terminus of Eya1 (Xu *et al.*, 1997). In contrast, the C-terminus of Eya1 did not show reporter gene activation when fused to the Gal4-BD.

To further narrow down the N-terminal region which is responsible for transactivation, different deletion constructs of the N-terminus were analyzed for their ability to autoactivate the reporter gene *lacZ.* N-terminal deletion mutants were generated using a PCR-based strategy and introduced into the yeast two-hybrid bait vector pGBT9. *S. cerevisiae* KFY1 cells transformed with these constructs and grown for 3 days were analyzed for *lacZ* expression by colony-lift filter assay.

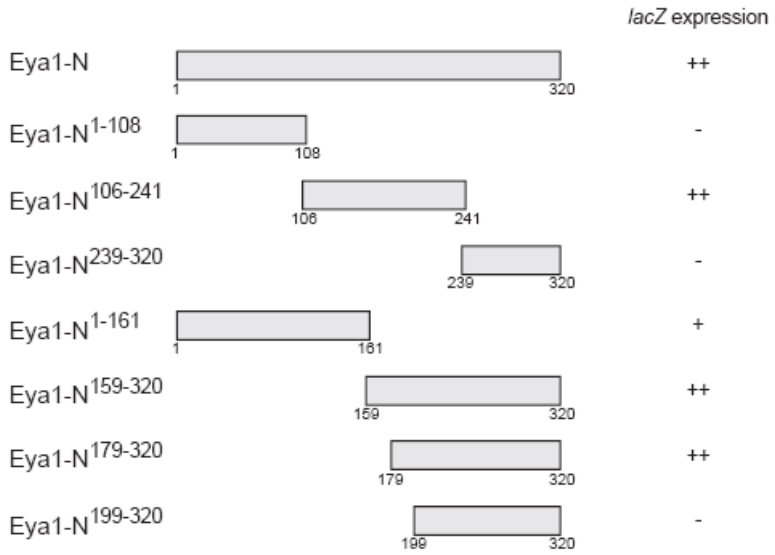

Fig 3.14. The transactivation activity of Eya1 is located between amino acids 109 and 198. Schematic overview of N-terminal Eya1 deletion mutants (left) and results of autoactivation assay (right). *S. cerevisiae* KFY1 cells were transformed with indicated expression constructs and grown for 3 days. Expression of *lacZ* by the transformants was determined by colony-lift filter assay and graded on a scale from no expression (-) to high expression (++).

Studies in yeast revealed that the N-terminal Eya1 deletion fragments comprising amino acids 1-108 and 199-320 did not autoactivate reporter gene expression when fused to the Gal4-BD, whereas larger fragments did (Fig. 3.14). These results indicate that the transactivation activity of Eya1 is located between amino acids 109 and 198 of the protein.

In summary, autoactivation studies demonstrated that both full length Eya1 and the N-terminus of Eya1 were not suited as bait constructs for the yeast two-hybrid screening procedure since both led to high activation of the reporter gene *lacZ*. It

was shown that this autoactivation is due to the transactivation activity of Eya1, which resides between amino acids 109 and 198 in the N-terminus of the protein. In contrast, the C-terminus of Eya1 including the conserved Eya domain did not autoactivate reporter gene expression and, thus, could be used as bait for screening of a mouse embryo cDNA library.

3.3.1.1 Screening of a mouse embryo cDNA library

In an attempt to identify interaction partners of Eya1, a yeast two-hybrid screen was performed using the C-terminus of Eya1 (Eya1-C) as a bait and a pretransformed cDNA library of an eleven-day old mouse embryo as a source of interacting proteins. 35 positive clones were isolated from approximately 2 million transformants. To exclude false-positive results, plasmid-DNA was extracted and subjected to re-transformation into yeast either alone or together with the bait followed by colony-lift filter assay. Retransformation analysis revealed that 25 out of 35 positive clones were false-positives since the interaction could not be reproduced. Ten potential interaction partners could be confirmed by retransformation assay and respective plasmid-DNA was sent to sequencing. Interestingly, one of the isolated cDNAs turned out to contain part of the *Six2* coding region including the conserved Six domain, which has been shown to mediate the interaction with Eya (Pignoni *et al.*, 1997). Four clones corresponded to so far unknown ESTs (Expressed Sequence Tags) or genomic regions located outside of known coding regions, and were not subjected to further analysis. Another five clones corresponded to fragments of Sipl1, Ezh2, LAGi, and the ribosomal protein L17 which was isolated twice. An overview of these four potential interaction partners is shown in Table 3.2.

Table 3.2. Potential Eya1 interaction partners isolated by yeast two-hybrid analysis

potential interaction partner	interaction strength	fragment of coding region
Sipl1 (Shank-interacting protein like 1)	++	1-1143 (full-length; containing intron 7)
Ezh2 (enhancer of zeste homolog 2)	++	1175-2241
LAGi (potential LAG1 interactor homolog)	++	233-1284
ribosomal protein L17	+	1-552 (full length)

3.3.1.2 Verification of potential interaction partners of Eya1

Surprisingly, the cDNAs of all four isolated potential interaction partners were not in frame with the Gal4-AD. Previous studies indicated that in *S. cerevisiae* translational frame-shifting can occur (Atkins *et al.*, 1991; Weiss, 1991). It is therefore conceivable that, although the cDNAs were not in frame with the Gal4-AD, frameshifting has led to synthesis of the proper protein. To analyze whether this was the case, all four potential interaction partners were cloned in frame to the Gal4-AD using a PCR-based strategy and gene-specific primers in case of Sipl1 or a mutagenesis approach in case of Ezh2, LAGi and L17. In-frame constructs were tested again for an interaction together with the bait Eya1-C by re-transformation of the yeast strain KFY1 and colony-lift filter assay.

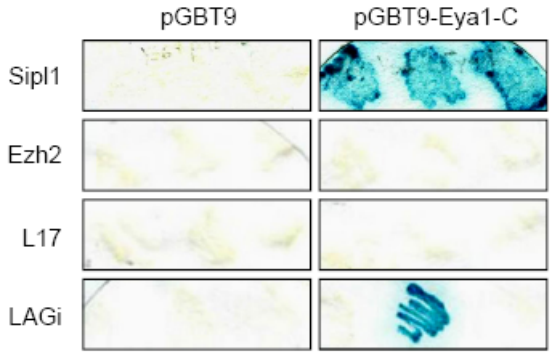

Fig. 3.15. In-frame constructs of Sipl1 and LAGi still interact with the C-terminus of Eya1. Co-transformation of *S. cerevisiae* KFY1 with pGBT9-Eya1-C or pGBT9 as negative control and the in-frame prey construct for each of the indicated potential interaction partners. Three colonies of each transformation plate were tested for expression of *lacZ* by colony-lift filter assay.

For two of the potential interaction partners, namely Sipl1 and LAGi, an interaction in re-transformation assay could be detected, whereby the Eya1-Sipl1 interaction was much stronger and more robust compared to that of Eya1 and LAGi (Fig. 3.15). Thus, the interaction of Eya1 with Sipl1 was confirmed and subjected to further analysis.

Sequencing revealed that the cDNA clone of Sipl1, which was isolated from the mouse embryonic cDNA library used for yeast two-hybrid screening, contained the intron located between exon 7 and 8 leading to a premature STOP codon and, thus, a truncated protein. To verify the interaction of Eya1 with full-length Sipl1, the coding region of Sipl1 obtained from an I.M.A.G.E clone was introduced in the Gal4-AD-encoding prey vector using a PCR-based strategy. The bait construct

containing Eya1-C and the prey construct containing Sipl1 were introduced yeast and subsequently analyzed for an interaction by colony-lift filter assay.

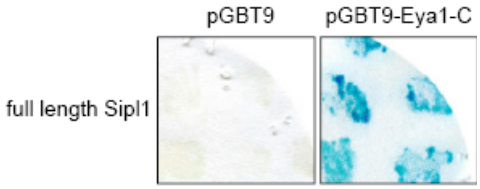

Fig. 3.16. Full-length Sipl1 interacts with the C-terminus of Eya1 in yeast. Co-transformation of *S. cerevisiae* KFY1 with pGBT9-Eya1-C or pGBT9 as negative control and pGADT7 containing the full length coding region of Sipl1. Five colonies of each transformation plate were tested for expression of *lacZ* by colony-lift filter assay.

Indeed, colony-lift filter assay using full-length Sipl1 as a prey showed clear interaction with the C-terminus of Eya1 (Fig. 3.16).

3.3.2 Characterization of the Eya1-Sipl1 interaction

Sipl1 was previously identified as an interaction partner of Shank which functions as a scaffold protein at post-synaptic densities (Lim *et al.*, 2001). The Sipl1 protein consists of 380 amino acids containing two conserved domains within its C-terminal part: an Ubl (Ubiquitin-like) domain and a Ran-BP2 (Ran binding protein2)-type zinc finger (ZnF). An overview of the protein domain structure of Sipl1 is given in Fig. 3.17. So far, there is no data available on protein function.

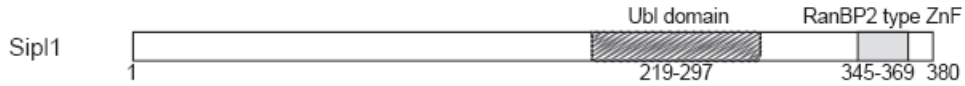

Figure 3.17. Sipl1 protein domain structure. Protein sequence was analyzed for conserved domains using the Conserved Domains database of NCBI (http://www.ncbi.nlm.nih.gov).

3.3.2.1 Eya1 and Sipl1 interact in mammalian cells

To verify the interaction of Eya1 and Sipl1 in mammalian cells, Co-immunoprecipitation analysis was performed. Therefore, Cos-7 cells were co-transfected with expression constructs for HA-tagged Eya1 (HA-Eya1) and Flag-tagged Sipl1 (Flag-Sipl1). Since initial attempts had failed to detect the Eya1-Sipl1 interaction in mammalian cells and since I could show that Eya1 levels are enhanced by proteasomal inhibitors (see Fig. 3.8, Fig. 3.9), cells were treated with 1 µM MG132 24 h post-transfection. Cells were harvested 24 h later in HEPES lysis buffer and subjected to immunoprecipitation. HA-Eya1 was precipitated using anti-HA antibody, whereas in the reciprocal experiment Flag-Sipl1 was precipitated

using anti-Flag antibody. Precipitates and cell lysates were analyzed by SDS-PAGE and immunoblotting.

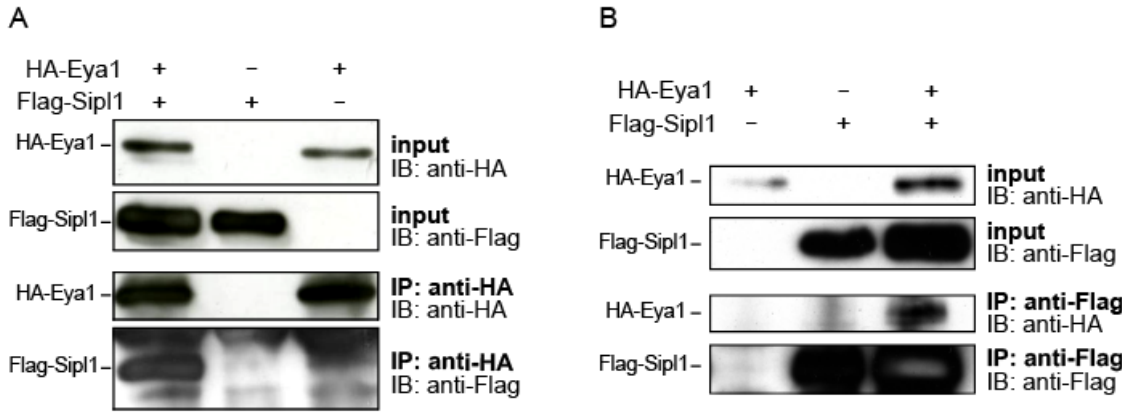

Fig. 3.18. Eya1 and Sipl1 interact in mammalian cells. Cos-7 cells were transfected with pHM6-Eya1 and pcDNA-Flag-Sipl1 or each construct alone. 24 h post-transfection cells were treated with MG132 (1 µM) for another 24 h followed by lysis of cells in HEPES lysis buffer and immunoprecipitation with (A) anti-HA (12CA5) or (B) anti-Flag antibody. An aliquot of cell lysates before immunoprecipitation and the precipitated complexes were analyzed by 10% SDS-PAGE and immunoblotting using anti-HA (6E2) antibody for detection of HA-Eya1 and anti-Flag antibody for detection of Flag-Sipl1.

As can be seen in Fig. 3.18 A, after immunoprecipitation with anti-HA antibody comparable amounts of HA-Eya1 were detected from cell lysates transfected with HA-Eya1 alone or together with Flag-Sipl1, but not from cell lysates transfected with Flag-Sipl1 confirming the specificity of the detected signal. Furthermore, it could be shown that Flag-Sipl1 co-precipitated together with HA-Eya1. In the reciprocal experiment (Fig. 3.18 B), a signal for HA-Eya1 was detectable after precipitation of Flag-Sipl1. These results indicate that the two proteins interact with each other in mammalian cells.

3.3.2.2 Eya1 and Sipl1 bind directly to each other

Both, yeast two-hybrid system and co-immunoprecipitation can show that two proteins of interest interact with each other in one protein complex. But whether the interaction is direct or indirect, can not be answered by applying these techniques since endogenous proteins of yeast or mammalian cells could serve as bridging factors mediating the interaction. To analyze whether the Eya1-Sipl1 interaction is of direct manner, GST pulldown assay was employed. Purified GST-Sipl1 fusion protein or GST alone as a negative control were coupled to agarose beads and incubated with *in vitro* synthesized HA-Eya1. Presence of Eya1 was detected by SDS-PAGE followed by immunoblotting using anti-HA antibody.

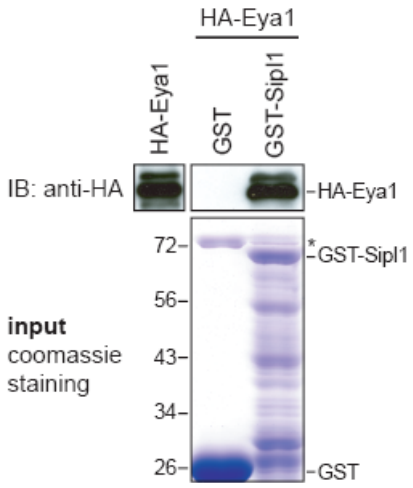

Fig. 3.19. Eya1 and Sipl1 interact directly with each other. *In vitro* synthesized HA-Eya1 was incubated with recombinant GST-Sipl1 or GST which had been coupled to agarose beads before (upper right panel). After thorough washing beads were boiled for 5 min in 2x Laemmli loading buffer and loaded on a 10% SDS-PAA gel. HA-Eya1 was detected by immunoblotting using anti-HA (6E2) antibody. An aliquot (4%) of *in vitro* synthesized HA-Eya1 was included in the immunoblot analysis (upper left panel). Input of GST fusion proteins was controlled by SDS-PAGE and coomassie staining (lower panel). Asterisk indicates bacterial protein co-purifying with GST.

GST and GST-Sipl1 were efficiently purified from bacterial cells as shown after SDS-PAGE and coomassie staining (Fig. 3.19). In the case of GST-Sipl1, a ladder of lower molecular weight proteins could be observed which probably represents degradation products. A signal for interacting HA-Eya1 was detected after incubation with GST-Sipl1, but not with GST alone. This result demonstrates that, indeed, Eya1 directly interacts with GST-Sipl1.

3.3.2.3 Interaction of Sipl1 with other Eya family members

The C-terminus of Eya1 which was used as a bait for the yeast two-hybrid screening procedure contains the so-called Eya domain, which is highly conserved in other Eya family members as well. An alignment of the Eya domains of murine Eya1-Eya4 is shown in Fig. 3.20.

As previously described, the alignment of the Eya domains of the mouse homologs Eya1-4 shows that Eya4 is most closely and Eya3 most distantly related to Eya1 (Fig. 3.20) (Zimmerman *et al.*, 1997; Borsani *et al.*, 1999). Overall, there is conservation of about 80% within this region.

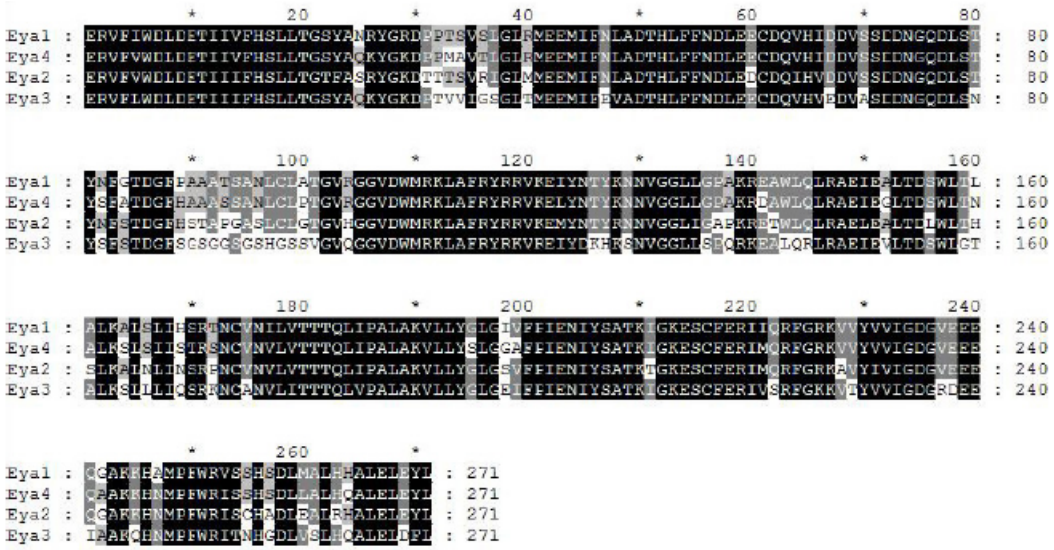

Fig. 3.20. Alignment of the conserved Eya domains of mouse Eya1-4. Alignment of the Eya domains of mouse Eya1-4 was performed using the ClustalW program (www.ebi.ac.uk/clustalw). The order of the alignment indicates the degree of conservation compared to Eya1 from high (Eya4) to low (Eya3).

The novel interaction partner Sipl1 was identified from a yeast two-hybrid screen using the C-terminal region of Eya1 including the conserved Eya domain as a bait. Hence, it could be possible that Sipl1 also interacts with other Eya family members. To examine whether this is the case, β-gal liquid assay was performed using C-terminal fragments of Eya1-Eya4 as baits and full-length Sipl1 as a prey.

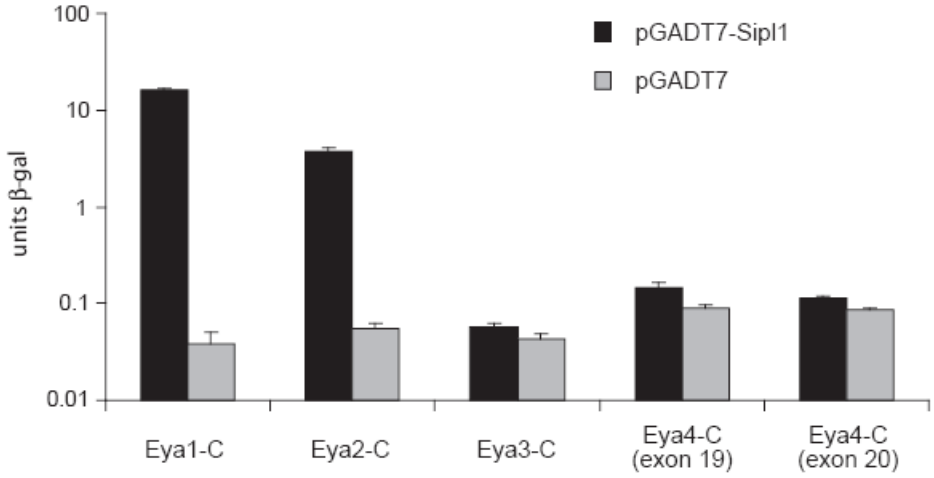

Fig. 3.21. Sipl1 interacts with Eya1 and Eya2, but not with Eya3 and Eya4. Co-transformation of *S. cerevisiae* KFY1 with pGADT7-Sipl1 or empty vector and pGBT9 containing C-terminal fragments of Eya1-4, respectively. After 3 days of growth, β-gal liquid assay was performed from 3 pooled colonies in triplicates. Error bars represent standard deviation.

β-gal liquid assay showed that Sipl1 interacts not only with the C-terminus of Eya1, but also with the C-terminus of Eya2 (Fig. 3.21). The interaction with Eya3 and two alternative splice forms of Eya4 could not be detected. The results were surprising

in that way that Eya2 is more distantly related to Eya1 compared to Eya4. However, comparable expression of the bait constructs for Eya2, Eya3 and Eya4 needs to be confirmed.

3.3.2.4 Localization of binding sites

To localize the respective binding site in each of the interaction partners, Eya1 and Sipl1, β-gal liquid assay was performed. For Eya1, several deletion mutants were generated using a PCR-based strategy, and cloned into the yeast two-hybrid bait plasmid pGBT9. Each deletion construct was used for transformation of yeast together with the *Sipl1*-expressing prey plasmid. Colonies were allowed to grow for 3 days, and expression of the reporter gene *lacZ* was determined by β-gal liquid assay. The interaction strength of wild type Eya1-C and Sipl1 was determined in parallel as a positive control.

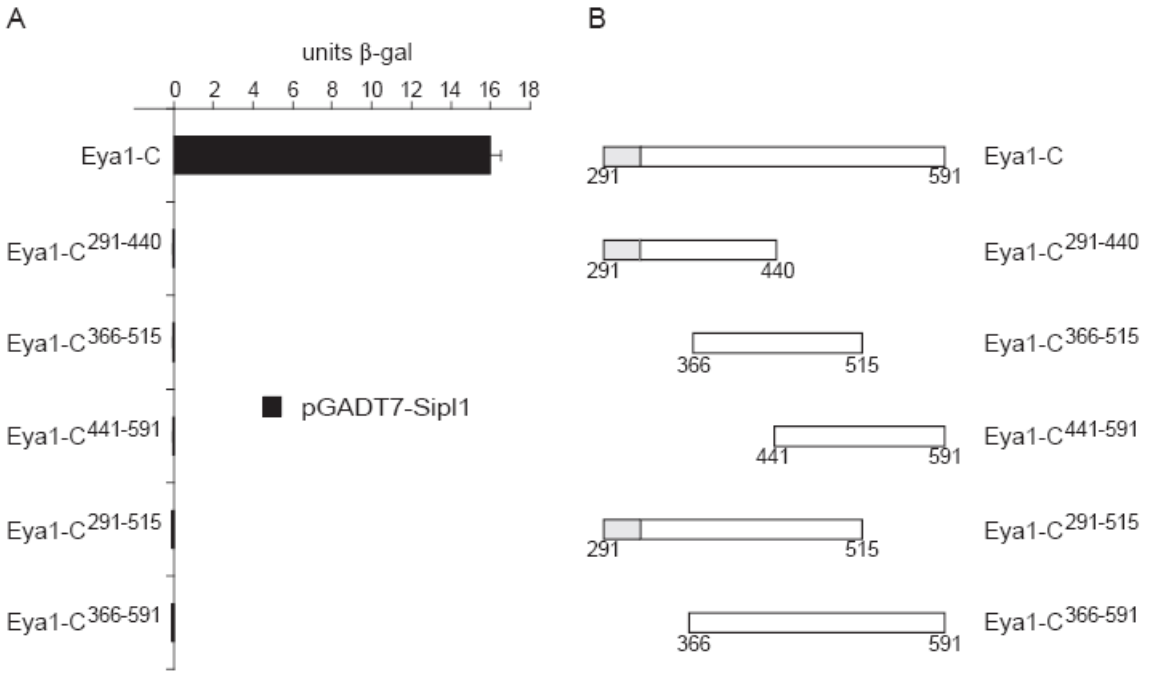

Fig. 3.22. The whole C-terminus of Eya1 is required for the interaction with Sipl1. *S. cerevisiae* KFY1 cells were co-transformed with yeast expression constructs for Eya1 deletion fragments and Sipl1 as indicated. After 3 days of growth β-gal liquid assay was performed from 3 pooled colonies. Each sample was measured in triplicates. (A) Results of β-gal liquid assay. Error bars represent standard deviation. (B) Schematic overview of C-terminal Eya1 deletion fragments used in A.

As seen in Fig. 3.22, the interaction between the C-terminus of Eya1 and Sipl1 lead to strong *lacZ* expression as determined by the amount of β-gal units produced. Deletion of any part of the C-terminus of Eya1 led to complete loss of the interaction suggesting that the interaction with Sipl1 requires the full-length

C-terminus of Eya1. An explanation for this result might be that Sipl1 binds to a structural motif within the Eya1-C-terminus which is composed of amino acids located in distinct regions of the Eya domain.

A similar experiment was performed to narrow down the minimal Eya1 binding site in the Sipl1 protein. Different deletion mutants of Sipl1 were created using PCR and gene-specific primers, and cloned into the Gal4-AD-encoding prey vector pGADT7. Each was introduced into yeast together with the bait vector for Eya1-C. Expression of *lacZ* was determined by measuring the amount of produced β-gal units in a β-gal liquid assay.

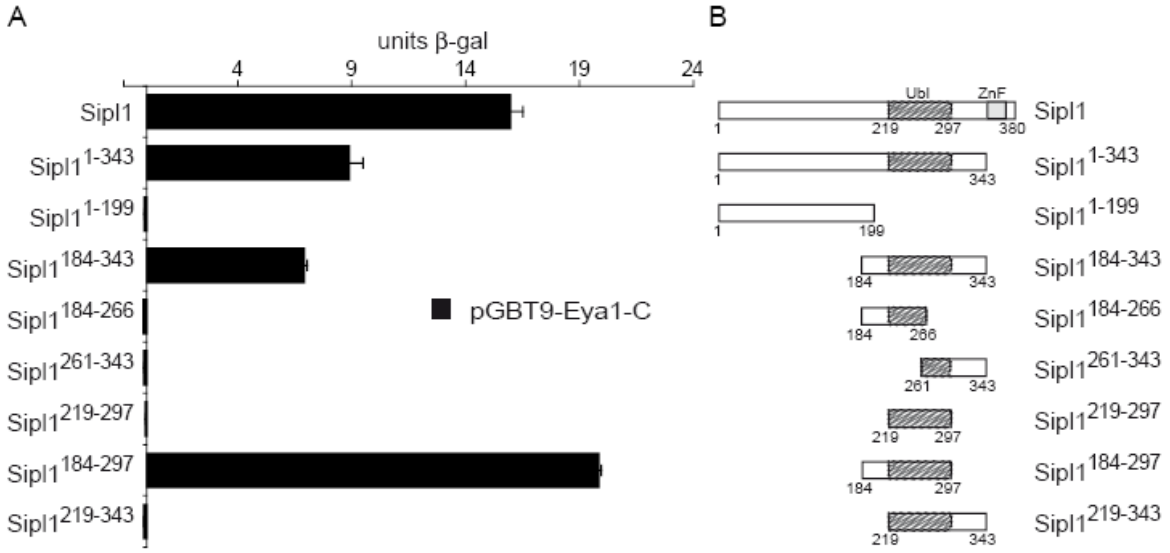

Fig. 3.23. Eya1 binds to the conserved Ubl domain of Sipl1. *S. cerevisiae* KFY1 cells were co-transformed with yeast expression constructs for the Sipl1 deletion fragments and the C-terminus of Eya1. After 3 days of growth β-gal liquid assay was performed from 3 pooled colonies and measured in triplicates. (A) Results of β-gal liquid assay. Error bars represent standard deviation. (B) Schematic overview of Sipl1 deletion mutants used in A.

Binding studies showed that the zinc finger in the very C-terminus of Sipl1 is not necessary for its interaction with Eya1, but absence of this region weakens the interaction to about 50% compared to full length Sipl1 (Fig. 3.23). Furthermore, it could be shown that the interaction is mediated via the C-terminal part of Sipl1 since the fragment containing amino acids 1-199 did not interact with Eya1, but the fragment containing amino acids 184-343 did. Finally, the minimal Eya1 binding region could be located to the complete Ubl domain and approximately 30 amino acids upstream of it, which might be required for correct folding of this domain.

3.3.3 Interaction of Eya1 with Rbck1, a Sipl1-related protein

The ubiquitin-like domain of Sipl1, which has been shown to mediate the interaction with Eya1, is a conserved domain present in other proteins as well. One of those is Rbck1 (RBCC protein interacting with PKC 1) (Tokunaga *et al.*, 1998). The N-terminus of Rbck1 is highly similar to the C-terminus of Sipl1 sharing both conserved regions, the Ubl domain and the Ran-BP type zinc finger (ZnF). In the C-terminus of Rbck1 two additional conserved domains can be found (Fig. 3.24 A). One is a coiled-coil domain, the other a RING-IBR region consisting of two zinc finger domains and a cysteine/histidine-rich motif (C/H) in between. RING-IBR domains are mainly present in proteins with E3 ubiquitin ligase activity (Tian *et al.*, 2007). Recently, it has been shown that Rbck1 acts as an E3 ubiquitin ligase (Yamanaka *et al.*, 2003; Tatematsu *et al.*, 2008). In addition to that, it is known that Rbck1 possesses a transactivation activity and functions as a transcriptional co-activator (Cong *et al.*, 1997; Tatematsu *et al.*, 1998).

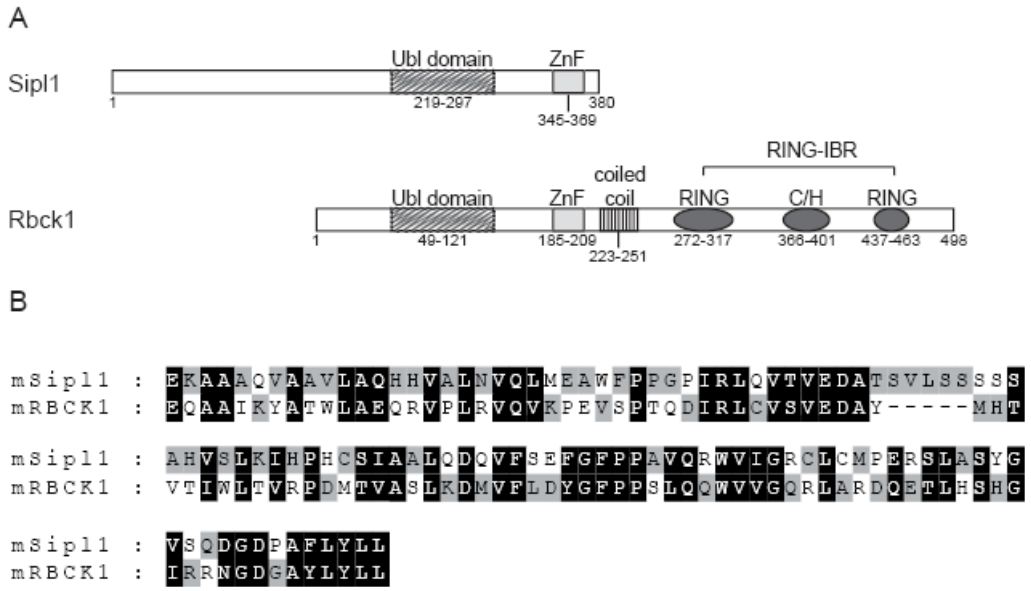

Fig. 3.24. Rbck1 is a Sipl1-related protein. (A) Schematic alignment of protein domain structures of Sipl1 and Rbck1. (B) Alignment of the minimal Eya1 binding region of Sipl1 (aa 184-297) to the corresponding region of Rbck1 using ClustalW (www.ebi.ac.uk/clustalw**).** Black boxes indicate identical amino acids. Ubl, ubiquitin-like; ZnF, zinc finger; RING, really interesting new gene; IBR, in between RING; C/H, cysteine/histidine-rich.

An alignment of the minimal Eya1 binding region of Sipl1 with the corresponding region of Rbck1 shows 42% identities in protein sequence within this region (Fig. 3.24 B). Based on this fact, one can assume that Eya1 also interacts with Rbck1. To address this question, Rbck1 was cloned into the yeast two-hybrid prey vector pGADT7 and checked for interaction with the C-terminus of Eya1 by yeast two-

hybrid analysis. β-Gal liquid assay showed weak but clearly detectable interaction between Rbck1 and the C-terminus of Eya1 (Fig. 3.25).

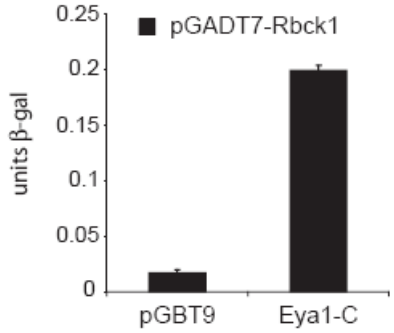

Fig. 3.25. Eya1 interacts with the Sipl1-related protein Rbck1. *S. cerevisiae* KFY1 were co-transformed with pGADT7-Rbck1 and pGBT9-Eya1-C or empty vector pGBT9. After 3 days of growth 3 pooled colonies were analyzed for expression of the reporter gene *lacZ* by β-gal liquid assay. Each measurement was preformed in triplicates. Error bars represent standard deviation.

To further verify the interaction of Eya1 and Rbck1, GST pulldown and co-immunoprecipitation analyses were performed according to the experiments shown before regarding the Eya1-Sipl1 interaction.

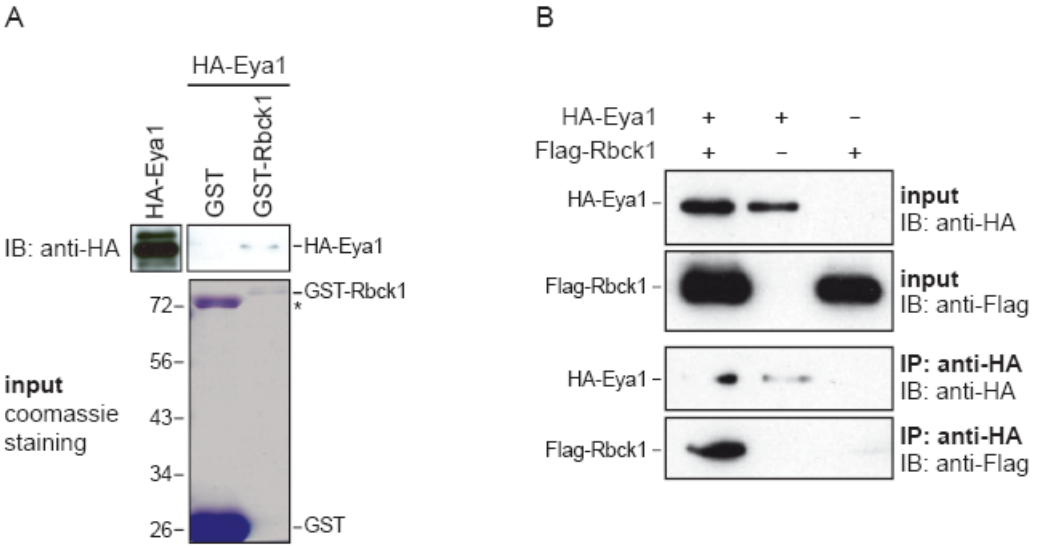

Fig. 3.26. Eya1 interacts with Rbck1 in GST pulldown and co-immunoprecipitation. (A) GST pulldown was performed by incubating *in vitro* synthesized HA-Eya1 with recombinant GST-Rbck1 or GST alone as a control (upper right panel). HA-Eya1 was detected by 10% SDS-PAGE and immunoblotting using anti-HA (6E2) antibody. The input of *in vitro* synthesized HA-Eya1 (upper left panel) and GST-fusion proteins (lower panel) was determined in parallel. The asterisk indicates bacterial protein co-purifying with GST. (B) For co-immunoprecipitation analysis Cos-7 cells were transfected with pHM6-Eya1 and pcDNA-Flag-Rbck1 or each construct alone, and treated with MG132 (1 μM) 24 h post-transfection. 24 h later cells, were lysed in HEPES lysis buffer and HA-Eya1 was precipitated using anti-HA (12CA5) antibody. An aliquot of cell lysates before immunoprecipitation and precipitated complexes were analyzed by 10%-SDS-PAGE and immunoblotting using anti-HA (6E2) antibody for detection of HA-Eya1 and anti-Flag antibody for detection of Flag-Rbck1.

GST pulldown analysis showed that HA-Eya1 is precipitated by GST-Rbck1, but not by GST alone, indicating that Eya1 and Rbck1 interact directly with each other *in vitro* (Fig. 3.26 A). Furthermore, the interaction of Eya1 and Rbck1 could be confirmed in mammalian cells by co-immunoprecipitation (Fig. 3.26 B). Comparable amounts of HA-Eya1 were precipitated by anti-HA antibody, and a clear signal for co-precipitated Flag-Rbck1 was only visible in presence of HA-Eya1 leading to the conclusion that, indeed, the two proteins interact in mammalian cells.

In summary, two novel interaction partners of Eya1 were identified: Sipl1 and Rbck1. It could be shown that the interaction with Eya1 is mediated via the Ubl domain present in both proteins. Thus, it is likely that Eya1 also interacts with other proteins containing this conserved domain. Both Sipl1 and Rbck1 were subjected to further analysis regarding the functional consequences of their interaction with Eya1.

3.4 FUNCTIONAL RELEVANCE OF THE INTERACTIONS

3.4.1 Cellular localization of Eya1, Sipl1, and Rbck1

A pre-requisite for an interaction between two proteins is co-localization of both proteins within the same compartment of a cell. Localization of Eya1 has been extensively studied, and it has been shown that Eya1 is localized in the cytoplasm. Furthermore, interaction of Eya1 with Six proteins, as for example Six2, leads to it's translocation into the nucleus where the Eya1-Six complex activates gene expression (Ohto *et al.*, 1999). In order to determine if Eya1 and its novel interaction partners Sipl1 and Rbck1 are located within the same cellular compartment, an immunofluorescence approach was employed. Therefore, Cos-7 cells were co-transfected with an EGFP-fusion construct of Eya1 (EGFP-Eya1) and an RFP-fusion construct of Sipl1 (RFP-Sipl1) or Rbck1 (RFP-Rbck1), respectively. To check the influence of Six2-mediated translocation of Eya1 on the localization of Sipl1 and Rbck1, each experiment was performed in presence or absence of Six2.

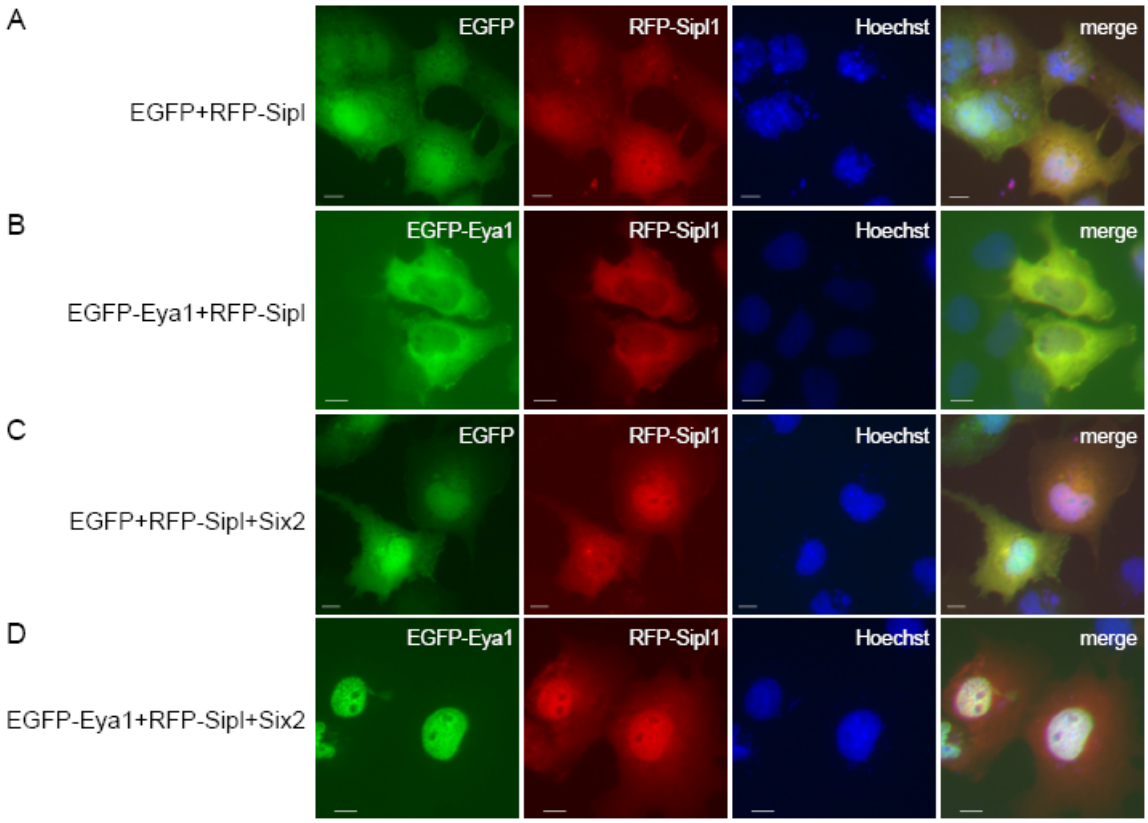

Fig. 3.27. Eya1 and Sipl1 are localized in the cytoplasm and translocated into the nucleus in presence of Six2. Cos-7 cells were grown on coverslips and co-transfected with indicated expression constructs. 40 h post-transfection cells were fixed, nuclei were stained with Hoechst dye, and fluorescence-labeled proteins were visualized by microscopy using an Axiovert 135 TV microscope (Zeiss). (A) Localization of RFP-Sipl1 in presence of EGFP. (B) Localization of RFP-Sipl1 in presence of EGFP-Eya1. (C) Effect of Six2 on localization of RFP-Sipl1. (D) Effect of Six2-mediated translocation of EGFP-Eya1 on localization of RFP-Sipl1. Scale bar: 10 µm.

Sipl1 protein alone was mostly localized in the cytoplasm and to less extent also in the nucleus (Fig. 3.27 A). Presence of Eya1 led to clear accumulation of Sipl1 in the cytoplasm (Fig. 3.27 B). Furthermore, Six2 mediated translocation of both Eya1 and Sipl1 into the nucleus arguing for an interaction between the proteins (Fig. 3.27 D). Interestingly, in some cells also in absence of Eya1, Six2-mediated translocation of Sipl1 into the nucleus was observed (Fig. 3.27 C). One reason for this could be that endogenous Eya1 serves as a bridge between Sipl1 and Six2, thereby allowing translocation of the complex. RT-PCR analysis showed that, indeed, Cos-7 cell express endogenous *Eya1* (Jürgen Tomasch, diploma thesis, 2007). Another possibility for the Six2-mediated translocation of Sipl1 is direct interaction of the two proteins.

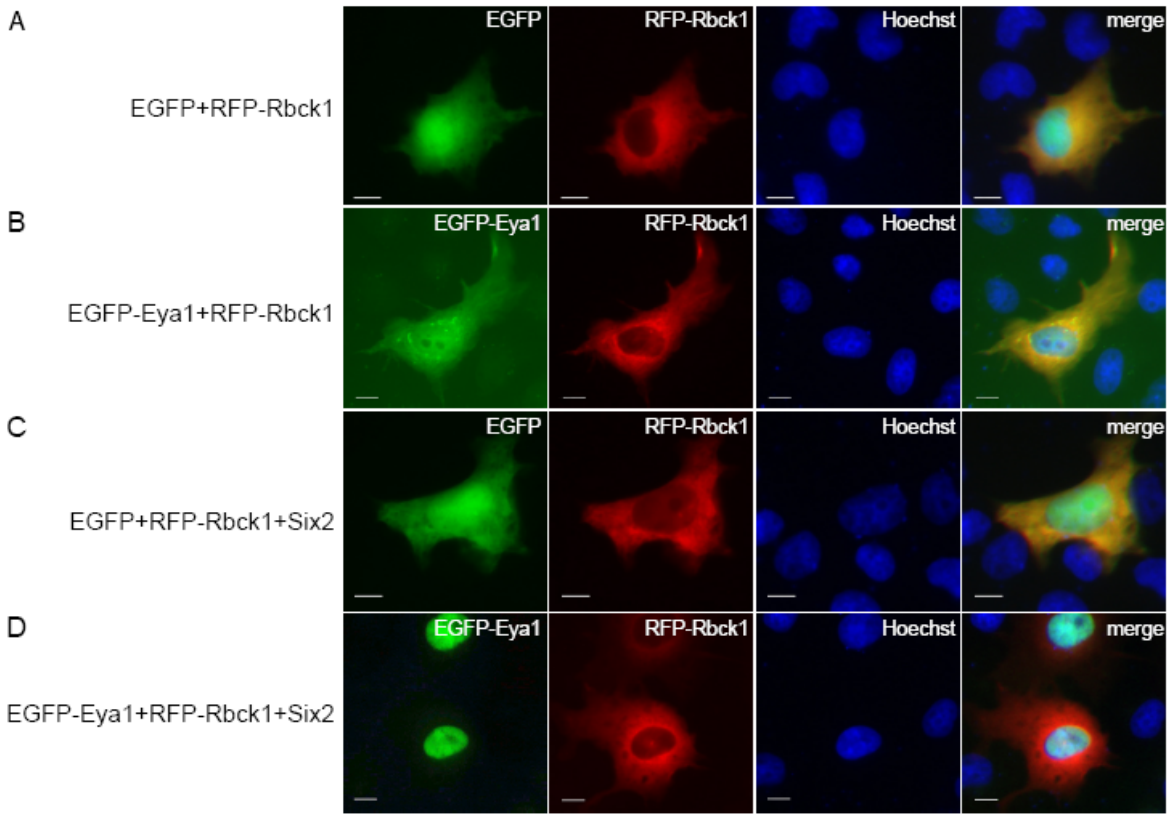

Fig. 3.28. Eya1 and Rbck1 are localized in the cytoplasm. Cos-7 cells were co-transfected with the indicated expression constructs. 40 h post-transfection cells were fixed, and nuclei were stained with Hoechst dye. Protein localization was analyzed by fluorescence microscopy using an Axiovert 135 TV microscope (Zeiss). (A) Localization of RFP-Rbck1 in presence of EGFP. (B) Localization of RFP-Rbck1 in presence of EGFP-Eya1. (C) Effect of Six2 on localization of RFP-Rbck1. (D) Effect of Six2-mediated translocation of EGFP-Eya1 on localization of RFP-Rbck1. Scale bar: 10 µm

As Eya1, Rbck1 was localized in the cytoplasm (Fig. 3.28). In contrast to Sipl1, its localization did not change in presence of Six2 indicating that the Eya1-Rbck1 interaction only occurs in the cytoplasm.

3.4.2 Co-expression of *Eya1* and *Sipl1* or *Rbck1* in mouse

An important point for the relevance of an interaction *in vivo* is tissue-specific coexpression of the interaction partners. In a first attempt to address the functional relevance of the interactions of Eya1 with its novel interaction partners Sipl1 and Rbck1 *in vivo*, expression studies were performed. RNA from several tissues of a 13.5 day old mouse embryo was analyzed for expression of *Sipl1* and *Eya1* by RT-PCR. Primers used for the detection of *Sipl1* expression were located in exon 6 and 8, primers for *Eya1* in exon 3 and 11. In parallel, expression of the house-keeping gene *Tbp (TATA-box binding protein)* was monitored for control of input.

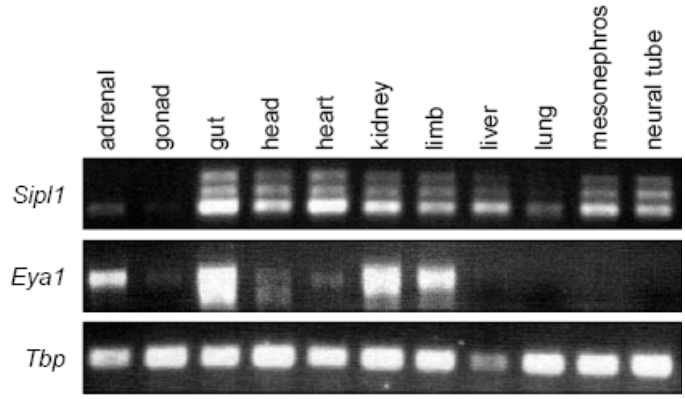

Fig. 3.29. *Sipl1* and *Eya1* are co-expressed in several tissues of a developing mouse embryo. Indicated tissues of a 13.5 day old embryo were used for RNA isolation and cDNA synthesis. *Sipl1* and *Eya1* expression was analyzed by RT-PCR using gene-specific primers. For control of cDNA input *Tbp* expression was determined from the same samples.

Sipl1 expression was detected in every tissue tested, whereby adrenal, gonad and lung showed lowest expression, and heart and gut highest expression (Fig. 3.29). Furthermore, *Eya1* expression could also be detected in several of those tissues, as for example gut, kidney and limb. Thus, RT-PCR analyses show that *Sipl1* and *Eya1* are co-expressed in several tissues of a developing mouse embryo.

A second approach to examine expression of *Sipl1* and *Rbck1* during mouse embryonic development was carried out by in RNA *in situ* hybridization on paraffin sections of a female mouse embryo of embryonic day 13.5. Therefore, gene-specific probes for *Sipl1* and *Rbck1* were used in either sense or antisense orientation to make sure that a specific signal is detected. For comparison, *Eya1* expression was determined in parallel.

In the case of *Sipl1*, high overall background staining was observed in the sense control (Fig. 3.30 A). However, a specific signal for *Sipl1* expression significantly higher than background was detectable in the kidneys, the dorsal root ganglia and a spot-like pattern most likely representing parts of the sympathetic nervous system (SNS) of the developing mouse embryo (Fig. 3.30 B). The sense control for *Rbck1* expression showed only slight background staining in the dorsal region of the neural tube (Fig. 3.30 C). In contrast, using an antisense probe against *Rbck1*, bright staining was detected in nearly every region of the section suggesting ubiquitous expression of *Rbck1*. Especially high signals for *Rbck1* expression were detected in kidneys, pancreas, stomach and the SNS (Fig. 3.30 D). Analysis of *Eya1* expression by *in situ* hybridization on comparable sections showed that also *Eya1* is highly expressed in developing kidneys and the SNS (Fig. 3.30 E, F).

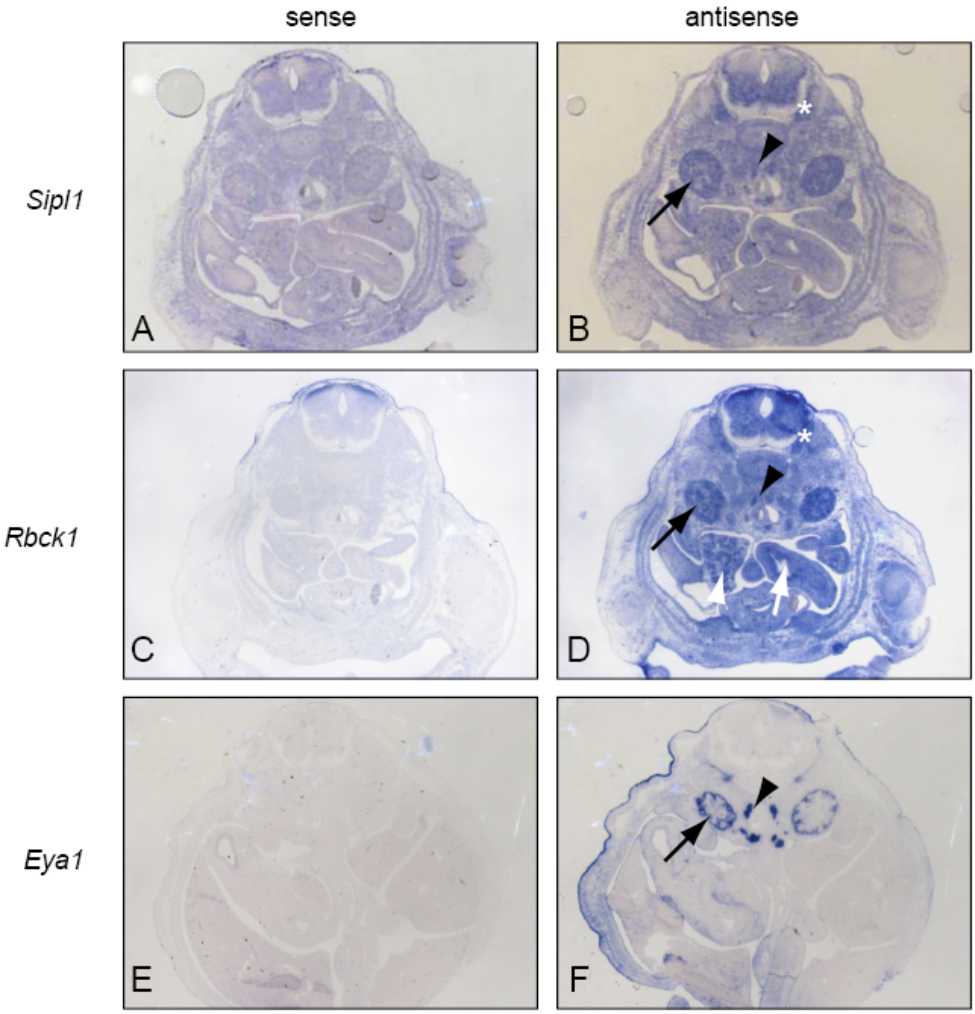

Fig. 3.30. Analysis of *Sipl1*, *Rbck1* and *Eya1* expression by *in situ* hybridization. Analysis of the expression of *Sipl1* (A,B), *Rbck1* (C,D) and *Eya1* (E,F) by *in situ* hybridization on sections of the kidney region of a 13.5 day old female mouse embryo using gene-specific probes in sense (A,C,E) or antisense (B,D,F) orientation. Expression in the kidney is indicated by black arrow, in the sympathetic nervous system (SNS) by black arrowhead. White arrow marks expression in stomach, white arrowhead expression in pancreas, asterisk expression in the dorsal root ganglion.

In summary, RNA *in situ* hybridization demonstrated that both *Sipl1* and *Rbck1* are expressed in several tissues of a mouse embryo including regions overlapping with *Eya1* expression.

3.4.3 Effect of Sipl1 and Rbck1 on transactivation function of Eya1

Eya proteins have been shown to act as co-activators for the Six transcription factors. A question, which arose during the course of this work, was whether the interaction of Sipl1 or Rbck1 influences this function. Based on already reported data, a transactivation assay was established using a luciferase reporter construct containing 6 MEF3 (myogenic enhancing factor 3) sites in the background of a minimal TATA promoter. Both Six1 and Six4 have been shown to bind to MEF3

sites which are naturally occurring in the regulatory regions of muscle-specific genes, e.g. *Myogenin*. The MEF3 consensus sequence is 5'-TCAGGTT-3' (Spitz *et al.*, 1998). Binding of Six1 and Six4 to these sites leads to activation of transcription with Six4 having the stronger effect compared to Six1 (Spitz *et al.*, 1998). Furthermore, Six4-mediated transactivation is enhanced by interaction with Eya1 or more efficiently by interaction with Eya2 (Fan *et al.*, 2000; Ruf *et al.*, 2004). As shown before, Sipl1 also interacts with Eya2, which can also be assumed for Rbck1 because the binding region of the two proteins is conserved. In order to analyze the effect of the two interaction partners on the ability of Eya2 to act as a co-activator for Six4, luciferase reporter assays were performed. Therefore, Cos-7 cells were transfected with the appropriate expression constructs, the MEF3-luciferase reporter construct and a renilla reporter construct as an internal control. 48 h post-transfection, reporter activity was determined using the Dual-Luciferase Reporter Assay System (Promega).

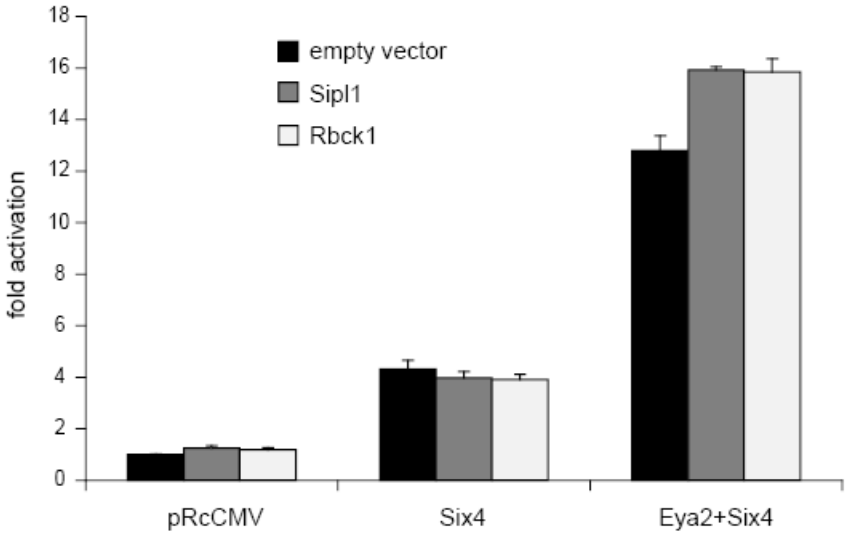

Fig. 3.31. Sipl1 and Rbck1 enhance Eya2-Six4 mediated transactivation. Cos-7 cells were seeded in 6-well format and co-transfected with pGL3-MEF3/TATA or pGL3-TATA reporter construct, indicated expression constructs, and Renilla control-plasmid. Each transfection was performed in triplicates. 48 h after transfection, cell lysates were prepared for measurement of luciferase activity. After normalization to Renilla activity, foldness of reporter gene activation of samples containing pGL3-MEF3/TATA was calculated relative to respective samples containing pGL3-TATA. Activity of the reporter alone was set to 1. Graph represents one of three experiments showing similar results. Error bars indicate standard deviation.

In line with the already published data, it was observed that Six4 alone activated the reporter to about 4 fold, and this activation was further enhanced to about 12 fold in presence of Eya2 (Fig. 3.31). Presence of Sipl1 or Rbck1 did not influence transactivation by Six4 alone, but enhanced transactivation by Eya2 and Six4 to

about 16 fold indicating that this effect is mediated via interaction of Sipl1 and Rbck1 with Eya2.

3.4.4 Identification of orthologs of *Sipl1* and *Rbck1* in zebrafish

To further address the functional importance of the Eya1-Sipl1/Rbck1 interaction the zebrafish model system was used. The zebrafish has several advantages compared to other model organisms. First of all, it is a vertebrate and, thus, more closely related to mouse or human compared to other established models, e.g. *Drosophila* and *C. elegans*. Second, development is fast and can easily be monitored by microscopy. And finally, gene-specific knockdown can be achieved quite simply by injection of morpholinos directed against the gene of interest. Aim of the zebrafish studies was to investigate the role of *Sipl1* or *Rbck1* during embryonic development in relation to *Eya1*. In 1999, Sahly *et al.* identified a zebrafish ortholog of *Eya1*, which has recently been shown to be involved in ear development (Sahly *et al.*, 1999; Kozlowski *et al.*, 2005). The zebrafish experiments were performed in collaboration with Frank Bollig and Christina Ebert. At the beginning of this study no zebrafish orthologs of *Sipl1* or *Rbck1* had been characterized. Using bioinformatics analysis, three putative orthologs could be identified (Fig. 3.32).

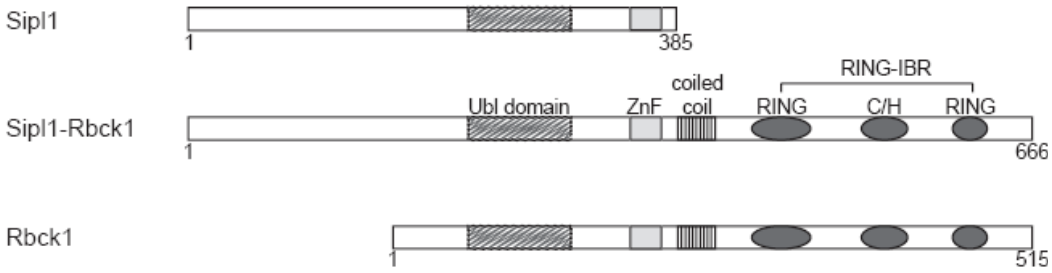

Fig. 3.32. Protein domain structure of zebrafish orthologs of Sipl1 and Rbck1. Protein domains of each of the orthologs were predicted using the Conserved Domains database of NCBI (http://www.ncbi.nlm.nih.gov).

Zebrafish s*ipl1* is an ortholog of mouse *Sipl1* and encodes a protein which contains both the conserved regions the Ubl domain and the RanBP-type ZnF in its C-terminal part. Zebrafish *rbck1* is an ortholog of mouse *Rbck1* with the respective proteins also sharing all conserved domains: the Ubl domain, the RanBP-type Znf, the coiled-coil region and the RING-IBR domain. In addition to that, a third ortholog, called s*ipl1-rbck1*, could be identified, which seems to be a fusion of both, *sipl1* and *rbck1* (Fig. 3.32). Bioinformatics analysis showed that a

similar fusion gene seems to be present in other fish species as well, as for example stickleback and fugu (data not shown). The coding regions of all three zebrafish orthologs were cloned by RACE (sequences are listed in the appendix). In the case of *sipl1-rbck1*, primers were designed based on known ESTs. For *sipl1* and *rbck1* no ESTs were available and primers used for RACE were located within regions highly conserved to the mouse orthologs. Obtained cDNA sequences were translated into protein sequences and aligned to the minimal Eya1 binding region of the respective mouse proteins using the ClustalW program (www.ebi.ac.uk/clustalw). Results are shown in Table 3.3.

Table 3.3. Amino acid sequence conservation of the minimal Eya1 binding region between zebrafish and mouse

	identities within the Eya1-binding region compared to	
Zebrafish protein	**mouse Sipl1**	**mouse Rbck1**
Sipl1	50%	43%
Sipl1-Rbck1	49%	38%
Rbck1	28%	41%

Alignment of the minimal Eya1 binding region of mouse Sipl1 or Rbck1 to each of the identified zebrafish proteins revealed that both zebrafish Sipl1 and Sipl1-Rbck1 are more closely related to mouse Sipl1, whereas zebrafish Rbck1 is more closely related to mouse Rbck1 within this region (Table 3.3). Therefore, zebrafish Sipl1 and Sipl1-Rbck1 are further designated as Sipl1 orthologs, and zebrafish Rbck1 as an Rbck1 ortholog.

3.4.4.1 Co-expression of *Eya1* and *Sipl1*/*Rbck1* orthologs in zebrafish

In order to analyze the expression pattern of the *Sipl1* orthologs during zebrafish embryonic development in comparison to that of *Eya1*, whole-mount RNA *in situ* hybridization was performed as described in 2.2.6.3 using zebrafish embryos of different stages (35-72 hpf) and gene specific probes.

As already described by Sahly *et al.*, high levels of *eya1* expression were detected in all three stages in the region of the branchial arches and the otic vesicle which corresponds to the developing ear (Fig. 3.33) (Sahly *et al.*, 1999). Zebrafish *sipl1* was expressed at high levels in the whole zebrafish head at earlier stages (35

hpf), while at later stages (72 hpf) its expression was more restricted to the region of the midbrain-hindbrain barrier and the otic vesicle of the zebrafish embryo. In contrast, the expression pattern of *sipl1-rbck1* was more restricted with high expression in the region of the midbrain-hindbrain barrier and the region of the developing ear.

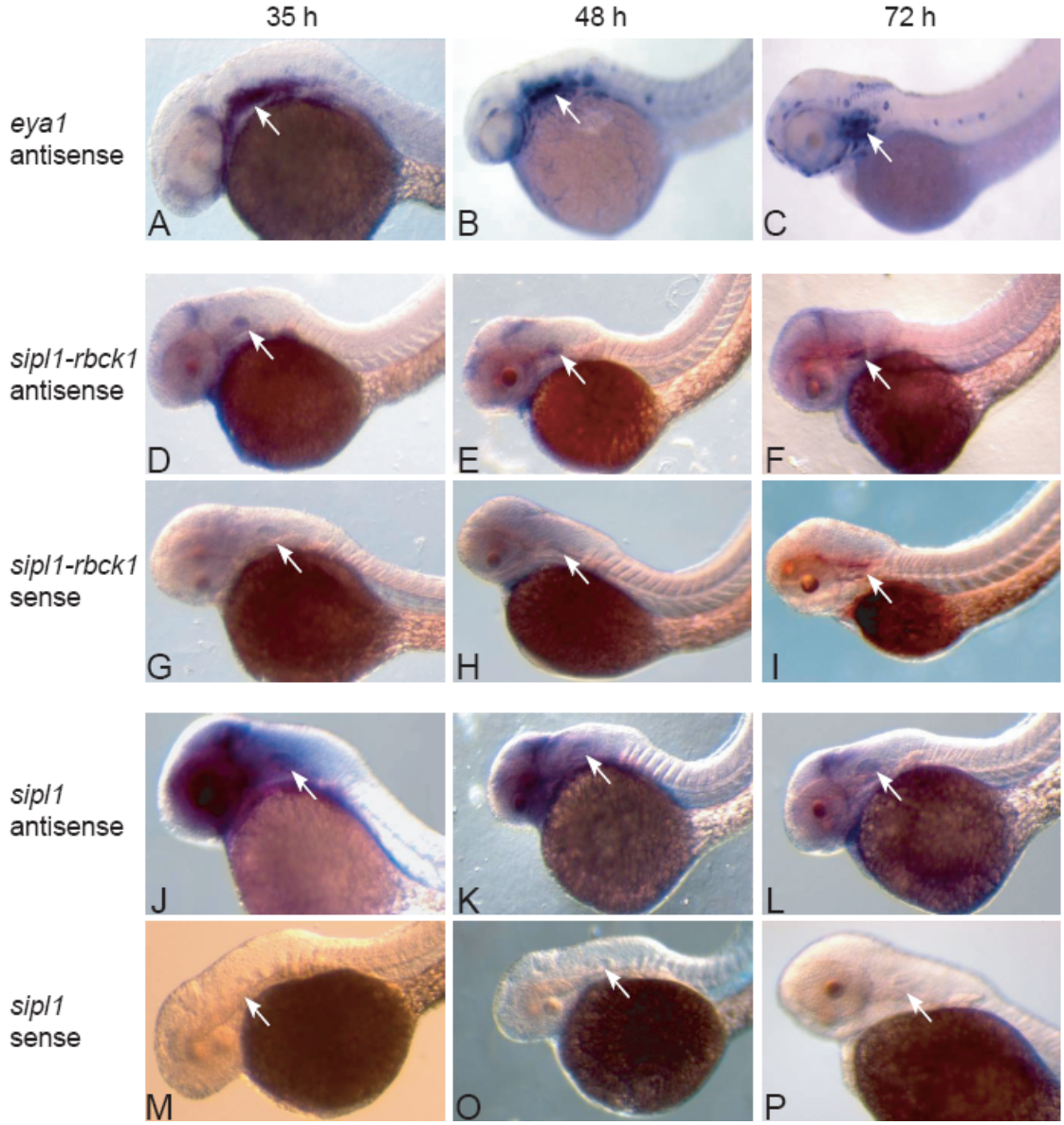

Fig. 3.33. Analysis of *eya1*, *sipl1* and *sipl1-rbck1* expression in zebrafish by whole-mount *in situ* hybridization. Analysis of the expression of *eya1* (A-C), *sipl1* (D-I), and *sipl1-rbck1* (J-P) by whole-mount *in situ* hybridization on different stages of zebrafish embryos using gene-specific probes in sense (G-I, M-P) or antisense (A-C, D-F, J-L) orientation. Position of the otic vesicle is indicated by white arrow.

Taken together, analysis of the expression of *eya1* and the *Sipl1*-orthologs *sipl1* and *sipl1-rbck1* showed that all 3 are co-expressed in the developing ear of the zebrafish embryo.

To have a closer look at this region, RNA *in situ* hybridization was performed on transverse sections of the ear region of a zebrafish embryo at 72 hpf. Again, gene-specific RNA probes for *sipl1* and *sipl1-rbck1* were tested in comparison to an *eya1* probe.

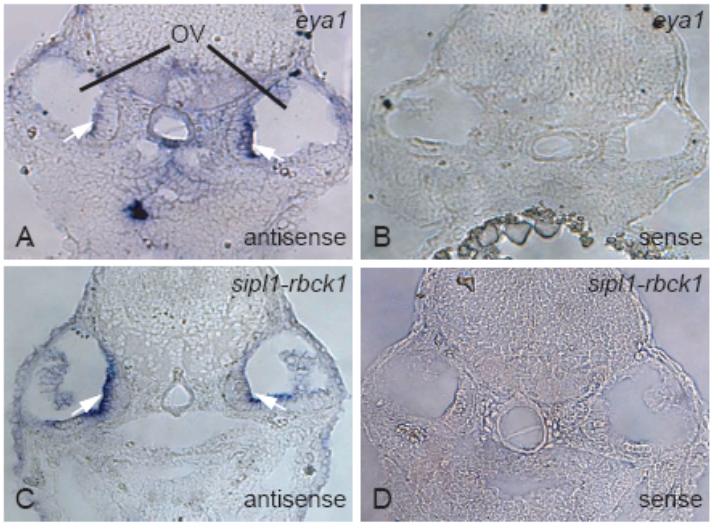

Fig. 3.34. *eya1* and sipl1-rbck1 are co-expressed in the developing inner ear of the zebrafish embryo. Analysis of expression of *eya1* (A,B) and *sipl1-rbck1* (C,D) in the otic vesicle of a 72 h old zebrafish embryo was performed by *in situ* hybridization on paraffin sections using gene-specific probes in sense (B,D) or antisense (A,C) orientation. Expression in the sensory epithelium of the otic vesicle is indicated by white arrow. ov, otic vesicle.

As can be seen from Fig. 3.34, *eya1* is expressed in a defined region of the inner ear which corresponds to the sensory epithelium. A signal for *sipl1* expression was detected in this region, but was also present in the sense control indicating that this signal is not specific (data not shown). In contrast, for *sipl1-rbck1* a clear signal could be detected in a similar region, which was not present in the sense control. In conclusion, both *eya1* and *sipl1-rbck1* are expressed in an overlapping region of the inner ear of a developing zebrafish embryo which is compatible with a possible interaction between the respective proteins.

To get an idea about the expression pattern of the *Rbck1* ortholog during zebrafish embryogenesis, whole-mount RNA *in situ* hybridization was performed using 72 h old embryos and a gene-specific probe.

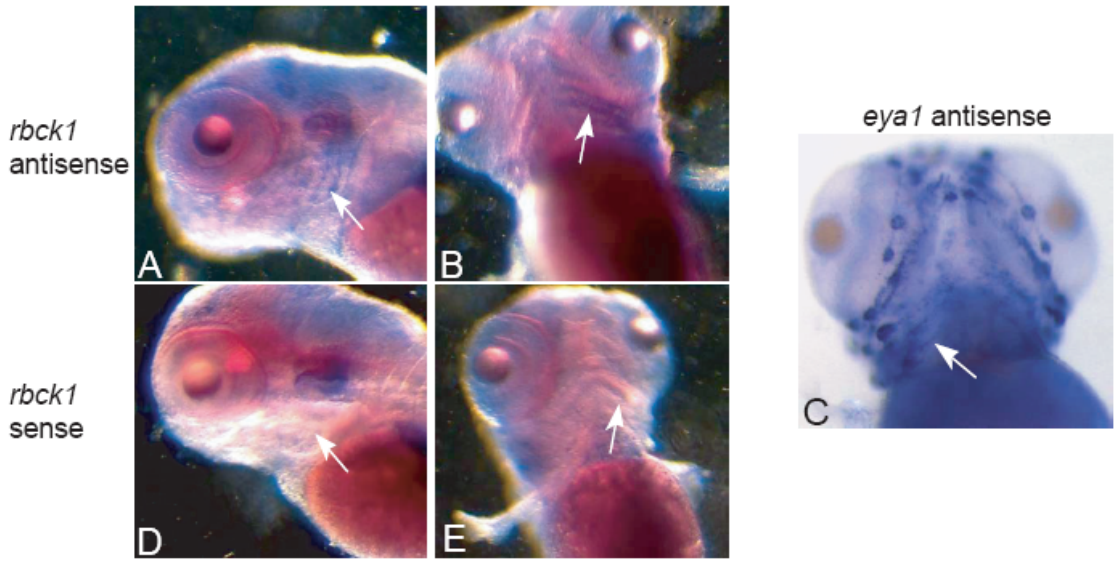

Fig. 3.35. Comparison of *eya1* and *rbck1* expression in zebrafish by whole-mount *in situ* hybridization. Comparison of the expression pattern of *rbck1* (A, B, D, E) and *eya1* (C) by whole-mount *in situ* hybridization on 72 hours old zebrafish embryos using gene-specific probes in sense (D,E) or antisense (A, B, C) orientation. Position of branchial arches is indicated by white arrow.

In the case of *rbck1*, the sense control showed high unspecific staining (Fig. 3.35). However, a specific signal, which was not present in the sense control, was detected in the region of the branchial arches of the zebrafish embryo. A closer look on *eya1* expression showed that also *eya1* is expressed in this region of the developing zebrafish.

Taken together, it has been demonstrated that the identified *Sipl1* and *Rbck1* orthologs are expressed in different tissues during zebrafish embryonic development. While both *sipl1* and *sipl1-rbck1* are co-expressed with *eya1* in the developing ear, *rbck1* shows overlapping expression with *eya1* in the region of the branchial arches.

3.4.4.2 Knockdown of *Sipl1/Rbck1* orthologs in zebrafish

To gain insight into the importance of a gene of interest during embryonic development, a common approach is the knockdown of the respective gene. Knockdown of genes in zebrafish can be achieved by the injection of morpholino antisense nucleotides (morpholinos) into 1- or 2-cell stage embryos. Morpholinos are nucleic acids analogs which can mediate knockdown of gene expression by binding to complementary regions in the mRNA. They can be designed to block translation initiation (by targeting the 5'-untranslated region) or to modify pre-mRNA splicing (by targeting splice junctions). To address the question whether the

two *Sipl1* orthologs, *sipl1* and *sipl1-rbck1*, are involved in developmental processes a morpholino-mediated knockdown approach was employed. cDNA sequences of *sipl1* and *sipl1-rbck1* were aligned with zebrafish genomic DNA by performing a BLAT search in the UCSC genome browser to determine intronic sequences of the respective gene (http://genome.ucsc.edu/). According to this data, morpholinos were created which were directed against splice donor sites of the respective mRNA. Sequences and target site of morpholinos used in this work are listed in Table 2.5.

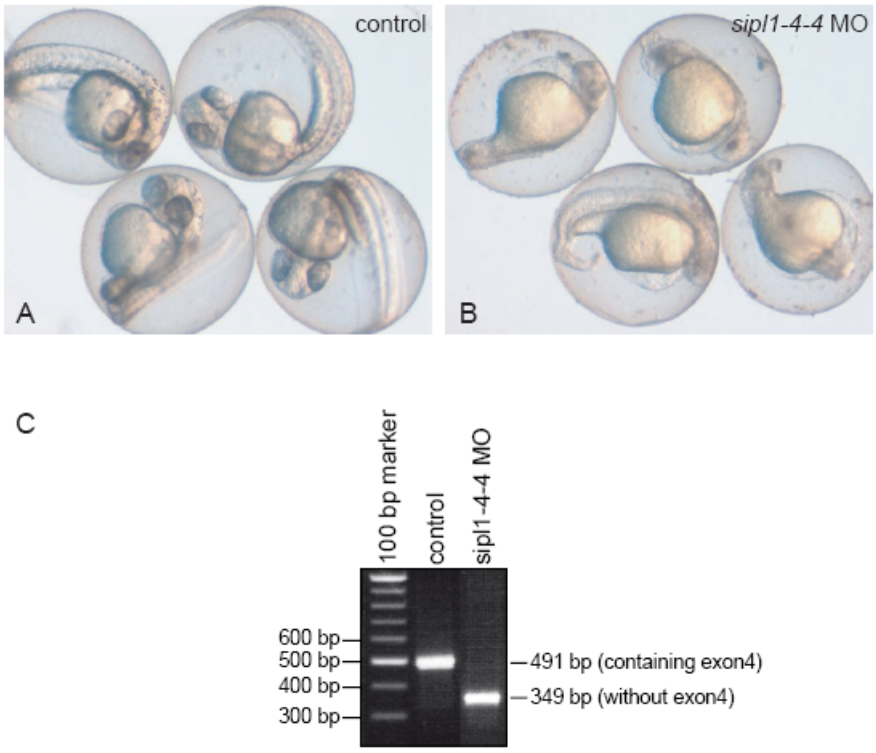

Fig. 3.36. Morpholino-mediated knockdown of *sipl1* expression in zebrafish results in severe malformations of the embryo. (A) Non-injected control-embryos at 35 hpf. (B) Embryos injected with morpholino directed against the exon4-intron4 splice donor site of *sipl1* (sipl1-4-4 MO) at 35 hpf. (C) Confirmation of knockdown by RT-PCR using primers located in exon 2 and exon 6 of *sipl1*, respectively.

As shown by RT-PCR analysis (Fig. 3.36 C), injection of *sipl1*-morpholinos directed against the splice donor site of exon 4 led to efficient knockdown of *sipl1* expression via exclusion of exon 4 from the mRNA leading to a frameshift and an early stop codon. Knockdown of zebrafish *sipl1* resulted in complete malformation of the zebrafish embryos (Fig. 3.36 B). Both, head and tail region of the embryos are shortened in size, and structures, as for example the eye, are either completely absent or severely malformed. This data is in line with the more

ubiquitous expression pattern of *sipl1* during zebrafish embryonic development suggesting a more general role of this gene in embryonic development.

In contrast to *sipl1*, knockdown of sipl1-rbck1 using two independent splice donor site morpholinos led to a BOR (branchio-oto-renal) syndrome-like phenotype mainly affecting the region of the branchial arches and the ear of the zebrafish embryo (Fig. 3.37).

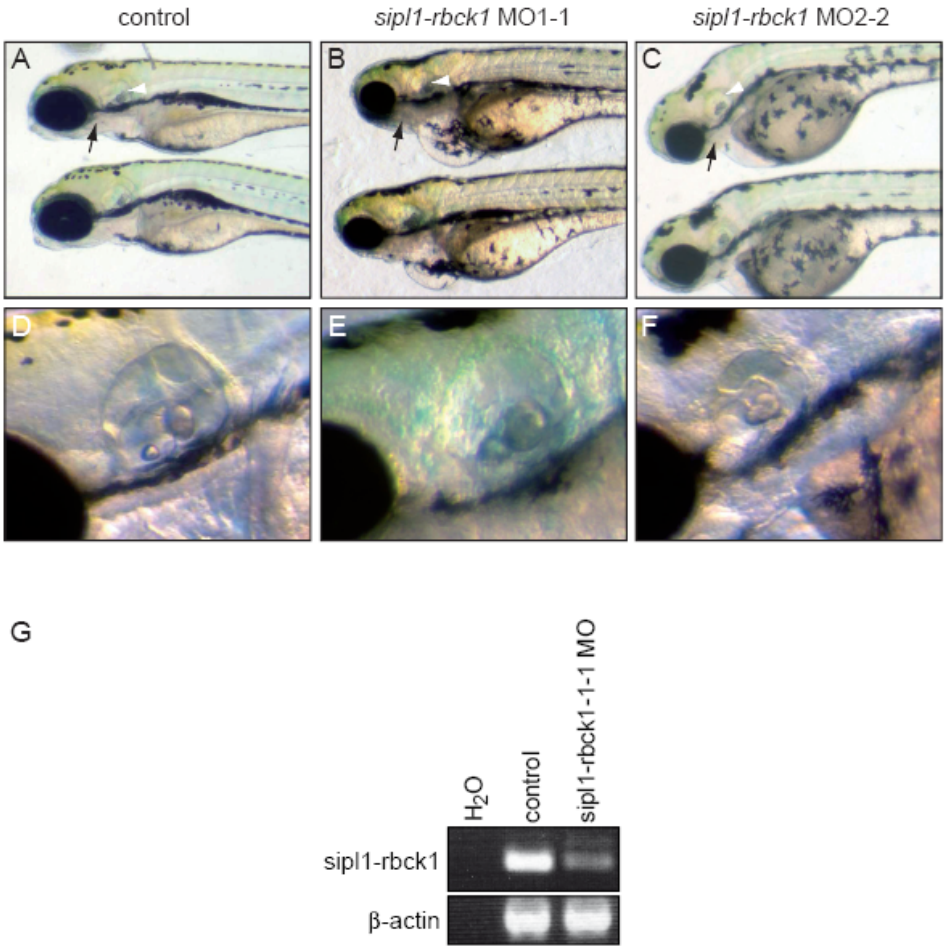

Fig. 3.37. Morpholino-mediated knockdown of *sipl1-rbck1* expression in zebrafish affects the development of the ear and the lower jaw. (A) Non-injected control-embryos at 4 dpf. (B) Embryos injected with morpholino directed against the exon1-intron1 splice donor site of *sipl1-rbck1* (sipl1-rbck1 MO1-1) at 6 dpf. (C) Embryos injected with morpholino directed against the exon2-intron2 splice donor site of *sipl1-rbck1* (sipl1-rbck1 MO2-2) at 4 dpf. Black arrow indicates region of the branchial arches. White arrowhead indicates position of the otic vesicle. (D) Lateral view of the otic vesicle of a non-injected control embryo at 4 dpf. (E) Lateral view of the otic vesicle of an embryo injected with *sipl1-rbck1* MO1-1 at 6 dpf. (E) Lateral view of the otic vesicle of an embryo injected with *sipl1-rbck1* MO2-2 at 4 dpf. (G) Confirmation of knockdown by RT-PCR shown exemplarily for *sipl1-rbck1* MO1-1 using primers located in exon 1 and exon 3 of *sipl1-rbck1*, respectively. *β-actin* expression was determined from the same samples to control input.

As seen in Fig. 3.37, the region of the branchial arches including the lower jaw seems to be shortened after knockdown of *sipl1-rbck1*. A closer look at the region of the otic vesicle of the morphant zebrafish embryos revealed that knockdown of

sipl1-rbck1 inhibited proper development. The otic vesicle of knockdown embryos is smaller in size and structures are not properly formed in comparison to control embryos (Fig. 3.37 D-F). This phenotype is partly similar to the phenotype of embryos after *eya1* knockdown, which has been shown to mainly affect the developing ear leading to reduced size and improper structure formation. Knockdown was confirmed by RT-PCR analysis (Fig. 3.37 G).

In summary, data from zebrafish knockdown experiments provided evidence that both, *sipl1* and *sipl1-rbck1* are involved in processes essential for embryonic development. While *sipl1* seems to have a more general role during embryonic development, *sipl1-rbck1* is mainly involved in development of the ear and the jaw which is derived from the branchial arches. Strikingly, knockdown phenotypes as well as expression pattern of *sipl1-rbck1* and *eya1* show a large overlap in the region of the zebrafish ear leading to the notion that either the genes or the respective proteins might act together during ear development.

3.5 ASSOCIATION OF *SIPL1* AND *RBCK1* WITH HUMAN DISEASE

Mutations in human *EYA1* have been shown to be associated with branchio-oto-renal (BOR) syndrome which is a severe human disease associated with kidney defects, branchial arch anomalies and hearing loss. BOR syndrome is a autosomal dominant disorder and occurs with a prevalence of 1:40000 in the general population (Fraser *et al.*, 1980). In about 40% of cases mutations in *EYA1* have been found (Chang *et al.*, 2004). In addition to that, some cases have been associated with mutations in *SIX1* and *SIX5* which both encode for interaction partners of Eya1, and it has been shown that most of the BOR-associated mutations identified in those genes lead to weakening of the interaction with Eya1 (Ruf *et al.*, 2004; Hoskins *et al.*, 2007). In the course of this work, two novel Eya1-interaction partners, Sipl1 and Rbck1, have been identified. Furthermore, it has been shown that each is co-expressed together with *Eya1* in certain tissues as for example kidney and ears. Strikingly, knockdown of an ortholog of *Sipl1* in zebrafish leads to a BOR-like phenotype of the zebrafish embryo. Thus, an interesting question was whether mutations in the respective human genes *SIPL1* or *RBCK1* are associated with BOR syndrome.

3.5.1 Screening of BOR patients for mutations in *SIPL1* or *RBCK1*

In collaboration with Friedhelm Hildebrandt from the University of Michigan, material from 91 BOR patients was screened for mutations in *SIPL1* and *RBCK1*. For *SIPL1* exons 2-8 were sequenced and one heterozygous mutation c.1093>T leading to substitution of the arginine at position 365 to cysteine (R365C) was found (Fig. 3.38 A). The arginine residue affected by the mutation is conserved in human, mouse and both zebrafish orthologs, Sipl1 and Sipl1-Rbck1.

For *RBCK1* 10 out of 12 exons were sequenced and also one mutation c.682>G was identified leading to substitution of the glutamine at position 228 to glutamate (Fig. 3.38 B). The affected residue is conserved between human and mouse, but not in zebrafish.

Both mutations were absent in 86 European healthy control individuals.

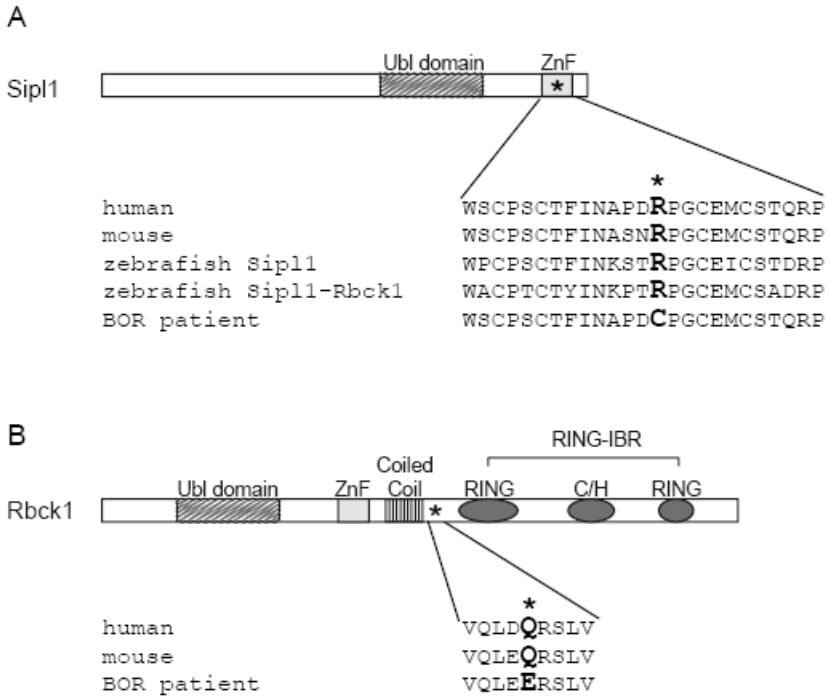

Fig. 3.38. BOR-associated mutations in *SIPL1* and *RBCK1*. (A) Position of potential BOR-associated *SIPL1*-mutation R365C within the conserved ZnF region of the protein is indicated by asterisk. Alignment of the amino acid sequences shows that the affected residue is conserved between human, mouse and zebrafish. (B) The potential BOR-associated *RBCK1* mutation Q228E is located in between the coiled coil domain and the conserved RING finger region of the protein as indicated by asterisk. The mutated residue is conserved in human and mouse.

3.5.2 The BOR-associated mutation *Sipl1*^{R365C} comprises the interaction with Eya1

As a first attempt to analyze the effect of the BOR-associated mutations of *SIPL1* and *RBCK1* on the function of the respective proteins, interaction studies were

performed. Using site-directed mutagenesis the mutations were introduced into the respective expression constructs for mouse Sipl1 or Rbck1 used before for yeast two-hybrid analysis, and tested for their influence on the interaction with Eya1 by β-gal liquid assay.

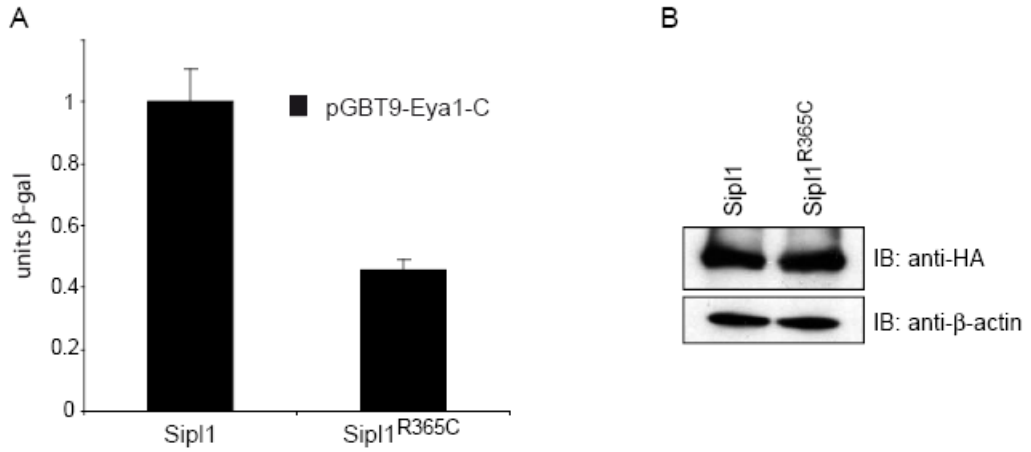

Fig. 3.39. The BOR-associated *Sipl1* mutation R365C compromises the interaction with Eya1. *S. cerevisiae* KFY1 were co-transformed with pGBT9-Eya1-C and pGADT7-Sipl1 or its mutant variant pGADT7-Sipl1^{R365C} (R358C in mouse, respectively). After 3 days of growth all colonies of each transformation plate were pooled and analyzed by β-gal liquid assay and immunoblot. (A) Results of β-gal liquid assay. Amount of β-gal units produced was calculated from results of three independent transformations. Each measurement was performed in triplicates. Error bars represent standard deviation. (B) Samples used in A were analyzed for protein levels by 10%-SDS-PAGE and immunoblot. Sipl1 was detected using anti-HA antibody. Equal loading was confirmed by detection of β-actin.

Using yeast two-hybrid analysis an influence of the *RBCK1* mutation Q228E (Q258E in mouse, respectively) on the interaction with Eya1 could not be detected probably due to the weakness of the interaction between the two proteins (data not shown). The mutation of *SIPL1* which was isolated from a BOR patient, however, weakened the interaction with Eya1 to about 50% compared to the wild type protein which was not due to differences in expression levels as confirmed in parallel by immunoblot (Fig. 3.39).

4 DISCUSSION

The overall goal of this work was to unravel the mechanisms by which Eya1 regulates organ development in more detail. This was addressed by two approaches: First of all, BOR-associated Eya1 mutations were analyzed for their effect on Eya1 function. Second, novel interaction partners of Eya1 were identified in a yeast two-hybrid screen and characterized.

An indispensable pre-requisite to analyze the function of a protein of interest at the physiological level is the availability of specific antibodies. In the first part of this work, two Eya1-specific antibodies generated in our lab were examined for their ability to specifically detect endogenous Eya1 protein.

4.1 IMPORTANCE OF EYA1-SPECIFIC ANTIBODIES

Jürgen Tomasch, a former diploma student in our group, had generated two Eya1-specific antibodies and characterized both regarding their ability to detect Eya1 protein in immunoblot, immunoprecipitation, and immunofluorescence using cells overexpressing *Eya1* (Jürgen Tomasch, diploma thesis, 2007). A still open question was whether the two antibodies are specific for Eya1, or whether they also recognize the homologs Eya2-4. Results from this work showed that the antibodies specifically detect Eya1, but not Eya2-4, in immunoprecipitation or immunoblot, respectively. The next step was to use these antibodies for the detection of the endogenous Eya1 protein. For this purpose, two murine cell lines, mK3 and mK4, have been analyzed. Both are clonal cell lines with mK3 representing an early, uninduced and mK4 a later, induced stage of the metanephric mesenchyme of the developing kidney (Valerius *et al.*, 2002). Several studies have shown that *Eya1* is expressed in both these stages, and involved in the reciprocal inductions between the metanephric mesenchyme and the ureteric bud, which initiate formation of the mature kidney (Xu *et al.*, 1999; Sajithlal *et al.*, 2005). In contrast, microarray analysis by Valerius *et al*. detected expression of *Eya1* only in mK4 cells (Valerius *et al.*, 2002). However, RT-PCR analysis done in this work confirmed *Eya1* expression in both cell lines with low levels in mK3 and high levels in mK4 (Fig. 3.2). After immunoprecipitation using one antibody (anti-Eya1.1) and detection using the other antibody (anti-Eya1.2), endogenous Eya1 protein was detected in mK4, but not in mK3 cells. The specifity of this signal was

verified using an RNA interference approach. Taken together, the Eya1-specific antibodies have been shown to be suitable for detection of endogenous Eya1 protein in mK4 cells when used in combination for immunoprecipitation and immunoblot. Strikingly, results in this work demonstrate the first successful detection of endogenous Eya1 protein by immunoprecipitation and immunoblot, providing an important tool for the analysis of Eya1 function *in vivo*. In fact, interaction of Eya1 with all so far described interaction partners, such as Six and Dach or inhibitory Gα subunits, has only been verified *in vitro*. Furthermore, the observation that Eya1 is translocated to the nucleus or the cell membrane by interaction with Six proteins or inhibitory Ga subunits, respectively, is based on *in vitro* data (Ohto *et al.*, 1999; Fan *et al.*, 2000). Using the Eya1-specific antibodies described in this work and an appropiate cell line or tissue, it would now be possible to confirm the respective interactions and their effect on the subcellular localization of Eya1 at the physiological level.

Eya1 has been suggested to act as a co-activator of several transcription factors, as Six, Pax, or Hox during the development of various organs, but only few target genes have been identified so far (Spitz *et al.*, 1998; Brodbeck, 2003; Chai *et al.*, 2006; Gong *et al.*, 2007). Chromatin-immunoprecipitation (ChIP) using an Eya1-specific antibody represents a powerful tool to identify novel target genes of Eya1-containing transcription activation complexes under physiological conditions. Moreover, it is not clear how the transcriptional activity of Eya1 is regulated. Transcriptional activity can be enhanced or repressed by various post-translational modifications as for example phosphorylation, sumoylation or acetylation (Polevoda and Sherman, 2002; Gill, 2003; Gardner and Montminy, 2005). Several studies indicated that the transactivation function of Drosophila Eya is positively regulated via phosphorylation at two MAPK phosphorylation sites (Hsiao *et al.*, 2001; Silver *et al.*, 2003). Interestingly, these sites are conserved in Eya1 as well, but phosphorylation of these sites and associated regulation of Eya1 function has not been investigated so far. In a first attempt this issue could be addressed *in vitro*, but for the verification of the physiological relevance the use of Eya1-specific antibodies is inevitable.

In addition to that, the Eya1-specific antibodies generated in our lab provide a useful tool for the identification of novel Eya1-interacting proteins by co-immunoprecipitation from mK4 cells or animal tissues followed by mass

spectrometry. The great advantage of this approach is that interacting proteins would be isolated under physiological conditions. The identification of novel interaction partners of Eya1 could provide important insights in the molecular mechanisms by which Eya1 mediates its central function in organogenesis.

4.2 CHARACTERIZATION OF DISEASE-ASSOCIATED EYA1 MUTANTS

To further investigate the molecular mechanisms by which Eya1 regulates organogenesis, seven disease-associated *EYA1* mutations, all substitutions located within the conserved Eya domain, were analyzed for their effect on Eya1 function. Among them four mutations were identified from BOR patients, two mutations from BO patients, and one mutation from a patient suffering from ocular defects (see Table 3.1). Several studies indicated that some of the BOR-associated Eya1 mutations compromise the interaction with known interaction partners of Eya1 (Buller *et al.*, 2001; Ozaki *et al.*, 2002). In the course of this work, the disease-associated Eya1 substitutions were examined for their influence on the interaction with Six1 and Gαi2, two described interaction partners of Eya1, in a yeast two-hybrid approach.

Interestingly, the mutant R514G, which is associated with ocular defects in humans, does not affect any of the interactions with known Eya1 interaction partners addressed in this work or in previous studies (Buller *et al.*, 2001; Ozaki *et al.*, 2002). This finding suggests that other factors are probably involved in the regulation of eye development together with Eya1.

Furthermore, both BO-associated mutants, D396G and R407Q, do not affect the interaction of Eya1 with Six1. Regarding the interaction with Gαi2 they showed different effects: D396G led to about 50% loss of interaction, whereas R407Q stabilized the interaction to about 2-fold. It is described that interaction with active Gαi2 leads to translocation of Eya to the cell membrane (Fan *et al.*, 2000). Thus, stabilization as well as loss of interaction might cause an aberrant subcellular localization of Eya1 which could be involved in the onset of BO-syndrome.

The BOR-associated substitutions L472R and L550P lead to weakening or even complete loss of the interaction with both Six1 and Gαi2. It has been reported before that binding of Six1 and Gαi2 is mutually exclusive, suggesting that the two proteins bind to an overlapping region of Eya1 (Fan *et al.*, 2000). In contrast, two

mutants, S454P and D396G, inhibited the interaction with Gαi2, but had no effect on the interaction with Six1. An explanation for this could be that the subregions of the Eya domain which mediate binding of Gαi2 and Six1 are only partially overlapping. Another possibility is that the binding of one interaction partner leads to a conformational change of the Eya domain which prevents binding of the respective other interaction partner. Interestingly, the two BOR-associated substitutions, S454P and L472R, have also been shown to weaken or abolish the interactions with other known interaction partners of Eya1 including Six5, Dach1, and Gαz (Ozaki *et al.*, 2002). Studies on Drosophila Eya implicated that the homologs of Six5 and Dach1, So and Dach, bind to distinct regions of the Eya domain (Bui *et al.*, 2000). Because of this Ozaki *et al*. assumed that introduction of the mutations S454P and L472R leads to a gross conformational change of Eya1 protein structure thereby disturbing interactions mediated via the Eya domain. To support this hypothesis, Ozaki *et al*. analyzed the protease digestion patterns of the proteins. Interestingly, digestion with trypsin or V8 protease resulted in differences in the digestion patterns of wild type Eya1 and the mutants S454P and L472R. Ozaki *et al*. concluded that in the mutants S454P and L472R the accessibility of protease sites is changed because of an altered protein conformation (Ozaki *et al.*, 2002). An independent study performed by Zhang *et al*. showed similar results. They examined the protein structures of wild type Eya1 and the mutant L472R *in silico* by molecular modelling. Again, structural differences were observed (Zhang *et al.*, 2004). Results from this work showed that, similarly to L472R, the substitution L550P inhibits the interaction with both, Six1 and Gαi2, indicating that the respective mutation also results in an altered protein conformation. Strikingly, data presented in this work demonstrate that the three BOR-associated Eya1 mutants, S454P, L472R and L550P, are present at lower protein levels in mammalian cells. Reason for this might be that the incorrect folding of the respective proteins mediates enhanced protein degradation which might represent a novel mechanism for the cause of BOR-syndrome.

4.3 EYA1 IS DEGRADED VIA THE PROTEASOMAL PATHWAY

It has been demonstrated that Eya1 is degraded via the proteasomal pathway. Proteins which are degraded by the proteasome are labeled for their degradation by attachment of ubiquitin to structurally exposed lysine residues. Ubiquitination is

mediated by the sequential action of 3 enzymes: The ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2), and the ubiquitin ligase (E3), whereby the E3-ubiquitin ligase controls both the specificity and timing of substrate ubiquitination. Eya1 ubiquitination occurs in two distinct regions of the Eya domain, each containing a cluster of 6 lysine residues. Moreover, results from Amna Musharraf indicate that Eya1 protein accumulates in presence of Six1 and Six2 (Amna Musharraf, unpublished data). An underlying mechanism was provided by results obtained in this work, indicating that presence of Six1 prevents ubiquitination of Eya1. But still many questions remain to be answered as for example: How is the inhibition of ubiquitination mediated? Is Six-mediated stabilization of Eya1 relevant *in vivo*? It is known that Six1 mediates translocation of Eya1 into the nucleus, where both proteins act together to activate target gene expression (Ohto *et al.*, 1999). Interestingly, Six1 binds to the conserved Eya domain of Eya1 where also ubiquitination of the protein has been demonstrated to occur. Thus, it is possible that interaction with Six1 prevents Eya1 ubiquitination because an important ubiquitination site is not accessible. Another explanation could be that ubiquitination and subsequent degradation of Eya1 exclusively occur in the cytoplasm of the cell and nuclear translocation of Eya1 by Six proteins sequesters the protein away from the place of ubiquitination. Evidence for the latter hypothesis is provided by the observation that both Six- and homeo-domain of Six1 are necessary to stabilize Eya1 (Amna Musharraf, unpublished data). Both, Six- and homeo-domain have also been described to be essential for nuclear translocation of Eya, whereas for interaction with Eya the Six domain alone is sufficient (Pignoni *et al.*, 1997; Ohto *et al.*, 1999). A similar mechanism is known to regulate protein levels of the tumor suppressor p53. Activity of p53 is primarily regulated by its protein stability. Under normal growth conditions, p53 is present at extremely low levels because upon synthesis the protein is rapidly degraded via the proteasomal pathway. p53 translocates between the cytoplasm and the nucleus by its intrinsic nuclear localization signal (NLS) and nuclear export signal (NES) sequences. Blocking nuclear export leads to stabilization of p53 indicating that degradation occurs exclusively in the cytoplasm (Freedman and Levine, 1998). DNA damage and other stress signals lead to accumulation of p53 in the nucleus where it activates target gene expression. This nuclear accumulation is induced by inhibition of nuclear export and, hence, inhibition of cytoplasmic

degradation, and can be achieved by various mechanisms, as post-translational modifications or interaction with other proteins (Ashcroft and Vousden, 1999; Sionov *et al.*, 2001). Interestingly, sequence analysis revealed that Eya1 contains a leucine-rich NES motif similar to that of p53 in its C-terminus (Fig. 4.1).

NES consensus	**L** X_{1-3} **L** X_{2-4} **L** X **L**	
human p53	**F** R E **L** N E A **L** E **L**	aa 341-350
human Eya1	**L** - A **L** - K A **L** S **L**	aa 448-455
mouse Eya1	**L** - A **L** - K A **L** S **L**	aa 480-487

Fig. 4.1. Eya1 contains a leucine-rich NES similar to that of p53. Alignment of the predicted Eya1 NES with the NES of human p53. Eya1 NES was predicted using the NetNES 1.1 program (www.cbs.dtu.dk/services/NetNES/). Conserved residues are indicated in bold type. In the NES consensus sequence, X can be any amino acid and L (leucine) can be substituted by other hydrophobic residues.

Whether the NES of Eya1 is functional and mediates nuclear export of the protein remains to be elucidated and could be addressed by localization studies using an Eya1 construct harbouring mutations within the NES. In this regard, it would be interesting to analyze whether nuclear localization of Eya1 is sufficient for its stabilization independent of its interaction with Six. However, an important point is to show that stabilization of Eya1 by interaction with Six proteins is relevant *in vivo*. If so, regulation of Eya1 stability could represent a novel mechanism for the regulation of Eya1 activity. Moreover, identification of the E3-ubiquitin ligase which mediates Eya1 ubiquitination and therefore its degradation could further clarify the regulation of Eya1 protein stability.

4.4 IDENTIFICATION OF NOVEL EYA1-INTERACTING PROTEINS

A central aspect of this work was the identification of novel interaction partners of Eya1. For this purpose, the classic Gal4-based yeast two-hybrid system has been employed. Eya1 should be used as a bait for the screening of a cDNA library of an 11 day-old mouse embryo. Several studies implicated that Eya1 is required for initiation of the development of several organs at this stage of mouse embryogenesis, as for example kidney, ear, thymus and muscle (Xu *et al.*, 1999; Xu *et al.*, 2002; Grifone *et al.*, 2007). It was not possible to use full-length Eya1 as bait for the screening procedure due to autoactivation of the reporter genes when fused to the Gal4-BD. The reason for this is the transactivation activity located

within the N-terminal domain of Eya1 (Xu *et al.*, 1997). In line with this assumption, also a bait construct containing the N-terminal part of Eya1 autoactivated the system to a high degree. The region responsible for transactivation was narrowed down to amino acids 109-198. In contrast, a bait construct containing the Eya1-C-terminus did not lead to autoactivation of the reporter genes and, hence, was used for the screening procedure. From approximately 2 million transformants, 10 potential interaction partners were identified. Two of them could be confirmed by further analysis in yeast. One was Six2, an already known interaction partner of Eya1 (Buller *et al.*, 2001). Isolation of Six2 confirmed that the approach designed in this work indeed was suitable to identify Eya1 interacting proteins. The other was Sipl1 (Shank-interacting protein like 1; also termed Sharpin) which is a novel interaction partner of Eya1.

4.4.1 Sipl1 as a novel interaction partner of Eya1

Sipl1 was first described as an interaction partner of Shank1 in rat (Lim *et al.*, 2001). Shank1 is one of three vertebrate Shank homologs, which have been shown to function as scaffold proteins in the formation and maintenance of postsynaptic densities by integrating neurotransmitter receptors into the cortical cytoskeleton (Sheng and Kim, 2000). Sipl1 protein is enriched in the postsynaptic densities and forms a complex with Shank in brain. Complex formation of Shank and Sipl1 is mediated via the C-terminal part of Sipl1 while its N-terminus is important for homomultimerization. Interaction of Shank with Sipl1 has been suggested to mediate cross-linking of Shank proteins (Lim *et al.*, 2001). However, *Sipl1* is not only expressed in the brain, but also in many other adult tissues, as for example heart, muscle, kidney and spleen (Lim *et al.*, 2001). Homologs of *Sipl1* have been identified in human and mouse, where they have been implicated in enteric nervous system function (Daigo *et al.*, 2003). Furthermore, recent studies indicated that mutations in the mouse *Sipl1* gene result in multiorgan inflammation, immune system dysregulation and dermatitis (Seymour *et al.*, 2007). The mechanisms by which Sipl1 is involved in these processes are unclear. In the course of this work, Sipl1 has been isolated as an interaction partner of Eya1 from a yeast two-hybrid screen of an embryonic cDNA library. The interaction between the two proteins was verified *in vitro* by GST pulldown assay and co-immunoprecipitation. Both Sipl1 and Eya1 are localized in the cytoplasm and

translocate into the nucleus in presence of Six protein, indicating a complex formation of the proteins. It should be noted, that Six-mediated Sipl1 accumulation in the nucleus was observed to some degree also in absence of co-transfected Eya1, which could either be caused by the interaction with endogenous Eya1 or by direct interaction of Sipl1 and Six. The Sipl1 protein possesses two conserved domains in its C-terminal part: an Ubl domain and a Ran-BP2 type zinc finger. Both these domains are conserved in other proteins as well, as for example Rbck1 (RBCC protein interacting with PKC 1; also termed HOIL-1, XAP3, or UIP28). In fact, the N-terminus of Rbck1 is highly similar to the C-terminus of Sipl1 and was previously considered to be an independent domain, termed as Rbck1 homology domain (Lim *et al.*, 2001). Binding studies in yeast demonstrated that the Eya1-Sipl1 interaction is mediated via the Eya domain of Eya1 and the Ubl domain of Sipl1. Based on the fact that the Ubl domain shows high similarity between Sipl1 and Rbck1, it was assumed that Rbck1 can also interact with Eya1.

4.4.2 Rbck1 as a novel interaction partner of Eya1

Indeed, the interaction of Rbck1 and Eya1 could be detected by yeast two-hybrid analysis, GST pulldown and co-immunoprecipitation experiments. As mentioned above, the N-terminus of Rbck1 is similar to the C-terminus of Sipl1 including both conserved domains the Ubl domain and the RanBP2 type ZnF. In addition to that, in the C-terminus of Rbck1 a coiled coil region and a RING-IBR domain can be found. RING-IBR-containing proteins have been shown to be involved in E3 ubiquitin ligase activity (Marin and Ferrus, 2002). Rbck1 has been described to act as an E3 ubiquitin ligase mediating the degradation of several unrelated proteins, as IRP2 (iron regulatory protein-2), PKC (protein kinase C), Bach1, and TAB2/3 (TAK1-binding protein 2/3) (Yamanaka *et al.*, 2003; Nakamura *et al.*, 2006; Tian *et al.*, 2007; Zenke-Kawasaki *et al.*, 2007). In addition to that, Rbck1 includes a transactivation activity and has been shown to act as a transcriptional co-activator upon HBV (hepatitis B virus) infection (Cong *et al.*, 1997). Within this work, localization studies in transfected Cos-7 cells revealed that Rbck1 is a cytoplasmic protein. In contrast to Sipl1, Rbck1 was not translocated into the nucleus by Six2 neither in presence nor in absence of Eya1. Tatematsu *et al.* compared localization of overexpressed and endogenous Rbck1 in HEK293 cells, showing that there are striking differences. Overexpressed Rbck1 was exclusively detected

in the cytoplasm, whereas endogenous Rbck1 was present in both, cytoplasm and nucleus (Tatematsu *et al.*, 2005). Thus, localization of overexpressed Rbck1 does not necessarily reflect localization of the endogenous protein, which could be affected by presence of Eya1 and/or Six2.

4.4.3 Physiological relevance of the Eya1-Sipl1/Rbck1 interaction

Both *Sipl1* and *Rbck1* are expressed together with *Eya1* in many tissues of a mouse embryo, which is in line with the hypothesis that the proteins act together during embryonic development. To further underline this assumption studies in zebrafish have been performed. A zebrafish ortholog of *Eya1* was identified by Sahly et al. in 1999. Results by Sahly *et al*. indicated that there is a remarkable similarity in both structure and expression pattern between *Eya1* orthologs of higher and lower vertebrates (Sahly *et al.*, 1999). In zebrafish, *eya1* expression was detected in several organs during embryogenesis, as for example the ear, the branchial arches and the somites. In fact, the expression pattern of *eya1* during zebrafish embryogenesis reflects the expression pattern of its mouse ortholog. One has to point out that, in contrast to mammalian *Eya1*, zebrafish *eya1* is not expressed during renal development. This is due to the fact that teleosts do not form a metanephros which has been described to be the major site of *Eya1* expression during mammalian kidney development (Kalatzis *et al.*, 1998; Sahly *et al.*, 1999).

In the course of this work, one *Sipl1* ortholog, one *Rbck1* ortholog, and one ortholog, termed *sipl1-rbck1*, which seems to be a fusion of both, have been identified in zebrafish. Based on the conservation within the minimal Eya1 binding region, zebrafish Sipl1 and Sipl1-Rbck1 were shown to be more closely related to mouse Sipl1 whereas zebrafish Rbck1 is more closely related to mouse Rbck1 within this region. Expression analysis by *in situ* hybridization revealed that *sipl1* is widely expressed during zebrafish embryonic development, especially in the head region of the embryo. In contrast, *sipl1-rbck1* expression was restricted to the region of the midbrain-hindbrain area and the developing ear. A more detailed analysis of the expression in the developing zebrafish ear revealed that *sipl1-rbck1* and *eya1* are co-expressed in a region corresponding to the sensory epithelium of the inner ear. Knockdown of *sipl1-rbck1* expression by injection of each of two independent morpholinos led to a similar phenotype with malformations of the

zebrafish ear and the lower jaw which is derived from the branchial arches. In fact, the observed phenotype resembles the characteristic symptoms of BOR syndrome in human. Strikingly, *eya1* has also been reported to be essential for proper formation of the zebrafish ear as shown by knockdown experiments in previous studies (Kozlowski *et al.*, 2005). Conclusively, the data presented is compatible with the assumption that the two zebrafish proteins, Eya1 and Sipl1-Rbck1, interact with each other during embryonic development.

In contrast, knockdown of *sipl1* resulted in severe malformations of the whole zebrafish embryo with truncated head and tail region, and improperly developed brain and eyes. This phenotype reflects the more ubiquitious expression pattern of *sipl1* during zebrafish embryogenesis and suggests a more general role for this gene in embryonic development. Analysis of the *rbck1* expression pattern during zebrafish embryogenesis also revealed an overlap with that of *eya1*. *In situ* hybridization showed that both are expressed in the branchial arches of the developing zebrafish. Knockdown of *rbck1*, which was performed by my colleague Christina Ebert, resulted in malformation of the zebrafish head especially in the region of branchial arches (Christina Ebert, unpublished data).

However, the interaction between the zebrafish orthologs of Eya1 and Sipl1 or Rbck1 needs to be confirmed. An elegant approach to address the importance of the interactions *in vivo* would be a comparison of the ability of wild type mRNA or mutant mRNA which harbours a deletion of the interaction site to rescue the knockdown phenotype.

The zebrafish studies performed in this work provide a first insight into the physiological importance of the Eya1-Sipl1/Rbck1 interactions. The physiological importance of the interactions in the context of mouse and human embryogenesis is not clear. The endogenous interaction between the proteins during mammalian embryogenesis needs to be verified. From *Eya1* knockout analysis in mice it is known that *Eya1* is essential for the development of many organs, as kidney, ear, thymus etc. (Xu *et al.*, 1999; Xu *et al.*, 2002). Knockout studies of *Sipl1* or *Rbck1* in mice could clarify the role of the genes in organ development. Furthermore, they could provide first hints for the importance of the Eya1-Sipl1/Rbck1 interactions in organogenesis. Unfortunately, by now, no knockout mice for *Sipl1* or *Rbck1* are available.

4.4.4 Functional consequences of the Eya1-Sipl1/Rbck1 interaction

Eya proteins have been shown to act as co-activators in several transcriptional activation complexes which mediate activation of gene expression during embryonic development. One of the best studied examples is the activation of the myogenin promoter by complex formation of Eya2 and Six1/4. Six1 and Six4 bind to conserved MEF3 sites within the *Myogenin* promoter, thereby activating gene expression (Spitz *et al.*, 1998). This activation is significantly increased by interaction with Eya2 (Fan *et al.*, 2000). Furthermore, it has been described that binding of other co-factors to Eya2, as for example Dach2, can even further enhance the transactivation potential of the Eya2-Six4 complex (Pascal Maire, personal communication). It has been shown that Sipl1, and presumably Rbck1 as well, can interact with Eya2. Furthermore, it has been demonstrated that interaction of Eya2 with Sipl1 increases Eya2-Six4-mediated transactivation. Presumably, Sipl1 has a comparable effect on the transactivation function of Eya1. Interestingly, also Rbck1 enhanced the transactivation potential of the Eya2-Six4 complex, although localization studies showed that Rbck1 is a cytoplasmic protein. This discrepancy between the transactivation and localization studies might be due to the use of different Rbck1 fusion constructs (Flag-Rbck1 or RFP-Rbck1, respectively). In fact, Rbck1 has been described before to function as a co-activator of transcription upon HBV infection (Cong *et al.*, 1997). Furthermore, endogenous Rbck1 has been shown to shuttle between the cytoplasm and the nucleus (Tatematsu *et al.*, 2005). Therefore, it is conceivable that Rbck1 indeed acts together with Eya2 and Six4 in the activation of target gene expression in the nucleus.

Based on the results from transactivation analyses the following model can be proposed: Eya1/2 bind to Six proteins leading to translocation of the complex into the nucleus where the proteins activate expression of organ-specific genes. Sipl1 and Rbck1 bind as co-factors to Eya1/2 thereby enhancing the transactivation potential of the complex (Fig. 4.2).

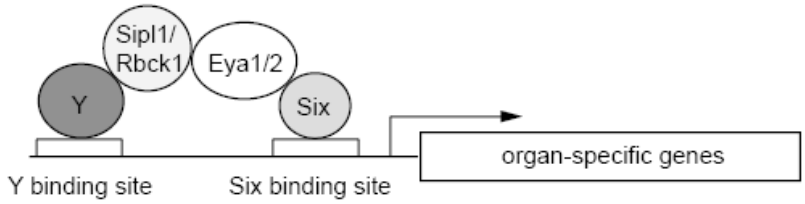

Fig. 4.2. Model for an Eya-Six-Sipl1/Rbck1 complex involved in the activation of organ-specific genes. Eya1/2 directly interacts with Six which mediates binding to the promoter region of the respective genes. The transactivation activity of the complex is increased by interaction of Eya1 with Sipl1 or Rbck1 which potentially interact with another adjacent transcription factor/complex Y (modified from Relaix and Buckingham, 1999).

Another interesting question is, whether the interaction of Eya1 with Sipl1 or Rbck1 has an effect on the binding of other known interaction partners to Eya1. Localization studies performed in this work suggest that the three proteins Eya1, Sipl1 and Six2 form one complex since both Eya1 and Sipl1 are translocated into the nucleus in presence of Six2. This hypothesis was further underlined by results from transactivation experiments showing that Sipl1 acts synergistically together with Eya2 and Six4 in activation of gene expression. However, biochemical analysis is necessary to verify that Sipl1 and Six proteins can bind simultaneously to Eya1. Interestingly, Eya1 and Rbck1 have a common interaction partner: CBP (CREB binding protein). CBP is a well-characterized co-activator that functions as a key-integrator in various transcription activating complexes (Agalioti *et al.*, 2000). CBP has been described to act as a linker for the interaction between mammalian Eya and Dach proteins thereby mediating target gene activation (Ikeda *et al.*, 2002). Strikingly, interaction with CBP has also been implicated in the regulation of Rbck1 transactivation function (Tatematsu *et al.*, 2005). It is tempting to speculate that CBP is also part of the Eya1-Rbck1 complex and presumably involved in target gene activation.

As described above, Rbck1 has been shown to function as an E3 ubiquitin ligase for several unrelated proteins. Interestingly, most of the described targets bind to the conserved Ubl domain of Rbck1. Results from this work implicated that also the interaction with Eya1 occurs via the Ubl domain of Rbck1 leading to the possibility that Eya1 is a target of Rbck1 E3 ubiquitin ligase activity. Preliminary studies in cell culture did no show evidence for enhanced Eya1 degradation in presence of Rbck1 (data not shown). Also the results regarding the ability of Rbck1 to enhance Eya-Six-mediated transactivation speak against a role of Rbck1 in the degradation of Eya1. But, similar to other Rbck1 targets, Eya1 could be

degraded in a context-specific manner dependent on a specific post-translational modification of the protein. Another possibility is that Rbck1 function requires the association with adaptor proteins, as it was described for the degradation of activated PKC (Nakamura *et al.*, 2006). However, the hypothesis that Rbck1 might act as an E3 ubiquitin ligase involved in the degradation of Eya1 has to be validated by further experiments.

A poorly understood feature of Eya proteins is their activity as phosphatases. The phosphatase activity of Eya1 has been indicated to be essential for normal kidney development since only *EYA1* mutations associated with BOR syndrome, but not with BO-syndrome or ocular defects, abolish dephosphorylation of target peptides *in vitro* (Rayapureddi *et al*., 2006; Musharraf *et al*., in press). To date, nothing is known about the regulation of this function. Also, *in vivo* targets have not been identified so far. In this regard, it would be interesting to examine whether Sipl1 and Rbck1 have an effect on the activity of Eya1 as a phosphatase or whether they are target proteins themselves.

Of course, it is also possible that Eya1 influences the functions of its interaction partners Sipl1 and Rbck1. No protein function of Sipl1 has been described so far. Identification of a specific activity of Sipl1 could provide important insights into the role of the Eya1-Sipl1 interaction. In contrast, Rbck1 has been demonstrated to function as a transcriptional activator and as an E3-ubiquitin ligase. A possible effect of interacting Eya1 on these activities remains to be elucidated.

4.4.5 *SIPL1* and *RBCK1* mutations in BOR syndrome

Mutations in the human *EYA1* gene are associated with branchio-oto-renal (BOR) syndrome. Interestingly, also mutations in *SIX1* and *SIX5* have been identified from BOR patients (Ruf *et al.*, 2004; Hoskins *et al.*, 2007). Both *SIX1* and *SIX5* encode interaction partners of Eya1. All BOR-associated *SIX1* mutations lead to weakening of the interaction with Eya1 (Ruf *et al.*, 2004). Strikingly, two of the four so far identified BOR-associated *SIX5* mutations also compromise the interaction with Eya1 (Hoskins *et al.*, 2007). In the course of this work, Sipl1 and Rbck1 were identified as novel interaction partners of Eya1. Hence, an intriguing question was whether mutations in human *SIPL1* or *RBCK1* are associated with BOR syndrome. Indeed, one *SIPL1* as well as one *RBCK1* mutation was identified from screening of 91 patients suffering from BOR syndrome. These finding suggests that both

genes play an essential role in the development of the organs affected in BOR patients, which are the ears, the kidneys and the branchial arch derivatives. Strikingly, knockdown of the zebrafish orthologs of *Sipl1* and *Rbck1* affects the development of the same organs. In particular, knockdown of the *Sipl1* ortholog *sipl1-rbck1* results in a BOR-syndrome-like phenotype, including malformations of the ear and the lower jaw which is derived from the branchial arches. The knockdown of *rbck1* expression leads to malformations of the zebrafish head, especially in the region of the branchial arches.
Binding studies in yeast demonstrated that the *SIPL1* mutation leads to loss of the interaction with Eya1. In contrast, an effect of the *RBCK1* mutation on the Eya1-Rbck1 interaction could not be detected. However, how the mutations influence the functional consequences of the interactions remains to be determined. It has been shown that both Sipl1 and Rbck1 act together with the Eya-Six complex to activate gene expression. In this regard, it would be interesting to analyze if the BOR-associated mutants of Sipl1 and Rbck1 are still able to do so.

In summary, it has been demonstrated that Sipl1 and Rbck1 are two novel interaction partners of Eya1. The interaction with Eya1 is mediated via the conserved Ubl domain of Sipl1 and Rbck1. Furthermore, the respective zebrafish orthologs of *Sipl1* and *Rbck1* are expressed together with that of *Eya1* in several tissues during vertebrate embryogenesis. Strikingly, the *Sipl1* ortholog *sipl1-rbck1* and *eya1* are co-expressed in the developing zebrafish ear and essential for its development which is in line with the hypothesis that the respective proteins interact with each other during organogenesis. Both Sipl1 and Rbck1 have been shown to act as co-activators of the Eya-Six complex in transactivation studies providing a first hint for the functional consequences of the interaction. Furthermore, by screening of BOR-patient material one *SIPL1* as well as one *RBCK1* mutation were identified. Further experiments regarding the functional consequences of the interaction of Sipl1 or Rbck1 with Eya1 should clarify the importance of the interaction during human organogenesis and reveal how the BOR-associated mutations in the respective genes lead to disease.

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APPENDIX A: OLIGONUCLEOTIDES

Generation of Eya1 mutants for *in vivo* ubiquitination assays

N-term-STOPf	5'-CCCGGATTCTGACCTTTAAAGAGTGTTCATCTGGGAC-3'
N-term-STOPr	5'-GTCCCAGATGAACACTCTTTAAAGGTCAGAATCCGGG-3'
K301Af	5'-CGAGGTTCAGATGGGGCGTCACGTGGCCGA-3'
K301Ar	5'-TCGGCCACGTGACGCCCCATCTGAACCTCG-3'
Δ426-491f	5'-ACTGGTGTCCGAGGTACGAACTGTGTGAAT-3'
Δ426-491r	5'-ATTCACACAGTTCGTACCTCGGACACCAGT-3'
H489stop-f	5'-GCCCTCTCCCTCATCTAGTCCCGGACGAAC-3'
H489stop-f	5'-GTTCGTCCGGGACTAGATGAGGGAGAGGGC-3'

Generation of Eya1 fragments for cloning into pGBT9:

(1) Bait constructs for the yeast two-hybrid analysis

YmEya1f	5'-ACGTGAATTCGAAATGCAGGATCTAACCAGC-3'
YmEya1Df	5'-ACGTGAATTCTCCGAGCGGCTGCGTCG-3'
YmEya1r	5'-ACGTGTCGACTTACAGGTACTCTAATTCCAAG-3'
YmEya1r2	5'-ACGTGAATTCGAAATGCAGGATCTAACCAGC-3'

(2) Localization of the transactivation domain

mEya1-N106f	5'-ACGTGAATTCGCTGCATATGGGCAAACACAG-3'
mEya1-N159f	5'-ACGTGAATTCCAGACGGGATTTCTTAGCTATG-3'
mEya1-N179f	5'-ACGTGAATTCTACAGCTACCAGATGCAAGG-3'
mEya1-N199f	5'-ACGTGAATTCAATTCACTCACCAACTCCTCC-3'
mEya1-N239f	5'-ACGTGAATTCATGACGAGCAGTAACACCAG-3'
mEya1-N108r	5'-ACGTGTCGACTTAATATGCAGCCATAGTTTGTGAG-3'
mEya1-N161r	5'-ACGTGTCGACTTATCCCGTCTGTCCAGGTGAC-3'
mEya1-N241r	5'-ACGTGTCGACTTAGCTCGTCATGTAGTGTGCTG-3'
mEya1-N320r	5'-ACGTGTCGACTTAAAGGTCAGAATCCGGGGGA-3'

(3) Localization of the Sipl1-binding site

mEya1-N366f	5'-ACGTGAATTCTTCAACTTGGCAGACACACATC-3'
mEya1-N441f	5'-ACGTGAATTCAAAGAGATCTACAACACCTACA-3'
mEya1-N440r	5'-ACGTGTCGACTTATACTCGTCTGTAGCGGAAGG-3'
mEya1-N515r	5'-ACGTGTCGACTTATCCATATAGCAGGACTTTTGCC-3'

Generation of Eya2 C-terminal fragment for cloning into pGBT9:

YmEya2Df	5'-ACGTGAATTCACAGAGAGGCCACATCGAG-3'
YmEya2r	5'-ACGTGTCGACTTATAGATACTCCAGTTCCAGGG-3'

Generation of Eya3 C-terminal fragment for cloning into pGBT9:

YmEya3Df	5'-ACGTGAATTCGATGCTGATGATCAGGCCAG-3'
YmEya3r	5'-ACGTGTCGACTTAGAGGAAGTCAAGCTCTAAAG-3'

Generation of Sipl1 fragments for cloning into pGADT7:

(1) Full-length Sipl1 for yeast two-hybrid analysis

Sipl1fw	5'-ACGTGAATTCATGTCGCCGCCCGCCGG-3'
Sipl1rv	5'-ACGTCTCGAGCTAGGTGGAAGCTGCAGCA-3'

(2) Localization of the Eya1-binding site

Sipl1-184f	5'-ACGTGAATTCGATGAGAAAGCAGCGGCCC-3'
Sipl1-219f	5'-ACGTGAATTCCTACAAGTCACAGTTGAAGA-3'
Sipl1-261f	5'-ACGTGAATTCCCACCAGCTGTGCAGCGC-3'
Sipl1-199r	5'-ACGTCTCGAGCTAATGATGCTGCGCCAGG-3'
Sipl1-266r	5'-ACGTCTCGAGTTAGCGCTGCACAGCTGGTGG-3'
Sipl1-297r	5'-ACGTCTCGAGTTAGAGCAAGTAAAGAAAAGCAG-3'
Sipl1-343r	5'-ACGTCTCGAGTTACTGAGAGGGACTTGGGAGA-3'

Generation of the Sipl1 mutant R365C by site-directed mutagenesis:

mSipl1-R365Cf	5'-CAATGCCTCAAACTGCCCTGGCTGTGAGATG-3'
mSipl1-R365Cr	5'-CATCTCACAGCCAGGGCAGTTTGAGGCATTG-3'

Generation of the full-length hSix1 fragment for cloning into pGADT7:

YhSix1f	5'-ACGTGGATCCTGCCGTCGTTTGGCTTTAC-3'
YhSix1r	5'-ACGTCTCGAGTTAGGACCCCAAGTCCACCAGAC-3'

Generation of the full-length Gαi2 fragment for cloning into pGADT7:

Galphai2f	5'-ACGTGGATCCGCTGCACCGTGAGCGCC-3'
Galphai2r	5'-ACGTCTCGAGTCAGAAGAGGCCACAGTCC-3'

Introduction of the Gαi2-activating mutation Q205L into pGADT7-Gαi2:

Galphai2Q205Lf	5'-GATGTGGGTGGACTGCGGTCTGAGCG -3'
Galphai2Q205Lr	5'-CGCTCAGACCGCAGTCCACCCACATC -3'

Generation of the full-length Rbck1 fragment for cloning into pGADT7:

YmRBCK1f	5'-ACGTGAATTCGCCCTGAGCCTTGCCCG-3'
YmRBCK1r	5'-ACGTCTCGAGTTAGTGGCAGTTTTGACAGCT-3'

Generation of the Rbck1 mutant Q228E by site-directed mutagenesis:

mRbck1-Q228Ef	5'-TGCAGCTGGAGGAGAGGAGCCTGGTG -3'
mRbck1-Q228Er	5'-CACCAGGCTCCTCTCCTCCAGCTGCA -3'

In-frame cloning of potential interaction partners by mutagenesis:

in-frame1-fw	5'-CATGGAGGCCCCGGGATCCGAATTC-3'
in-frame1-rv	5'-GAATTCGGATCCCGGGGCCTCCATG-3'

Oligonucleotides used for qRT-PCR from mouse tissues:

mmeya1.1-for	5'-TGGCCCTACCCCTTCCCCAC-3' (J. Tomasch, diploma th.)
mmeya1.1-rev	5'-TGACAATCCACTTTCCGTCTT-3' (J. Tomasch, diploma th.)
actb_1f	5'-TGTTACCAACTGGGACGACA-3' (J. Klattig, PhD thesis)
actb_2r	5'-GGGGTGTTGAAGGTCTCAAA-3' (J. Klattig, PhD thesis)

Oligonucleotides used for RT-PCR from mouse tissues:

YmEya1f	5'-ACGTGAATTCGAAATGCAGGATCTAACCAGC-3'	
YmEya1r2	5'-ACGTGAATTCGAAATGCAGGATCTAACCAGC-3'	
Sipl1_exon6f	5'-CCTGTGTATGCCTGAACGAA-3'	
Sipl1_exon8r	5'-AGAGGATCCCAAGCACAGG-3'	
Tbp-1f	5'-GGCCTCTCAGAAGCATCACTA-3'	(J. Klattig, PhD thesis)
Tbp-2r	5'-GCCAAGCCCTGAGCATAA-3'	(J. Klattig, PhD thesis)

Oligonucleotides used for RT-PCR from zebrafish:

sipl1-exon2f	5'-ATGAGCTGCGTCTCCTCAAG-3'
sipl1-exon6r	5'-GCACAAACACTGAGAGATGATCC-3'
sipl1-rbck1-e1f	5'-TATACGGCCGCTATGTCTCC-3'
sipl1-rbck1-e3r	5'-GGAGGTAGTCGCCCTTCTTC-3'

Oligonucleotides used for generation of mouse-specific riboprobes:

Sipl1fw	5'-ACGTGAATTCATGTCGCCGCCCGCCGG-3'
Sipl1rv3	5'-ACGTCTCGAGCTAGTCGAGGAAGTGCACGCTG-3'
mRBCK1_fw1	5'- CCCTCAGGGTGCAAGTAAAA-3'
mRBCK1_rv2	5'- CTCAAGGTGCTTCGGTTCTC-3'

Oligonucleotides used for generation of zebrafish-specific riboprobes:

dr_eya1-F3	5'-GGACTATCCTTCCTACCCGACG-3'	(Kozlowski *et al*., 2005)
dr_eya1-R4	5'-GTGGCAGCAGCGTGGAATCCG-3'	(Kozlowski *et al*., 2005)
sipl-f1	5'-GTGGGCTCCGACTCTCTG-3'	
sipl-r1	5'-GCACAAACACTGAGAGATGATCC-3'	
sipl1-rbck1-e2f	5'-AGTTTGGCAACACCTCCACA-3'	
sipl1-rbck1-e5r	5'-CAATTGTGGAGTGTGGGAAG-3'	
rbck1_fw1	5'-TATGGCTTCCATCCGTCTCT-3'	
rbck1_rv2	5'-TCCAGCATCTCTGTGGTCTG-3'	

Cloning of the full-length coding sequences of the zebrafish orthologs:

sipl1Chr12s	5’-ACGTGGATCCGCCACCATGTCGACGAGCACGGGCTG-3’
sipl1Chr12as	5’-ACGTCTCGAGCTCTGTGTGTTTACGCCGCAC-3’
sipl1-rbck1f	5’-ACGTGGATCCACCATGTCGCTGAGCTCCGGCG-3’
sipl1-rbck1r	5’-ACGTCTCGAGTTAGTGACAGTTCTGGCATTTTG-3’
rbck1f	5’-ACGTGAATTCGCTGCTCTTGATGCCTCTAG-3’
rbck1r	5’-ACGTCTCGAGTTAGTGGCAGTTCTGGCATTG-3’

APPENDIX B: ZEBRAFISH *SIPL1*

CODING SEQUENCE

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   1 ATGTCGACGA GCACGGGCTG CAGTACGGTG CTGATGTCGG TGCGGGTCTC
  51 CGTGCGGCCG CTGCCGGTGG GCTCCGACTC TCTGCGGCTG CAGCTCAGCA
 101 TGAACCCGAA CCGCGCGGGG CTCTTCACCC TCACACTGAG ACACACCGAC
 151 CGCGGGGGAC GTAGCGTGTC TCTGGCAGAG TTCGACCTGC GCTCGGTGCA
 201 GTATGAGCTG AAGTCTCCGC GCTGTCATGA GCTGCGTCTC CTCAAGCCGC
 251 CGCACGACTG TCTGAGCTTC AGCTTCCGCA GCGAGCAGGA GGCGCAGGAG
 301 TGGGCGACCG TCGTCATGTC CTCACTGCGG GAGTCACACA GAGTTGCCAG
 351 TATTTGTCAG GAAGGCCTGC AGTATGTGAA GAGTGGAGAG AAAAGCGCAG
 401 TCCTGTCTCT GTCAATGAAA GAGGAGCTGT GTGTGGAGCT TTCCAGAGCG
 451 ATAGAGGCTG GAGATGCTCA GGCCGCTGCG CGTTACGCCA CAGATCTGGC
 501 CCAGCAGCAG ATGACGCTCA GCATTCAACC AGCGCCGCGC GACACCGACG
 551 ACAAAGACAT CAGCTTGGCT GTGATAGTGG AAGATGCGTC GTCTTCCTGC
 601 TGTGTGACGG TGAAAATCCA CCCGCACGTG ACCGTCGCCT CGCTAAAGCA
 651 GCAGATGTTT GTGGAGTACG GTTTTCACCC GCGGGTGCAG CGCTGGATCA
 701 TCTCTCAGTG TTTGTGCTCT GACAGTCGCT CGGTGTCGTC CTATGGCGTC
 751 TGCAGGGATG GAGACACTGC GTTCCTCTAC CTGTTATCCG CAGGTCACGC
 801 CAGCCTCAGC CAGCAGCAGG AGAACGGGCT CTCCGTGCCC ACAGCGGCTC
 851 CAGCAAACGC TTCACTTTCA GCCCCGGCGG GCGGCGGCAG CAGCGGGCAC
 901 GACTGGAGGG CGTACAGCAC TCTACCACCA CGCTTCAGCC ACGCCAGCAC
 951 AGGCAGCGGC GGCTCAGAAA AGCCCAGCGT CACTGACATC ATTAACCTGG
1001 AGATGCTGCA ACTCGGAGGC TCCAAACTCA AGTCCAGTAA CACACAGTCA
1051 GGCTGGCCCT GCCCGTCCTG TACGTTCATA AATAAATCTA CGAGACCCGG
1101 CTGTGAAATC TGCAGCACTG ACCGGCCCAA TCCTCCACAT CACACTCATC
1151 TTCAACAGGA GAAGTCAAGA AGATCCAACC AGTGA
```

APPENDIX C: ZEBRAFISH *SIPL1-RBCK1*

CODING SEQUENCE

```
   1 ATGTCGCTGA GCTCCGGCGG GTGGACTCGA GCATCTCCGC CGGCCCAGTC
  51 CTCGTCGTCT CACCTCGGCC ACGAGGCCTC GCAGTCGGCA TGCAGCACTG
 101 TTTTGATGTC GGTGAAGGTG TCGGTATGCC ATTCCGGTAT ACGGCCGCTA
 151 TGTCTCCCCG GAGCAGGAGA CGAATCTCTC CGTCTTCAGC TGAGCATGGA
 201 CCCCGGGAAA GCAGGGGAAT TCCGCCTCGC GCTGCGGGAT ATCAGCGCTA
 251 CAGCGGCCGG GCGCAGTGTG TTTATTGCTG AATTTGACCT CAAGACTGTT
 301 CAATATGAAG TCAAGACGCC ACTGTGCCAT GAACTGAGTT TGGCAACACC
 351 TCCACATGAC CGTATCTCGT TCAAATTTCG GTGTGAACAG GAAGCGCAGG
 401 AGTGGGCCAC CGTGGTGATG TCATCCCTCC TGGCAATTAG TTCCTCCACT
 451 GAAGAAGGGC GACTACCTCC TCCACCTCTG GCCACACAGA GCAAAGCACC
 501 CATGCCACGC ACAGAGGAGA TCTGTGCGGA GCTGGTCAGT GCGATAGAGG
 551 CAGGTGATGT ACGGTCTGCA TCAGTCTGTG CATCGTCTTT AGCTAAACAG
 601 AAAGCTGCTC TGAGCATTCA GCCCTCGAAA CGAAACTACA CAGACTCTGA
 651 AGTTTGCTTG GCTGTGGTGG TGGAAGATGC ATCCTCTTCC TGTTGTGTCT
 701 CGGTGAAAGT CTTCCCACAC TCCACAATTG GTGCTCTCAA ACAGCAGGTC
 751 TTCTCAGACT ATGGTTTTCA TCCACGTGTT CAGCGCTGGG TCATTGGTCA
 801 GTCTTTGTGC TCTGACCATC GCTCTCTGGC ATCCTATGGA GTCCAGCGAG
 851 ATGGAGACAC TGCGTTCCTT TATCTCATTT CTGCCCGTCA GGCTCGTCTC
 901 AGTCGAGGGA TCTACCAGCA AGATCAGGAA AGTGCTTTAC TCATGGTGCC
 951 AACGACTCAC CAGGCCCACC AAGAAGCAGT TAGCAATGGG CCAGCAGCAC
1001 TCAACACAGC CTCAAGACCA TACAGCACCC TGCCTACAAG ACTTCATAAC
1051 AGCCATAATA CTCTGAGTAA CAATGCTGGA GGATCAGAGA GGTTGGGTTT
1101 AAGTGATATT CGTGACCTGA TCAACCTTGA GCTGCCACAG CTAAATGAAG
1151 CCCTGGGTCC CAACAGAACA AGCATACAGC CGGGATGGGC CTGCCCGACC
1201 TGTACGTATA TTAATAAACC AACCCGTCCA GGCTGTGAAA TGTGCAGCGC
1251 AGACAGACCT GAAGGATACA CTGTTCCTGG CAACTACAGA CCAGATGCTT
1301 TAGAGCTGCG ACGCATTCAG CAAGAAAAAG AAGCAATAAG GCAGTACCAA
1351 CAGGCCAGGG AAACAGAGCG CAGAGAGAAC TTTGCTCGCC TGGTACAGAT
1401 GGACGGGCAG GATCTGGTGC CAAACCCGGA GCGAGTGGAG TGCAGGATCT
1451 GTTACGTGGA GCTGGAGTCT GGTGAAGGAG TGCTCCTGAG AGAGTGTTTG
1501 CACTGCTTCT GCAAAGAGTG TCTGCGCTCT GTGATTCTGA TGTCGGAGGA
1551 CCCTCAGGTG GCTTGTCCAT ACAGAGACGA GTCCTACGCC TGTGACTGCG
1601 TCCTGCAGGA AAGAGAAATT CGAGCTCTGG TGTCAGTGGA TGATTATCAG
1651 CACTGGCTGC AGAGGGGTCT GTCTGTGGCG GAGTCTCGCT GTGAGGGCAG
1701 TTATCACTGC GCTACTGCCG ACTGTCCTGG CTGGTGTGTT TATGAGGACA
1751 CTGTCAACAC TTTCCACTGT CCAGTGTGCA AGAAACAAAA CTGCCTGCTC
1801 TGCAAGGCTA TTCATGAAGG GATGAACTGT AAGCAGTATC AGGATGATCT
1851 TACAGCTCGA GCCATCAATG ACTCTGCAGC TCGAAGGACC AGAGACCTGC
1901 TGAAGACTCT TGTTAATTCT GGAGAGGCGA TGCATTGCCC CCAGTGCGGA
1951 ATCATTGTCC AGAAGAAGGA GGGCTGTGAT TGGCTGCGCT GTACCGTCTG
2001 CCACACTGAG ATCTGCTGGG TCACCAGAGG GCCGCGCTGG GGGCCCAAAG
2051 GTCCAGGAGA CATAAGTGGA GGCTGCCGCT GCAATGTCAA CAAGCAGAGA
2101 TGCCATCCAA AATGCCAGAA CTGTCACTAA
```

APPENDIX D: ZEBRAFISH *RBCK1*

CODING SEQUENCE

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   1 ATGGCTGCTC TTGATGCCTC TAGAAATGTA ACGGAAGCGG AGGATGCCGC
  51 TCGACTTCTT AGTGACGCCA TCAACTCCAG AGACAAAGAT GAAGCAACTA
 101 AATACCTGAA TCAACTTCTG GATCTGAAAC TCCCAGTGAG CGTCAAAATT
 151 AATCCAGATG CATATTCTCA AGACAACATC AGATTAAGAG TTGGAGTGGC
 201 TGATGCTGAA TCAGAACATC ACATCCCAAT AACAGTGATG GTACCAGTTT
 251 ACATGACAAT ATCCGAGCTG AAGGAAAAGA TCAACGGTGA CTATGGCTTC
 301 CATCCGTCTC TCCAGCGCTG GGTGATCGGG AAGCGTCTCG CCCAGGACAA
 351 AGAAACCCTT TATTTTTATG GGATCCGAAA CCACGATGAT TCTGCCTTCC
 401 TCTTCATCCG CTCTGCTCAG TCTGTCAATC TGCGCCGCGA ACAAGAGAGA
 451 AGAGAGAAAG AGGAGAGGCA GATCGATGTT ATAATGGAGA CCATTGAACG
 501 TCCTCTTCAA CGTCCACCAG AGCGCATCAC TAGAGGAAAC ACTCGCCCTG
 551 CTCTACCTCC AAAACCCAAA TTTCTGGATG GTTGGGCATG TCCTCAGTGC
 601 ACGTACCTGA ATAAACCGAC ACGTCCAGGT TGTGAGATGT GCAGCACGGC
 651 GAGACCCGAC AACTATCAGG TGCCGGACTT GTACCAGCCG GACGAGTCTG
 701 AAACCAGGAG ACTACAGCAG GAGGAACTCG CCAGCCTGCA GTATGAGCAG
 751 TCTCTGTTAC AAGAGGAAGA AAGAAATTTC CTTGAACGGC AGAGGAATTA
 801 CGAAGAACTC CTGCAAACAG ATGCACACAG CCTGGTGGGA AACACAGATC
 851 AGCTGGAGTG CGCCATTTGT TTTGGCACCA TCATGCCAGG AGAGGGCGCC
 901 GTTTTAAGAG AGTGCCTTCA TAGCTTCTGC AGGGACTGTC TGAAAGGGAC
 951 CGTAGTGAAC TGCCTGGACG CTGAGGTTTG TTGTCCTTAT GGAGACAACG
1001 CTTATGCCTG TAACTGCAAA CTCCAAGATC GAGAGATCAA ATCTCTTCTC
1051 ACTCAGGACG AGTACCAAAA ATTCCTAGAA CTGCGGCTGA ATATCGCTGA
1101 GTCCCGCAGT GAGAACAGTT ACCATTGCAA AACCCCGGAC TGCGCTGGCT
1151 GGTGCATCTT CGAAGATGAC GTTAACGAAT TTAAGTGCGA CATCTGCAAT
1201 GAGACCAACT GCCTCCTCTG CAAGGCTATT CATAAAGGAA TGAACTGCAA
1251 GGAGTACCAG GACGACCTAC GTGTGAGAGC GCAGAACGAT GAAGCGGCTC
1301 GGCAGACCAC AGAGATGCTG GATCAACTGC TAAAGAATGG CGAGGCGATG
1351 AACTGTCCCA AATGTCAGGT GATCGTCCAG AAGAAAGACG GCTGTGACTG
1401 GATCTGTTGT CTAATGTGCA AAACCGAGAT CTGCTGGGTG ACCAAACAAG
1451 CCCGATGGGG ACCTTTGGGT GCTGGAGACA CATCAGGCGG ATGCAAATGT
1501 CGAGTAAACG GAGTCCTTTG TCATCCGCAA TGCCAGAACT GCCACTAA
```

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Jena, 30.06.2008 Kathrin Landgraf

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Poster:

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SELBSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel, Literatur und persönlichen Mitteilungen angefertigt habe.

Ich versichere, dass ich diese Arbeit noch an keiner anderen Hochschule eingereicht habe, um ein Promotionsverfahren eröffnen zu lassen. Die Hilfe eines Promotionsberaters wurde von mir nicht in Anspruch genommen. Dritte haben von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit der vorgelegten Dissertation stehen.

Die Promotionsordnung der Biologisch-Pharmazeutischen Fakultät ist mir bekannt.

Jena, 30. Juni 2008 Kathrin Landgraf