Eya4 Induces Hypertrophy via Regulation of p27kip1

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Background—E193, a heterozygous truncating mutation in the human transcription cofactor Eyes absent 4 (Eya4), causes hearing impairment followed by dilative cardiomyopathy.

Methods and Results—In this study, we first show Eya4 and E193 alter the expression of p27kip1 in vitro, suggesting Eya4 is a negative regulator of p27. Next, we generated transgenic mice with cardiac-specific overexpression of Eya4 or E193. Luciferase and chromatin immunoprecipitation assays confirmed Eya4 and E193 bind and regulate p27 expression in a contradictory manner. Activity and phosphorylation status of the downstream molecules casein kinase-2α and histone deacetylase 2 were significantly elevated in Eya4- but significantly reduced in E193-overexpressing animals compared with wild-type littermates. Magnetic resonance imaging and hemodynamic analysis indicate Eya4-overexpression results in an age-dependent development of hypertrophy already under baseline conditions with no obvious functional effects, whereas E193 animals develop onset of dilative cardiomyopathy as seen in human E193 patients. Both cardiac phenotypes were aggravated on pressure overload. Finally, we identified a new heterozygous truncating Eya4 mutation, E215, which leads to similar clinical features of disease and a stable myocardial expression of the mutant protein as seen with E193.

Conclusions—Our results implicate Eya4/Six1 regulates normal cardiac function via p27/casein kinase-2α/histone deacetylase 2 and indicate that mutations within this transcriptional complex and signaling cascade lead to the development of cardiomyopathy. (Circ Cardiovasc Genet. 2015;8:752-764. DOI: 10.1161/CIRCGENETICS.115.001134.)

Key Words: dilated cardiomyopathy • Eya4 • molecular biology • p27Kip1 • signaling pathways

To date, 8 heterozygous mutations in human Eyes absent 4 (Eya4) have been described, all of which present with isolated sensorineural hearing loss (SNHL).1-4 We recently identified a heterozygous truncating Eya4 mutation designated E193, which presents SNHL accompanied by late-onset dilative cardiomyopathy (DCM).5 Unlike the wild-type Eya4 gene, which consists of 21 exons and encodes a protein of 638 amino acids, E193 presents a 4,846-bp deletion and encodes an eponymous frameshift after amino acids residue 193, resulting in 29 new residues and a premature termination signal (Figure 1A). Eya family members, 4 of which exist, are transcriptional cofactors which interact with Six and Dach protein families of real transcription factors in a complex network which is involved in several cellular and developmental processes.6 Eyas possess a divergent N-terminal domain with transactivating and serine/threonine phosphatase activity.7,8 The conserved C-terminal Eya domain contains a tyrosine phosphatase domain and mediates protein–protein interactions. Unlike Drosophila, vertebrate Eyas lack nuclear localization sequence and DNA-binding motif. Six family members were shown to induce nuclear translocation of Eya proteins and to direct them to specific promoter sequences where they act as a complex can exert activator/repressor activity.9,10 Albeit considerable evidence that Eyas are important for cardiac function, only little is known about underlying molecular mechanisms. Eya1 mutations are associated with isolated cardio-facial syndrome,11 Eya2 was shown to regulate mTOR, a critical mediator of physiological hypertrophy,11 and Eya3 mutant mice have been described to have heart problems.12

Clinical Perspective on p 764

We show here that Eya4 together with Six1 negatively regulates the gene for the cyclin-dependent kinase inhibitor p27kip1 (p27) in vitro and in vivo. We hypothesize that the heterozygous truncated E193 variant has a dominant-negative effect over Eya4, thereby relieving Eya4/Six1-mediated transcriptional repression of p27. p27, usually associated with cell cycle regulation and proliferation, is highly expressed in cardiomyocytes and discussed to function as a regulator for the development of cardiomyopathy. We show here that Eya4 together with Six1 negatively regulates the gene for the cyclin-dependent kinase inhibitor p27kip1.
of hypertrophy.\textsuperscript{15,16} It was just recently shown that p27 together with its substrate casein kinase (CK)2\(\alpha\)' represents a feedback loop in differentiated cardiomyocytes and is crucial for hypertrophic growth.\textsuperscript{15} Furthermore, CK2\(\alpha\) isoforms have been reported to activate histone deacetylase 2 (HDAC2), which in turn results in the development of myocardial hypertrophy.\textsuperscript{17}

In the present study, we found that Eya4 overexpression represents a feed-forward loop in cardiomyocytes with its substrate casein kinase (CK)2\(\alpha\)' reported to activate histone deacetylase 2 (HDAC2), which in turn results in the development of myocardial hypertrophy.\textsuperscript{17}

In the present study, we found that Eya4 overexpression results in the development of hypertrophy, whereas overexpression of E193 leads to a DCM-like phenotype. Our results suggest that Eya4/Six1 act as transcriptional repressor of p27 expression, thereby releasing p27-mediated inhibition on CK2\(\alpha\) activity. Subsequently, HDAC2 is phosphorylated and activated, which allows a hypertrophic response. E193, together with Six1, showed an opposite effect on p27, CK2\(\alpha\) activity, and HDAC2 activation. Our data indicates that an intercoordinated Eya4/Six1 signaling cascade is crucial in cardiac physiology, and an imbalance leads to the development of cardiac phenotypes, such as hypertrophy or DCM.

**Methods**

\(\alpha\)MHC-pH\(\alpha\)-Eya4 and -E193 transgenic (TG) mice, the latter corresponding to the truncating mutation described by Schoenberger et al.,\textsuperscript{17} were generated and maintained on a FVB/N\textsuperscript{CrI} background. Transaortic constriction (TAC) was achieved based on standard procedures. All of the procedures followed were reviewed and accepted by the local ethics committee and performed in accordance with the institutional guidelines for animal studies. Analysis of human material was done after review and approval by the local ethics committee. Customized adenovirus was commercially obtained. Cardiomyocytes from neonatal rats (NRCMs) and adult mice were isolated, cultured, and treated as previously described.\textsuperscript{18}

**Statistical Analysis**

Values are presented as mean±SEM. Normality of data was tested using the Shapiro–Wilk test. The nonparametric Mann–Whitney test was used to test for significance between groups with sample sizes of \(\leq 5.\) In spite of non-normality according to Shapiro–Wilk test and variance heterogeneity according to Levene’s test, statistical significance between groups with large sample sizes was calculated using either 1-way ANOVA (when comparing 2 groups) or 2-way ANOVA tests (when comparing multiple groups with 2 factors) followed by Holm–Sidak post hoc testing. Statistical analyses were performed using SigmaPlot 12.0 and SPSS. \(P\) values \(< 0.05\) were considered significant.

All molecular methods were done using established protocols and are described in more detail in the Methods section in the Data Supplement.

**Results**

**Cellular Localization of Eya and E193 Proteins**

Endogenous Eya4 and Six1 expression was analyzed by reverse transcriptase polymerase chain reaction and Western blot (WB) analysis in NRCMs (Figure 1A and IB in the Data Supplement) and adult cardiomyocytes (Figure IIA in the Data Supplement) of 2 experimental settings. Immunofluorescence microscopy showed Eya4 is distributed in cytoplasm and nucleus of both cell types under basal conditions, whereas Six1 is predominantly nuclear (Figure IC and IIB in the Data Supplement). On angiotensin II stimulation or TAC, Eya4 was translocated to the nucleus with only traces remaining in the cytoplasm; Six1 was solely nuclear (Figure IC and IIB in the Data Supplement).

We next investigated cellular localization of the truncated E193 protein (Figure 1A) in comparison to Eya4, alone and in context with Six1, using the overexpression approach. NRCMs were infected with recombinant adenovirus (pAd) encoding DsRed-Eya4 (DsR-Eya4) or -E193 (DsR-E193), alone or in combination with GFP-Six1 (Figure 1B). After 48 h, DsR-Eya4 was detected in cytoplasm and nucleus and completely translocated on GFP-Six1 coexpression (Figure 1B, panels 1 and 3). DsR-E193 was cytoplasmic and, surprisingly, nuclear; coexpression of GFP-Six1 only showed little effect on localization, suggesting E193 translocates irrespective of Six1 (Figure 1B, panels 2 and 4). Noteworthy, Six1-knockdown resulted in a solely cytoplasmic localization of Eya4, whereas E193 was again cytoplasmic and nuclear, proving Six1-independent translocation (data not shown). GFP-Six1 alone showed nuclear localization (data not shown) as seen with endogenous Six1 in stimulated NRCMs (Figure IC in the Data Supplement) and adult cardiomyocytes (Figure IIB). WB (Figure 1C) using nuclear and cytoplasmic fractions confirmed these results: DsR-Eya4 alone was detectable in both cellular compartments, whereas GFP-Six1 coexpression resulted in low cytoplasmic and high nuclear DsR-Eya4 levels; DsR-E193 was cytoplasmic and nuclear under both experimental conditions. Interaction studies revealed DsR-E193 and -Eya4 both were able to immunoprecipitate Six1, albeit to a different extent (Figure 1D).

**p27 Expression in Response to Eya4 or E193 Overexpression In Vitro**

We investigated a possible influence of Eya4 gain-of-function on endogenous Eya4 and p27 expression. NRCMs were infected with adenovirus encoding human E193, Eya4, or murine shRNA_Eya4. Quantitative reverse transcriptase polymerase chain reaction and WB showed transcription and protein expression of Eya4 were not altered on E193 overexpression (Figure 2A and 2B). On Eya4 overexpression, transcript level of endogenous Eya4 was not altered, whereas the total amount of Eya4 protein significantly increased compared with untreated controls. Of note, the detected Eya4 protein band consists of both endogenous and TG Eya4 protein. As expected, Eya4 shRNA significantly blunted Eya4 transcript and protein levels. Concomitant analysis of p27 expression demonstrated a significant reduction in transcript and protein levels on Eya4 overexpression (Figure 2A, middle panel; Figure 2B, middle panel), whereas E193 induced an increase. In parallel, Eya4 knockdown also caused a significant increase in p27 transcript and protein levels. Six1 protein levels were unaltered in either experimental setting, indicating Eya4 does not influence Six1 expression in vitro (Figure 2B, right panel).

Next, we measured \([\text{H}^\text{3}]-\text{leucine}\) incorporation as a surrogate parameter for forced protein synthesis and hypertrophy. Adenoviral delivery of E193 and shRNA_Eya4 both prevented an elevated amino acids uptake (Figure 2C) in NRCMs, indicating a lower rate of protein synthesis as compared with uninfected controls, yet we saw elevated amounts of atrial natriuretic peptide (ANP) and \(\beta\)-myosin heavy chain (\(\beta\)-MHC) compared with controls (Figure IIIA and IIIB in the Data Supplement). pAd-Eya4 led to a significant increase in \([\text{H}^\text{3}]-\text{leucine}\) uptake and, therefore, protein synthesis along with an increase in ANP and \(\beta\)-MHC (Figure IIIA and IIIB). In addition, cardiomyocyte cross sectional areas were slightly but significantly reduced on E193 overexpression and Eya4 knockdown, but significantly increased on
Eya4 overexpression when compared with untreated controls (Figure 2D). Our results imply that an overexpression of Eya4 is sufficient to trigger cellular cardiac hypertrophy and support a role for Eya4 in the regulation of cardiac physiology.

It is known that Eyas are directed to target genes by Six proteins. We found 2 putative Six1-binding sites in the promoter region of the p27 gene, at −2604 and −1794, respectively (Figure 2E). We recloned the human p27 promoter region fused to the luciferase reporter gene into an adenoviral expression vector and performed reporter assays. Introduced into NRCMs, p27PF elicited substantial luciferase activity, which was significantly enhanced on pAd-E193, but significantly suppressed by coinfection with pAd-Eya4. To investigate which putative Six1 consensus site is crucial for the Eya4-mediated suppression on p27 promoter activity, triplet deletions were introduced in either binding site to pAd-p27PF_-2604 and pAd-p27PF_-1794. Upon infection, pAd-p27PF_-1794 transcriptional activity was significantly enhanced, even after coinfection with pAd-Eya4; pAd-E193 did not further enhance promoter activity. Coinfection of pAd-p27PF_-2604 with pAd-Eya4 or pAd-E193 resulted in transcriptional activity as upon coinfections with the WT promoter. Additionally, we performed a luciferase reporter assay with pAd-p27PF, alone or in combination with pAd-E193 or Eya4, after treating cells with shRNA for Six1. Transcriptional activity in either experimental setting was as seen on infection with pAd-p27PF_-1794 (data not shown). We, therefore, speculate that Eya4 negatively regulates p27 expression via the −1794 site and that transcriptional inhibition is dependent on nuclear translocation of Eya4 and abundance of functional Eya4/Six1 complex, the latter not being the case with E193. We further examined binding of Eya4 to the −1794 site effect compared with WT littermates (Figure 3F). We used Western blot (WB) analysis on crude cell lysate from NRCMs, untreated and on infection with pAd-E193, -Eya4, or -shRNA_Eya4 and probed with antibody against phosphorylated (p)HDAC2. As depicted in Figure 2G, pHDAC2 was hardly detectable on E193 overexpression and Eya4 knockdown, but significantly enhanced in pAd-Eya4-infected NRCMs. Concomitantly, we observed a significant decrease in Ckt activity in E193 and shRNA_Eya4 overexpressing cells and an increase on Eya4 overexpression compared with untreated control cells (Figure 2H).

**Animal Model**

We generated 2 TG mouse lines using α-MHC-Eya4 and -E193 expression cassettes termed HA-Eya4 and HA-E193 (Figure 3A). WB analysis with HA antibody proved cardiac expression of both transgenes in the respective animals (Figure 3B), but in no other tissues (data not shown). Next we determined the expression of Six1, total Eya4, and HA-E193 in hearts of TG mice and control littermates (Figure 3C). In HA-E193 lysate, α-Eya4 antibody detected endogenous Eya4 (52 kDa) and the HA-E193 transgene (22 kDa). In lysates of HA-Eya4 TG mice, we obtained a prominent band compared with WT littermates, representing both endogenous and TG HA-Eya4. There was no difference concerning Six1 expression in either genotype. Fluorescence microscopy with anti-HA confirmed stable expression of either transgene and demonstrated that HA-Eya4 and HA-E193 were evenly distributed in cytoplasm and nucleus of isolated adult cardiomycocytes of TG animals (Figure 3D). WB analysis for p27 confirmed that, as seen in vitro, HA-E193 overexpression resulted in an increase in p27 levels, whereas HA-Eya4 overexpression resulted in significantly reduced p27 protein levels (Figure 3E) compared with WT controls. Noteworthy, when generating the gain-of-function mice, we obtained 2 TG lines for E193 and Eya4 each (data not shown). WB analysis showed different expression levels for HA-E193 and -Eya4 in either line. When performing expression analysis for p27 protein levels, both HA-E193 lines showed indistinguishable, yet significantly higher p27 protein levels than WT littermates, whereas in both HA-Eya4 lines, p27 levels were equally, but significantly reduced compared with WT animals. Because both lines of either TG model were indistinguishable in regards to p27 expression, we focused on the line with the higher expression levels of HA-E193 or Eya4 for further analysis.

The in vivo chromatin immunoprecipitation confirmed the results of the in vitro experiment whereupon an overexpression of E193 results in less Eya4/Six1 complexes at the −1794 site of the p27 promoter, whereas overexpression of Eya4 had the opposite effect compared with WT littermates (Figure 3F). We used magnetic resonance imaging to visualize cardiac structures in detail and determine hemodynamics (Figure 3G and 3K). Nine-month-old HA-E193 mice presented a cardiac phenotype similar to human E193 patients: wall thinning (Figure 3H), increased heart dimension because of left ventricular dilation, significantly reduced cardiomycocyte cross sectional areas, increased fibrosis, and contractile dysfunction (Figure 3I–3K). These findings resemble human DCM and indicate that a disturbed Eya4/Six1 signaling cascade leads to an age-dependent cardiomyopathy. In
Figure 2. Overexpression of pAd-Eya4, -E193, and -shRNA_Eya4 in vitro. A, Relative mRNA expression of endogenouseya4, p27kip1, and adenovirus-mediated transgenes in cardiomyocytes from neonatal rats (NRCMs) as detected by quantitative reverse transcriptase polymerase chain reaction (RT-PCR). NRCMs were adenovirally infected with pAd-E193, -Eya4, or -shRNA_Eya4. Values are normalized to GAPDH and presented as mean±SEM of 3 individual experiments performed in triplicate. *P<0.05 vs untreated control (ctr.), n=3. n.d., not detected. B, Representative Western blot (WB) and densitometric analysis of Eya4, p27, and Six1 protein expression on adenoviral overexpression of E193, Eya4, or shRNA_Eya4. Data are presented as mean±SEM relative to GAPDH. Relative expression in control (ctr) is set to 100%. *P<0.05 vs ctr, n=3. C, [3H]-leucine incorporation was measured as a surrogate parameter for protein synthesis and hypertrophy after infection with pAd-E193, -Eya4, or -shRNA_Eya4. Value of ctr is set to 100%. Data are mean±SEM of 3 (Continued)
Figure 2. Continued. individual cell preparations. \*P<0.05 vs untreated ctr, n=3. 4. Cardiomyocyte cross sectional areas (CCSA) of at least 450 cardiomyocytes of 3 individual cell preparations on adenoviral delivery of E193, Eya4, or Eya4-shRNA. Data are means\pm SEM. \*P<0.05 vs untreated ctr. E. Wild-type (WT) pAd-p27PF and generation of mutated plasmids pAd-p27PF-\_1749 and pAd-p277PF-\_2604 (top). The promoter activities of adenovirus-mediated p27PF, p27PF-\_1749, and p27PF-\_2604 were determined by measuring luciferase activity in single- and cotransfection reporter assays: \*P<0.05 vs pAd-p27PF, n=5. F. Quantitative (RT-PCR)-amplified p27-1794 promoter bands in chromatin of NRCMs infected with pAd-E193, -Eya4, or -Eya4-shRNA demonstrated Eya4 and Six1 enrichment at the p27-1794 promoter. Untreated NRCMs (ctr) and mouse IgG served as negative controls, and input chromatin as positive control. Immunoprecipitation (IP) was conducted with Eya4- and Six1-antibodies. G. Crude lysates from NRCMs, untreated (ctr), and adenovirally overexpressing E193, Eya4, or shRNA_Eya4 were used for a CK2 in vitro kinase activity assay with synthetic peptide (p-CK2 sp). Value of ctr is set to 100%. Data are means\± SEM. \*P<0.05 vs untreated ctr, n=3. H. Representative WB and quantitative analysis of phosphorylated histone deacetylase (HDAC2) in crude lysates of untreated NRCMs (ctr) or on adenoviral overexpression of E193, Eya4, or shRNA_Eya4. Relative expression of ctr is set to 100%. Data are means\± SEM relative to GAPDH. \*P<0.05 vs ctr, n=3. Eya4 indicates Eyes absent 4.

contrast, magnetic resonance imaging revealed hypertrophied left ventricles in 9-month-old HA-Eya4 mice at baseline compared with control littermates (Figure 3G). Histological examination confirmed onset of hypertrophy as determined by a significant increase in heart dimension and wall thickness, prominently enlarged cardiomyocyte cross sectional areas, and distinct cardiac fibrosis without considerably compromised cardiac function (Figure 3H–3J). Heart weight to tibia length ratio was also significantly increased. Therefore, HA-Eya4 overexpression appeared to induce a mild but significant cardiac hypertrophy already at baseline without affecting cardiac function. Results obtained by imaging and histomorphology are summarized in Figure 3K. To gain a better insight into the progression of phenotypes and prove molecular changes described above preceded functional changes, we observed the expression of transgenes, p27, and pHDAC2 protein levels in WT and TG mice for a time course of 90 days (Figure IVA–IVC in the Data Supplement). In addition, we also examined the expression of ANP and β-MHC as representatives for fetal cardiac genes. Expression of both transgenes was moderate at birth but increased after day 14, paralleled by an increase in p27 protein levels in HA-E193 animals or rather a decrease in HA-Eya4 mice compared with WT littermates. Accordingly, pHDAC2 levels declined in HA-E193, but increased in HA-Eya4 animals. ANP and β-MHC were clearly detectable upon birth in all genotypes monitored as a result of the embryonic gene expression pattern. After a transient dip at around day 14, reactivation of ANP and β-MHC expression occurred in both TG lines as was seen in an increase in protein levels, whereas in WT mice, neither protein was detectable at these time points.

Eya4 Aggravates Heart Failure Induced by Pressure Overload

Next, 12-week-old mice were subjected to TAC. After 4 weeks, cardiac structural and functional changes were monitored. At 4 months, both TG lines showed onset of cardiac phenotypes as mentioned earlier. Of note, changes in cardiac phenotype in the unstressed hearts were less pronounced in the 16-week-old mice compared with the 9-month-old animals described earlier. An age-dependent comparison of phenotypes is summarized in Figure IVD and IVE. In WT mice, left ventricular wall thickness and myocardial mass increased on TAC compared with sham-operated controls (Figure 4A and 4B). However, in HA-E193 mice, TAC induced a slight thinning rather than an increase in wall thickness. In contrast, in HA-Eya4 mice, wall thickness and myocardial mass abruptly increased during TAC compared with sham-operated HA-Eya4 and WT TAC mice (Figure 4A and 4B). Similarly, cardiomyocyte cross sectional areas in WT and HA-Eya4 mice enlarged during TAC (Figure 4C), whereas they diminished in HA-E193 mice. Fibrosis was augmented already in sham-operated TG animals compared with WT, and it significantly increased in all 3 groups under TAC (Figure 4D). The structural changes in HA-E193 mice upon TAC were associated with a significant reduction in contractile function (Figure 4E) suggesting transition to heart failure conditions, whereas in HA-Eya4 mice, it was less pronounced by pressure overload. Collectively, these data imply E193 circumvents the development of cardiac hypertrophy on pressure overload and indicate Eya4 plays a crucial role in maintaining cardiac physiology. Detailed magnetic resonance imaging data are summarized in Figure 4E.

Eya4 Regulates p27 Expression and Influences p27/CK2α/HDAC2 Signaling In Vivo

Eya proteins act as transcriptional cofactors in a physical complex with Six1.11 WB of heart extracts revealed HA-E193 overexpression significantly upregulates p27 already at 4 months, whereas HA-Eya4 significantly suppresses p27; these molecular events are more pronounced in consequence of pressure overload (Figure 5A). As mentioned earlier, it was recently described that p27 and CK2α are mutual regulators15 and CK2α phosphorylates HDAC2. WBs probed with α-pHDAC2 showed a significant increase in pHDAC2 levels in HA-Eya4 TG hearts compared with WT in both sham-operated and TAC animals, which were greatly reduced in HA-E193 hearts, even on TAC (Figure 5B). This again suggests that Eya4 is able to regulate the phosphorylation status of HDAC2 by directly suppressing the expression level of p27, thereby activating CK2α. We confirmed this with an in vitro kinase assay with a synthetic peptide of CK2 and heart lysates from all 3 genotypes from both experimental settings (Figure 5C). As depicted, CK2 activity was significantly increased in 4-month-old HA-Eya4 mice, whereas in HA-E193 hearts, it seemed abolished in both experimental settings. We further analyzed binding abilities of HA-Eya4 and HA-E193 to the p27 promoter with an electrophoretic mobility shift assay (Figure 5D, left panel). Accordingly nuclear extracts isolated from TG and WT mice, sham-operated or TAC treated, were incubated with a biotin-labeled probe corresponding to the −1794 site within the p27 promoter. All extracts used gave rise to a single shifted band, which was most prominent in HA-Eya4 mice under both experimental settings and almost abolished in HA-E193 animals. The addition of Eya4 (Figure 5D, middle panel) or Six1 antibody (Figure 5D, right panel) yielded a specific supershift band; addition of unlabeled probe completely competed out a shift of band in WT extracts (Figure 5D, middle panel, lane 4, right panel, lane 1) and TG models (data not shown). This indicates Eya4, together with Six1, specifically and synergistically binds to the p27 promoter at −1794, whereas E193 seems to prevent the Six1
Figure 3. Generation of transgenic (TG) mice and phenotypic analysis under basal conditions at 9 months of age. A, Schematic of HA-Eya4 and HA-E193 expression cassettes under control of the α-myosin heavy chain (MHC) promoter. B, Representative WB with anti-HA on crude myocardial extracts from wild-type (WT) and TG mice to prove transgene expression. GAPDH served as loading control. C, Representative WB of Eya4 and Six1 levels in crude cardiac lysates from WT and TG animals. The Eya4 antibody used was directed against the N-terminal region of human and murine Eya4 and therefore recognizes endogenous and overexpressed Eya4 and E193, respectively. GAPDH served as loading control. Black arrows: full length Eya4; open arrow: E193 isoform. D, Representative adult cardiomyocytes from TG mice. Immunofluorescence staining confirmed expression of HA-tagged transgenes and determined cellular distribution; (Continued)
p27-binding capacity. Based on our findings, we propose a model for Eya4/Six1 in maintaining cardiac physiology (Figure 5E): in the absence of stimuli, Eya4 is cytoplasmic and nuclear; nuclear Eya4/Six1 complexes are in a more inactive state regarding p27 expression. After stimulation, Eya4 is translocated where it as a Eya4/Six1 complex binds the p27 promoter at ~1794, thereby suppressing p27 expression, which subsequently results in a decline in p27 abundance. Decreasing p27 levels lead to activated CK2α and subsequent phosphorylation of HDAC2, which then enables a hypertrophic response. E193—albeit able to bind Six1 as demonstrated by IP (Figure 1D)—translocates to the nucleus independent from Six1 and in the absence of stimuli. Abundance of E193/Six1 disrupts Eya4/Six1-mediated suppression of p27 expression, resulting in elevated p27 levels, even after stimulation, therefore less active CK2α and, subsequently, unphosphorylated, inactive HDAC2, blocking hypertrophic growth.

E215, a Novel Truncating Eya4 Mutation

In addition to individuals with the E193 mutation,7 we identified a patient with similar phenotype. Family history revealed 2 additional individuals with SNHL and DCM, one of which had died of terminal heart failure. The status of another individual was uncertain (Figure 6A). Sequence analyses of the Eya4 transcript obtained by reverse transcriptase polymerase chain reaction of lymphocyte mRNA showed that it encoded a heterozygous C→T base pair transition after amino acids 215, resulting in a premature termination signal (Figure 6B).

We, therefore, designate this new truncated Eya4 protein E215. WB of myocardial biopsies of the affected individual identified a p27 protein expression in crude heart lysates from WT and TG animals. Relative protein expression in WT is set to 100%. Data are mean±SEM relative to GAPDH. 7P<0.05 vs WT, n=5 for all groups. Representative chromatin immunoprecipitation (ChiP) assay demonstrating diverse Eya4 or Six1 enrichment at the p27-1794 promoter in cardiomyocytes of WT and TG animals. Data are presented as quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)–amplified p27-1794 promoter bands. Mouse IgG served as negative and input chromatin as positive control. Immunoprecipitation (IP) was conducted with Eya4 and Six1 antibodies. G, Magnetic resonance images (MRI) of hearts showing short axis views. H, Representative images of longitudinal heart sections stained with hematoxylin and eosin (HE); scale bar 1 mm. I, Representative fluorescent photomicrographs of left ventricular (LV) heart sections stained with labeled wheat germ agglutinin to determine cardiomyocyte cross sectional areas (CCSA); scale bar 20 μm. J, Representative images of LV cardiac sections stained with picrosirius red (PSR) show increased fibrosis in both TG lines; scale bar 50 μm.

Eya4 transcript obtained by reverse transcriptase polymerase chain reaction of lymphocyte mRNA showed that it encoded a heterozygous C→T base pair transition after amino acids 215, resulting in a premature termination signal (Figure 6B). Sequence analyses of the Eya4 transcript obtained by reverse transcriptase polymerase chain reaction of lymphocyte mRNA showed that it encoded a heterozygous C→T base pair transition after amino acids 215, resulting in a premature termination signal (Figure 6B). We, therefore, designate this new truncated Eya4 protein E215. WB of myocardial biopsies of the affected individual identified a p27 protein expression in crude heart lysates from WT and TG animals. Relative protein expression in WT is set to 100%. Data are mean±SEM relative to GAPDH. 7P<0.05 vs WT, n=5 for all groups. Representative chromatin immunoprecipitation (ChiP) assay demonstrating diverse Eya4 or Six1 enrichment at the p27-1794 promoter in cardiomyocytes of WT and TG animals. Data are presented as quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)–amplified p27-1794 promoter bands. Mouse IgG served as negative and input chromatin as positive control. Immunoprecipitation (IP) was conducted with Eya4 and Six1 antibodies. G, Magnetic resonance images (MRI) of hearts showing short axis views. H, Representative images of longitudinal heart sections stained with hematoxylin and eosin (HE); scale bar 1 mm. I, Representative fluorescent photomicrographs of left ventricular (LV) heart sections stained with labeled wheat germ agglutinin to determine cardiomyocyte cross sectional areas (CCSA); scale bar 20 μm. J, Representative images of LV cardiac sections stained with picrosirius red (PSR) show increased fibrosis in both TG lines; scale bar 50 μm.

Discussion

Truncating mutation E193 causes DCM and heart failure preceded by SNHL.7 Although most previously described DCM-causing mutations affected structural proteins, this mutation affects the human transcriptional coactivator gene Eya4.7 Data first obtained in heart function of zebrafish pointed to an unrecognized and crucial role of Eya4/Six1-mediated transcriptional regulation for normal heart morphology and function.

Eya proteins interact with the transcription factor Six to translocate to the nucleus and form active transcription complexes.11 Mutations within human Eya1 lead to defects of cranial-facial, musculoskeletal, and kidney development.10,11 Although a role for Eya1 in cardiac physiology and function to date has not been described, Eya2, Eya3, and Eya4 have been specified to affect cardiac function.7,11,14 It was recently demonstrated that Eya4 regulation of the Nas+/K+/ATPase is crucial for the maintenance of cardiac function in zebrafish;20 a mouse model of Eya4 deficiency additionally revealed abnormal anatomy in the middle ear cavity and the Eustachian tube, leading to otitis media.21 However, cardiac abnormalities were not reported in this model.

Transcriptional targets of Eya4/Six1 have been investigated in the developmental context, but the identification of target genes in cardiomyocytes has been void. Interestingly, it has been reported that Six6 represses p27, thus controlling retinal and pituitary precursor cell proliferation.22 We, therefore, speculated that Eya4 might influence p27 expression in cardiomyocytes via Six1, another member of the Six family. Indeed, we identified one functional Six1-binding site in the p27 promoter at ~1794. In analogy to data obtained from studies with Eya3,22 we expected Eya4/Six1 would relieve suppression on the p27 promoter. Surprisingly, Eya4 suppressed p27 expression in our experimental settings in vitro and in vivo. In contrast, the truncated E193 mutant seemed to have a dominant negative effect over Eya4 in regards to p27 expression, resulting in constitutive elevated p27 levels in vitro and in vivo. On a molecular level, Eya4 lacks a DNA-binding site; it dimerizes and interacts with Six1, whereupon it is translocated to the nucleus. In contrast, albeit it does interact with Six1, E193 also translocates Six1-independent. The functional Six1 consensus site at ~1794 in the p27 promoter is consistent with the transcriptional repression of p27 by Eya4 interaction with Six1. In contrast, E193 significantly decreased Six1 DNA-binding capacity, even in the presence of endogenous Eya4. Consequently, Six1-mediated suppression of p27 is released, and p27 levels remain high.

It has been reported before that Six1 has the ability to act as both, activator or repressor of expression; this ability is in part attributed to the recruitment of diverse cofactors like Eya or dachshund (Dach) and the cellular context.22–24 Various Six family proteins were shown to act as repressors on target genes on association with the Groucho (Gro) family of corepressors,25,26 an effect which can be lifted by cotransfection with Eya.10
Figure 4. Overexpression of HA-Eya4 aggravates pressure overload-induced hypertrophy in vivo, which is not seen in HA-E193 transgenic (TG) animals 16 weeks of age. A, Magnetic resonance imaging (MRI) sections of hearts showing short axis views of sham (top row) and transaortic constriction (TAC)-treated mice (bottom row). B, HE-stained representative longitudinal heart sections from wild-type (WT) and TG mice of both experimental settings; scale bar 1 mm. C, Representative cardiac longitudinal sections stained with wheat germ agglutinin for LV cardiomyocyte cross sectional areas (CCSA); scale bar 20 μm. D, Picrosirius red (PSR) staining of left ventricular (LV) heart sections from WT and TG mice that underwent TAC for 4 weeks; scale bar 50 μm. E, Assessment of heart weight/tibia length (HW/TL) ratios, LV enddiastolic volume (LVEDV), ejection fraction (EF), interventricular septum thickness (IVS), cardiomyocyte cross sectional areas (CCSA), and collagen content; *P<0.05 vs WT sham, n=6 for all groups. Data are mean±SEM. Eya4 indicates Eyes absent 4.
Figure 5. Overexpression of HA-Eya4 and HA-E193 in vivo results in altered p27 and pHDAC2 levels in transgenic (TG) mice compared with wild-type (WT) littermates. A, Representative Western blot (WB) and quantification results showing response of p27 (Continued)
Eya proteins are predicted to have a functional Gro-binding domain and, though not yet confirmed in vivo, therefore, could also function as corepressor or coactivator in a context-dependent manner. Although the precise interplay of Eya, for example, Eya4 with Six and other cofactors in cardiomyocytes, remains to be determined, there is data on Eya3 describing a role in reversing Six1-Dach–mediated repression of target genes in vitro.22

Findings here tempt to speculate that the intrinsic phosphatase activity of Eya3 is a key player in permitting the recruitment of other coactivators, thereby switching the function of Six and perhaps Eya itself from repression to activation and vice versa. This might explain the effects of the C-terminal truncating mutations (E193 and E215) described here. In both the C-terminal, tyrosine phosphate domain remains.

The cyclin-dependent kinase inhibitor p27 is usually discussed in context with cell growth and division. Antiproliferative signals lead to accumulation and stabilization of p27, which can then inhibit CK2α and cause cell cycle arrest.28,29 Although highly expressed in terminally differentiated and postmitotic adult cardiomyocytes, a function of p27 in these cells beyond cell cycle arrest has not been fully examined. Levels of p27 significantly decline in response to hypertrophic stimuli in vivo.30 Recently, Hauk et al demonstrated that p27-deficient mice develop age-dependent cardiac hypertrophy and show an aggravated hypertrophic response to TAC, supporting the idea that p27 regulates myocardial hypertrophy17 and subsequently elevated protein levels, even in the presence of nuclear E193 could then saturate Six1-binding capacity for Six-independent way, perhaps as a passive process, because truncated E215 protein, albeit at different amounts. Additional in vivo loss-of-function studies to verify data obtained from these patients’ heart samples revealed both full length Eya4 and truncated E193 animals, which averted myocardial CK2α activity on phosphorylation of HDAC2,15 supports our hypothesis on a causal relationship between Eya4/Six1 and p27/CK2α/HDAC2 in regulating cardiac hypertrophy. Even more, we speculate the cardiac phenotype in HA-E193 animals emerges from a dominant negative effect of E193 over Eya4 in regards to p27 expression. Meaning that abilities to suppress p27 and to hypertrophy are important for a normal cardiac response to hemodynamic challenges and, conversely, the inability to suppress p27 (or presumably continuous high p27 levels) ultimately causes cardiac phenotypes, such as DCM. Overexpressed Six1 was mainly nuclear in our experimental setting, which is in line with the literature; E193 was located in cyto- and nucleoplasm in either experimental setting. Eya4 alone was cytoplasmic and nuclear; Six1 coinfected resulted in predominantly nuclear Eya4. Co-IP experiments demonstrate Eya4 and, to a lesser extent, E193 physically interact with Six1. Thereupon, we suggest that Eya4 and E193 are translocated via interaction with endogenous Six1. However, from the strong nuclear enrichment of E193 alone and on stimulation, even after Six1 knockdown, we hypothesize it is translocated in a more Six-independent way, perhaps as a passive process, because proteins below 40 kDa do not depend on shuttle proteins or the importin transport machinery to enter the nucleus. Abundance of nuclear E193 could then saturate Six1-binding capacity for Eya4, resulting in less functional Eya4/Six1 complexes on the p27 promoter and, therefore, less suppression of p27 expression and subsequently elevated protein levels, even in the presence of stimuli when the cell demands for hypertrophic growth to compensate for demands in myocardial workload.

Figure 5. Continued. Protein expression to transaortic constriction (TAC) in 16-week-old WT and TG mice. Relative expression in WT sham is set to 100%. Data are presented as mean±SEM. *P<0.05 vs WT sham, n=6 for all groups. B, WB and densitometric data reflecting levels of phospho-CK2α synthetic peptide (p-CK2α sp) among WT and TG mice in both experimental settings. CK2α活性 in WT sham was taken as 100%. Data are presented as mean±SEM. *P<0.05 vs WT sham, n=6 for all groups. C, Representative radiogram and densitometry regarding CK2α activity on phosphorylation of HDAC2,15 supports our hypothesis on a causal relationship between Eya4/Six1 and p27/CK2α/HDAC2 in regulating cardiac hypertrophy. Even more, we speculate the cardiac phenotype in HA-E193 animals emerges from a dominant negative effect of E193 over Eya4 in regards to p27 expression. Meaning that abilities to suppress p27 and to hypertrophy are important for a normal cardiac response to hemodynamic challenges and, conversely, the inability to suppress p27 (or presumably continuous high p27 levels) ultimately causes cardiac phenotypes, such as DCM. Overexpressed Six1 was mainly nuclear in our experimental setting, which is in line with the literature; E193 was located in cyto- and nucleoplasm in either experimental setting. Eya4 alone was cytoplasmic and nuclear; Six1 coinfected resulted in predominantly nuclear Eya4. Co-IP experiments demonstrate Eya4 and, to a lesser extent, E193 physically interact with Six1. Thereupon, we suggest that Eya4 and E193 are translocated via interaction with endogenous Six1. However, from the strong nuclear enrichment of E193 alone and on stimulation, even after Six1 knockdown, we hypothesize it is translocated in a more Six-independent way, perhaps as a passive process, because proteins below 40 kDa do not depend on shuttle proteins or the importin transport machinery to enter the nucleus. Abundance of nuclear E193 could then saturate Six1-binding capacity for Eya4, resulting in less functional Eya4/Six1 complexes on the p27 promoter and, therefore, less suppression of p27 expression and subsequently elevated protein levels, even in the presence of stimuli when the cell demands for hypertrophic growth to compensate for demands in myocardial workload.

Eya signaling is also of relevance in human disease. Previously, 2 families were described with truncating E193 mutations.7,32 We additionally identified a heterozygous truncating mutation in the Eya4–gene resulting in an E215 mutant. The affected patients present SNHL and DCM. Analysis of one of these patients’ heart samples revealed both full length Eya4 and truncated E215 protein, albeit at different amounts. Additional in vitro experiments favor the hypothesis of a dominant-negative effect of truncated Eya proteins with the described consequences on p27/CK2α/HDAC2 and myocardial function. Limitation of our work is the fact that we do not provide additional in vivo loss-of-function studies to verify data obtained by using our gain-of-function TG mice in regards to the in vivo function of Eya4 and E193. We therefore cannot completely rule
out the possibility that although proper controls were exerted that the findings reported in this article are a result of nonphysiological levels of Eya4 or E193 protein. However, the role of Eya4 as an important regulator for cardiac function was firmly supported by results from various Eya4 in vitro knockdown experiments depicted throughout Figure 2, respectively.

In summary we demonstrated that the Eya4/Six1 complex acts as a transcriptional suppressor on p27, which results in the development of hypertrophy. Loss of the transcriptional inhibition on p27 through overexpression of HA-E193 caused the development of age-related DCM in vivo via suppressed CK2α activity and subsequent inhibition of HDAC2 phosphorylation, underlining the importance of a fine-tuned Eya4/Six1 signaling cascade for maintaining normal cardiac function.

Acknowledgments
We thank Professor Toshiyuki Sakai (Kyoto, Japan) for providing the p27PF.

Sources of Funding
This work was supported by research grants from the Interdisciplinary Center for Clinical Research IZKF (E-37, E-40), the German Research Foundation DFG (WI 3677/1-1, SFB 688/Z02), the Federal Ministry of Education and Research BMBF (01EO1004/C2, 01EO1004/D11, 03V0147) and the Rudolf-Virchow-Center of the University of Wuerzburg.

Disclosures
None.

References


**CLINICAL PERSPECTIVE**

Mutations in the human Eyes absent 4 (Eya4) gene that cause loss of large parts of the C-terminal part of the protein result in DCM and heart failure preceded by sensorineural hearing loss. Eya4 is one of 4 vertebrate orthologs of the Drosophila gene eyes absent (eya). Eya proteins are transcriptional coactivators that lack a DNA-binding domain but interact with the transcription factor Six. We now demonstrated that Eya4 is not only responsible for the development of heart failure when mutated. Eya4 signalling is obviously also activated in acquired heart disease and, therefore, seems to be crucially involved in normal cardiac function, especially in response to hemodynamic stress. Interestingly, transgenic Eya4 overexpression caused myocardial hypertrophy, whereas overexpression of the truncated and dominant negative Eya4 isoform E193 caused DCM. Eya4 seems to act as transcriptional repressor on p27 expression. The cyclin-dependent kinase inhibitor p27 is a potent inhibitor of cell growth and division. In cancer cells, p27 is downregulated, thereby promoting tumor growth. In failing heart, p27 protein levels are hardly detectable. Recent findings from other groups illustrate a role for p27 as negative regulator of myocardial hypertrophy. The truncated and dominant negative isoform E193 obviously released transcriptional repression of Eya4 on p27. Further signalling via p27/CK2-α and histone deacetylase 2 then caused age-dependent development of DCM. With its molecular and pathophysiological similarities in dividing cancer cells and enlarging cardiac myocytes, it might be possible to further dissect disease mechanisms and to identify new pharmacological treatment options for heart failure patients.
Eya4 Induces Hypertrophy via Regulation of p27kip1

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Circ Cardiovasc Genet. 2015;8:752-764; originally published online October 23, 2015;
doi: 10.1161/CIRCGENETICS.115.001134

Circulation: Cardiovascular Genetics is published by the American Heart Association, 7272 Greenville Avenue,
Dallas, TX 75231
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Print ISSN: 1942-325X. Online ISSN: 1942-3268

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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SUPPLEMENTAL MATERIAL
Supplementary Methods

Animal Models and Treatment: All of the protocols were reviewed and accepted by the local ethics committee and performed in accordance with the institutional local ethics committee.

cDNAs clones encoding HA-tagged full length human Eya4 (HA-Eya4) or the truncating mutation E193 (HA-E193) were commercially obtained, the latter corresponding to the human mutation identified and first described by Schoenberger et al. It encodes the 193 N-terminal amino acids of wild-type E193, followed by 29 new aa and a termination signal. Both constructs were amplified by PCR, digested and separately cloned into a mammalian expression vector enclosing the mouse α-MHC promoter. After digestion, DNAs containing the α-MHC promoter and either HA-Eya4 or HA-E193 were purified and microinjected into fertilized FVB eggs. PCR analysis was used to identify founder mice and subsequent progeny.

For analysis of age-dependent effects upon cardiac morphology and physiology, mice were kept under non-challenged conditions until they reached 9 months of age. Before hearts were excised, mice were anesthetized with tribromoethanol/amylene hydrate (0.1ml/10mg BW). Hearts were arrested in diastole using ice-cold KCl solution, fixed at -80°C in Tissue-Tek® OCT™ (Sakura) and divided into longitudinal sections for protein and histologic analysis.

Chronic pressure-overload LV hypertrophy was induced by transverse aortic constriction (TAC) in wild type (WT) and transgenic (TG) mice 12 weeks of age. The animals were anesthetized with isoflurane and ventilated with a tidal volume of 0.1ml and a respiratory rate of 120 breaths per minute (Harvard Apparatus). A longitudinal cut was made in the proximal portion of the sternum to visualize the aortic arch. The transverse aortic arch was ligated between the innominate and left common carotid arteries with an overlaid 27-gauge needle. After leaving a discrete region of constriction the needle was removed. For sham surgery the procedure was identical but without aortic ligation. 4 weeks after surgery, animals were anesthetized with tribromoethanol/amylene hydrate (0.1ml/10mg BW) and hearts were excised for further analysis.

MRI and Hemodynamic Analysis: All studies conformed to the “Guide for the Care and Use of Laboratory Animals” and were approved by the institutional local ethics committee.
MRI was performed using a Bruker Biospec 70/20 scanner, with a field strength of 7.05 tesla and horizontal bore. For improved performance and image quality, the scanner was equipped with a G60 microscopy gradient system and a custom radiofrequency transmit coil in birdcage design. Investigations in the MR scanner were performed under inhalation anesthesia using oxygen-enriched room air with 1.5-2% isoflurane. Cardiac morphology was determined running a free breathing ECG-triggered multi-slice cine-fast low-angle shot (FLASH) imaging sequence. The heart was covered in total utilizing stacks of 8-10 contiguous images in various orientations including left ventricular long-axis and short-axis orientations. Spatial image resolution was 0.117x0.117mm with a slice thickness of 1mm. A temporal resolution of 20 frames per heart cycle was achieved for acquisition of cine-loops, with 2-4 averages per frame to increase signal-to-noise ratio. Total measurement time per animal was ~90 minutes.

Following image acquisition, the image frames were then analyzed using a dedicated custom analysis program in interactive data language (IDL). After the end-diastolic and end-systolic frames had been visually predefined, the cardiac compartments and surrounding tissue were semi-automatically segmented, allowing for quantification of myocardial wall thicknesses, myocardial mass, ventricular diameters, left and right ventricular end-diastolic and end-systolic volumes, stroke volume, cardiac output, and ejection fraction.

For additional categorization of regional myocardial function, the myocardial areas with significant thinning, hypokinesia, dyskinesia, or akinesia were visually evaluated for every slice.

**Morphometric Analysis:** Body weight and tibia length were measured at organ collection, after MRI and hemodynamic analysis. The Tissue-Tek® OCT™ embedded hearts were cryocut into 10µm sections. Hematoxylin eosin (HE) and picrosirius red (PSR) stainings were performed according to standard protocols. Cell size measurements from cardiac tissue of WT and TG mice were evaluated in 10µm cryosections stained with Alexa Fluor® 350-conjugated wheat germ agglutinin (Molecular Probes®, Life Technologies™) and DAPI (4′,6′-Diamidino-2-phenylindole dihydrochloride, Sigma-Aldrich). The area of 100 randomly selected cardiomyocytes, with nearly circular capillary profiles and nuclei, was manually traced using the “Image J” software (NIH); six hearts were analyzed per genotype and experimental setting.
Preparation and Cultivation of Neonatal Rat Cardiomyocytes (NRCMs): NRCMs from hearts of 1-3-days old Wistar rats (Charles River Laboratories, Germany) were isolated as previously described. Cells were resuspended in minimum essential medium (MEM, Gibco) with 5% fetal calf serum (FCS) for preplating and plated in MEM/5 on 6-well plates or chamber slides at an assay dependent density. Medium for NRCMs was supplemented with 5-Bromo-2'-deoxyuridine (BrdU) (0.1 mM) to suppress fibroblast growth. Fibroblast contamination was usually around 4-7% as regularly determined by immunohistochemical staining for Troponin T.

Immunofluorescence Staining of Isolated Adult Cardiomyocytes: Stable expression and subcellular distribution of HA-Eya4 and HA–E193 in vivo was determined by immunostainings. The protocol was reviewed and accepted by the local ethics committee and performed in accordance with the institutional local ethics committee. After anesthesia hearts of adult TG mice (sham and TAC treated) were excised and mounted on a Langendorff apparatus. Upon washing with a Ca-free solution the LV was enzymatically digested by coronary perfusion with Liberase TH (Roche). Isolated cardiomyocytes were plated in 1 mM Ca containing Tyrode solution on laminin-coated X-well tissue culture chambers (Sarstedt). After fixation, permeabilizing and blocking, α-Eya4 (sc-15106) or α-HA antibody (sc-905, Santa Cruz Biotechnology) was used O/N at 4°C, followed by a 1h incubation with Alexa Fluor® 594 conjugated secondary antibody (Molecular Probes®, Life Technologies™). Nuclei were stained with DAPI according to standard protocols.

Adenoviral Expression Constructs and Gene Transfer: The customized adenoviral plasmids encoding DsRed-tagged human Eya4 (pAd-DsR-Eya4) and E193 (pAd-DsR-E193), untagged human E215 (pAd-E215) and GFP-tagged murine Six1 (pAd-GFP-Six1) were obtained from GeneArt® (Life Technologies™). The E193 construct corresponds to the human E193 mutant identified and described by Schoenberger et al. Adenovirus encoding shRNA against murine Eya4 (pAd-shRNA_Eya4), murine Six1 (pAd-shRNA-Six1) and control non-targeting adenovirus were obtained from Sirion Biotech (Martinsried, Germany). Adenovirus encoding the human p27kip1 promoter-luciferase fragment (pAd-p27PF) was generated by subcloning the respective DNA fragment into an adenoviral plasmid. Viral particles were generated using the ViraPower Adenoviral Expression System according to manufacturer’s instructions. Adenoviral in vitro gene transfer was performed according to manufacturer’s protocol at an assay dependent multiplicity of infection. Cells were harvested 48-72 hours after infection or visualized by microscopy.
**Immunohistochemistry:** For detection and visualization of endogenous Eya4 and Six1 primary NRCMs were isolated and seeded into X-well tissue culture chambers (Sarstedt) as mentioned above. 48h after isolation cells were fixed, permeabilized and blocked before O/N incubation at 4°C with primary antibodies to Eya4 (T21) (sc-15106) and Six1 (sc-9127; both Santa Cruz Biotechnologies), followed by a 1h incubation with Alexa Fluor® 594 conjugated secondary antibody (Molecular Probes®, Life Technologies™). Nuclei were stained with DAPI according to standard protocols.

**Microscopy:** Stained sections or cells were viewed with a Kyocera BZ8000 or the Leica TCS SP5 Broadband Confocal.

**Nuclear and Cytoplasmic Protein Extraction:** Nuclear and cytosolic proteins were extracted using the NE-PER® kit (Thermo Fisher) according to manufacturer’s protocol.

**Westernblot (WB) and Coimmunoprecipitation (Co-IP) Analysis:** Protein expression quantification was made on homogenates from either infected cultured cells or heart tissue from transgenic mice. Protein samples were subjected to SDS-PAGE and Western blotting and probed with purified primary antibodies O/N followed by a 1hr incubation with horseradish peroxidase-conjugated (HRP) secondary antibodies (GE Healthcare Life Sciences). Bands were visualized by enhanced chemiluminescence (ECL) (GE Healthcare Life Sciences). Semi-quantitative analysis was made using the ImageQuant Software (Biometra). Antibodies used included: α-Eya4 (ab47990, Abcam), α-p27 (ab7961, Abcam), α-GAPDH (MAB374, Chemicon International), α-H1 (ab11079, Abcam), α-HA (sc-7392, Santa Cruz Biotechnology), α-pHDAC2 (ab75602, Abcam), α-ANP (AB5490, Millipore) and HRP-coupled secondary antibodies.

WB analysis of human material for analysis was done after review and approval by the local ethics committee. Human EYA4 protein expression analysis was made on homogenates from heart tissue from affected and unaffected individuals subjected to SDS-PAGE and Western blotting. The antibody (α-EYA4 (111-125), sigma-aldrich) used gave a 52 kDa product from wildtype, and an additional 24 kDa product from the mutated EYA4 in the affected patient. Of note, there is currently no explanation in the literature for the discordance in size of the wild type band observed and the calculated size of approximately 70 kDa of EYA4, unknown post-translational modifications and structure of the protein may affect its running pattern.
For Co-IP experiments NRCMs were infected with DsRed-tagged Eya4 and E193, either alone or in combination with GFP-tagged Six1 and immunoprecipitated using the Dynabeads® Protein G Immunoprecipitation Kit according to the respective protocol (Life Technologies™). Beads were cross-linked O/N to α-RFP (ab125244, Abcam) antibody at 4°C before incubation with the antigen containing samples. For elution beads were boiled at 95°C, and supernatants were subjected to SDS-PAGE and WB analysis. α-GFP antibody (#632592, Clontech) and a species specific HRP-conjugated second antibody were used for detection and visualization with ECL. Supernatants of cells overexpressing GFP-Six1, DsRed-tagged Eya4 and -E193 alone were used as loading controls for the specificity of the GFP antibody.

For Co-IP of endogenous proteins Dynabeads® Protein G was cross-linked to α-Eya4 T21 (sc-15106, Santa Cruz Biotechnology) at 4°C before incubation with the antigen containing samples. For elution beads were boiled at 95°C, and supernatants were subjected to SDS-PAGE and WB analysis. α-Six1 antibody (sc-9127, Santa Cruz Biotechnology) and a species specific HRP-conjugated second antibody were used for detection and visualization with ECL.

Site-directed Mutagenesis and Luciferase Assay: Deletions were introduced in the promoter region of the p27 promoter by using the QuikChange® site-directed mutagenesis system (Stratagene) and verified by DNA sequencing (Eurofins MWG Operon). NRCMs were transfected with the respective plasmids using the Lipofectamine™ 2000 & Plus reagent system (Life Technologies™). Luciferase activity was determined using a Luciferase Assay System according to manufacturer’s protocol (Promega).

[^H]-Leucine Incorporation: NRCMs were isolated as described, infected with respective adenovirus at an MOI of 150 and stimulated with Ang II (10µmol/l). 92h after transfection [3H]-leucine (Amersham Biosciences) was added at an activity of 0.0925 MBq. After another 4h cells were washed twice with 1x PBS, lysed with 1% SDS and harvested. DNA-concentration was determined using the Quant.iT™dsDNA HS Assay Kit (Life Technologies™) according to manufacturer’s protocol. Proteins were precipitated with 500µl chilled 10% TCA for 30min, pelleted at 13.000g and resolved in 500µl 1% SDS. After 30min at 30°C, 5ml scintillation fluid was added and the amount of incorporated [3H]-marked leucine was measured as counts/ml/min (CPM) using a β-counter. The CPM:DNA ratio was calculated for each sample. The change in protein synthesis is expressed as a percentage of the CPM:DNA concentration ratio in unstimulated cells which was taken as 100%.
Quantitative RT-PCR (qRT-PCR): Total RNA was extracted using a RNA fibrous tissue kit (Qiagen, Hilden) and cDNA synthesized from 1µg of RNA using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA). qRT-PCR was performed with commercial and customized TaqMan probes (Life Technologies™). Target gene mRNA levels were normalized to GAPDH which served as housekeeping gene for comparison.

Chromatin Immunoprecipitation Analysis (ChIP): Proteins and DNA in non-treated NRCMs and cultures infected with pAd-CMV-Eya4, -E193 and Eya4-shRNA were cross-linked with 1% formaldehyde (v/v) in PBS/Na-butyrate. Chromatin was isolated, sheared by sonication on ice yielding chromatin fragments of 100-600 bp in length and cleared for large complexes by centrifugation. Protein G Dynabeads® (Life Technologies™) was used to pull down antibody-antigen-DNA complexes immunoprecipitated with antibodies against α-Eya4 T21 (sc-15109) or α-Six1 (M120) X (sc-9127 X, both Santa Cruz Biotechnology). RNAPolII (Sigma) and IgG were also included as positive and negative controls, respectively. Immunoprecipitated DNA was recovered using Chelex-100. Input and immunoprecipitated DNA were analysed and amplified by qPCR using primers spanning the promoter regions of p27 or GAPDH. Melting curves were determined to rule out signals from primer dimers or other non-specific DNA. All samples were run in triplicate from 3 independent experiments; data were normalized to percent input. The size of the PCR products was confirmed on a 2% agarose gel stained with ethidium bromide.

Heart lysates from WT and TG mice were treated with 1% formaldehyde in PBS/Na-butyrate. Chromatin was sheared by sonication on ice. Chromatin-protein complexes were immunoprecipitated with α-Eya4 (T21) (sc-15107) or α-Six1 (M120) X (sc-9127 X, both Santa Cruz Biotechnology) and pulled down with Dynabeads® Protein G (Life Technologies™). RNAPolII (Sigma) and IgG were included as positive and negative controls, respectively. Following elution with Chelex-100, DNA (Input and immunoprecipitated DNA) was subjected to qPCR. Melting curves were determined to rule out signals from primer dimers or other non-specific DNA. All samples were run in triplicate from 3 independent experiments; data were normalized to percent input. The size of the PCR products was confirmed on a 2% agarose gel stained with ethidium bromide.

Heart lysates from human samples were treated as described for lysates from transgenic mice. IP was performed with α-Eya4 (ab93865, Abcam).
Sequences of primers used are available upon request.

**Electrophoretic Mobility Shift Assay (EMSA) and Supershift Assay:** EMSA was carried out using the LightShift® Chemiluminescent EMSA Kit (Thermo Fisher) according to manufacturer’s protocol. End-biotinylated target dsDNA containing the Six1 consensus site was prepared using the Biotin 3’ End DNA Labeling Kit (Thermo Fisher) according to manufacturer’s instructions. Nuclear protein extracts were prepared using the NE-PER® kit (Thermo Fisher) according to manufacturer’s protocol. For the competition experiment, nuclear extracts were incubated with 200-fold excess of unlabeled target DNA. For supershift assays, nuclear extracts were pre-incubated with an Eya4-specific antibody directed against the C-terminus.

Sequences of primers used are available upon request.

**In vitro Casein Kinase Activity Assay:** The Casein Kinase 2 activity assay was performed with components of a commercially available kit (Millipore) and 50µg protein/assay. Heart lysate (50µg protein/assay) from sham and TAC-treated WT and TG mice, heart lysate from human patient and control, or crude cell lysate from adenovirally infected NRCMs, 0.185MBq [γ-32p]ATP, 5µl substrate peptide (1mM) (RRRDDDSDDD), 7µl reaction buffer, and 2µl PKA inhibitor cocktail (2µM) were mixed and kept at 30°C for 30 min. Reactants were electrophoresed and transferred by standard SDS-PAGE and WB method. X-ray film was exposed to the gel, kept at -80°C overnight and developed.

**RNA isolation and RT-PCR of human samples:** RNA was isolated from human blood using a commercially available kit (Vivantis). RT-PCR reactions were carried out using the Qiagen One-Step RT-PCR kit to amplify the open reading frame. The base pair substitution was by sequence analysis. Sequences of primers used are available upon request.

**Statistical Analysis:** Values are presented as mean±SEM. Normality of data was tested using the Shapiro-Wilk test. The non-parametric Mann-Whitney test was used to test for significance between groups with sample sizes of 5 or less. In spite of non-normality according to Shapiro-Wilk test and variance heterogeneity according to Levenes test, statistical significance between groups with large sample sizes was calculated using either 1-way ANOVA (when comparing two groups) or 2-way ANOVA (when comparing multiple groups with two factors) tests followed by Holm-Sidak post hoc testing. Statistical analyses were carried out using SigmaPlot 12.0 and SPSS. P values <0.05 were considered significant.
Supplementary References


Supplementary Figure Legends

**Supplementary Figure 1:** Endogenous Eya4 and Six1 expression in primary cardiomyocytes isolated from neonatal rats (NRCMs). **A,** qRT-PCR analysis of endogenous eya4 and six1 expression in two individual isolations. **B,** Western blot analysis of endogenous Eya4 and Six1 protein levels in NRCMs. GAPDH is shown as loading control. The results presented are of three individual isolations. **C,** Immunofluorescence microscopy showing expression and cellular distribution of endogenous Eya4 (red) and Six1 (green) in non-stimulated and Angiotensin II treated NRCMs. Scalebar 50µm. Merge represents overlay with Dapi-stained nuclei. **D,** Lysate from NRCMs was immunoprecipitated with α-Eya4 antibody. Western blot was performed using an α-Six1 antibody. Pooled non-treated lysate (input) from two isolations served as positive control.

**Supplementary Figure 2:** Endogenous Eya4 and Six1 proteins in adult cardiomyocytes isolated from sham or TAC treated WT mice. **A,** Western blot analysis of endogenous Eya4 and Six1 protein levels in LV extracts of sham and TAC treated animals. GAPDH is shown as loading control. **B,** Immunofluorescence microscopy showing expression and cellular distribution of endogenous Eya4 (red) and **C,** Six1 (green) in adult cardiomyocytes isolated from sham (upper panels) or TAC treated (lower panels) WT mice. Scalebar 50µm. Merge represents overlay with Dapi-stained nuclei.

**Supplementary Figure 3:** Adenovirally delivered Eya4, E193 or Eya4-shRNA influence the expression of the immediate early genes **A,** β-MHC and **B,** ANP (normalized to GAPDH) *in vitro.* Data regarding β-MHC expression are presented as mean±SEM relative to total MHC expression. *P<0.05 vs untreated control (ctr), n=3. Data shown in B are presented as mean±SEM relative to GAPDH. Expression level in ctr is set to 100%. *P<0.05 vs untreated ctr, n=3.

**Supplementary Figure 4:** Constitutive overexpression of Eya4 or E193 lead to alterations in protein levels of p27kip1, pHDAC2 and selected cardiomyopathy associated proteins in TG animals already before cardiac phenotypes are detectable. Western blotting was performed on heart lysates from **A,** WT, **B,** HA-E193 and **C,** HA-Eya4 TG animals at different time points. Data are presented as mean±SEM relative to GAPDH, n=5. Data regarding β-MHC expression are presented as mean±SEM relative to total MHC expression, n=5. **D,** Differences between WT and TG animals regarding HW/TL.
and E, CCSA were detectable starting at the age of three months and increased in an age dependent manner.
Suppl. Fig. 1

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<td>ctrl-3</td>
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C

\( \theta \) Angi

\( + \) Angi

D

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\( \theta \) Angi

\( + \) Angi

merge
Suppl. Fig. 2

A

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B

[Images of fluorescence microscopy results for Eya4, Dapi, and merge for Split, TAC, and Six1 conditions.]