Gene regulation in hearts of Hey-mutant mouse embryos and monitoring of sub-cellular Hey1 distribution

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades der
Julius-Maximilians-Universität Würzburg

vorgelegt von

Eva Englberger
aus Deggendorf

Würzburg, 2012

eingereicht bei der Fakultät für Biologie am: 24.07.2012

Mitglieder der Promotionskommission:

Vorsitzender: Prof. W. Rössler

1. Gutachter: Prof. M. Gessler
2. Gutachter : Prof. R. Benavente

Tag des Promotionskolloquiums:

Doktorurkunde ausgehändigt am:

Dedicated to…

… my parents,

for always supporting me, in any case;

… Björn,

your love and support helped me so much –

with you, everything is so easy;

… me,

there were times I had doubts,

there were times I thought I couldn’t go on,

but in the end,

I did it -

MY WAY.

„Considering the wonderful frame of the human body, this infinitely complicated engine, in which to the due performance of the several functions and offices of life, so many strings and springs, so many receptacles and channels are necessary, and all to be in their right frame and order; and in which, besides the infinite, imperceptible and secret ways of mortality, there are so many sluices and flood-gates to let death in, and life out, it is next to a miracle we survived the day we were born."

(Puckle, 1798)

STUFEN

(Hermann Hesse)

Wie jede Blüte welkt
und jede Jugend dem Alter weicht,
blüht jede Lebensstufe,
blüht jede Weisheit auch und jede Tugend
zu ihrer Zeit und darf nicht ewig dauern.

Es muss das Herz bei jedem Lebensrufe
bereit zum Abschied sein und Neubeginne,
um sich in Tapferkeit und ohne Trauern
in and're, neue Bindungen zu geben.
Und jedem Anfang wohnt ein Zauber inne,
der uns beschützt und der uns hilft zu leben.

Wir sollen heiter Raum um Raum durchschreiten,
an keinem wie an einer Heimat hängen,
der Weltgeist will nicht fesseln uns und engen,
er will uns Stuf' um Stufe heben, weiten!

Kaum sind wir heimisch einem Lebenskreise
und traulich eingewohnt,
so droht Erschlaffen!
Nur wer bereit zu Aufbruch ist und Reise,
mag lähmender Gewöhnung sich entraffen.

Es wird vielleicht auch noch die Todesstunde
uns neuen Räumen jung entgegen senden:
des Lebens Ruf an uns wird niemals enden.
Wohlan denn, Herz, nimm Abschied und gesunde!

Abbreviations

Amp	ampicillin
APS	ammoniumpersulfate
AV	atrioventricular
bHLH	basic helix-loop-helix
cDNA	copy DNA
ChIP	chromatin immunoprecipitation
CMV	Cytomegalovirus
D10	DMEM + 10% FBS + 1% penicillin/streptomycin
DEPC	diethylpyrocarbonate
DKO	double KO
Dll	Delta-like ligand
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	dimethylsulfoxide
DNA	desoxy ribonucleic acid
Dox	doxycyclin
dTNPs	deoxynucleotide triphosphate
DTT	dithiotreitol
ECGS	endothelial cell growth serum
EDTA	ethylendiaminetetraacidic acid
EGF	epidermal growth factor
EGFP	enhanced GFP
EMT	epithelial-mesenchymal transition
EtOH	ethanol
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FITC	fluoresceine isothyiocyanate
FS	Flag-Strep tag
FSG	fishskingelatin

GFP	green fluorescent protein
GO	gene ontology
HBMEC	human brain microvascular endothelial cells
HEK	human embryonic kidney
iEP	IRES-EGFP-puro
IP	immunoprecipitation
IRES	internal ribosome entry site
ISH	*in situ* hybridization
KO	knock out
LTR	long terminal repeat
MAML	mastermind-like
MAPK	mitogen-activated protein kinase
MOPS	3-(N-Morpholino)-1-propane sulfonic acid
NES	nuclear export signal
NLS	nuclear localization signal
NPC	nuclear pore complex
p.a.	per analysis
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	phosphate buffered saline tween
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEI	polythyleneimine
PFA	para-formaldehyde
PI	propidium iodide
PMSF	phenylmethanesulfonylfluoride
POD	peroxidase
qRT-PCR	quantitative reverse transcription PCR
REST	relative expression sequence tool
RNA	ribonucleic acid

SB	sodium borate
SDS	sodium dodecyl sulfate
SHH	sonic hedgehog
TBS	Tris buffered saline
TE	Tris-EDTA
TEMED	tetramethylethylenediamin
tRNA	transfer RNA
VSD	ventricular septum defect
WISH	whole mount ISH
wt	wildtype

Table of Contents

Table of Contents 11
Summary 14
Zusammenfassung 16
1. Introduction 19
1.1 Heart Development 19
1.2 Notch signaling pathway 20
1.2.1 Canonical Notch signaling 20
1.2.2 Target genes of Notch and their importance in development 22
1.2.2.1 Hes- and Hey-protein family 22
1.2.2.1.1 Expression patterns of Hey genes 23
1.2.2.1.2 Phenotypes of Hey knock out mice and ActH1 mice 23
1.2.2.2 Targets of the Hey proteins 25
1.2.3 Interaction of Notch signaling with other pathways 25
1.3 Nuclear transport 26
1.4 Aim of the study 28
2. Material 29
2.1 Chemicals 29
2.2 Plasmids 29
2.3 Restriction Enzymes for Cloning 30
2.4 Primer 31
2.5 Antibodies for Western Blot 32
2.6 Antibodies for immunoflurescent staining 32
2.7 RNA (whole mount) *in situ* hybridization probes 33
2.8 Cell culture reagents and cell lines 33
2.9 Buffers 34
2.9.1 Buffers for PCR 34
2.9.2 Buffers for ISH 35
2.9.3 Buffers for Western Blot and IPs 36
2.9.4 general buffers 38
2.10 Mice 39

3. Methods 40
3.1 Mouse genotyping.. 40
3.2 Heart tissue preparation 41
3.3 RNA extraction and cDNA synthesis... 41
3.4 Quantitative real time-PCR (qRT-PCR).. 42
3.5 Cloning of virus constructs 43
3.6 Cell culture.. 43
3.7 Immunofluorescence.. 44
3.8 Generation of stable cells lines 44
3.9 *In vitro* assay for analyzing possible influences on the localization of Hey proteins 45
3.10 Generation of nuclear and cytoplasmic protein lysates.. 46
3.11 Cell cycle synchronization and propidium iodide FACS for cell cycle analysis 47
3.12 Tissue preparation for *in situ* hybridization 48
3.13 *In situ* hybridization (ISH) and whole mount ISH (WISH) 49
3.13.1 DNA purification for generating ISH probes.. 49
3.13.2 *In vitro* transcription and RNA precipitation of ISH probes.. 49
3.14 ISH protocol 50
3.15 WISH protocol 51
3.16 Immunoprecipitation (IP) 52
3.17 SDS-Page and Western Blot.. 53
4. Results 55
4.1 Analysis of gene expression in cardiac tissue in different Hey mutant mouse embryos at different stages of development 55
4.1.1 Gene expression profile in ventricular tissue of Hey2-KO embryos (E14.5) 55
4.1.2 Gene expression profile in ventricular tissue of Hey1/L-DKO embryos (E14.5) 57
4.1.3 Gene expression profile in ventricular tissue of ActH1 embryos (E14.5)............................... 58
4.1.4 Gene expression profile in ventricular tissue of Hey2-KO embryos (E17.5) 59
4.1.5 Gene expression profile in ventricular tissue of Hey1/L-DKO embryos (E17.5) 60
4.1.6 Summary of all genotypes and developmental stages at developmental stage E14.5........... 61
4.2 Validation of gene expression using *in situ hybridization* 62
4.2.1 *In situ hybridization* on sections of Hey2-KO embryos (E14.5 or E17.5)............................... 62
4.2.2 *Whole mount in situ hybridization* on Hey2-KO embryos (E10.5 or E11.5) 63
4.3 Possible influences on the nuclear localization of Hey 65
4.3.1 Treatment of HeLa-201-Cherry-mHey1 cells with inhibitors of important intracellular pathways 65

4.3.2 Treatment of HeLa cells with stimulating agents 67
4.3.3 Distribution of Hey in nucleus and cytoplasm 67
4.3.4 Possible influences of CaMKII on Hey localization 69
4.3.5 Hey localization during the cell cycle 69
4.4 Interaction of Hey with Importinα and β 71
4.4.1 Interaction studies with Importinα 71
4.4.2 Interaction studies with Importinβ 72
5. Discussion 74
5.1 Gene regulation in different Hey mutant mice at different timepoints in development 74
5.1.1 Results from quantitative real time PCR analyses 74
5.1.2 Validation of regulated genes with ISH and WISH 77
5.2 Localization studies for Hey1 78
5.2.1 Influences in cell culture experiments 78
5.2.2 Distribution of Hey in nucleus and cytoplasm 80
5.3 Interaction studies of Hey with nuclear transport proteins 81
6. Bibliography 84
Appendix 90
Publications and meetings 91
Eidesstattliche Erklärung 92
Lebenslauf 93
Danksagung 95

Summary

In the present study, different aspects of the Notch target genes Hey1 and Hey2 have been investigated. The first part dealt with gene regulation in ventricular tissue of different Hey-mutant mice at two developmental stages. The second aspect attempted to answer questions on the nuclear transport of Hey proteins and their interaction with the transport proteins Importinα/β. The final part of the study was focused on possible influences on the localization of Hey1 within the cell.

Hey2-KO mouse embryos show a strong cardiac phenotype of ventricular septum defects and hypertrophy as compared to wild type littermates. Tissue samples from heart ventricles derived from Hey2-wt and –KO mouse embryos at embryonic day 14.5 and 17.5 were analyzed with the help of quantitative real-time PCR to determine differentially regulated genes of the two different genotypes that might be caused by the lack of Hey2. At the earlier timepoint, a clear up-regulation of several genes was obtained when compared to wild type littermates. Among the regulated genes were important heart-developmental factors such as Tbx2, the vegf receptors 1, 2 and 3 and Sema6D as well as PlxnD1, two members of the semaphorin-plexin system. Later in development, this clear regulation pattern was more or less lost. Hey1/L-DKO embryos served as controls for both developmental stages as they phenocopy the Hey2-KO animals. With this comparison, it could be concluded that the gene regulation obtained for Hey2-KO embryos was in fact due to the lack of Hey2 and not influenced by morphological changes within the heart. In contrast, ActH1 mice over-expressing the Hey1 gene in a global manner, showed a counter-regulation of genes up-regulated at E14.5 stage as most genes were down-regulated in this analysis. Furthermore, validation of the obtained gene expression data with (whole mount) *in situ* hybridization was attempted, however, only for Sema6D and Smad6, this regulation was evident. All other tested RNA probes did not show distinct signals or any differences between wt and KO, thought in part to be due to the decreased sensitivity of this method compared to real-time qRT-PCR.

The nuclear transport of Hey proteins has not been elucidated to date and in the present study, some aspects of the transport processes were investigated. Importinα as well as β

have been identified earlier by mass spectrometry as possible interaction partners of Hey proteins. As both factors are involved in nuclear transport, it was examined if there was an interaction with Hey1, and what the nature of that interaction might be. However, a direct interaction of Importinα or Importinβ with Hey1 could not be verified using immuno-precipitation leaving open the question how nuclear transport of Hey proteins then works.

Hey proteins are normally located in the nucleus where they can fulfill their role as transcription factors. The question of whether the localization of Hey1 could be modified in HeLa cells was evaluated. This was performed in cells that stably expressed mCherry-coupled Hey1 which were treated with EGF, TGFα or inhibitors of different important signaling pathways like the MAPK cascade. However, Hey1 localization was unchanged and only detectable in the nucleus. This finding also occurred when plasmids containing CaMKII (mutants) were transfected into the cells indicating that there is no influence on Hey1 localization. Moreover, the nuclear localization signal of Hey1 did also not change throughout all cell cycle phases supporting these results. On the other hand, Western Blot analyses of nuclear and cytoplasmic fractions of Hey-expressing cells revealed almost equal amounts of the Hey proteins in both compartments leading to the question of biochemical relevance of cytoplasmic Hey1.

From the data obtained throughout this study, it can be concluded that the lack of Hey2 changes gene expression patterns in ventricular tissue of mouse embryos during several stages of embryonic development leading to great differences in gene expression compared to wild type littermates. Furthermore, the nuclear transport mechanism for Hey1 is still unclear as no interaction with Importin proteins could be established. Finally, inhibition or stimulation of signaling pathways did not show any influences on the sub-cellular localization of Hey1 which did also not change throughout all cell cycle stages.

Zusammenfassung

Die vorliegende Arbeit beschäftigt sich mit drei unterschiedlichen Aspekten der Notch-Zielgene Hey1 und Hey2. Im ersten Teil wurde untersucht, inwieweit sich Mutationen in verschiedenen Hey-Genen beziehungsweise deren Abschalten auf die Genexpression in ventrikulären Geweben von Mausembryonen an zwei verschiedenen Zeitpunkten in der Embryonalentwicklung auswirken. Der zweite Abschnitt der Arbeit widmete sich der Fragestellung, wie Hey-Proteine in den Kern hinein oder aus ihm heraus transportiert werden und ob die Hey-Proteine dabei mit Importinα/β interagieren. Als letzter Punkt wurde die Frage behandelt, ob es möglich ist, die (nukleäre) Lokalisation von Hey1 zu beeinflussen.

In Herzgewebe von Hey2-Wildtyp- und –KO-Embryonen, das an zwei verschiedenen Tagen der Embryonalentwicklung in der Maus (E14.5 und E17.5) entnommen worden war, wurde mittels quantitativer real-time RT-RCR ermittelt, ob das Abschalten von Hey2 einen Einfluss auf die Genexpression hat, denn Hey2-KO-Embryonen zeigen deutliche phänotypische Veränderungen im Herzen (Ventrikel-Septum-Defekt, Hypertrophie) im Vergleich zu Wildtyp-Embryonen. Zum früheren Zeitpunkt in der Entwicklung konnte eine deutliche Hochregulation einiger Gene gezeigt werden, unter ihnen wichtige Mitspieler in der Herzentwicklung wie Tbx2, die vegf-Rezeptoren 1 bis 3 und Sema6D sowie PlxnD1, die beide zum Semaphorin-Plexin-System gehören. Die Analyse des späteren Entwicklungsstadiums ergab keine solch klare Genregulation mehr. Als Kontrolle dienten Hey1/L-DKO-Embryonen, die phänotypisch den Hey2-KO-Tieren sehr ähnlich sind, und daher zeigten, dass die resultierenden Veränderungen im Genexpressionsmuster in Hey2-KO-Mäusen durch das Fehlen von Hey2 bedingt sind und nicht aufgrund der morphologischen Veränderungen zustande gekommen sind. Ventrikuläres Gewebe von ActH1-Mäusen, die eine globale Überexpression von Hey1 aufweisen, zeigte eine deutlich gegensätzliche Genexpression zum Zeitpunkt E14.5, da hier viele Gene herunter reguliert waren. Weiterhin wurde versucht, diese Genregulationsdaten mittels (whole mount) *in situ*-Hybridisierung zu bestätigen. Allerdings war dies nur für Sema6D und Smad6 ansatzweise möglich; alle anderen verwendeten RNA-Sonden zeigten keine deutlichen Signale und damit keinen Unterschied zwischen Hey2-Wildtyp- und –KO-Tieren, was

durch eine geringere Sensitivität dieser Methode verglichen mit der quantitativen real-time RT-RCR bedingt sein könnte.

Die Kerntransportprozesse bezüglich der Hey-Proteine sind noch nicht ausreichend erforscht. In dieser Arbeit wurde versucht, einen Teil davon zu beleuchten. Importinα und β wurden bereits mittels Massenspektrometrie als mögliche Interaktionspartner für Hey1 bestimmt und spielen eine wichtige Rolle während des Kernimports. Demzufolge stellte sich die Frage, ob die Hey-Proteine tatsächlich mit ihnen interagieren und falls sie dies tun, wie diese Interaktion aussehen könnte. Allerdings konnte weder für Importinα, noch für Importinβ mittel Immunpräzipitation nachgewiesen werden, dass eine direkte Interaktion mit Hey1 stattfindet, was die Frage aufwirft, wie die Transportprozesse für die Hey-Proteine ablaufen, wenn diese nicht über die klassischen Importin-vermittelten Mechanismen stattfinden.

Die Hey-Proteine sind üblicherweise im Zellkern lokalisiert, wo sie ihre Aufgabe als Transkriptionsfaktoren wahrnehmen und erfüllen können. Dennoch stellte sich die Frage, ob die Lokalisation von Hey beeinflusst werden kann, indem HeLa-Zellen, die stabil eine mCherry-fusionierte Version von Hey1 exprimieren, mit Wachstumsfaktoren wie EGF und TGFα stimuliert werden oder mit Inhibitoren verschiedener anderer wichtiger Signalwege wie der MAPK-Kaskade behandelt werden. Allerdings änderte sich die nukleäre Lokalisation von Hey1 dadurch genauso wenig wie durch Transfektion der Zellen mit Plasmiden, die CaMKII (Mutanten) exprimieren können, was darauf hinweist, dass kein Einfluss auf die Lokalisation von Hey1 erfolgt. Auch während des gesamten Zellzykluses war Hey1 immer im Zellkern lokalisiert, was diese Ergebnisse weiter unterstützt. Allerdings zeigten Western Blot-Analysen von Kern-Cytoplasma-Fraktionierungen, dass die Hey-Proteine auch zu einem nicht unerheblichen Anteil im Cytoplasma vorliegen, was die Frage aufwirft, welche Funktion sie dort haben könnten und wie sie dorthin gelangen.

Die gezeigten Ergebnisse lassen darauf schließen, dass das Fehlen von Hey2 die Genexpression im Ventrikel von Mausembryonen während der (Herz-)Entwicklung verändert, was zu deutlichen Unterschieden im Genexpressionsmuster verglichen mit Wildtyp-Embryonen führt. Weiterhin ist der Kerntransportmechanismus für Hey1 nach wie vor unklar, da keine

direkte Interaktion von Hey1 mit Importproteinen nachgewiesen werden konnte. Darüber hinaus wurde die nukleäre Lokalisation von Hey1 weder durch Inhibition oder Stimulation anderer wichtiger intrazellulärer Signalwege, noch durch Expression möglicher Effektoren beeinflusst und diese hat sich auch während aller Phasen des Zellzykluses nicht verändert.

1. Introduction

Heart development is one of the key processes during embryogenesis – with severe consequences if it fails. In newborns, congenital heart defects represent the main proportion with about a 1% incident rate in all human live births (Hoffman&Kaplan, 2002). One signaling pathway that is highly involved in cardiac development is the Notch pathway (Nemir&Pedrazzini, 2008; de la Pompa, 2008) that has great influence on proper formation of the vertebrate heart.

1.1 Heart Development

Mouse heart development begins as early as gastrulation stage (around embryonic day E7.0) with cells from the mesodermal layer later forming the heart. The so-called cardiac crescent then develops into the heart tube and the myocardial, pericardial and endothelial parts, along with the atrioventricular canal, occur. During heart development, cells from the secondary heart field invade and populate the outflow tract and the primordium of the later right ventricle. Then, the so-called looping of the heart tube starts and the typical heart structure is established (around E11.0). Thereafter, atrial and ventricular parts can be distinguished and they are separated by the atrioventricular canal. From now on, atria and ventricles develop separately (Anderson, 2003; Moorman, 2004; Bruneau, 2002).

Formation of the ventricles is caused by ballooning of the outer curve of the two ventricles forming their apical parts. During this ballooning process, formation of the ventricular septum starts. The septum has a muscular and a membranous part that developed from different sources, with the membranous section being responsible for closing the gap between both regions. If this is unsuccessful, severe heart defects are the consequence such as ventricular septum defects (Christoffels, 2004; High&Epstein, 2008; Savolainen, 2009).

Formation of the heart is a highly regulated process influenced by many signaling pathways interacting with each other. Several factors take part in the genetic interplay accompanying heart development such as the earliest cardiac marker Nkx2.5 or members of the MEF2

family that also influence the whole heart development process (Srivastava&Olson, 2000). In addition to Notch signaling, the Bmp/Tgfβ-pathway is also involved in heart development. **Figure 1** depicts the interplay of these two signaling cascades in the heart when atrio-ventricular canal (AV canal) formation takes place. Its' proper development is dependent on the presence of T-box factor 2 (Tbx2) which inhibits chamber-specific gene expression within that tissue. Tbx2 itself is regulated by bone morphogenic protein 2 (Bmp2) that is also located to the AV canal. The fact that both cardiac factors are only restricted to the AV canal is an interesting observation and may be due to Notch signaling influences. The Notch target genes Hey1 and Hey2 may thereby play a great role (High&Epstein, 2008; Rutenberg, 2006; Bruneau, 2002; Niessen, 2008; de la Pompa, 2008).

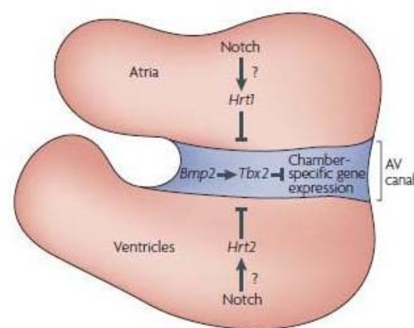


Figure 1: interacting pathways in heart development

(High&Epstein, 2008)

Another important factor in heart development is GATA4 that itself is known to interact with the Hey proteins thereby influencing the expression of the atrial marker ANF (Fischer, 2005). This highlights the great importance of Notch signaling during heart formation.

1.2 Notch signaling pathway

1.2.1 Canonical Notch signaling

The Notch signaling pathway is a highly conserved signaling cascade that plays fundamental roles both in development and disease. During embryonic development, it guides cellular processes involved in cell differentiation as well as proliferation and apoptotic programs.

What is characteristic for Notch signaling is its' necessity of neighboring cells for activation as the Notch receptor as well as the Delta-like or Jagged ligands are membrane-bound (Kopan&Ilagan, 2009; Andersson, 2011).

In mammals, there are four members of the Notch receptor family (Notch 1 to 4). The receptors are composed of an extracellular domain that harbors several epidermal growth factor (EGF)-like repeats that convey the interaction with the ligands, a single transmembrane domain and an intracellular part that conducts the stimulus to the nucleus of the cell. The mammalian Notch signaling has five ligands: Jagged 1 and 2 (Jag1, Jag2) and Delta-like 1, 3 and 4 (Dll1, Dll3, Dll4). As mentioned above, these ligands are also membrane-bound via a transmembrane domain and they also harbor EGF-like repeats in their extracellular part (Niessen&Karsan, 2007; Kopan&Ilagan, 2009).

Canonical Notch signaling is conveyed in the following manner: after ligand binding, the Notch receptor is cleaved in a two-step process leading to a free Notch intracellular domain (NICD) that can translocate to the nucleus of the cell where the NICD binds to the RBPJκ-repressor complex. Due to that, the repressor complex is converted into an activating signal by recruiting co-activators (MAML and CBP). Only then the transcription of target genes of the Notch signaling pathway is possible (see **Figure 2**; Fischer&Gessler, 2007).

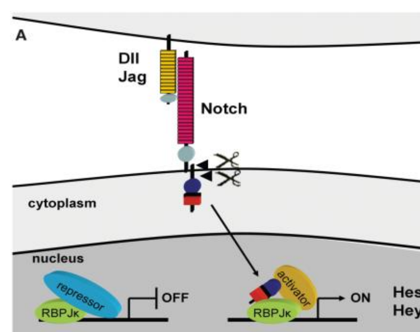


Figure 2: canonical Notch signaling

(Fischer&Gessler, 2007)

1.2.2 Target genes of Notch and their importance in development

1.2.2.1 Hes- and Hey-protein family

In *Drosophila melanogaster*, this basic helix loop helix (bHLH) protein family was first described. The nomenclature of the mammalian counterparts is related to this as they are called hairy/Enhancer-of-split related (Hes) (with YRPW motif (Hey)). In mammals, there are three different Hey proteins: Hey1, Hey2 and HeyL. For the Hes proteins, seven members have been described. All of them – Hey as well as Hes – act as transcriptional repressors.

At the amino terminus, the members of this protein family harbor a basic domain that is responsible for DNA binding - most likely to classical E-box sequences (CACGTG) - followed by a helix loop helix motif. This motif is important for protein dimerization. Hey and Hes proteins can form homodimers as well as heterodimers with other members of the protein family. The last conserved part of the Hes and Hey proteins is the Orange domain that also plays a role in the dimerization process. In contrast, the carboxy terminus of the proteins is less conserved. For Hey1 and Hey2, this part is quite similar as shown in **Figure 3.** There are two conserved motifs: YRPW and TE(I/V)GAF. HeyL, on the other hand, has the same TEIGAF sequence as Hey1, however, it is preceded by a YHSW motif. In Hes1, the TEIGAF motif is missing and the WRPW motif is more similar to the Drosophila sequence than to that of the Hey proteins (Fischer, 2003; Iso 2003; Davis, 2001).

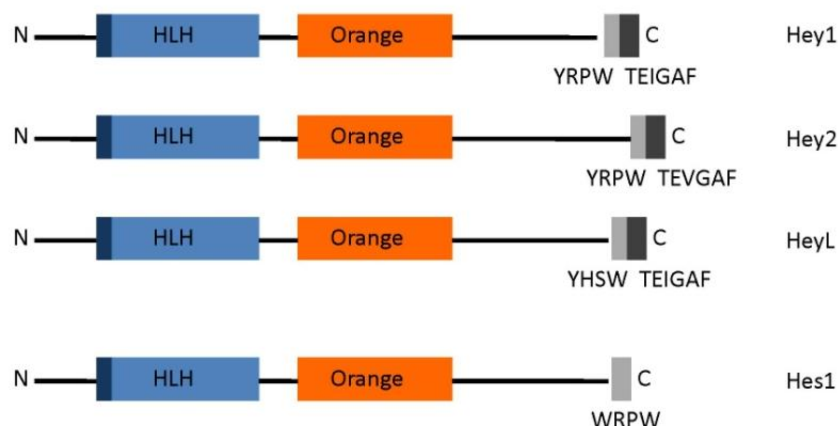


Figure 3: protein structure of Hey and Hes proteins

Hey and Hes proteins show a highly conserved protein structure, especially at the amino-terminal part; HLH: helix-loop-helix

1.2.2.1.1 Expression patterns of Hey genes

The Hey proteins show specific and very distinct expression patterns in several tissues during murine development. During heart formation, all three Hey proteins are co-expressed in the atrioventricular cushions. Additionally, Hey1 and Hey2 are restricted to specific cardiac areas as Hey1 is limited to the atria and Hey2 to the ventricular part of the heart (**Figure 4 A**) (Fischer, 2003; Fischer, 2007). Furthermore, all three Hey proteins can be detected in blood vessels (**Figure 4 B**) where Hey1 is expressed in the endothelial part and Hey2 and HeyL can be found in smooth muscle as well as endothelial cells (Fischer, 2003).

According to these limited and distinct expression patterns it is assumed that the single Hey proteins play specific roles during embryonic development. However, some redundant roles for all Hey proteins are also suggested.

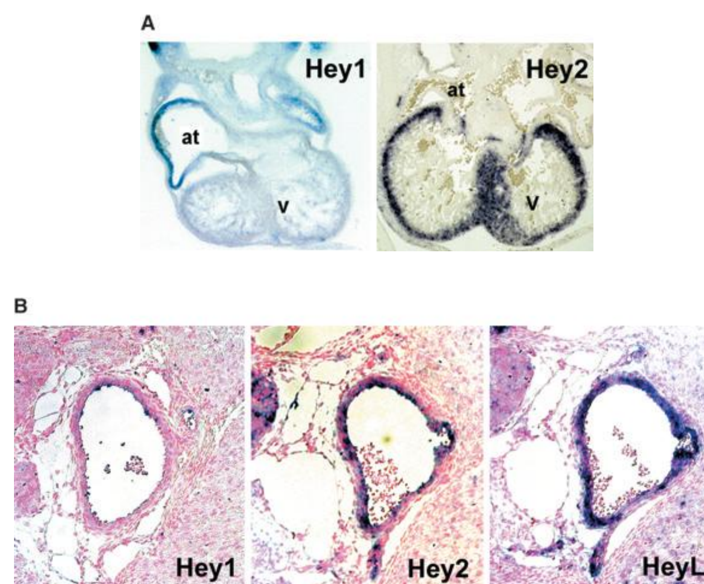


Figure 4: expression patterns of Hey proteins

A) *in situ* hybridization showing limited expression of Hey1 in atrial and Hey2 in ventricular tissue of mice hearts; B) expression of Hey1, Hey2 and HeyL in blood vessels (Fischer, 2003)

1.2.2.1.2 Phenotypes of Hey knock out mice and ActH1 mice

The importance of the Hey proteins for normal development is shown in Hey and Hes knock out (KO) mouse models. Homozygous Hey1-KO animals develop normally showing no obvious abnormalities during embryogenesis and when heart development is investigated specif-

ically. Homozygous Hey2 knock outs on the other hand display several tissue-specific abnormalities with the most prominent phenotype becoming obvious in cardiac structures from embryonic day 13.5 (E13.5) onwards. These animals suffer from severe heart defects caused by ventricular septum defects (VSD) in which the membranous part of the septum does not grow out to close the gap to the muscular section of the septum. Furthermore, these mice typically show a massive biventricular hypertrophy which is thought to be the compensating process for the VSD. The same phenotype is evident in Hey1 and HeyL double knock out animals whereas the single HeyL-KO – similar to the single Hey1-KO – does not develop any obvious abnormalities. The combined loss of both Hey1 and Hey2 results in embryonic death at around E9.5/E10.5 due to vascular remodeling defects as well as hemorrhages and problems with arterial differentiation. Hes1-KO animals also show failure in heart development like VSD and overriding aorta at E15.5 as well as defects in secondary heart field proliferation, a reduced numbers of cardiac neural crest cells and problems in outflow tract development earlier during embryogenesis (Fischer, 2003; Wiese, 2010; Sakata, 2006; Sakata, 2002; Rochais, 2009).

Phenotypic effects caused by elevated Hey1 levels can be studied in the so-called ActH1-mice that are characterized by a global over-expression of Hey1 under the control of the β-actin promoter and have been generated by M. Susa. These mice display defects in bone formation but apart from this develop quite normal (Salie, 2010).

The fact that some Hey single knock outs do not show any phenotypes might be explained by the redundancy of the Hey family members in which, for example, Hey2 can compensate for the lack of Hey1 in blood vessels (Fischer, 2004). Some of the phenotypes described above are due to an impaired EMT process during heart development. These cardio-vascular phenotypes can also be observed in mice in which other members of the Notch signaling pathway have been knocked out such as the Notch receptors themselves (Timmerman, 2004).

1.2.2.2 Targets of the Hey proteins

The Hey proteins as targets of the Notch signaling pathway are themselves influencing a great variety of other factors. As previously mentioned, interactions with GATA4/6 and ANF have been described (Fischer, 2005). Microarray analysis and ChIP-Seq were applied to identify further targets of the Hey protein family. GO term analysis clustered the identified genes mainly to transcriptional regulation as well as developmental processes (Heisig, 2012). Additional interaction partners of Hey1 were identified by mass spectrometry such as ubiquitin ligases as well as proteins involved in nuclear transport or signal mediators of various important signaling pathways (Dr. Daniela Salat, personal communication).

1.2.3 Interaction of Notch signaling with other pathways

As previously mentioned, Notch signaling interacts with the Bmp/Tgfβ-pathway. This is not only true for heart development, but in a recent publication (Moya, 2012), it was described that Notch and the Smad1/5 component of the Bmp/Tgfβ-pathway together orchestrate tip and stalk cell fate in developing mouse retinas, too. Furthermore, during the EMT process in heart development, Notch interacts with another component of Bmp/Tgfβ signaling as Snail1/2 expression is influenced; these factors promote EMT-induced invasion of the cells into other tissues (MacGrogan, 2011; Niessen, 2008; von Gise&Pu, 2012).

There are several other examples in which Notch interacts directly or indirectly with other signaling cascades or factors such as the inhibitor of DNA binding (Id) proteins (Meier-Stiegen, 2010; Tokuzawa, 2010). A link to angiogenesis is given via the vascular endothelial growth factor receptors (vegfR) (Phng, 2009) as well as with FoxC transcription factors (Hayashi, 2008). Another system that is involved in the (cardiac) developmental processes is the plexin-semaphorin protein family that shows interactions with Notch signaling both in neural as well as heart development (Gitler, 2004). Furthermore, there is evidence for cross-talk between the Notch pathway and other important signaling cascades during developmental processes such as the sonic hedgehog (SHH) and Wnt pathway (Dyer, 2010; Srivastava&Olson, 2000).

1.3 Nuclear transport

Nuclear transport plays an important role in each cell. The transport process can be divided into two different types: active (and thereby directed) transport and passive diffusion of small molecules (up to about 30kDa) (Güttler&Görlich, 2011; Elion, 2002). The interchange between both cellular compartments is quite rapid with approximately one million molecules shuttled per second (Ribbeck&Görlich, 2001).

Active nuclear transport takes place via the so-called nuclear pore complexes (NPC) in which about 30 nucleoporins form a ring-like structure that has a diameter of about 40nm. The NPC has a cytoplasmic component, the mid-part is formed by the central core and finally, the nucleus-located basket acting as exit point of imported proteins. The nucleoporins exist of three different protein families: 1) transmembrane nucleoporins that anchor the NPC in the nuclear membrane, 2) FG-nucleoporins that can bind Importins (also called karyopherins) and 3) nucleoporins containing either WD repeats or a seven-bladed propeller motif. The structure of the NPC is highly conserved through different species from vertebrates to *Caenorhabditis elegans* and yeast (Sorokin, 2007) and is depicted in **Figure 5**.

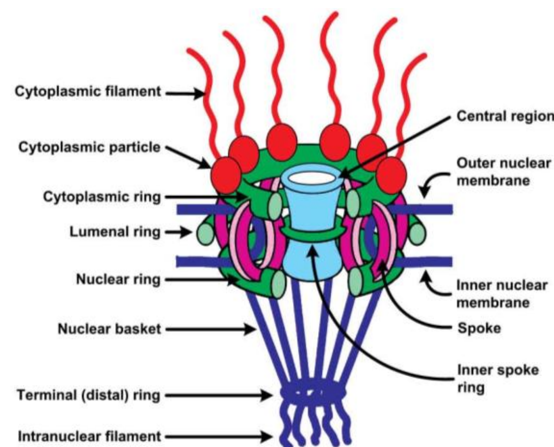


Figure 5: structure of the nuclear pore complex

(Sorokin, 2007)

Nuclear transport is mainly mediated by two different protein families: the RanGTPases and the Importins (Gittler&Görlich, 2011).

The Importins can be divided into an α and β sub-family. Thereby, Importinα acts as the Importinβ-dependent adapter molecule in nuclear import processes and binds to classical nuclear localization signals (NLS) within the protein that should be transported. Importins in general act in close collaboration with Ran. As depicted in **Figure 6**, Importinβ binds to RanGTP and in doing so, the metabolic energy supplied by the RanGTPase system can be used as the driving force for directed nuclear import. The nuclear concentration of RanGTP is more than 1000-fold higher as in the cytoplasm which leads to the unloading of the cargo and the Ran-Importin complex is re-directed to the cytoplasm where it disaggregates and is recycled for a new round of protein import. Nuclear export occurs in the opposite manner in which exportins bind in a high RanGTP-level to nuclear export signals (NES) of proteins and removes them from the nucleus. Each transport process is therefore irreversible and costs one GTP molecule (Gittler&Görlich, 2011; Nigg, 1997).

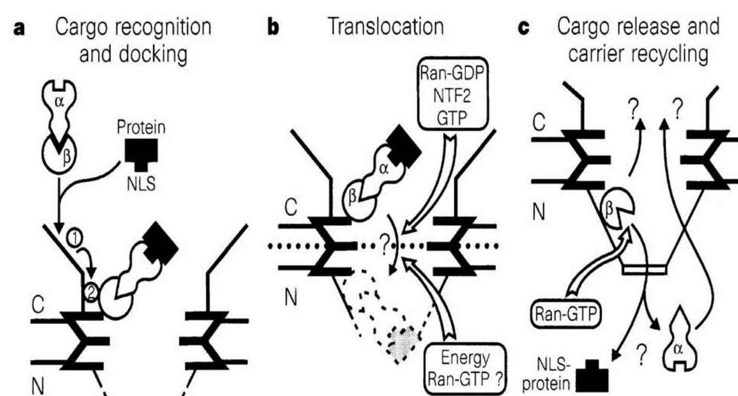


Figure 6: nuclear transport processes

A) Importins bind to the NLS of the protein-to-transport; B) Ran-concentration gradient is used as driving force to import the protein into the nucleus; C) the transported protein is released of the Importin complex which is re-directed to the cytoplasm for new transport processes (Nigg, 1997)

With regards to the Hey proteins, it is not only interesting to investigate nuclear transport processes but it would also be important to know if they can shuttle between the nucleus and the cytoplasm. Monitoring nuclear shuttling of various proteins can be easily done by using the so-called hetero-karyon-assay in which cells with two nuclei from different cell lines or species can be created (Gama-Carvalho&Carmo-Fonseca, 2006). The fusion of the cells is arranged by the addition of polyethylene glycol (PEG) to the cells which induces ag-

glutination of the cells and assists cell-cell contacts probably due to perturbations and re-arrangement of membrane lipids (Yang&Shen, 2007). Nevertheless, the hetero-karyon-assay might represent an appealing method to monitor shuttling of the Hey proteins.

1.4 Aim of the study

Several interesting hypotheses about the Hey proteins were the starting point for this study. Microarray analysis of cells over-expressing Hey1 or Hey2 revealed several new target genes for the Hey proteins (Heisig, 2012). To investigate if these targets can be validated *in vivo*, embryonic heart tissue of Hey-KO and –wt animals should be analyzed with quantitative re-al-time qRT-PCR for changes in the gene expression pattern. The obtained results showed several regulated genes in the KO animals that were attempted to be further investigated using *in situ* hybridization techniques.

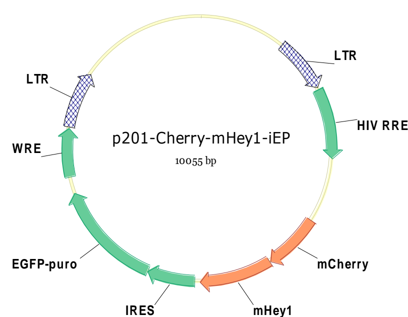
As second aim, the nuclear transport processes of the Hey proteins should be investigated as Importins and also Ran have been identified as possible interaction partners for Hey1 by mass spectrometry analysis in the same Hey1-overexpressing cell culture system used for the microarray study (the mass spectrometry work has been performed by Dr. Daniela Salat). Another interesting aspect is the sub-cellular localization of Hey proteins in the nucle-us. In this study, it should be analyzed if the localization of Hey1 could be influenced by blocking other important pathways in the cell like MAPK signaling or by interaction with the CaMKII system or if Hey1 could be displaced into the cytoplasm during the cell cycle.

2. Material

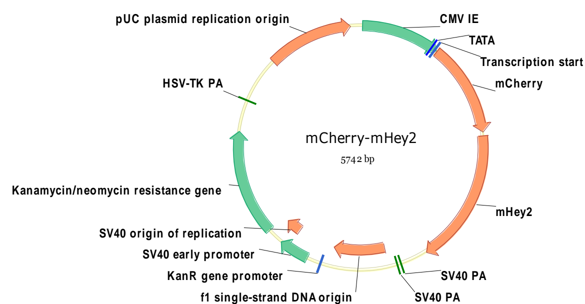
2.1 Chemicals

All chemicals, if not specifically noted, were purchased in p.a. quality from either Roth, Sigma-Aldrich or AppliChem.

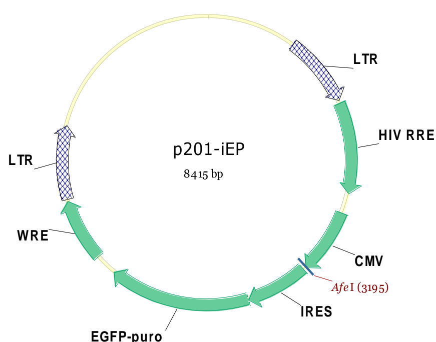
2.2 Plasmids



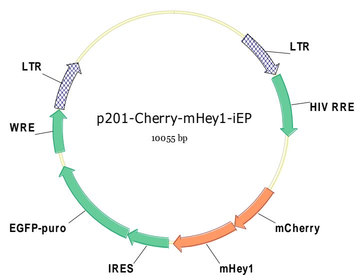
pmCherry-mHey1: donor plasmid for cloning of p201-Cherry-mHey1-iEP



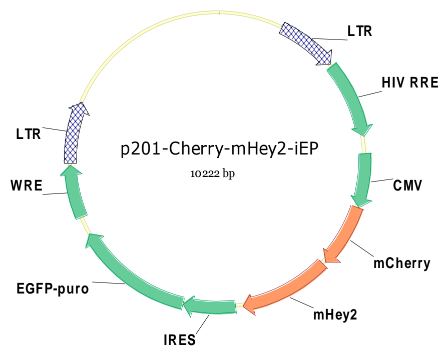
pmCherry-mHey2: donor plasmid for cloning of p201-Cherry-mHey2-iEP



p201-iEP: acceptor plasmid for cloning of p201-Cherry-mHey1-iEP or –mHey2-iEP



p201-Cherry-mHey1-iEP: lentiviral expression plasmid with CMV promoter, harboring an IRES element together with a fusionprotein of EGFP and puromycin for selection of transduced cells



p201-Cherry-mHey2-iEP: lentiviral expression plasmid with CMV promoter, harboring an IRES element together with a fusionprotein of EGFP and puromycin for selection of transduced cells

pCDNA3.1-3xMyc-plasmids harboring Importinα or β were a kind gift of Prof. E. Nigg (Biocenter, University of Basel, Switzerland) as well as a DsRed-Importinβ vector together with the Importinβ-antibody from Santa Cruz.

pCS2p, pCS2p-Flag and pCDNA3.1 were previously created in our laboratory and used as control plasmids as well as pEGFP constructs with different mutants of Hey1 or the wild type sequence. Furthermore, pCS2p-Flag-Hey constructs with full length Hey1 or the Hey1-delta basic variant as insert were employed.

pCS2p-CaMKII-wild type, -T286D and -K42M were kindly provided by Dr. M. Kühl (Howard Hughes Medical Institute, University of Washington School of Medicine, USA).

2.3 Restriction Enzymes for Cloning

All enzymes for cloning were bought from MBI fermentas.

Enzymatic reactions were carried out according to the manufacturer's protocols at 37°C in the appropriate buffer systems.

2.4 Primer

Table 1 shows all primer pairs used for real time PCR analysis. Usually, the PCR product was between 50 and 200bp. All primer sequences are given in 5' → 3' direction and were synthesized by Sigma. The lyophilized powder was dissolved in sterile H_2O at a final concentration of 100µM.

Table 1: primer pairs for real-time qRT-PCR

name	forward primer	reverse primer
HPRT	TGTTGTTGGATATGCCCTTG	ACTGGCAACATCAACAGGACT
Hey1	TGAGCTGAGAAGGCTGGTAC	ACCCCAAACTCCGATAGTCC
Hey2	TGAGAAGACTAGTGCCAACAGC	TGGGCATCAAAGTAGCCTTTA
HeyL	CCGACTGGGAGCCTTAGC	GTTTCTTCCTGGCTTGCATCT
ANF	GGGTAGGATTGACAGGATTGG	CACACCACAAGGGCTTAGGA
MLC-1A	CGCGAATTCAAGCTGGGGCTCTTTATTTC	CGCGGATCCGGGTAAAGCACGTTTCTC
MLC-2A	CGCGAATTCAGGCACAGAGTTTATTGAGG	CGCGGATCCGAGGAAGCCATCCTGAGT
PlxnD1	ACCCTGACACCCTGCATATCT	GTCGATGTGGTCCGTCTTCT
PlxnA2	TGTTTGATTTCCTGGATGAGC	ATCTGTGATGCTGCCCTTGT
Sema3C	TTGAGTGTGCTCCCAAGTCTC	AGCTATAATGCGCTCGTTCAGT
Sema6D	CTGAAGCTGGCGTGGTACTT	GACCACCTTTCTGTCCTCCTC
ID4	AGGGTGACAGCATTCTCTGC	TGGAATGACAAGACGAGACG
ID2	GGACATCAGCATCCTGTCCT	CTCCTGGTGAAATGGCTGAT
Dll4	AGCTGGGTGTCTGAGTAGGC	AGAAGGTGCCACTTCGGTTA
Jag1	GAGCTCAGCAGAGGAACCAG	GGGAACCCTGTCAAGGAAAT
Jag2	TGGAGGTGGCTGTGTCTTTC	CGTGTCCACCATACGCAGAT
Klf10	AGCCAACCATGCTCAACTTC	ATCCCCTCTCTGGGCTTTT
Egln1	GCAACGGAACAGGCTATGTC	CTTTAGCTCTCGCTCGCTCA
Insig1	GACGAGGTGATAGCCACCAT	TCGTCCTATGTTTCCCACTGT
Calm1	GCTGCAGGATATGATCAACG	GTCCGTCGCCATCAATATCT
Nkx2-5	AGCCCGAGGCCTACTCTG	AGATCTTGACCTGCGTGGAC
Bmi1	TGTCCAGGTTCACAAAACCA	GCCTTGTCACTCCCAGAGTC
TiParp	CCTTTTCCGTTCCTGTTTCA	TCTGGGCAGATGATTTGTGA
Tbx20	CAGCAGTCACAGCCTACCAG	GAATCGGTGTCGCTATGGAT
FoxC1	CAGAGACTCGCTTTCCTGCT	TCCCGTTCTTTCGACATAGG
vegfR3	CCCCAGGATCTCCACTAGGT	GCGGGATCCACGCAGAGTGATGTGTGGTC

flk1	GGCGGTGGTGACAGTATCTT	GTCACTGACAGAGGCGATGA
sflt1	CTGGGACGCATCTTTTCTTC	ACCAGGTAGACACCCGACAC
mflt1_fl	CATGAGCCTGGAAAGAATCAA	TATCTTCATGGAGGCCTTGG
Bmp4	AGGAGGAGGAGGAAGAGCAG	CACCTCATTCTCTGGGATGC
GATA4	TCAAACCAGAAAACGGAAGC	CTGCTGTGCCCATAGTGAGA
Smad6	GTGGAGCTGAAACCCCTGT	AGGAGGAGACAGCCGAGAAT
HNRPU	CGTTAAAAGACCGCGAGAAG	TGCCTTTTGACACACCGTAG
Mark3	GCAGTGCTGTAGGAGGGAAG	CATCTCTCGCTGCAAACGTA
Cited2	CATCGGCTGTCCCTCTATGT	CATATGGTCTGCCATTTCCA
Skil	CCAGTCTAAAGAGGCCACCA	CATGATCTTCCCCTTGTCGT
Nrg1	CTGTATCGCCCTGTTGGTGGT	CATTCTCTGGTGGTGGGTTTG
Notch3	CTCTCCAGCCTGCCTCTATG	AGCCGCATTCCTCAGTGTT
Prrx1	AACCCATCGTACCTCGTCCT	CAATGCTGTTGGCCATGTT
Nox4	CGATTCCGGGATTTGCTAC	GACTCCTCAAATGGGCTTCC
Klf10	AGCCAACCATGCTCAACTTC	ATCCCCTCTCTGGGCTTTT
ActH1 recomb	CAGCTTCTTTGCAGCTCCTT	ATTCTCGTCCGCGCTCTCCTTTTCC

2.5 Antibodies for Western Blot

The antibodies for Western Blots are given in **Table 2** with the appropriate dilutions.

Table 2: antibodies for Western Blot

name	species		dilution	brand
anti-Flag	mouse	monoclonal	1:2000	Cell Signaling
anti-Impbeta	rabbit	polyclonal	1:1000	Santa Cruz
anti-Myc	mouse	monoclonal	1:1000	selfmade
anti-GFP	goat	polyclonal	1:1000	BD Pharmigen
anti-Tubulin	mouse	monoclonal	1:1000	Sigma
anti-mouse POD	goat		1:5000	Chemicon
anti-rabbit POD	goat		1:5000	BioRad
anti-goat POD	rabbit		1:5000	Sigma

2.6 Antibodies for immunoflurescent staining

For the anti-Flag immunofluorescence, the monoclonal antibody from Cell Signaling mentioned above was used (dilution: 1:800) and the secondary antibody was the Alexa568 goat

anti-mouse (1:1000; Molecular Probes). For staining of phospho-ERK1/2 (1:1000), a polyclonal rabbit antibody from Cell Signaling was kindly provided by PD Dr. Svenja Meierjohann; the secondary antibody was Alexa488 goat anti-rabbit (Molecular Probes, 1:1000). For anti-Hes1 staining, a Hes1 antibody from Santa Cruz was used (1:200) with the Alexa594 goat anti-rabbit antibody as secondary dye (1:1000; Molecular Probes).

2.7 RNA (whole mount) *in situ* hybridization probes

The following RNA probes were used in ISH or WISH assays (**Table 3**):

Table 3: *in situ* hybridization RNA probes

	ISH	**WISH**
target	**dilution**	
ANF	1ng	1µg
Hey2	3ng	
Jag1	2ng	
Jag2	2ng	
MLC-1A	2ng	
MLC-2A	1ng	1µg
PlxnD1	2ng	1µg
Sema6D	2ng	
Tbx2	2ng	1µg
vegfR2 (= flk1)	1ng	1µg
vegfR3	1ng	1µg
Mark3	2ng	
Smad6	2ng	

2.8 Cell culture reagents and cell lines

All cell culture media and additives were obtained from Sigma, PAA or PAN. PBS, 0.25% trypsin/EDTA (1mM) and 0.2% gelatin (0.8g in 400ml Aqua dest.) were freshly prepared. PBS, gelatin and 150mM NaCl, necessary for transfections, were autoclaved before used.

HeLa cells were a kind gift of the AG Fischer, Chair of Biochemistry. This human cancer cell line was cultured in standard DMEM medium + 10% FBS + 1% penicillin/streptomycin (= D10

medium) and passaging of the cells was performed using trypsin. The same culture conditions were used for NIH3T3 cells (donated from Dr. Toni Wagner, AG Schartl).

Human embryonic kidney cells (HEK293(T)) were kept in the same medium and stable cells lines expressing Flag-Strep-tagged Hey or Hes (293-FS-mHey1/-mHey2/-Hes1) have been previously generated in our laboratory.

Human brain microvascular endothelial cells (HBMEC) were cultured on 0.2% gelatin-coated cell culture dishes in a medium containing M199 basal medium and 15% FBS, 1% penicillin/streptomycin, 1% glutamax100, 1% ECGS and 0.5% Heparin. Passaging was done using trypsin.

Inhibitors and stimulation agents for the cell culture experiment in which possible influences on the localization of Hey1 should be investigated were kindly provided from PD Dr. Svenja Meierjohann and purchased from the following companies:

Calbiochem: PP2, LY94002, Ilomastat, PTPI IV

ALC labs: AG1478, UO126

Peprotech: hEGF, TGFα.

2.9 Buffers

2.9.1 Buffers for PCR

BASE buffer	25mM NaOH 0.2mM EDTA pH 12
NEUTRAL buffer	40mM Tris-HCl pH5

10x PCR buffer

100mM	Tris-HCl pH8.85

500mM	KCl
15mM	$MgCl_2$
1%	TritonX-100
0.3mg/ml	BSA (acet.)

20x SB buffer

200mM NaOH

equilibrate to pH8 with boric acid

50x TAE buffer

2M	Tris-Ac pH 7.5 - 8
50mM	EDTA

DNA loading buffer

50%	glycerol
15%	ficoll
10mM	EDTA pH8
0.25%	bromphenol blue

2.9.2 Buffers for ISH

all RNA buffer and solutions were treated with DEPC or prepared using DEPC-H_2O

DEPC-H_2O

1l	H_2O
800µl	DEPC

incubate over night, autoclave

hybridization mix

2,5ml	deionizied formamide	50%
1,25ml	20x SSC, pH7	5x
50mg	blocking reagent (Roche)	1%
544µl	DEPC-H_2O	
50µl	0,5M EDTA	5mM
5µl	Tween-20	0.01%

50µl	CHAPS (10%)	0.1%
100µl	heparine	0.5mg/ml
500µl	10mg/ml yeast tRNA	1mg/ml

proteinase K solution
10mg/ml in 50mM Tris, pH8 + 5mM EDTA

20x SSC

3M	NaCl
0.3M	NaCitrat x2 H_2O

equilibrate to pH7 with citric adic, add DEPC, autoclave

4% PFA/PBS

40g	paraformaldehyde
100ml	10x PBS
ad 1l	DEPC-H_2O

equilibrate to pH7

2x **MABT** buffer

100mM	maleic acid
150mM	NaCl
0.1%	Tween-20

equilibrate to pH7.5, add DEPC, autoclave

2.9.3 Buffers for Western Blot and IPs

2x protein loading buffer

0.1M	Tris-HCl pH6.8
4%	SDS
0.25%	bromphenol blue
25%	glycerol
200mM	DTT

RIPA buffer

50mM	Tris-HCl pH8
150mM	NaCl
1%	Nonidet P-40
0.5%	deoxycholate
0.1%	SDS

SDS running buffer

25mM	Tris-HCl
192mM	glycine
1%	SDS

blotting buffer

25mM	Tris-HCl
150mM	glycine
10%	methanol

Western Blot developing solution

100mM	Tris-HCl pH8
250mM	luminol
90mM	coomaric acid
0.03%	H_2O_2 (35%)

hypotonic buffer 1

10mM	Tris-HCl pH8
1.5mM	$MgCl_2$
10mM	KCl
10%	glycerol

hypotonic buffer 2

10mM	Tris-HCl pH8
1.5mM	$MgCl_2$

10mM	KCl
0.1%	Triton-X100
10%	glycerol

low salt buffer

100mM	Tris-HCl pH8
1.5mM	$MgCl_2$
10%	glycerol

high salt buffer

100mM	Tris-HCl pH8
1.5mM	$MgCl_2$
840mM	KCl
10%	glycerol

dialysis buffer

100mM	Tris-HCl pH8
0.3%	Triton-X100
10%	glycerol

2.9.4 general buffers

10x PBS

140mM	NaCl
2.7mM	KCl
8mM	Na_2HPO_4
1.8mM	KH_2PO_4
pH7.5	

TE

10mM	Tris-HCl pH8
1mM	EDTA

10x TBS

140mM	NaCl
2.7mM	KCl

250ml	1M Tris-HCl pH7.5
ad 1l	H_2O

LB medium

10g	tryptone
5g	yeast extract
10g	NaCl
ad 1l	H_2O

autoclaved before use
for **LB + Amp** add 1mg/ml ampicillin after autoclaving
for **agar plates** add 15g selcetion agar (Gibco), autoclave; add selection antibiotic afterwards

2.10 Mice

Mice from BL/6 genetic background were kept according to guidelines in the German Law.

Pregnant females were identified by checking for the typical cervical plug and afterwards separated from the other mice.

ActH1 mice were generated by M. Susa (Novartis Institutes for BioMedical Research, Basel).

3. Methods

3.1 Mouse genotyping

Tails from mouse embryos were prepared for genotyping with polymerase chain reaction (PCR) by digesting them in BASE buffer for 15min at 95°C. Afterwards, the samples were cooled down at room temperature and mixed with an equal volume of NEUTRAL buffer. Next, genotyping PCR was pipetted using the following primer pairs and PCR protocol (**Table 4**). Finally, the PCR samples were separated on 1% agarose gels in SB buffer to decide on the genotype (300V, 15 to 20min).

Table 4: PCR program and primer pairs for mouse genotyping

PCR program:

94°C	5min	
94°C	30sec	35x
60°C	30sec	35x
72°C	45sec	35x
72°C	7min	
14°C	paused	

H_2O	25,26µl
10x PCR buffer	3µl
25mM dNTPs	0,3µl
primer I	0,08µl
primer II	0,08µl
primer III	0,08µl
polymerase	0,2µl
template DNA	1µl
	30µl

genotype	primer name	primer sequence	product size
Hey1	**M13**	CGCCAGGGTTTTCCCAGTCACGAC	wt: 225bp
	Transini 1	ATGGTTGAGTTTTAACCGGAGACTGAGCGT	KO: 299 bp
	clik-race	ATTCTCGTCCGCGCTCTCCTTTTCC	
Hey2	**Hey 2 ko test 3`**	TCGGTGAATTGGACCTCATCACTGAGC	wt: 250bp
	Hey 2 ko test 5`	GCTGTCTCAAGGCCTCAACAGCATTG	KO: 299bp
	Z3L	ATCGGTGCGGGCCTCTTCGCTATTA	
HeyL	**mHeyL ex 2r**	TGTTGCACACACTTCACCCCTCT	wt: 129bp
	mHeyL ex2l b	GGATCCTTCAGCTCTGAGAAA	KO: 210bp
	M13	CGCCAGGGTTTTCCCAGTCACGAC	
ActH1	**b-act1**	TTCCTTTGTCCCCTGAGCTT	wt: 240bp
	b-act2	CACGATGGAGGGGAATACAG	ActH1: 169bp
	b-act3	CAGCCCAAGCTGATCCTCTA	
ActH1a	**b-act-in**	CCGAGGTGACTATAGCCTTCTTT	active: 300bp
	clik race	ATTCTCGTCCGCGCTCTCCTTTTCC	

3.2 Heart tissue preparation

Pregnant mice were sacrificed at either embryonic day E14.5 or E17.5. The embryos were pealed out of the yolk sac and their tails were used for genotyping. Afterwards, the embryos were immediately stored in PBS on ice. Next, the preparation of the heart was performed and the ventricular parts were cut off and immediately transferred to a vial containing 500µl TriFast (peqlab) and homogenized. For the E17.5 embryos, the left and the right ventricle were separately taken and mixed with TriFast. All samples were kept on ice and as soon as possible stored at -80°C or directly used for RNA extraction.

3.3 RNA extraction and cDNA synthesis

Total RNA was extracted from tissue samples of mouse embryos of either E14.5 or E17.5 developmental stage using TriFast according to the manufacturer's instructions. After thawing, the samples were mixed with an equal volume of chloroform and vortexed, before they were centrifuged for 20min at 13300 rpm. The aqueous phase was transferred to a new vial and mixed with 1µl glycogen (10mg/ml) and 500µl isopropanol and incubated at -20°C for 1h. Afterwards, the samples were centrifuged again and the pellet was washed with 70% ethanol. The pellet was finally dried at 57°C for 5 to 10min and dissolved in 20µl pre-warmed DEPC-H_2O. The amount of total RNA was quantified using a NanoDrop spectrophotometer and the RNA was either directly used for cDNA synthesis or stored at –80°C.

cDNA synthesis was carried out using the Revert Aid First-Strand cDNA synthesis Kit (MBI fermentas). 1µg of RNA was transcribed into cDNA using 1µl of oligo d(T)-primer and incubated at 70°C for 5min. In the second step, 4µl of 5x reaction buffer, 2µl 10mM dNTPs and 1µl RNase-inhibitor were added and the samples were kept at 37°C for further 5min. Finally, the samples were mixed with 1µl Reverse Transcriptase (42°C (60min), 70°C (10min)). Afterwards, the volume was adjusted to 200µl with H_2O dest. and the cDNA samples were stored at -20°C.

3.4 Quantitative real time-PCR (qRT-PCR)

Using qRT-PCR, gene expression of the house keeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) and several target genes were analyzed in the heart tissue samples. Quantification is relying on the increase of fluorescence of SybrGreen, a fluorescent dye that can intercalate into double stranded DNA. Different expression levels can be calculated by normalization of each individual sample to the house keeping gene. **Table 5** shows the PCR mix as well as the program used.

Table 5: real-time qRT-PCR program and PCR mix

qRT-PCR program:

			H_2O	17,5µl	
95°C	3min		10x PCR buffer	2,5µl	
95°C	15sec	40x	25mM dNTPs	0,25µl	
60°C	20sec		FITC (1:2000)	0,25µl	diluted in H_2O, 0,45% DMSO
55 - 95°C	every 10sec plus 1°C		SybrGreen (1:2000)	0,75µl	
16°C			primer I	0,75µl	
			primer II	0,75µl	
			Polymerase	0,25µl	
			template cDNA	3µl	
				26µl	

For some primer pairs, 1µl DMSO or 1,5µl Ethylenglycol were added, with reduced volume of water, to improve PCR efficiency. For all primer pairs, see material part.

To verify the correct amplification of PCR products, the samples were loaded on DNA agarose gels which also showed possible primer dimers generated during the PCR process.

Calculation of expression differences was done using the REST software (relative expression software tool, Qiagen) which additionally provided statistical data.

3.5 Cloning of virus constructs

In order to generate stable cell lines expressing mCherry-coupled Hey1- or Hey2-constructs, lentiviral constructs for infection of standard cell lines such as HeLa or HBMEC cells were cloned. The p201-iEP plasmid served as host vector which contains the constitutively active CMV-promoter as well as LTR sites and an IRES-EGFP-puromycin resistance cassette.

To create p201-Cherry-mHey1-iEP and p201-Cherry-mHey2-iEP, the original p201-iEP construct was digested with 1U/µg of Eco47III (= AfeI). The mCherry-mHey1 part was cut out of the vector pmCherry-mHey1 with the restriction enzymes HindIII and NheI. For mCherry-mHey2, the digestion was performed using BamHI and also NheI. Afterwards, the inserts were ligated with the vector and transformed into chemo-competent DH5α *E. coli* and plated on ampicillin selection agar plates.

Test-PCR of the grown clones was performed using the following primer pair: pCMV-1 and Cherry-rev with a PCR program similar to the one for mouse genotyping. Positive clones were sent for sequencing (GATC Konstanz) using the pCMV-1 primer (for both constructs).

3.6 Cell culture

HeLa and HEK293(T) cells were kept in DMEM medium containing 10% FBS and 1% penicillin/streptomycin under normal cultivation conditions and spilt at a density rate of about 90%. HBMEC cells were cultured and passaged according to the same conditions, but on cell culture dishes coated with 0.2% gelatin and in another medium containing the following components: M199 basal medium, 15% FBS, 1% penicillin/streptomycin, 1% glutamax100, 1% ECGS and 0.5% Heparin. For immunofluorescent characterization, all cell lines were transferred to cover slides.

HEK293-FS-mHey1, -mHey2 and –Hes1cells were cultured in normal medium. To induce the expression of Flag-tagged Hey/Hes, 100ng of doxycycline were added to the medium (for 72h).

For some assays, it was necessary to transiently transfect the cells with selected plasmids. This was done using PEI (polyethylene imine). Transfection was conducted by adding 8µg of plasmid DNA and 16µl of 1x PEI (DNA:PEI = 1:2) to a 70 – 80% confluent 10cm-cell culture dish. The success of the transfection was checked on the next day via fluorescence microscopy (Nikon).

3.7 Immunofluorescence

Cells were seeded at 60 – 70% density on cover slides and incubated over night. The next day, the cells were shortly rinsed with PBS and fixed in 4% PFA (15min, RT). After washing the cells with PBS (5min), 50mM NH_4Cl was added (10min) to inactivate the formalin and the cells were subsequently washed in PBS (2x 5min) before – for the staining of intracellular proteins only – the cells were permeabilized with 0.1% Triton100 in PBS for 7min and again washed in PBS. If not, the cells were immediately incubated in the blocking solution 0.2% fish skin gelatin (FSG) in PBS for 30min. Afterwards, the primary antibody was added, being diluted in the FSG solution to its final concentration (1h). The cells were then washed three times in FSG (each 5min) and the incubation with the secondary antibody from the Alexa family (diluted in FSG, 1h, in the dark) followed. As next steps, the samples were again washed with FSG (3x 5min) and then the nuclear staining with Hoechst33342 (1:10000 in FSG, 7min, in the dark) was performed. Before the cells were mounted in Mowiol, they were washed 3x 5min in PBS. Finally, the fluorescence was visualized with a Leica fluorescence microscope.

3.8 Generation of stable cells lines

Lentiviral constructs expressing mCherry-mHey1 or -mHey2 were used for the generation of stable HeLa and HBMEC cell lines.

To achieve this, HEK293T cells were seeded at a density of about 70% and then transfected as described above. The transfection mixture contained 6µg of the selected virus construct

DNA, 4.5µg of pPAX2 (packaging plasmid) and 3µg of CMV-VSVg (envelope plasmid). On the next day, 500mM NaButyrat was added and the medium was changed after 6 – 8h. Two days later, the medium of the transfected cells that now contained the produced virus was harvested and either stored at -80°C or immediately put on the cells that should be infected.

HeLa and HBMEC cells were infected with the virus and 8µg/ml polybrene. After 6 – 8h, the medium was changed and after 24h, selection of infected cells was started by adding 1µg/ml puromycin. When the selection was finished, individual clones were chosen by fluorescence microscopy and displaced with a pipette tip as individual clones to new culture medium.

3.9 *In vitro* assay for analyzing possible influences on the localization of Hey proteins

To check if the localization of Hey proteins could be influenced *in vitro*, HeLa and HBMEC cell lines expressing the mCherry-mHey1 construct were treated with different stimulatory or inhibitory agents as well as plasmids expressing CaMII kinase (wild type or deletion mutants).

The cells were treated with inhibitory agents that affected other signaling pathways like the MAPK pathway that could possibly interact with Notch signaling and therefore might influence the localization of Hey as nuclear factor. In **Table 7** the inhibitors are listed together with their concentration (diluted in DMSO) and caused effects.

Table 6: inhibitors for the localization assay

agent	effect on	final concentration	time
PP2	Src-family kinases	20nM	2h
AG1478	EGFR	20nM	2h
LY294002	PI3K	10nM	2h
UO126	MEK	10nM	2h
Ilomastat	some MMPs	10nM	2h
PTPI IV	Tyr-Phosphatases	20nM	2h

The cells were treated with the reagents, as given in the table, for 2h and afterwards, the localization of Hey was checked with the help of fluorescence microscopy as Hey was cou-

pled with the fluorescent dye mCherry. As negative control, cells were treated with the solvent DMSO.

As stimulating treatment, hEGF (100ng/ml) and TGFα (10nM) were mixed into the cell culture medium. For this part of the assay, the HeLa and HBMEC cells were previously starved over night (1% FBS) and then stimulated with 100ng/ml of either drug for 20min. Afterwards, the localization of Hey1 was again checked by fluorescence microscopy.

The third part of the assay was to transiently transfect HeLa-p201-Cherry-mHey1 cells with the Ca^{2+}/Calmodulin-dependent kinase II (CaMKII) constructs that either contained the wild type form or deletion mutants of CaMII kinase. With the transfected cells, a time series was analyzed under the fluorescence microscope with time points 0h, 30min, 1h, 2h, 4h, 8h, 24h.

All described experiments were at least performed twice.

3.10 Generation of nuclear and cytoplasmic protein lysates

In order to monitor the localization of Hey1, nuclear and cytoplasmic extracts were generated in the following manner: cells were harvested in ice-cold PBS and centrifuged for 5min (1400rpm, 4°C). Subsequently, the cells were washed in 4x packed cell volume (PCV) hypotonic buffer 1 (centrifugation as described above) and afterwards resuspended in 3x PCV hypotonic buffer 2. In this buffer, the cells could swell for about 5min, before they were homogenized using a syringe and a fine needle (24G, No. 17; Neoinject). Cell lysis was checked with trypan blue staining and decided as successful if at least 90% of the cells were destroyed. Afterwards, the cells were centrifuged for 15min (2800rpm, 4°C) and the generated supernatant was pipetted into a new vial. This was the cytoplasmic lysate. The nuclear pellet was washed with 3x packed nuclear volume (PNV) hypotonic buffer 1 (900rcf, 3min, 4°C) and afterwards 1x PNV of the low salt buffer was pipetted to it. As the next step, 2x PNV of high salt buffer were given to the pellet, in two steps and under vortexing of the sample. Afterwards, the sample was incubated at 4°C under rotation for 30min. As the last step, both lysates were centrifuged at maximum speed (4°C, 30min) and the supernatants were pipetted

into new vials and for the nuclear lysates mixed with 2x volumes of dialysis buffer (in two steps, under vortexing). The lysates were stored at -20°C until the Western Blot was performed.

3.11 Cell cycle synchronization and propidium iodide FACS for cell cycle analysis

To determine if Hey1 localization may change during the cell cycle from nucleus to cytoplasm, HeLa-p201-Cherry-mHey1-iEP cells were synchronized and afterwards analyzed with FACS to check for cell cycle state and with fluorescence microscopy to visualize Hey localization.

Cell synchronization was achieved by serum starvation of the cells for 72h. Afterwards, the cell cycle was re-induced by addition of 20% FBS to the medium and the cells were harvested for propidium iodide (PI) fluorescent-assisted cell sorting (FACS) and microscopy analysis at different time points. HeLa cells undergo the whole cell cycle in about 30 hours. Therefore, to get samples of every cell cycle stage, samples were taken at timepoint 0 (before induction by FBS) and at 6, 12, 18, 24 and 30 hours (see **Figure 9**).

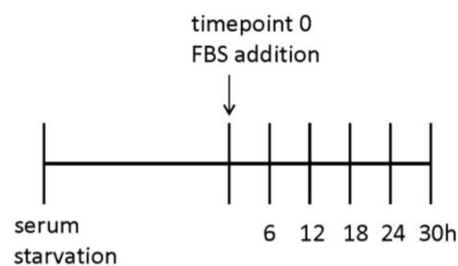


Figure 7: cell cycle synchronization of HeLa-p201-Cherry-mHey1 cells

and harvesting scheme for PI FACS and microscopy analysis of Hey1 localization

To determine the cell cycle stage of the cells at the different timepoints, PI FACS was performed. Therefore, the HeLa cells were harvested using trypsin as described above and then washed in ice-cold PBS. Afterwards, the cells were resuspended in 1ml ice-cold PBS and giv-

en dropwise and under vortexing into 4ml 100% EtOH (final concentration: 80%). Finally, the cells were fixed at -20°C for at least over night.

About 1 – 2h before FACS analysis, the cells were centrifuged (10min, 1500rpm, 4°C) and washed once in ice-cold PBS. The cell pellet was subsequently resuspended in 400µl 38mM NaCitrat, 15µl of a 1mg/ml stock PI (54µM) and 1µl of a 10mg/ml stock RNase A (24µg/ml). The FACS samples were then incubated at 37°C for 30min and finally analyzed with the FACS technique in which the amount of DNA is measured into which PI can intercalate. Analysis of cell cycles phases was done with the help of the DIVA-software (BD Pharmigen).

3.12 Tissue preparation for *in situ* hybridization

Pregnant mice were sacrificed as described above and the embryos of E14.5 developmental stage were washed in ice-cold PBS and afterwards transferred to 4% para-formaldehyde (PFA) (over night, 4°).

The next day, the samples were washed twice in PBS (each 10min) and once in 0.9% NaCl (10min). Afterwards, the tissue was dehydrated in an increasing isopropanol row, starting from 30% to 100% (each step for 2h). After this, an isopropanol/chloroform (1:1) mixture was added for the same time span, followed by pure chloroform and chloroform/paraffin (1:1). Finally, the samples were incubated three times for 2h each in pure paraffin before they were embedded in paraffin blocks and kept at 4°C until being sectioned. The sectioning was done using a Leica-microtome, with sectioning the tissue into 7µm slices.

For whole mount *in situ* hybridization, the embryos were – after the PFA fixation – trans-ferred through an increasing methanol row and stored in 100% methanol at -20°C until used.

In all cases, wt and KO samples were stained in parallel.

3.13 *In situ* hybridization (ISH) and whole mount ISH (WISH)

3.13.1 DNA purification for generating ISH probes

To create an RNA *in situ* probe, the plasmid containing the desired sequence (usually based on the pCS2p vector) was digested with the appropriate restriction enzyme (10µg, 37°C, 2 – 3h). Afterwards, the DNA was purified according to the following protocol: the volume was adjusted to 200µl with DEPC-H_2O and then, 100µl TE-saturated phenol were added as well as 100µl chloroform:isoamylalcohol (24:1). The sample was mixed by vortexing, incubated for 30min and afterwards centrifuged (5min, maximum speed). The aqueous upper phase was transferred to a new vial and an equal volume of chloroform:isoamylalcohol (24:1) was add-ed and the sample was again mixed and centrifuged. Again, the upper phase was pipetted into a new vial and 1/10 volume of 3M NaAcetat and 2.5 volumina of 100% EtOH were pipet-ted to it. Afterwards, the DNA was precipitated at -80°C (30min) followed by a centrifugation step (10min, maximum speed, 4°C). Finally, the DNA pellet was washed in 70% EtOH and dried at room temperature before it was dissolved in 20µl DEPC-H_2O (30min, RT).

3.13.2 *In vitro* transcription and RNA precipitation of ISH probes

To transcribe the DNA into RNA, 2µl of the linearized plasmid were mixed with 12.5µl DEPC-H_2O and 2µl transcription buffer and incubated at room temperature for 15min. All following steps were performed on ice. 1.5µl Dig-labeling Mix (Roche), 0.8µl RNAsin and 1.2µl of the appropriate RNA polymerase were added (Promega) and the sample was incubated at 37°C for 2h. Afterwards, 1µl of DNase was pipetted to the transcribed probe and 2µl of the final sample were loaded on a RNA test gel (agarose in DEPC-H_2O with MOPS and formaldehyde) to control the transcription process.

The RNA was precipitated by adding 7µl 7.5M NH_4Acetat and 75µl ice-cold EtOH 100% and incubated at -80°C (30min) and afterwards centrifuged (30min, maximum speed, 4°C). The pellet was dissolved in 20µl DEPC-H_2O and 7µl 7.5M NH_4Acetat as well as 75µl ice-cold EtOH 100% and again precipitated and centrifuged as described above. This RNA pellet was then washed in ice-cold 70% EtOH and shortly dried at room temperature before it was dissolved

in 100µl DEPC-H_2O (10 – 15min, 50°C). Finally, the RNA probe was diluted 1:10 in hybridization buffer judging from band size on the RNA gel on the amount of transcribed probe.

3.14 ISH protocol

The sections on the slides were de-paraffinized by incubation at 65°C for 30min and subsequently transferred to chloroform (2x 10min). Now a decreasing ethanol row was conducted (100% ethanol to 30%) followed by a washing step with PBS (2x 5min) and a re-fixation in 4% PFA (30min). Next, the slides were washed again in PBS (2x 5min) and afterwards the proteinase K digestion was performed (10µg/ml; 10min). After another washing in PBS (1x 5min), the slides were re-transferred to PFA for another 30min followed by 2x 5min PBS washing. Finally, the tissue was incubated in 2x SSC (2x 2min) and Tris/glycine buffer (2x 15min) before the denaturated RNA probe (diluted 1:10 in hybridization mix + tRNA) was pipetted on the sections (final concentration of the probe 1:100). The slides were incubated over night at 70°C in a wet-chamber (paper towels soaked in 5x SSC).

The next day, the samples were washed 3x 20min in 5x SSC and 40min at 60°C with 0.5x SCC/20% formamide. Subsequently, the slides were transferred to a new washing buffer and cooled down to 37°C for approximately 15min before they were washed in NTE at the same temperature for 15min. RNase A digestion was performed next (10µg/ml in NTE buffer, 37°C, 15min) and afterwards a further washing in NTE (15min, 37°C) and 0.5x SSC/20% formamide (30min, 60°C) was added. The final washing step was an incubation in 2x SSC for 30min before the sections were blocked in 1% blocking reagent (Roche) in 1x MABT for 1h at room temperature. Meanwhile, the antibody was pre-absorbed at 4°C in the same blocking solution (1:5000, Roche). Finally, the antibody was given to the sections and incubated over night at 4°C.

The third day started with washing in TBST (4x 10min, 3x 20min) and NTMT (2x 10min). Afterwards, the sections were transferred to NTMT + 2mM Levamisol (10min) before the incubation in the BM Purple Substrate (Roche) took place. BM Purple Substrate was mixed with 2mM Levamisol and 0.1% Tween-20 before being pipetted on the sections. These were incubated at room temperature or 4°C until staining was considered to be finished. The reaction

was stopped by washing the sections in NTMT (2x 15min) and PBS (10min). After a short rinse in Aqua bidest., the tissue was embedded in Kaiser's glycerol gelatin (Merck).

3.15 WISH protocol

Starting the whole mount ISH protocol, the tissue was first re-hydrated up to 25% methanol and afterwards washed in PBST (2x 10min) before the samples were digested with proteinase K (37°C, 15min, 20µg/ml). The digestion was stopped afterwards with 0.2% glycine in PBST for 5min, followed by a washing step with PBST (2x 5min) and the re-fixation with 4% PFA/0.2% glutaraldehyde in PBST and another washing in PBST (2x 5min). Pre-hybridization was performed by incubating the samples for 1h at 70°C in hybridization mix. The RNA probe was afterwards added, after being heated for 5min at 100°C. The hybridization was performed overnight.

On the next day, the samples were rinsed a few times with Wash I (2x SSC, 50% formamide) and afterwards washed 2x 15min with each Wash I and Wash II (2x SSC, 50% formamide, 1% Tween-20) solution (70°C) and finally with PBST (3x 5min). As the next step, the samples were incubated for at least 1h at 4°C in MABT + 2% blocking reagent while the anti-DIG alkaline phosphatase antibody was pre-absorbed for the same time in blocking solution (1:3000 dilution). Afterwards, the antibody was added to the samples in a final dilution of 1:6000 (0.025U) and the mixture was kept overnight at 4°C.

The following day, the samples were washed first with PBST (6x 10min) and NTMT (3x 10min) and then incubated in BM Purple AP substrate in the dark until the color reaction was finished. The coloring was stopped by washing the samples in H_2O bidest. followed by a post-fixation step in 4% PFA overnight at 4°C. Finally, the samples were washed again with water and then transferred through an increasing glycerol row until they were kept in 80% glycerol for photos and storage at 4°C.

3.16 Immunoprecipitation (IP)

To analyze possible interaction partners of Hey1 that might influence the nuclear localization of Hey and had been identified in a mass spectrometry assay by Dr. Daniela Salat, IPs were performed in HEK293T cells that were transiently transfected as described above with the appropriate plasmid constructs.

1. Interaction of Hey with Importinβ (Impβ): For this assay, the DsRed-Importinβ vector was used in HEK293-FS-Hey1 cells. Cells were either stimulated with doxycycline (Flag-Hey1 expression), stimulated and transfected with the Impβ plasmid or not induced (negative control). IP was done against the Strep-tag of Hey1.
2. Interaction of Hey with Myc-tagged Impα: Two different plasmid types for Hey1 and Hey1-delta basic were used: pEGFP-constructs and pCS2p-Flag plasmids. IP was performed against the Myc-tag of Impα.

The next step of the procedure was to prepare cell lysates from the transfected cells. Therefore, cells were harvested in ice-cold PBS and centrifuged (2000rpm, 5min, 4°C) and afterwards the cell pellet was dissolved in lysis buffer (TBS + 1% Triton100; PMSF (50µg/ml), complete protease inhibitor complex (25x stock, Roche)) and the cell solution was incubated for 45min at 4°C, rotating. Subsequently, the cells were centrifuged (30min, 4°C, 13300rpm) and the generated supernatant was transferred to a new vial. 100µl of the lysate was stored at -20°C to serve as input control for the IP samples in the Western Blot analysis.

For Flag- or HA-IPs, M2-Flag-Agarose beads (Sigma-Aldrich) or monoclonal anti-HA-Agarose beads (Sigma-Aldrich) were washed three times with lysis buffer (3000rpm, 1min, 4°C). Afterwards, the protein lysates were mixed with 30µl of these beads and incubated over night at 4°C on a rotator. On the next day, the beads were washed three times with lysis buffer and afterwards transferred into the SDS-gel loading buffer. To conduct the IPs for Impβ or Strep-tagged proteins, StrepTactin beads (IBA) that have been washed three times with lysis buffer were mixed with the lysate. Afterwards, the IP was performed as described.

IPs for Myc-tagged proteins were performed in the following manner: the lysates were mixed with 15µl Myc-antibody (9E10P4) and incubated over night as described above. On the next day, Protein A Agarose beads that have been washed three times with lysis buffer

were added to the samples and incubated for further 3h. Finally, the IP samples were washed five times with lysis buffer and the pelleted beads were dissolved in SDS loading buffer.

For some IPs, RIPA buffer was used instead of lysis buffer.

3.17 SDS-Page and Western Blot

Using sodium dodecyl sulfate polyacrylamide gel electrophoreseis (SDS-PAGE) and the Western Blot technique, the protein lysates were analyzed.

To prepare a SDS gel (12%), the following components were mixed (**Table 7**):

Table 7: components of protein gels

	Running gel	**Stacking gel**
H_2O	1.5ml	2ml
Tris buffer	2.85ml	375µl
30% acrylami-de	3ml	500µl
20% SDS	37.5µl	15µl
10% APS	75µl	30µl
TEMED	10µl	7.5µl

Running gel with pH 8,8 Tris-HCl
Stacking gel with pH 6,8 Tris-HCl

The gels could polymerize at room temperature and were afterwards loaded with the protein samples. The protein lysates were heated with 4x loading buffer for 5min (95°C) and afterwards loaded on the gel. The gel was then run for about 1.5h at 200V, 35mA per gel and 50W. Upon completion, blotting was performed in which the proteins were transferred from the gel onto a nitrocellulose membrane (35min, 25V, 400mA, blotting buffer-soaked gel and membrane). Afterwards, the membrane was blocked for 1h at RT in 3% milk buffer (in PBS; for GFP-antibody 5%) and then incubated with the primary antibody (diluted in milk buffer; 1h, RT or overnight at 4°C). After washing the membrane with PBS (3x 10min), the secondary

antibody was added (diluted 1:5000 in milk buffer, 1h, RT) followed by another washing with PBS. The peroxidase reaction was developed in the ECL solution.

For some Western Blots, it was necessary to strip the nitrocellulose membrane to be able to use two different antibody systems on one membrane. Therefore, after developing the first system, the membrane was washed in 100mM glycine (pH 2.5) and 100mM Tris-HCl (pH 8.8) buffer (each for 10min) followed by a second blocking step and then going on with the standard protocol.

4. Results

4.1 Analysis of gene expression in cardiac tissue in different Hey mutant mouse embryos at different stages of development

Hey2-KO animals show severe heart defects such as VSD and hypertrophy of the right ventricle. In the present study, it was analyzed if the lack of Hey2, which is only expressed in the ventricles, causes changes in the gene expression pattern of ventricular tissue of wt- and KO-embryos at E14.5 and E17.5. The two other Hey factors, Hey1 and HeyL, are only expressed in the atrium (Hey1) or are more or less absent in cardiac tissue (HeyL). However, Hey1/L-double knock out animals exhibit the same cardiac morphological abnormalities as Hey2-KO embryos and therefore served as controls for our experiments.

Possible differences in gene expression were monitored using real-time qRT-PCR. Changes in gene expression were calculated for Hey2-KO samples compared to wt and for Hey1/L-DKO compared to double heterozygous animals using the REST software (Qiagen).

Target genes for the analysis were chosen dependent on two criteria: 1) the gene was identified as a target of Hey in a microarray analysis in HEK293 cells massively over-expressing Hey1 or Hey2 and 2) ChIP-Seq analysis revealed a promoter-located peak for the selected gene (Heisig, 2012).

4.1.1 Gene expression profile in ventricular tissue of Hey2-KO embryos (E14.5)

As previously mentioned, Hey2-KO embryos are characterized by massive heart abnormalities. To see if knocking out the Hey2 gene has any influences on gene expression in cardiac tissue which are not caused by the morphological changes within the heart that are also occurring in control Hey1/L-DKO embryos, ventricular samples of Hey2-wt as well as –KO animals at developmental stage E14.5 were analyzed. As shown in **Table 8** (with significantly regulated genes shown in grey), several genes were up-regulated in the KO situation compared to wild type animals. For example, a (highly) significant change could be detected for

members of the vegf-receptor family (vegfR1 (= flt1), vegfR2 (= flk1) and vegfR3) as well as two components of the plexin-semaphorin system (PlxnD1, Sema6D). On the other hand, atrial markers such as ANF and MLC-1A/2A also showed increased expression along with Tbx2.

Table 8: gene regulation in E14.5 Hey2-KO mice

gene	x-fold	p-value
Mark3	11,01	0,000
ANF	7,67	0,000
mflt1	5,59	0,000
PlxnD1	5,13	0,000
sflt1	4,23	0,000
Sema6D	4,10	0,000
Tbx2	3,78	0,000
flk1	3,52	0,002
Nrg1	2,84	0,001
vegfR3	2,69	0,007
TiParp	2,57	0,001
MLC-1A	2,46	0,000
Jag2	1,98	
MLC-2A	1,93	0,001
Hey1	1,90	
ID4	1,82	0,029
Dll4	1,63	
Smad6	1,58	0,027
ID2	1,47	
Nox4	1,23	
HNRPU	1,20	
Jag1	1,16	
Egln1	1,10	
Calm1	1,05	
Bmp4	0,92	
Cited2	0,83	
HeyL	0,62	
Nkx2-5	0,61	
Tbx20	0,46	
Insig1	0,42	
Bmi1	0,31	

wt: n = 16
KO: n = 16

4.1.2 Gene expression profile in ventricular tissue of Hey1/L-DKO embryos (E14.5)

Hey1/L-DKO animals, compared to double heterozygous littermates, were analyzed to determine if the regulation pattern observed for the Hey2-KO phenotype was due to the lack of the Hey2 gene or caused instead by the structural and morphological changes in the heart. As Hey1/L-DKO individuals phenocopy these abnormalities such as VSD and hypertrophy, they were assessed as controls for the Hey2-KO analysis, to differentiate between Hey2-regulated genes and expression changes that could be influenced as a result of the morphological abnormalities in the embryonic heart. However, only ID4 and Sema6D displayed significant up-regulation in these animals. Both genes have also been up-regulated in the Hey2-KO phenotype. On the other hand, several other factors like the vegf-receptors are unaltered (see **Table 9**).

Table 9: gene rgulation in E14.5 Hey1/L-DKO mice

gene	x-fold	p-value
ID4	5,76	0,009
Sema6D	3,80	0,026
vegfR3	2,55	
Mark3	2,52	
Nrg1	2,39	0,090
ANF	2,31	0,088
Nox4	2,25	
flk1	1,86	
MLC-1A	1,76	0,060
Tbx2	1,70	
PlxnD1	1,69	0,083
Smad6	1,62	
mflt1	1,59	
Jag2	1,50	
sflt1	1,48	
Cited2	1,36	
ID2	1,33	
Jag1	1,31	
MLC-2A	1,23	
Bmp4	1,18	
TiParp	1,03	
Insig1	0,99	
HNRPU	0,88	
Dll4	0,85	
Calm1	0,78	
Hey2	0,76	

het het: n = 15
DKO: n = 18

Nkx2-5	0,68
Egln1	0,67
Tbx20	0,54

4.1.3 Gene expression profile in ventricular tissue of ActH1 embryos (E14.5)

The opposite situation was found for the ActH1 mice in which there is a global over-expression of the Hey1 gene under the control of the β-actin promoter. These mice could therefore serve as counterparts to the Hey2-KO animals. In looking at gene regulation as a whole, it could indeed be seen that in these mice, most genes are counter-regulated compared to Hey2-KO embryos as it might have been expected (**Table 10**). A significant down-regulation could be observed for HNRPU and Sema6D, the latter being up-regulated in Hey2-KO animals.

Table 10: gene regulation in E14.5 ActH1 mice

wt: n = 11
act: n = 10

gene	x-fold	p-value
Insig1	4,11	
Calm1	2,97	
Hey1	2,77	0,001
ANF	2,16	
ID2	2,13	0,073
ID4	1,88	
TiParp	1,73	
Egln1	1,73	
MLC-2A	1,66	
MLC-1A	1,63	
Jag1	1,40	
Nrg1	1,10	
Bmi1	1,01	
Nox4	0,94	
HeyL	0,86	
Dll4	0,83	
flk1	0,80	
Klf10	0,76	
Hey2	0,67	
Mark3	0,66	
PlxnD1	0,66	
vegfR3	0,65	
Tbx2	0,64	
Smad6	0,63	

Cited2	0,56	
Tbx20	0,55	
mflt1	0,50	
Bmp4	0,47	0,067
Nkx2-5	0,46	
sflt1	0,36	
Sema6D	0,36	0,015
FoxC1	0,24	0,077
HNRPU	0,08	0,020

4.1.4 Gene expression profile in ventricular tissue of Hey2-KO embryos (E17.5)

When analyzing a later timepoint in development, E17.5 Hey2-KO animals no longer show the clear up-regulation pattern as seen in the E14.5 embryos. However, if the two ventricles are considered separately as depicted in **Table 11**, it becomes obvious that more genes are regulated in the right ventricle which is in fact more affected by the KO. In addition, **Table 11** shows that Tbx2 is up-regulated in both heart chambers as it has already been up-regulated in the E14.5 animals, potentially indicating an important role for this factor. A second gene that is up-regulated at both developmental stages is ID4. This is a HLH protein and might therefore be an interaction partner of the Hey proteins. Interestingly, the atrial factors ANF and MLC-1A/2A now show a down-regulation compared to the younger Hey2-KO animals.

Table 11: gene regulation in E17.5 Hey2-KO mice

gene	left ventricle	p-value	right ventricle	p-value
ID2	3,78	0,003	1,78	
ID4	3,33	0,008	2,00	
Tbx2	2,49	0,045	3,49	0,017
Bmp4	2,38		1,72	
Smad6	2,31		2,89	0,028
PlxnA2	2,24	0,091	1,25	
HNRPU	2,23	0,069	2,38	
ANF	2,02		0,13	0,001
Cited2	1,87		4,65	0,002
sflt1	1,68		1,39	
Sema6D	1,63		0,64	
Klf10	1,45		0,96	
TiParp	1,42		0,79	
vegfR3	1,37		0,88	
PlxnD1	1,28		0,63	

wt Vl	n = 22
wt Vr	n = 24
KO Vl	n = 25
KO Vr	n = 20

gene	left ventricle	p-value	right ventricle	p-value
MLC-1A	1,25		0,54	0,048
Nrg1	1,23		2,03	
mflt1	1,21		1,41	
Jag2	1,20		0,40	
Dll4	1,06		0,31	
Notch3	0,95		1,30	
FoxC1	0,95		0,58	
MLC-2A	0,86		0,40	0,020
Mark3	0,79		1,24	
Sema3C	0,79		0,94	
flk1	0,75		0,69	
Skil	0,71		1,36	
HeyL	0,68		0,79	
Hey1	0,66		1,26	
Nox4	0,52		0,42	0,047
GATA4	0,48		1,14	
Jag1	0,44		0,11	0,001
Prrx1	0,35		0,42	

4.1.5 Gene expression profile in ventricular tissue of Hey1/L-DKO embryos (E17.5)

The older Hey1/L-DKO embryos also served as controls for the Hey2-KO analysis. However, not many similarities were observed when compared with the results from the other genotype which was also previously noted for the younger animals (**Table 12**). This suggests that gene regulation is really due to the lack of Hey2 and not caused by morphological changes within the heart. Interestingly, in this analysis, more gene regulation was noted in the left ventricle – in contrast to the Hey2-KO animals. On the other side, also some down-regulated genes such as HNRPU and again Sema6D were obtained.

Table 12: gene regulation in E17.5 Hey1/L-DKO mice

gene	left ventricle	p-value	right ventricle	p-value
flk1	3,37	0,015	1,23	
mflt1	3,03	0,014	1,81	
sflt1	2,31	0,011	1,40	
Mark3	2,07	0,034	2,47	0,025
Bmp4	1,79		1,60	
Smad6	1,72		1,68	
Skil	1,61		1,14	
vegfR3	1,34		0,66	
Tbx2	1,24		0,77	

het het Vl n = 11
het het Vr n = 10

DKO Vl n = 19
DKO Vr n = 18

Nrg1	1,20		1,73	
ID2	1,14		1,46	
Notch3	1,12		0,66	
Cited2	1,05		1,64	
MLC-1A	1,01		1,14	
ID4	0,98		1,19	
Jag2	0,95		0,21	
Klf10	0,92		1,31	
GATA4	0,85		0,50	
PlxnA2	0,83		0,42	
MLC-2A	0,78		0,43	
HNRPU	0,75		0,44	0,034
PlxnD1	0,74		2,00	
Sema6D	0,74		0,33	0,035
Sema3C	0,72		0,63	
Prrx1	0,71		0,62	
ANF	0,59		0,72	
Nox4	0,57		0,61	
Hey2	0,49	0,024	0,28	0,001
FoxC1	0,44		1,24	
TiParp	0,36	0,007	0,24	0,001
Jag1	0,28	0,086	0,30	
Dll4	0,15	0,039	0,16	

4.1.6 Summary of all genotypes and developmental stages at developmental stage E14.5

Figure 8 depicts a summary overview of all gene expression studies at the E14.5 timepoint. There are some genes that are equally regulated – or in the case of ActH1 animals counter-regulated which is nicely shown in the diagram. Of all genes analyzed, only Sema6D was equally up-regulated in the Hey2-KO and Hey1/L-DKO embryos with an obvious counter regulation in ActH1 animals.

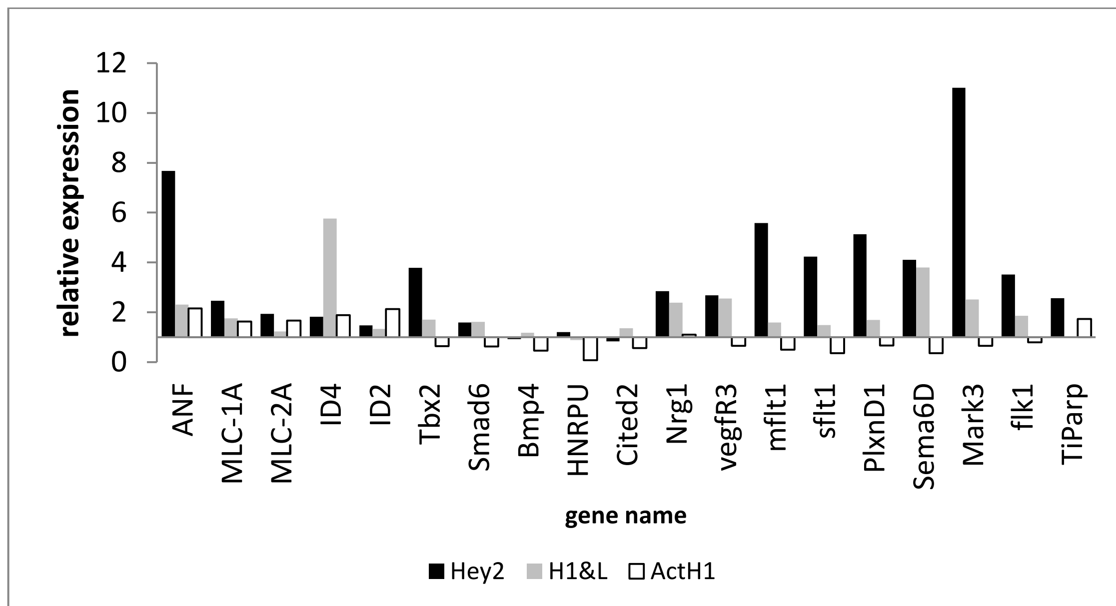


Figure 8: overview of all regulated genes in E14.5 mouse embryos

4.2 Validation of gene expression using *in situ hybridization*

4.2.1 *In situ hybridization* on sections of Hey2-KO embryos (E14.5 or E17.5)

Paraffin-embedded Hey2-wt or –KO embryos at the developmental stages E14.5 and E17.5 were sectioned and stained with RNA probes for genes that were (significantly) regulated in the quantitative real-time PCR analysis. **Figure 9** shows examples for the result of the *in situ* staining in which it was quite difficult to replicate the changes seen in the PCR assay.

For the atrial marker ANF, it was possible to show the typical expression in the atria of the hearts and also the ectopic expression in the ventricular parts of Hey2-KO individuals. As depicted below, staining was observed in the left ventricle concurring with the up-regulation noted in the quantitative real-time PCR analysis and with already published data (Fischer, 2004). *In situ* hybridization for Smad6 as well as Sema6D does at least in part reflect the gene expression data with stronger staining in the compact layer of both ventricular chambers in the KO mice compared to wild type littermates. However, there are many examples in which the regulation obtained in the expression analysis could not be repeated due to unspecific staining as exemplarily depicted for PlxnD1 and MLC-2A although the latter shows typical atrial staining patterns as expected for this gene.

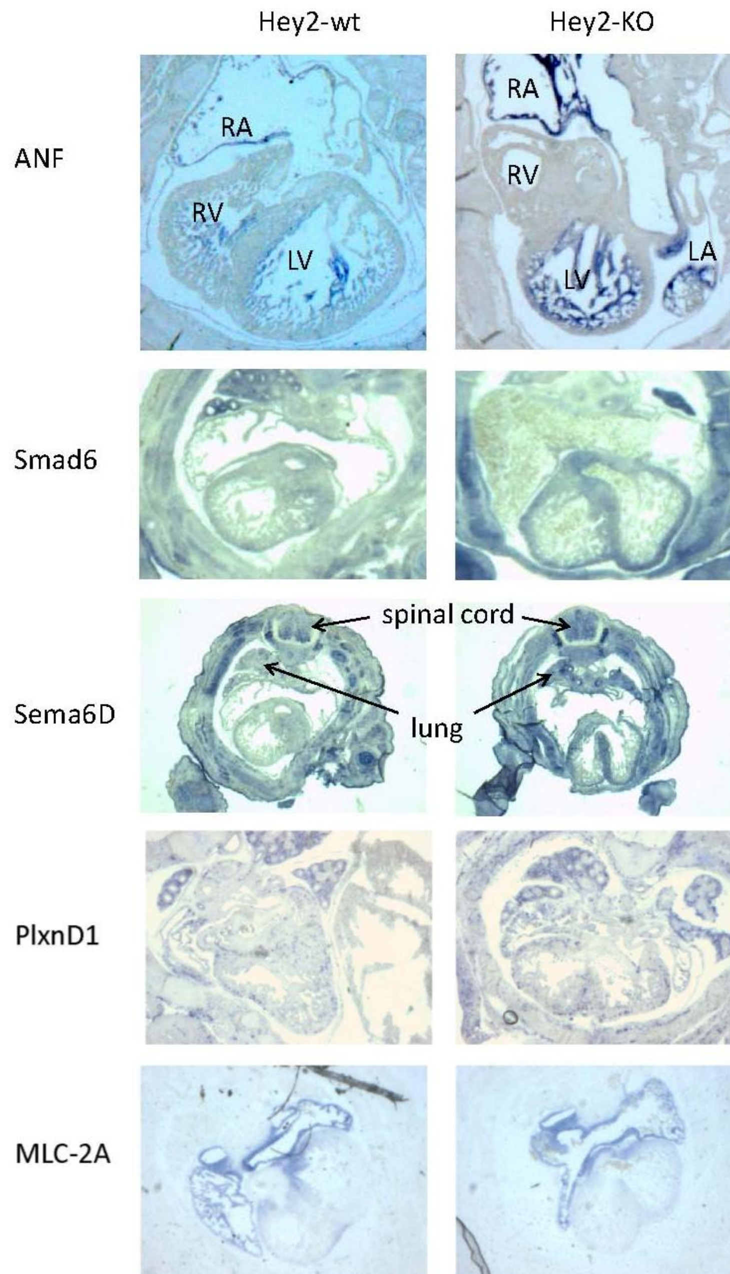


Figure 9: results of *in situ* hybridization on E14.5 Hey2-wt and -KO mice

Hey2-wt: left, Hey2-KO: right; from the top: ANF, Smad6, Sema6D, PlxnD1, MLC-2A

4.2.2 *Whole mount in situ hybridization* on Hey2-KO embryos (E10.5 or E11.5)

Some selected target genes of the quantitative real-time PCR assay were also investigated using *whole mount ISH*. Although only weakly stained, a nice staining pattern for PlxnD1 could be achieved with specific staining being visible in the endothelial cells between the somites and in the head (see **Figure 10**). However, no difference between Hey2-wt and –KO

embryos of E11.5 could be detected although qRT-PCR showed a significant difference of more than 5-fold for PlxnD1.

Figure 10: results of PlxnD1 WISH in E11.5 Hey2-wt and -KO mice

(Hey2-wt: left (head only) and middle), Hey2-KO right)

Furthermore, in staining experiments for Tbx2 or vegfR3, the differential gene regulation obtained by qRT-PCR could also not be evaluated in WISH samples (**Figure 11**).

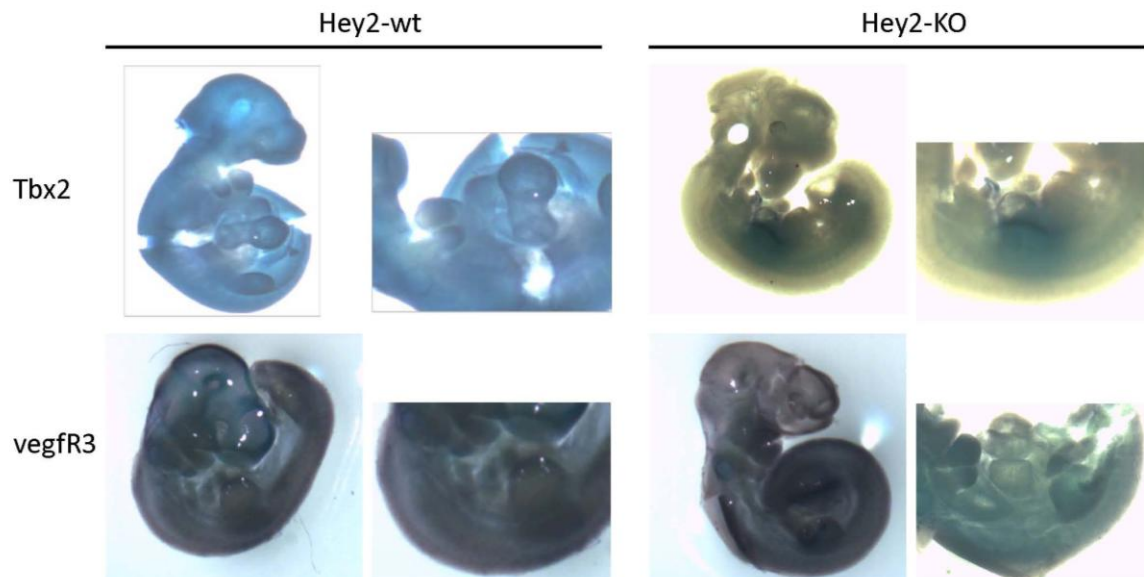


Figure 11: WISH for Tbx2 and vegfR3 on E11.5 Hey2-wt and -KO embryos

Tbx2: upper panel; vegfR3: lower panel; Hey2-wt: left, Hey2-KO: right

for details of the heart regions see right pictures

4.3 Possible influences on the nuclear localization of Hey

Stable cell lines (HeLa and HBMEC) expressing mCherry-fused Hey1 were generated to study possible influences on the localization of Hey1. The success of this was monitored by fluorescence microscopy showing mCherry-Hey-signals in the nucleus and GFP being visible in the complete HeLa cell (**Figure 12**). As selection for the mCherry-mHey2 construct was not as successful as expected, the following experiments were only carried out in the HeLa-p201-Cherry-mHey1 cells and partially additionally in HBMEC-p201-Cherry-mHey1 endothelial cells.

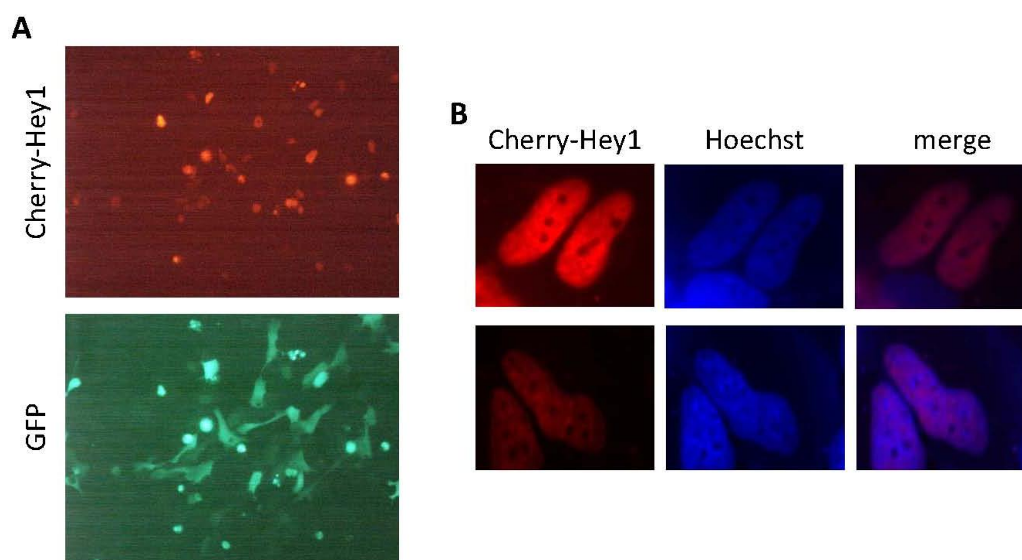


Figure 12: establishment of HeLa-p201-Cherry-mHey1 cells

A) microscopy expression control with nuclear mCherry expression (left) and GFP expression visible in the whole cell (right); B) confirmation of nuclear localization of mCherry-mHey1 by comparison to nuclei staining with Hoechst33342

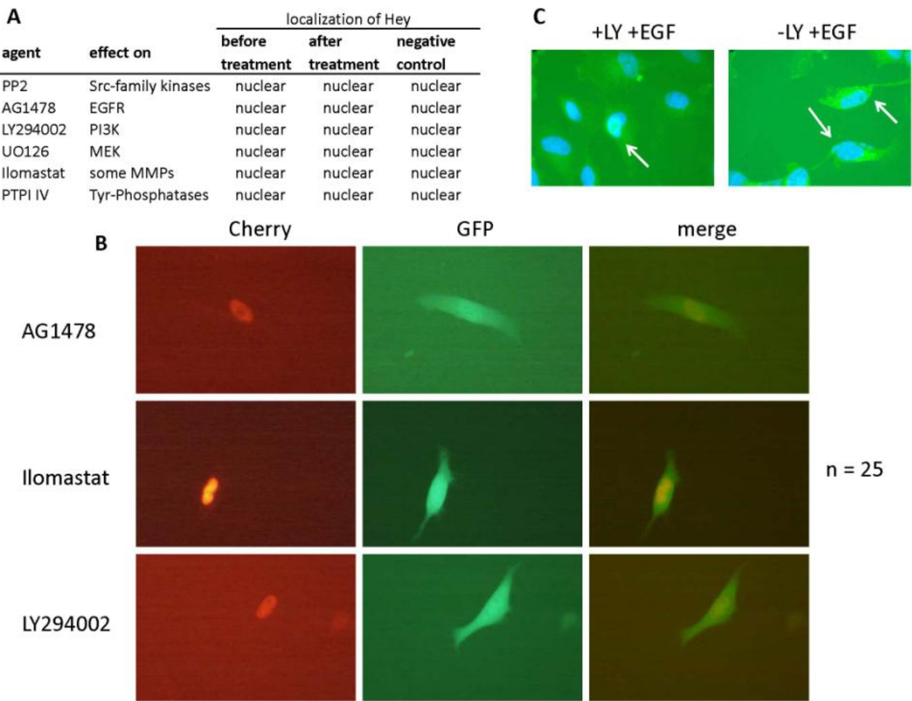
4.3.1 Treatment of HeLa-201-Cherry-mHey1 cells with inhibitors of important intracellular pathways

It is known that the Notch signaling pathway interacts with different other networks within the cell. Therefore, we wanted to see if inhibiting other important pathways like EGF signaling or signal transduction through kinases and phosphatases has any influence on the subcellular localization of Hey. However, as shown in **Figure 13** (panels **A** and **B**), there was no

change in the localization of Hey1 – it remained nuclear as clearly shown for the examples of AG1478 (EGF-receptor inhibitor), Ilomastat (inhibiting matrix metalloproteinases (MMPs)) and LY294002 which is affecting phosphatidyl inositole 3 kinase (PI3K) signaling. The same observations were made for the other three inhibitors: PP2 (inhibitor of Src-kinases), UO126 that has effects on MEK signaling and PTPI IV that inhibits tyrosine phsophatases.

To check if the assay methodology is working as a whole, LY294002 was chosen as it was described in the literature to be able to translocate ERK1/2 from the cytoplasm into the nucleus (Mut, 2012). Localization of endogenous phospho-ERK1/2 after stimulation with EGF was monitored via immunofluorescence and it could be shown, although pERK expression was quite weak, that in the absence of LY294002, more pERK can be found in the cytoplasm (**Figure 13 C**).

For the HBMEC-p201-Cherry-mHey1 cells treated with the same inhibiting agents, there was also no obvious change in the localization of Hey.



agent	effect on	localization of Hey: before treatment	after treatment	negative control
PP2	Src-family kinases	nuclear	nuclear	nuclear
AG1478	EGFR	nuclear	nuclear	nuclear
LY294002	PI3K	nuclear	nuclear	nuclear
UO126	MEK	nuclear	nuclear	nuclear
Ilomastat	some MMPs	nuclear	nuclear	nuclear
PTPI IV	Tyr-Phosphatases	nuclear	nuclear	nuclear

Figure 13: results of the localization studies on Hey1 in HeLa-p201-Cherry-mHey1 cells treated with different inhibitors of signaling pathways

A) table of all used inhibitors with caused effects and results on Hey1 localization; B) example pictures for AG1478, Ilomastat and LY294002 (Cherry: left, GFP: middle, merge: right) C) localization of phospho-ERK1/2 in the presence or absence of LY294002; immunofluorescence of serum-starved HeLa cells stimulated with LY294002 and hEGF for 1.5h

4.3.2 Treatment of HeLa cells with stimulating agents

In contrast to treating the cells with inhibiting agents, the growth factors human EGF (hEGF) and TGFα were added to the HeLa-p201-Cherry-mHey1 cells to see if they have any influence on the localization of Hey. As shown in **Table 13**, these factors also did not affect the localization of Hey1 which was observed only in the nucleus of the cells (n = 25).

When the assay was repeated in HBMEC-p201-Cherry-mHey1 cells, the same results were obtained.

Table 13: results of localization studies of Hey1 in HeLa-p201-Cherry-mHey1 cells treated with EGF and TGFα

	localization of Hey		
agent	**before treatment**	**after treatment**	**negative control**
hEGF	nuclear	nuclear	nuclear
TGFα	nuclear	nuclear	nuclear

This result fits to the observations described above for the inhibitory agents and it can be assumed that neither inhibiting nor stimulating possible interacting pathways of Notch signaling might influence the nuclear localization of Hey where it can fulfill its role as a transcription factor.

4.3.3 Distribution of Hey in nucleus and cytoplasm

Hey proteins act as transcriptional repressors. Therefore, they are assumed to be located in the nucleus of the cell as seen in (immuno-)fluorescence assays. However, it was the question if this could be further proven in nuclear and cytoplasmic protein lysates using the Western Blot technique. Dr. Daniela Salat could show that in HEK293 cells, an almost equal amount of Hey1, Hey2 and HeyL can be detected by Western Blot in the cytoplasm and the nucleus. To answer the question if this is a cell line specific effect, an EGFP-Hey1 wild type construct was co-transfected with a pCS2p-Flag-Hes1-Hey1 CTerm chimeria into HeLa and HEK293TD cells. The chimera, in which the complete Hes1 amino acid sequence including the Orange domain was fused with the C-terminal part of Hey1 harboring the TEIGAF- and the YRPW-motif, was applied to check if the translocation to the cytoplasm is caused by

Hey1, as Hes1 alone was only detectable in the nuclear fraction of HEK293 cells (personal communication with Dr. Daniela Salat). Immunofluorescence staining of the co-transfected cells showed that both proteins are exclusively located to the nucleus (**Figure 14 A**). Western Blot analysis of nuclear and cytoplasmic fractions on the other hand revealed that both Hey1wt-GFP and Hes1-Hey1 CTerm can be detected in both compartments in almost equal amounts (**Figure 14 B** and **C**).

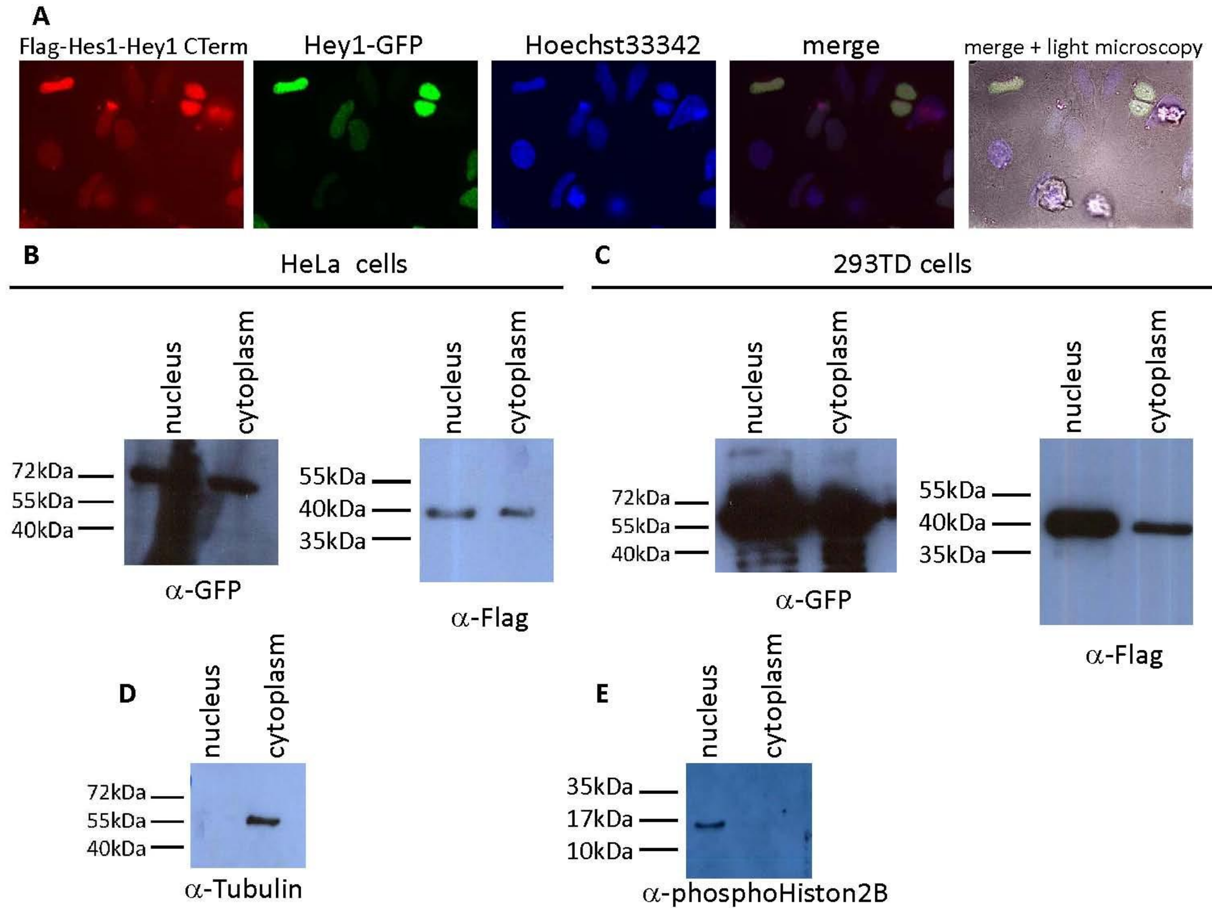


Figure 14: distribution of Hey1 and a chimeric Hes1-Hey1 CTerm protein in nucleus and cytoplasm

A) immunofluorescence for Hey1-GFP and Flag-tagged Hes1-Hey1 CTerm in HeLa cells; nuclear staining with Hoechst33342; B) and C) Western Blots of nuclear and cytoplasmic fractioning of HeLa and HEK293TD cells co-transfected with pEGFP-Hey1 wild type and pCS2p-Flag-Hes1-Hey1 CTerm; D) and E) control Western Blots with anti-Tubulin and anti-phosphoHiston2B antibodies

As controls for the successful fractioning, Western Blots against α-Tubulin – a cytoplasmic marker – and phosphoHistone2B (nuclear marker; kind gift of Dr. Steffi Herold, AG Eilers) were performed (**Figure 14 D** and **E**).

4.3.4 Possible influences of CaMKII on Hey localization

The HeLa-p201-Cherry-mHey1 cells were transiently transfected with three different CaMKII variants: wild type CaMKII, a C-terminal truncated version (K42M) that exhibits impaired binding affinity to interaction partners and another mutant in which the autophosphorylation reaction is no longer possible (T286D). Localization of Hey was monitored at different time points after transfection. As depicted in **Figure 15**, no change in the localization of Hey could be detected; it was only expressed in the nucleus (n = 20). As no change in Hey1 localization was observed, only the pictures till 1h after transfection are shown.

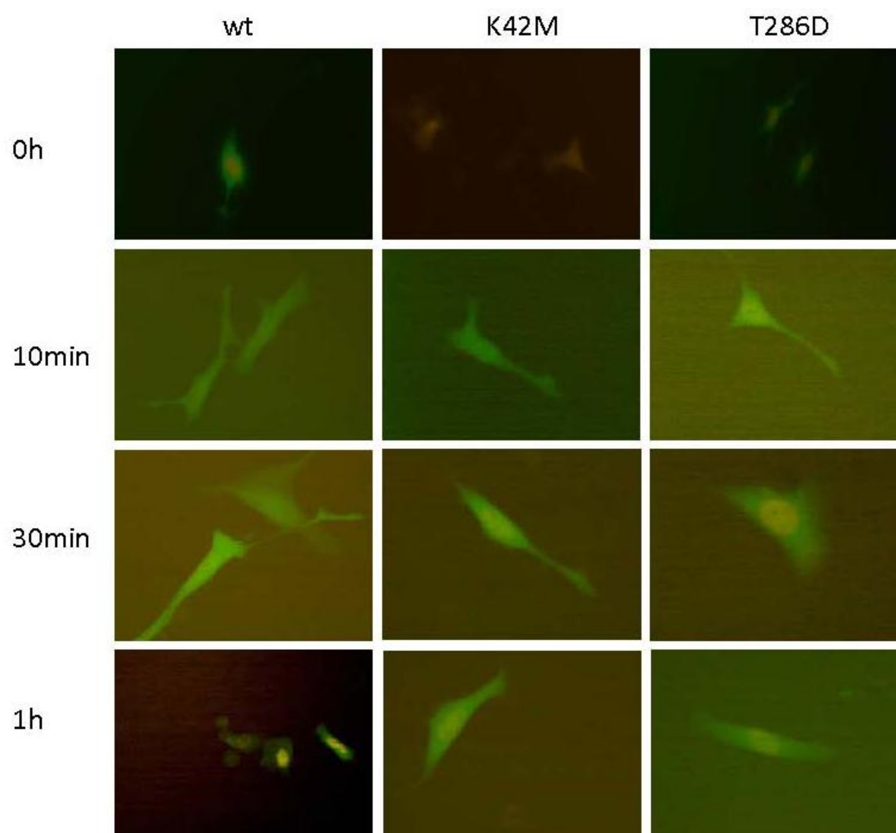


Figure 15: localization studies in HeLa-p201-Cherry-mHey1 cells transfected with CaMKII mutants

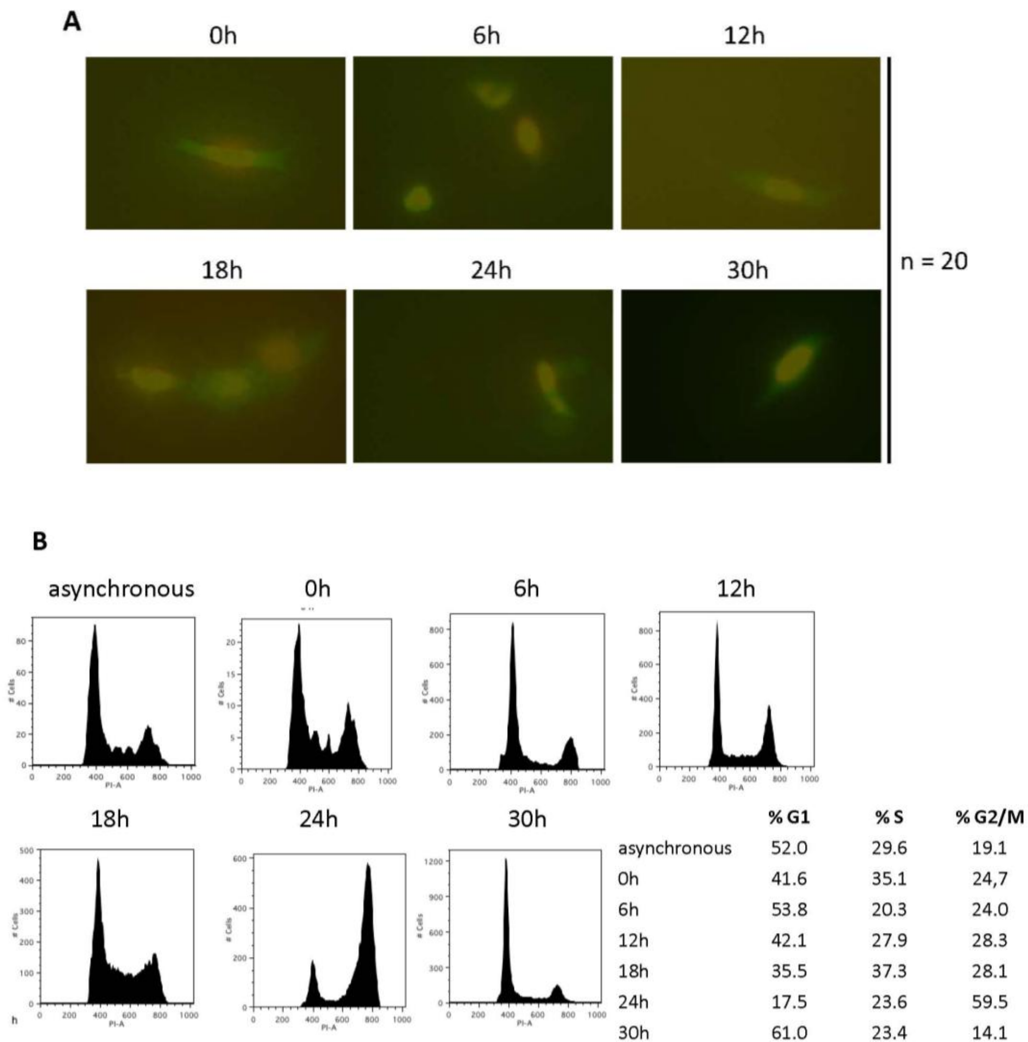
(CaMKII wt: left, K42M: middle, T286D: right) in a time course; merge of red and green channel

4.3.5 Hey localization during the cell cycle

During the cell cycle, a lot of different processes in the cell take place, especially in the nucleus where the chromosome set is doubled. Possibly, these cellular processes can influence

Hey localization. Therefore, we wanted to look at the localization of Hey in the HeLa-p201-Cherry-mHey1 cells in all cell cycle phases to see if it might be translocated to the cytoplasm during any one of the stages. To achieve this, serum starved cells were harvested at all cell cycle stages (6h, 12h, 18h, 24h and 30h after cell cycle induction) and analyzed by fluorescence microscopy. PI FACS analysis was performed to determine cell cycle stages.

At all analyzed time points, the mCherry-Hey signal could only be detected in the nucleus as already monitored in the other localization assays. However, the cell cycle seemed to be normal as shown by the PI FACS profiles. Therefore, it can be assumed that Hey localization is independent of the cell cycle (**Figure 16**).



	% G1	% S	% G2/M
asynchronous	52.0	29.6	19.1
0h	41.6	35.1	24,7
6h	53.8	20.3	24.0
12h	42.1	27.9	28.3
18h	35.5	37.3	28.1
24h	17.5	23.6	59.5
30h	61.0	23.4	14.1

Figure 1614: localization on Hey1 during the cell cycle in HeLa-p201-Cherry-mHey1 cells

A) Hey1 localization does not change during the different cell cycle stages; B) PI FACS profiles and percentage of cells in each cell cycle stage determined by PI FACS analysis

4.4 Interaction of Hey with Importinα and β

4.4.1 Interaction studies with Importinα

Dr. Daniela Salat identified by mass spectrometry possible interaction partners of Hey1 in HEK293-FS-mHey1 cells. One such candidate was Importinα that is involved in nuclear transport processes together with Importinβ and Ran. In IP experiments, it was investigated if this interaction could be confirmed. To achieve this, HEK293TD cells were transiently transfected with an EGFP-Hey1 construct containing either wild type-Hey1 or the delta-basic variant that could no longer be located in the nucleus together with Myc-tagged Impα (in pCDNA3-vector). Immunoprecipitation against the Myc-tag and Western Blot analyses with α-Myc and α-GFP antibodies could only show expression of Impα in the input samples as well as after IP; however, no GFP-tagged Hey1 could be detected in the IP samples (**Figure 17**).

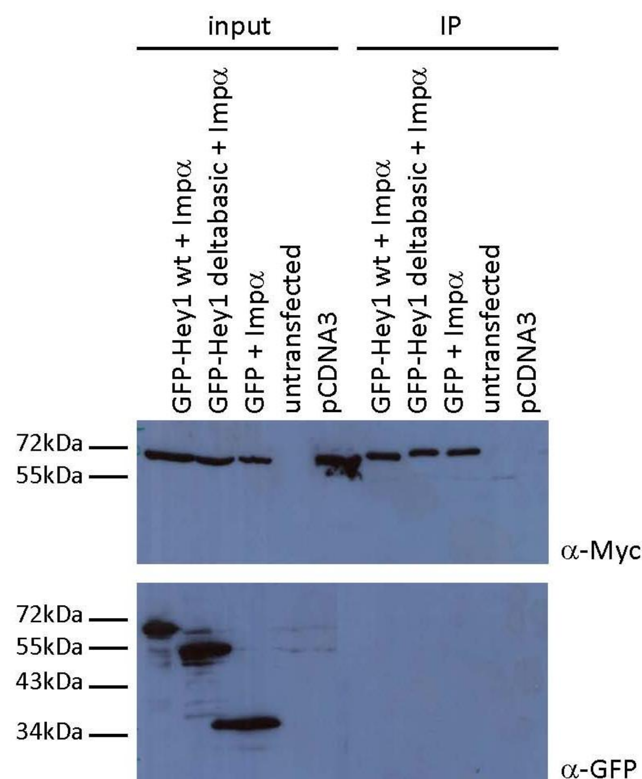


Figure 15: interaction studies of Hey1 and Importinα

Western Blots for input (left) and IP samples against Myc-tag (right) of interaction studies of GFP-tagged Hey1 with Myc-tagged Importinα in HEK293TD cells; upper panel: Myc-Western Blot; lower panel: GFP-Western Blot

In order to verify the non-interaction of Hey1 with Impα, the same experiment was repeated using pCS2p-Flag-Hey1 constructs (Hey1-wild type and Hey1-delta basic). As depicted below (**Figure 18**), it was not possible to show that Impα and Hey1 directly interact.

According to these results, it could be assumed that Importinα is no interaction partner of Hey1.

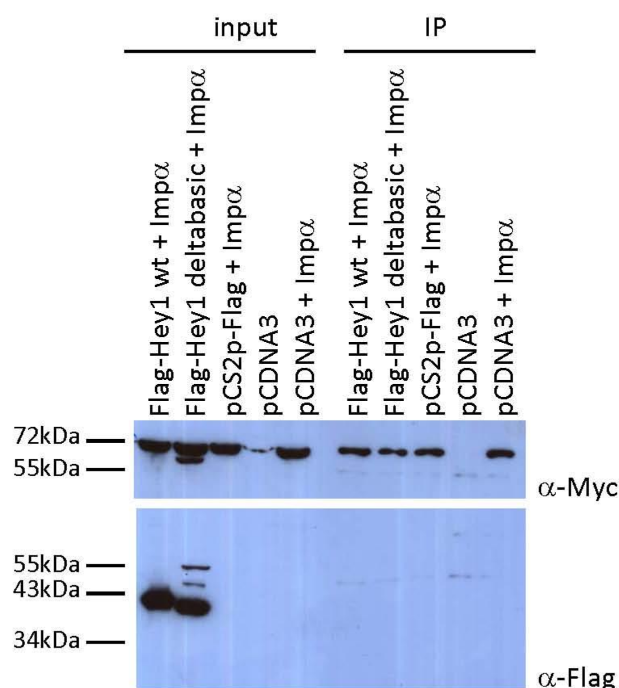


Figure 16: further interaction studies of Hey1 and Importinα

Western Blots for input (left) and IP samples against Myc-tag (right) of interaction studies of Flag-tagged Hey1 with Myc-tagged Importinα in HEK293TD cells; upper panel: Myc-Western Blot; lower panel: Flag-Western Blot

4.4.2 Interaction studies with Importinβ

In the mass spectrometry analysis, Importinβ has also been identified as a possible interaction partner for Hey1. Therefore, the interaction was studied in HEK293-FS-mHey1 cells as well as –mHey2 and –mHes1 cells. When an immunoprecipitation against the Strep-tag fused to Hey1 in doxycyclin-induced HEK293-FS-mHey1 cells was performed, the IP samples did not show any signal of endogenous Impβ (97kDa) as well as the transiently transfected DsRed-coupled version (125kDa) being verifiable in the input samples (**Figure 19**). However, Hey1 itself could nicely be detected in the α-Flag Western Blot after IP and the Impβ-

Western Blot positive control of transiently transfected HeLa cells showed reliability of the assay. Furthermore, confirmation of the IP itself was verified using a known interaction of Hey1, Hes1, as Hey1 could be detected in IP samples from doxycyclin-induced HEK293-FS-mHes1 cells that had been transfected with HA-tagged Hey1.

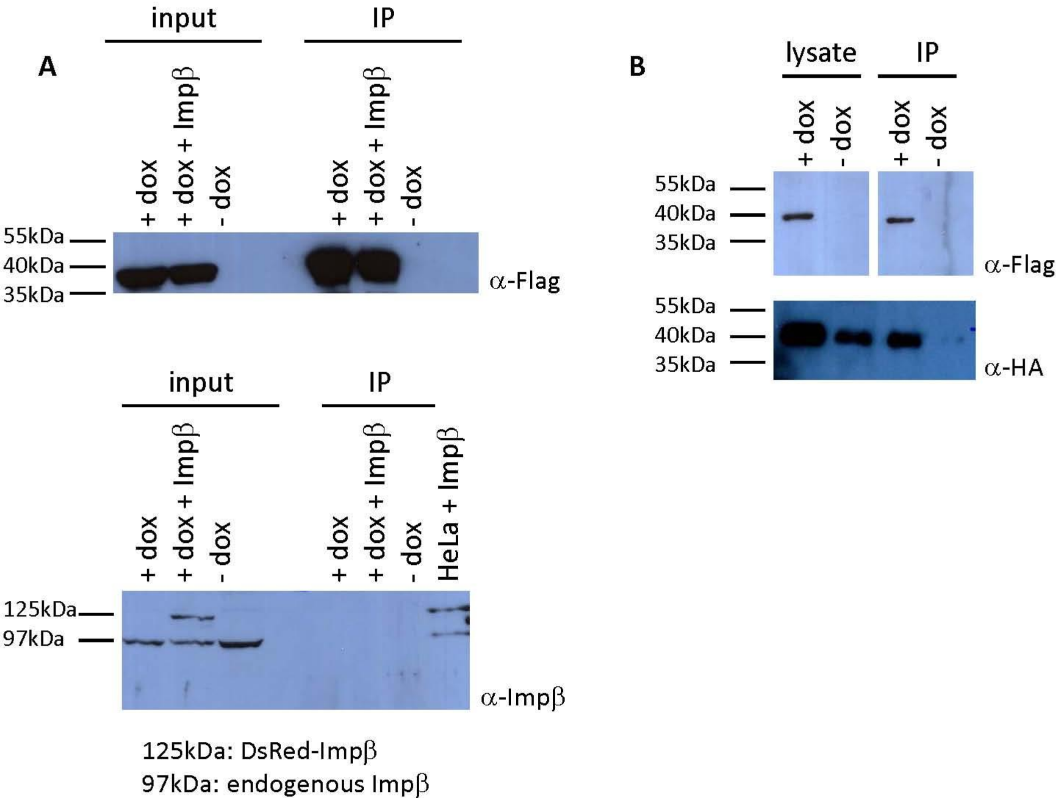


Figure 17: interaction studies of Hey1 and Importinβ

A) Western Blots for input (left) and IP samples against Strep-tag (right) of interaction studies of Flag-Strep-tagged Hey1 with DsRed-tagged Importinβ in HEK293-FS-mHey1 cells; upper panel: Flag-Western Blot; lower panel: Impβ-Western Blot; B) Western Blot for HA-tagged Hey1 in HEK293-FS-mHes1 cells as IP control experiment (Strep-IP); upper panel: Flag-Western Blot for Hes1 expression; lower panel: HA-Western Blot for Hey1 expression; induced and non-induced cells have all been transfected with the HA-Hey1 plasmid

The same results were obtained for HEK293-FS-mHey2 and –mHes1 cells (Western Blots not shown).

All these results hint towards the assumption that there seems to be no direct interaction of Hey1 with Importinβ as already previously obtained for Importinα.

5. Discussion

5.1 Gene regulation in different Hey mutant mice at different timepoints in development

5.1.1 Results from quantitative real time PCR analyses

The effects of several Hey knock outs at different developmental stages were analyzed using real-time qRT-PCR in the following mouse embryonic ventricular tissue samples: E14.5 – Hey2-KO, Hey1/L-DKO, ActH1 and E17.5 – Hey2-KO, Hey1/L-DKO. In these analyses, the Hey1/L-DKO animals served as phenotypic controls for the Hey2-KO individuals as they show the same morphological changes within the heart. Therefore, it can be assumed that the gene regulations obtained for the Hey2-KO animals at both developmental stages are due specifically to the lack of Hey2 and not caused by morphological disorders in the embryonic heart as the Hey1/L-DKO animals did not show these changes in gene expression. The ActH1 mice globally over-expressing Hey1 under the control of the β-actin promoter are the counterparts for the Hey-KO animals. In these embryos, a counter-regulation of several genes was obtained as expected.

It has already been published that differential gene expression patterns exist in atrial and ventricular tissue as well as in the left and the right ventricle. For the two ventricles, this is thought to be due to the fact that they stem from two different heart fields during heart development – primary heart field: left ventricle; secondary heart field: right ventricle (Ng, 2010). The difference in gene expression between the left and the right ventricle could also be observed in the E17.5 Hey2-KO and Hey1/L-DKO animals in the present study. Ng *et al.* (2010) describe in their publication that atrial cardiomyocytes express Hey1 and its downstream targets Gata4, Gata6, ANF, Bmp2 and Tbx2. Hey2 is the bHLH factor in ventricular cardiomyocytes and it influences the same factors but additionally also Cx40, Tbx5, MLC-1A and MLC-2A which was down-regulated in the real-time qRT-PCR assay in the Hey2-KO genotype. A similar picture of different markers within the heart and differences in the molecular regulation machinery of cardiac-chamber specific genes and their expression is drawn by

Small&Krieg (2004). The authors sum up the interplay of ANF and the MLC factors together with MEF2, Gata4/6, Nkx-2.5, Cx40 and Irx4 and they describe the differences in atrial and ventricular tissue. It is absolutely clear that cardiac development is based on an interplay of several factors (Srivastava&Olson, 2000; Christoffels, 2004; Olson, 2006). Therefore, it is not surprising that the KO of Hey2 leads to massive changes in the expression of other genes involved in the complex process of proper heart development such as ANF and Tbx2 and thereby causes the dramatic morphological phenotype in heart tissue.

Almost all regulated genes from the real-time qRT-PCR analysis have an identified promoter-located peak in the ChIP-Seq analysis performed by Heisig *et al.* (2012) in HEK293 cells over-expressing Hey1 or Hey2. The same primer set was also used on cDNA samples obtained from mouse embryonic stem (ES) cells that were infected with inducible Hey1 and Hey2 constructs. However, it was not possible to further validate the candidate genes of the tissue real-time qRT-PCR analysis in these cells (for detailed data, see **Appendix**). As the doxycyclin-induced ES cells are over-expressing Hey1, Hey1-GFP or Hey2, it might be assumed that gene regulation should be inverse to the Hey2-KO situation in the embryos. However, when looking at the expression data, significant over-expression of the inserted Hey gene could be observed, but this could not be translated to regulation of target genes such as the vegf receptors or Tbx2 and Smad6 that both were unexpectedly also up-regulated in the Hey1-over-expressing ES cells what makes it difficult to draw a final conclusion from this cell culture experiment. A possible explanation could be that gene regulation in ES cells is more complex and more relying on epigenetic influences (Günther, 2011) than in differentiated cells what might result in changes in gene expression patterns.

In the following, regulated genes of the described real-time qRT-PCR study are discussed with respect to interaction aspects with Hey proteins.

In the analysis of Hey2-KO embryos at developmental stage E14.5, a strong up-regulation was obtained for Tbx2. T-box factors in general play an important role during heart development and interact both with Bmp2 signaling as well as the Notch pathway (de la Pompa, 2008) and Nkx2.5 (Hoogars, 2007). Kokubo *et al.* (2007) postulate that formation of the

atrioventricular boundary is regulated by Hey1 and Hey2 as they repress Tbx2 expression in this process; Hey1 does this from the atrial section of the heart and Hey2 is working from the ventricles. There are further examples from the literature in which an interaction of Notch-Hey signaling and Bmp or the whole Bmp/Tgfβ signaling pathway during heart development have been described earlier (Ma, 2005; Zavadil, 2004). Therefore, it is not surprising that the KO of Hey2 results in up-regulation of Tbx2 and has influences on the whole regulatory signaling system during heart development as this might have been expected. In addition, there is a recent publication on the interplay of Hey and Hes1 with the Bmp/Tgfβ-pathway in developmental processes in the mouse retina (Moya, 2012) indicating another example of crosstalk between these two signaling cascades.

Another player in cardiac development is Id1 – as well as the other inhibitor of DNA-binding factors Id2, Id3 and Id4. Interestingly, double knock out animals of different Id-combinations show several cardiac abnormalities similar to Hey-KO embryos such as VSD (Fraidenraich, 2004). Therefore, it might not be surprising that Id2 and Id4 were up-regulated in Hey2-KO embryos compared to wild type littermates in our analysis. This is supported by the fact that especially Id4 has been identified to be a co-operation partner of Hey proteins in embryonic stem cells (Meier-Stiegen, 2010) and osteoblast differentiation (Tokuzawa, 2010) what hints towards an interplay of both HLH protein families in several developmental processes.

Up-regulation was also seen for members of the vegf-receptor family: flt1 (= vegfR1) and flk1 (= vegfR2). As described by Phng&Gerhardt (2009), the vegfRs co-operate with the Notch signaling pathway during angiogenesis with Dll4, flt1 and Notch mainly conveying signal conduction. Especially Dll4 is an essential factor influencing angiogenesis during embryonic vascular development as it was shown to regulate expression of both the soluble form of vegfR1 (= sflt1) and the vegf receptor 2 (= flk1) in HUVECs (Harrington, 2008) which were both up-regulated in the Hey2-KO embryos. Another hint for the fact that Notch signaling plays an important role in the development of the vascular system comes from the interplay of Hey2 with FoxC transcription factors as it is described that these transcriptional regulators can directly activate the Hey2 promoter (Hayashi&Kume, 2008). However, regulation of FoxC1 expression in the qRT-PCR analysis of the ventricular samples could not be detected. There-

fore, this might either be a one-way-regulatory system or FoxC1 is upstream of Hey2 in the signaling cascade.

Grego-Bessa *et al.* (2007) describe an effect of impaired Notch signaling on Neuregulin1 (Nrg1) and interestingly, this gene was up-regulated in the E14.5 Hey2-KO embryos used in our study. Another regulated gene that has been identified in the knock out animals is Gata4. It is known that this transcription factor and the Hey proteins are able to influence each other along with ANF which was also regulated in this analysis (Fischer, 2005). However, it was not possible to see an effect of the Hey2-KO on Gata4 in the real-time qRT-PCR analysis performed in the present study.

The zebrafish homologue of Hey2 is *gridlock*. *Gridlock* was shown to influence expression of ANF as well as Gata4 and Gata5 with the latter identified as an interaction partner in zebrafish (Jia, 2007). This undermines the conservation/importance of Notch signaling in developmental processes and its similar effects and influences throughout different species.

5.1.2 Validation of regulated genes with ISH and WISH

To further validate gene regulation at developmental stage E14.5, Hey2-wt and –KO embryos were analyzed by *in situ* hybridization. However, only the already published staining patterns for ANF and MLC-1A/-2A in the atria and the ventricular chambers could be reproduced (Fischer, 2005; Koibuchi&Chin, 2007) indicating reliability of the applied technique. For Smad6 and Sema6D, it was in part possible to monitor the up-regulation obtained in the real-time qRT-PCR analysis. For the whole mount ISH samples in E10.5/11.5 embryos, the staining for PlxnD1 showed weak expression. However, when comparing the result to published data it became obvious that the right expression pattern was obtained (Gitler, 2004) although it was not possible for us to monitor the expression changes generated from our qRT-PCR data (about 5-fold more in Hey2-KO than in Hey2-wt).

A possible reason why we did not obtain specific staining signals for all other regulated genes could be that the ISH technique is not as sensitive as real-time qRT-PCR is which is able

to detect slight differences between two samples. Moreover, ISH is much more susceptible to errors and changes in the experimental surroundings than this is true for PCR. At times, there are even expression gradients visible in one and the same section.

To improve the validation of the real-time qRT-PCR data by *in situ* hybridization will be a further experimental challenge to be concentrated on to provide additional support for the gene regulation data and one may be able to draw further conclusions on the influences of Hey factors on embryonic development and their consequences.

The second part of the study dealt with a totally different aspect of the Hey proteins as their biochemical properties were investigated. The results of these experiments will be discussed in the following paragraphs with special focus on Hey1 and its' sub-cellular localization and the nuclear transport processes that are associated with it.

5.2 Localization studies for Hey1

5.2.1 Influences in cell culture experiments

HeLa cells expressing mCherry-mHey1 were treated with effectors on important signaling pathways to analyze if these factors could influence the sub-cellular localization of Hey1 by changes in crosstalk from the Notch pathway with other signaling cascades. Cells stimulated with EGF and TGFα did not show any change in nuclear localization of Hey1 as wells as when inhibiting agents were added. However, it is known that Notch signaling interacts/crosstalks with other pathways (Andersson, 2011; Dyer, 2010) and the obtained results leave open the question if components of these other pathways are able - under yet unknown circumstances - to translocate the Hey proteins to the cytoplasm of the cell although it was not observed in the present study as Hey1 was always located in the nucleus. However, translocation of Hey to the cytoplasm induced by other signaling cascades would represent a possible way of inhibition of Hey function as it can fulfill its task of transcriptional regulation only in the nu-

cleus. With the use of the LY inhibitor and monitoring its effect on the cytoplasmic translocation of pERK1/2 as a control, it was possible to show that the applied assay was functioning. Therefore, it can be assumed that inhibiting these other signaling pathways does not influence the localization of Hey1 in the HeLa cells as well as in HBMEC-p201-Cherry-mHey1 endothelial cells indicating no cell type-specific effect.

The same cell culture system was applied to monitor effects of wild type or mutant CaMKII on the sub-cellular localization of Hey1. It is known that a specific isoform of CaMKII – the δc variant - is playing an important role in cardiomyocytes and can induce hypertrophy accompanied by an up-regulation of ANF (Zhang, 2002; Zhang 2003) which is similar to the Hey2-KO phenotype. In the cell culture assay, expressing any variant of CaMKII did not influence the localization of Hey1 although it cannot be excluded that under specific circumstances, Hey proteins and CaMKII could interact. This is in line with the results mentioned above for the inhibitors of different signaling pathways as well as the fact that up to now, no direct interaction of CaMKII and the Hey protein family has been described.

In conclusion from all findings, it seems as if influencing other pathways within the cell does not change the nuclear localization of Hey proteins. Therefore, Hey proteins always have the ability to conduct their transcriptional repression task what is indispensible for such a highly conserved and important signaling pathway such as Notch that governs essential developmental processes from the very beginning.

In all these assays, Hey proteins have always been located to the nucleus. To further validate these findings on protein level, nuclear and cytoplasmic fractions of different Hey-expressing cells were generated and the distribution of Hey in these fractions was analyzed.

5.2.2 Distribution of Hey in nucleus and cytoplasm

Sub-cellular distribution of Hey1 was monitored in HeLa and HEK293TD cells using both (immuno-)fluorescence methods and Western Blot analysis. Interestingly, Hey1-GFP could only be observed using fluorescence microscopy in the nucleus of HeLa cells, however, Western Blots revealed almost equal amounts of Hey1-GFP in the nucleus and the cytoplasm. Hes1 on the other hand was shown by Dr. Daniela Salat to be exclusively expressed in the nucleus when analyzing nuclear and cytoplasmic fractions by Western Blot. Therefore, the question was if this difference in localization could be depending on the divers C-termini of the two proteins. Therefore, a chimera of Hes1 fused with the C-terminus of Hey1 was applied and it showed the same result when immunofluorescencent staining for Flag-tagged Hes1-Hey1 CTerm was performed which monitored that the chimeric protein was also only located to the nucleus of the cells. However, this result is in contradiction to the observations from the Western Blot analysis where the chimeria could be detected in both the nuclear and the cytoplasmic fraction of HeLa as well as HEK293TD cells.

These differences in localization can be due to several reasons. The first possible explanation could be technical regarding the preparation of the nuclear and cytoplasmic lysates for Western Blot experiments as it harbors the possibility that parts of the nuclear fraction pollute the cytoplasmic sample. However, we could not detect α-tubulin traces in any of the nuclear fractions and using an antibody directed against phospho-histone2B, it could be shown that no signal of nuclear proteins is detectable in the cytoplasmic fractions. Furthermore, Hes1 alone was shown by Dr. Daniela Salat to be only expressed in the nucleus which also speaks against this technical problem and hints towards the assumption that the C-terminus of Hey1 could indeed be responsible for a possible translocation to cytoplasm.

On the other hand, the mistake can also be part of the immunofluorescence staining method as there are several studies available in the literature dealing with influences of solvents and permeabilizing agents on staining and sub-cellular localization of different proteins (Melan, 1999; Schnell, 2012). In these publications, it is discussed whether it is more useful to apply methanol instead of PFA for fixation for different proteins and if using detergents like Triton X-100 to permeabilize the cells is the best choice for studying protein localization within the cell. The authors suggest to better use fluorescence protein-coupled versions of the protein-

of-choice and to employ live cell imaging for localization studies. However, this technique has its own disadvantages as the tag can influence the characteristics of the protein, too. On the other hand, the localization studies concerning Hey1 in the HeLa-p201-Cherry-mHey1 cells by adding inhibitors or plasmids expressing CaMKII to the cells have been done using mCherry-coupled Hey1 and live cell imaging and they did not show a cytoplasmic translocation of Hey1. Therefore, the question why we can detect Hey1 in the cytoplasm in Western Blots is still open and needs additional experiments to be answered.

Microarray analysis and ChIP-Seq in Hey1 over-expressing HEK293 cells showed that some cell cycle-influencing proteins such as Cyclin-dependent kinases can be target genes of the Hey protein family (Heisig, 2012). Based on this, the localization of Hey1 during the cell cycle was analyzed in the HeLa-p201-Cherry-mHey1 cells. As described above for the pathway inhibitors, the mCherry-Hey1 signal was only detected in the nucleus during the whole time course of 30h after release into the cell cycle. However, there is up to now no literature available describing possible influences of different cell cycle stages on the localization of Hey proteins. On the other hand, there are examples of proteins that change their sub-cellular distribution during the cell cycle and therefore, it could also be assumed to be the case for the Hey proteins. For example, GNL1, a putative nucleolar GTPase, is only localized to the nucleus and nucleolus in G2 stage while it is cytoplasmic during all other cell cycle stages (Boddapati, 2012). From the present study however, it might be concluded that the cell cycle does not influence Hey distribution within the cell.

5.3 Interaction studies of Hey with nuclear transport proteins

Importinα and Importinβ have been identified as possible interaction partners of Hey1 using mass spectrometry (Dr. Daniela Salat). Both proteins are known to play important roles during nuclear transport processes.

Importinα and β interact during nuclear transport; thereby, Importinα binds to the nuclear localization signal of the protein-to-transport while Importinα itself is bound by Importinβ

(Güttler &Görlich, 2011). However, it was not possible to show any such Importin-Hey complexes using immunoprecipitation methods. This result raises the question why it is not feasible to detect these interactions of Hey with the Importins. There are several explanations: 1) the stability of the complex is very weak and it collapses as soon as the cellular constitution is destroyed when protein lysates are prepared. However, it was possible to monitor other interactions of Hey with for example GATA4/6 (Fischer, 2005) and in the present study, it could also be shown that Hey1 interacts with its known binding partner Hes1; therefore, this might not be the only reason why no interaction of Hey and Importins could be detected. On the other hand, interaction of Importinβ - together with Ran – could be shown for the mitotic spindle protein HURP1 (Sillje, 2006). Furthermore, it was recently published that the nuclear transport of WT1 is also dependent on interaction with Importinβ as well as Importinα (Depping, 2012) where both proteinacous interactions were monitored using GST-pull down assays as well as immunoprecipitation. Myb-binding protein 1a (Mybbp1a) has also been identified to interact with both Importins by GST-pull down assays (Keough, 2003); 2) another possible explanation might be that it is an indirect interaction of Hey1 with both Importins as such. Therefore, it would not be possible to detect the interaction using immunoprecipitation. If this is the case, it may be necessary to stabilize or somehow fix the complexes as performed in the ChIP technique by using PFA; 3) finally, it is also plausible that the nuclear transport of Hey does not require any transport proteins at all as it is postulated that small molecules ($\leq$ 30kDa) could use passive diffusion to leave and enter the nucleus of the cell (Güttler&Görlich, 2011; Elion, 2002). With a predicted protein size of about 34kDa, this could indeed be possible for Hey proteins. However, what would call for a directed and mediated transport is the fact that there is a predicted classical nuclear localization signal within the Hey1 protein (identified using ProteinPredict; http://www.predictprotein.org/) which could be recognized by Importinα. Furthermore, GFP-coupled Hey1 is definitely too large in kDa-size to passively diffuse in and out of the nucleus what further supports the search for a nuclear transport system for the Hey proteins. With this, it may be possible to explain the presence of Hey proteins in the cytoplasm, too, especially with regard to the fact that a Hey1-mutant lacking the basic domain (Hey1-delta basic) which is no longer able to bind to the DNA is preferentially located to the cytoplasm as shown by immunofluorescence in HEK293 cells (personal communication with Dr. Julia Heisig).

The other player in the nuclear transport system is Ran. RanGTP thereby serves as the driving force for the nuclear import process (Gittler&Görlich, 2011; Nigg, 1997; Sorokin, 2007). There are two different mutants of Ran available: RanQ69L that blocks nuclear import and RanT24N which resembles the cytoplasmic situation with low amounts of RanGTP (Silljé, 2006). Applying immunoprecipitation as well as immunofluorescence, it was analyzed if the Ran mutants could interact with Hey1 or influence its' sub-cellular localization. However, contradictory results were obtained that made it impossible to draw a clear conclusion as we were unable to answer the question if a potential interaction of Hey1 and Ran can take place in the cell (data not shown). Furthermore, Ran is often a false-negative hit in mass spectrometry analyses and was mostly proven to be no direct interaction partner of the analyzed protein (personal communication with Dr. Daniela Salat). Therefore, it is possible that Hey proteins and Ran do not interact and it requires further experiments to prove the obtained results.

Concluding from all results obtained for both Importinα and Importinβ, it appears that Hey1 is not interacting with these nuclear transport proteins. This leads to the question of how Hey is transported between nucleus and cytoplasm and if this is possibly influenced or mediated by other binding partners/transmitting proteins than Ran and Importinα/β. It is known that Notch1 signaling for example is mediated by Importin alpha 3, 4 and 7 (Hünninger, 2010) – but not the subunit 1 that was used in the present study and has been identified as a potential Hey1-interaction partner in the mass spectrometry analysis by Dr. Daniela Salat in Hey1-over-expressing cells. A second possibility would be that the whole transport process is depending on a different mechanism or third, as previously mentioned, passive diffusion takes place. However, this interesting point of Hey biochemistry needs to be further elucidated.

6. Bibliography

Anderson, R. H., Webb, S., Brown, N. A., Lamers, W., Moorman, A. (2003): Development of the heart: (2) Septation of the atriums and ventricles; *Heart and Education in Heart*, Vol. 89, 949 - 958

Andersson, E. R., Sandberg, R., Lendahl, U. (2011): Notch signaling: simplicity in design, versatility in function; *Development*, Vol. 138, 3593 - 3612

Boddapati, N., Anbarasu, K., Suryaraja R., Tendulkar, A. V., Mahalingam, S. (2012): Subcellular distribution of the human putative nucelolar TGPase GNL1 is regulated by a novel arginine/lysine-rich domain and a GTP binding domain in a cell cycle-dependent manner; *Journal of Molecular Biology*, Vol. 416, 346 - 366

Bruneau, B. G. (2002): Transcriptional Regulation of Vertebrate Cardiac Morphogenesis; *Circulation Research*, Vol. 90, 509 - 519

Christoffels, V. M., Burch, E. E., Moorman, A. F. M. (2004): Architectural Plan for the Heart: Early Patterning and Delineation of the Chambers and the Nodes; *Trends in Cardiovascular Medicine*, Vol. 14, 301 – 307

Davis, R. L., Turner, D. L. (2001): Vertebrate hairy and Enhancer of split related proteins: transcriptional repressors regulating cellular differentiation and embryonic patterning; *Oncogene*, Vol. 20, 8342 - 8357

de la Pompa, J. L. (2009): Notch Signaling in Cardiac Development and Disease; *Pediatric Cardiology*, Vol. 30, 643 – 650

Depping, R., Schindler, S. G., Jacobi, Ch., Kirschner, K. M., Scholz, H. (2012): Nucelar Transport of Wilms‘ Tumour Protein Wt1 Involves Importins α and β; *Cellular Physiology and Biochemistry*, Vol. 29, 223 – 232

Dyer, L. A., Kirby, M. L. (2009): The role of secondary heart field in cardiac development; *Developmental Biology*, Vol. 336, 137 - 144

Elion, E. A. (2002): How to Monitor Nuclear Shuttling; *Methods in Enzymology*, Vol. 351, 607 – 622

Fischer, A., Leimeister, C., Winkler, C., Schuhmacher, N., Klamt, B., Elmasri, H., Steidl, C., Maier, M., Knobeloch, K.-P., Amann, K., Helisch, A., Sendtner, M., Gessler, M. (2002): *Hey* bHLH Factors in Cardiovascular Development; *Cold Spring Harbor Symposia on Quantitative Biology*, Vol. LXVII, 63 – 70

Fischer, A., Gessler, M. (2003): *Hey* Genes in Cardiovascular Development; *Trends in Cardiovascular Medicine*, Vol. 13. 221 – 226

Fischer, A., Schuhmacher, N., Maier, M., Sendtner, M., Gessler, M. (2004): The Notch target genes Hey1 and Hey2 are required for embryonic vascular development; *Genes and Development*, Vol. 18, 901 – 911

Fischer, A., Klattig, J., Kneitz, B., Diez, H., Maier, M., Holtmann, B., Englert, C., Gessler, M. (2005): Hey Basic Helix-Loop-Helix Transcription Factors Are Repressors of GATA4 and GATA6 and Restrict Expression of the GATA Target Gene *ANF* in Fetal Hearts; *Molecular and Cellular Biology*, Vol. 25, 8960 - 8970

Fischer, A., Steidl, C., Wagner, T. U., Lang, E., Jakob, P. M., Friedl, P., Knobeloch, K.-P., Gessler, M. (2007): Combined Loss of Hey1 and HeyL Causes Congenital Heart Defects Because of Impaired Epithelial to Mesenchymal Transition; *Circulation Research*, Vol. 100, 856 – 863

Fischer, A., Gessler, M. (2007): Delta-Notch – and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors; Nucelic Acids Research, Vol. 35(14), 4583 – 4596

Fraidenraich, D., Stillwell, E., Romero, E., Wilkes, D., Manova, K., Basson, C. T., Benezra, R. (2004): Rescue of Cardiac Defects in *Id* Knockout Embryos by Injection of Embryonic Stem Cells; *Science*, Vol. 306, 247 - 252

Gama-Carvalho, M, Carmo-Fonseca, M. (2006): Heterokaryons: An Assay for Nucleocytoplasmic Shuttling; *Cell Biology*, Chapter 37, 277 – 283

Gitler, A. D., Lu, M. M., Epstein, J. A. (2004): PlexinD1 and Semaphorin Signaling Are Required in Endothelial Cells for Cardiovascular Development; *Developmental Cell*, Vol. 7, 107 - 116

Grego-Bessa, J., Luna-Zurita, L., del Monte, G., Bolós, V., Melgar, P., Arandilla, A., Garratt, A., N., Zang, H., Mukouyama, Y., Chen, H., Shou, W., Ballestar, E., Esteller, M., Rojas, A., Pérez-Pomares, J. M., de la Pompa, J. L. (2007): Notch Signaling Is Essential for Ventricular Chamber Development; *Developmental Cell*, Vol. 12, 415 – 429

Günther, M. G. (2011): Transcriptional control of embryonic and induced pluripotent stem cells; *Epigenomics*, Vol. 3, 323 – 343

Güttler, T, Görlich, D. (2011): Ran-dependent nuclear export mediators: a structural perspective; *The EMBO Journal*, Vol 30, 3457 – 3474

Harrington, L. S., Sainson, R. C., Williams, C. K., Taylor, J. M., Shi, W., Li, J. L., Harries, A. L. (2008):Regulation of multiple angiogenic pathways by Dll4 and Notch in human umbilical vein endothelial cells; *Microvascular Research*, Vol. 75, 144 - 154

Hayashi, H., Kume, T. (2008): Foxc Transcription Factors Directly Regulate Dll4 and Hey2 Expression by Interaction with the VEGF-Notch Signaling Pathways in Endothelial Cells; *PLoS One*, Vol. 3, 1 - 9

Heisig, J., Weber, D., Englberger, E., Winkler, A., Kneitz, S., Sung, W.-K., Wolf, E., Eilers, M., Wie, C.-L., Gessler, M. (2012): Target Gene Analysis by Microarrays and Chromatin

Immunoprecipitation Identifies HEY Proteins as Highly Redundant bHLH Repressors; *PLoS Genet*, 8(5), epub ahead

High, F. A., Epstein, J. A. (2008): The multifaceted role of Notch in cardiac development and disease; *Nature Reviews Genetics*, Vol. 9, 49 – 61

Hoffman, J. I., Kaplan, S. (2002): The incidence of congenital heart disease; *Journal of the American College of Cardiology*, Vol. 39, 1890 - 1900

Hoogars, W. M. H., Bernett., P., Moorman, A. F. M., Christoffels, V. M. (2007): T-box factors determine cardiac design; *Cellular and Molecular Life Sciences*, Vol. 64, 646 - 660

Hueninger, K., Krämer, A., Soom, M., Chang, I., Köhler, M., Depping, R., Kehlenbach, R. H., Kaether, Ch. (2010): Notch1 signaling is mediated by importins alpha 3, 4 and 7; *Cellular and Molecular Life Sciences*, Vol. 67, 3187 - 3196

Iso, T., Hamamori, Y, Kedes, L. (2003): Notch Signaling in Vascular Development; *Arteriosclerosis, Thrombosis and Vascular Biology*, Vol. 23, 543 - 553

Jia, H., King, I. N., Chopra, S. S., Wan, H., Ni, T. T., Jiang, Ch., Guan, X., Wells, S., Srivastava, D., Thong, T. P. (2007): Vertebrate heart growth is regulated by functional antagonism between Gridlock and Gata5; *PNAS*, Vol. 104, 14008 - 14013

Keough, R. A., Macmillan, E. M., Lutwyche, J. K., Gardner, J. M., Tavner, F. J., Jans, D. A., Henderson, B. R., Gonda, T. J. (2003): Myb-binding protein 1a is a nucelocytoplasmic shuttling protein that utilizes CRM1-depnedent and independent nuclear export pathways; *Experimental Cell Research*, Vol. 289, 108 – 123

Koibuchi, N., Chin, M. T. (2007): CHF1/Hey2 Plays a Pivotal Role in Left Ventricular Maturation Through Suppression of Ectopic Atrial Gene Expression; *Circulation Research*, Vol. 100, 850 - 855

Kokubo, H., Tomita-Miyagawa, S., Hamada, Y., Saga, Y. (2007): Hesr1 and Hesr2 regulate atrioventricular boundary formation in the developing heart through the repression of Tbx2; *Development*, Vol. 134, 747 - 755

Kopan, R., Ilagan, M. X. G. (2009): The Canonical Notch Signaling Pathway: Unfolding the Activation Mechanism; *Cell*, Vol. 137, 216 – 233

Lamar, E., Deblandre, G., Wettstein, D., Gawantka, V., Pollet, N., Niehrs, C., Kintner, C. (2001): Nrarp is a novel intracellular component of the Notch signaling pathway; *Genes and Development*, Vol. 15, 1885 - 1899

Ma, L., Lu, M.-F., Schwartz, R. J., Martin, J. F. (2005): *Bmp2* is essential for cardiac cushion epithelial-mesenchymal transition and myocardial patterning; *Development and disease*, 132, 5601 - 5611

MacGrogan, D., Luna-Zurita, L., de la Pompa, J. L. (2011): Notch Signaling in Cardiac Valve Development and Disease; *Birth Defects Research (Part A)*, Vol. 91, 449 – 459

Meier-Stiegen, F., Schanbeck, R., Bernoth, K., Martini, S., Hieronymus, T., Ruau, D., Zenke, M., Just, U. (2010): Activated Notch1 Target Genes during Embryonic Cell Differentiation Depend on the Cellular Context and Include Lineage Determinants and Inhibitors; *PLoS One*, Vol. 5, 1 – 18

Melan, M. A. (1999): Overview of Cell Fixatives and Cell Membrane Permeants; *Methods in Molecular Biology*, Vol. 115, 45 - 55

Moorman, A. Webb, S., Brown, N. A., Lamers, W. Anderson, R. H. (2004): Development of the heart: (1) Formation of the cardiac chambers and arterial trunks; *Heart and Education in Heart*, Vol. 89, 806 - 814

Moya, I. M., Umans, L., Maas, E., Pereira, P. N. G., Beets, K., Francis, A., Sents, W., Robertson, E. J., Mummery, C. L., Huylebroeck, D., Zwijsen, A. (2012): Stalk Cell Phenotype Depends on Integration of Notch and Smad1/5 Signaling Cascades; *Developmental Cell*, Vol. 22, 501 - 514

Mut, M., Lula, S., Demir, O., Kurnaz, I. A., Vural, I. (2012): Both mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinases (ERK) 1/2 and phosphatidylinositide-3-OH-kinase (PI3K)/Akt pathways regulate activation of E-twenty-six (ETS)-like transcriptions factor 1 (Elk1) in U138 glioblastoma cells; *International Journal of Biochemistry and Cell Biology*, Vol. 44, 302 – 310

Nemir, M., Pedrazzini, T. (2008): Functional role of Notch signaling in the developing and postnatal heart; *Journal of Molecular and Cellular Cardiology*, Vol. 45, 495 - 504

Niessen, K., Karsan, A. (2007): Notch signaling in the developing cardiovascular system; *American Journal of Physiology and Cellular Physiology*, Vol. 293, C1 – C11

Niessen, K., Karsan, A. (2008): Notch Signaling in Cardiac Development; *Circulation Research*, Vol. 102, 1169 – 1181

Nigg, E. A. (1997): Nucleocytoplasmic transport: signals, mechanisms and regulation; *Nature*, Vol. 386, 779 – 78

Ng, S. Y., Wong, C. K., Tsang, S. Y. (2010): Differential gene expressions in atrial and ventricular myocytes: insights into the road of applying embryonic stem cell-derived cardiomyocytes for future therapies; *American Journal of Cell Physiology*, Vol. 299, C1234 – C1249

Olson, E. N. (2006): Gene Regulatory Networks in the Evolution and Development of the Heart; *Science*, Vol. 313, 1922 - 1927

Phng, L.-K., Gerhardt, H. (2009): Angiogenesis: A Team Effort Coordinated by Notch; *Developmental Cell*, Vol. 16, 196 – 206

Ribbeck, K. Görlich, D. (2001): Kinetic analysis of translocation through nuclear pore complexes; *The EMBO Journal*, Vol. 20, 1320 - 1330

Rochais, F., Dandonneau, M., Mesbah, K., Jarry, T., Mattei, M.-G., Kelly, R., G. (2009): *Hes1* Is Expressed in the Second Heart Field and Is Required for Outflow Tract Development; *PLoS One*, Vol. 4

Rutenberg, J. B., Fischer, A., Jia, H., Gessler, M., Zhong, T. P., Mercola, M. (2006): Developmental patterning of the cardiac atrioventricular canal By Notch and Hairy-related transcription factors; *Development*, Vol. 133, 4381 – 4390

Sakata, Y., Kamel, C. N., Nakagami, H., Bronson, R., Liao, J. K. Chin, M. T. (2002): Ventricular septal defects and cardiomyopathy in mice lacking the transcription factor CHF1/Hey2; *PNAS*, Vol. 99, 16197 - 16202

Sakata, Y., Koibuchi, N., Xiang, F., Youngblood, J. M., Kamei, C. N. Chin, M. T. (2006): The spectrum of cardiovascular anomalies in CHF1/Hey2 deficient mice reveals roles in endocardial cushion, myocardial and vascular maturation; *Journal of Molecular and Cellular Cardiology*, Vol. 40, 267 – 273

Salie, R., Kneissel, M., Vukevic, M., Zamurovic, N., Kramer, I., Evans, G., Germin, N., Müller, M., Kinzel, B., Susa., M. (2010): Ubiquitous overexpression of Hey1 transcription factor leads to osteopenia and chondrocyte hypertrophy in bone; *Bone*, Vol. 46, 680 - 694

Savolainen, S. M., Foley, J. F., Elmore, S. A. (2009): Histology Atlas of the Developing Mouse Heart with Emphasis on E11.5 to E18.5; *Toxicologic Pathology*, Vol. 000, 1 – 20

Schnell, U., Dijk, F., Sjollema, K. A., Giepmans, B. N. G. (2012): Immunolabeling artifacts and the need for live-cell imaging; *Nature Methods*, Vol. 9, 152 - 158

Silljé, H. H. W., Nagel, S., Körner, R., Nigg, E. A. (2006): HURP Is a Ran-Importinβ-Regulated Protein that Stabilizes Kinetochore Microtubules in the Vicinity of Chromosomes; *Current Biology*, Vol. 16, 731 – 742

Small, E. M., Krieg, P. A. (2004): Molecular Regulation of Cardiac Chamber-Specific Gene Expression; *Trends in Cardiovascular Medicine*, Vol. 14, 13 - 18

Sorokin, A. V., Kim, E. R. and Ovchinnikov, L.P. (2007): Nucleocytoplasmic Transport of Proteins; *Biochemistry (Moscow)*, Vol. 72, 1439 – 1457

Srivastava, D., Olson, E. N. (2000): A genetic blueprint for cardiac development; *Nature Insight Progress*, Vol. 407, 221 - 226

Timmerman, L. A., Grego-Bessa, J., Raya, A., Bertrán, E., Pérez-Pomares, J. M., Díez, J., Aranda, S., Palomo, S., McCormick, F., Izpisúa-Belmonte, J. C., de la Pompa, J. L. (2004): Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation; *Genes and Development*, Vol. 18, 99 – 115

Tokuzawa, Y., Yagi, K., Yamashita, Y., Nakachi, Y., Nikaido, I., Bono, H., Ninomiya, Y., Kanesaki-Yatsuka, Y., Akita, M., Motegi, H., Wakana, S., Noda, T., Sablitzky, F., Arai, S., Korukawa, R., Fukuda, T., Katagiri, T., Schönbach, Ch., Suda, T., Mizuno, Y., Okazaki, Y (2010): Id4, a New Candidate Gene for Senile Osteoporosis, Acts as a Molecular Switch Promoting Osetoblast Differentiation; *PLoS Genetics*, Vol. 6, 1 – 15

Weerkamp, F., Luis, T. C., Naber, B. A., Koster, E. E., Jeannotte, L., van Dongen, J., J., Staal, F. J. (2006): Identification of Notch target genes in uncommitted T-cell progenitors: No direct induction of a T-cell specific gene program; *Leukemia*, Vol. 20, 1967 – 1977

von Gise, A., Pu, W. T. (2012): Endocardial and Epicardial Epithelial to Mesenchymal Transition in Heart Development and Disease; *Circulation Research*, Vol. 110, 1628 - 1645

Wiese, C., Heisig, J., Gessler, M. (2010): Hey bHLH Factors in Cardiovascular Development; *Pediatric Cardiology*, Vol. 31, 363 - 370

Yang, J, Shen, M. H. (2007): Polyethylene Glycol-Mediated Cell Fusion; *Methods in Molecular Biology*, Vol. 325, 59 – 66

Zavadil, J., Cermak, L., Soto-Nieves, N., Böttinger, E. P. (2004): Integration of TGF-β/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition; *The EMBO Journal*, Vol. 23, 1155 – 1165

Zhang, T., Johnson, E. N., Gu, Y., Morisette, M. R., Sah, V. P., Gigena, M. S., Belke, D. D., Dillmann, W. H., Rogers, T. B., Schulman, H., Ross, J. Jr., Brown, J. H. (2001): The Cardiac-specific Nucelar δ_B Isoform of Ca^{2+}/Calmodulin-dependent Protein Kinase II Induces Hypertrophy and Dilated Cardiomyopathy Associated with Increased Protein Phosphatase 2A Activity*; Journal of Biological Chemistry*, Vol. 277, 1261 - 1267

Zhang, T., Maier, L. S., Dalton, N. D., Miyamoto, S., Ross, J. Jr., Bers, D. M., Brown, J. H. (2003): The δ_c Isoform of CaMKII Is Activated in Cardiac Hypertrophy and Induces Dilated Cardiomyopathy and Heart Failure; *Circulation Research*, Vol. 92, 912 - 919

[1] the quotation at the end of the Danksagung is taken from the song "The way you make me feel" written by Bryan Adams

Appendix

real-time qRT-PCR data of embryonic stem (ES) cells stably over-expressing Hey1, Hey1-GFP or Hey2 (cells were established by Dr. Julia Heisig)

	ES-Hey1		ES-Hey1-GFP		ES-Hey2	
gene	**x-fold**	**p-value**	**x-fold**	**p-value**	**x-fold**	**p-value**
ANF	---		---		---	
MLC-1A	0,57	0,341	0,81	0,514	0,28	0,1
MLC-2A	1,27	0,661	1,77	0,352	0,64	0,802
Hey1	176,07	0,000	4096,00	0,000	0,22	0,096
Hey2	2,01	0,511	0,24	0,514	395,26	0,000
ID4	0,97	0,680	0,71	0,343	1,36	0,277
ID2	0,18	0,336	---		0,31	0,095
Tbx2	1,37	0,000	2,06	0,000	1,28	0,526
Smad6	2,07	0,000	1,93	0,000	4,54	0,029
Cited2	2,11	0,000	1,74	0,000	1,97	0,058 *
Nox4	2,18	0,000	1,47	0,667	1,06	0,842
vegfR3	1,70	0,834	0,65	0,343	---	
mflt1	1,78	0,834	1,64	0,000	---	
sflt1	---		0,91	0,837	---	
Mark3	0,90	0,511	0,85	0,325	2,59	0,082
Bmp4	0,78	0,842	0,60	0,325	1,27	0,59
HNRPU	1,22	0,667	0,60	0,662	0,33	0,612
PlxnD1	1,13	0,499	1,33	0,332	2,37	0,031
Sema6D	---		---		---	
flk1	---		---		---	
TiParp	1,35	0,329	0,97	0,679	0,89	0,504
Nrg1	1,89	0,166	1,44	0,679	2,04	0,031

Publications and meetings

Publication

Target Gene Analysis by Microarrays and Chromatin Immunoprecipitation Identifies HEY Proteins as Highly Redundant bHLH Repressors

Julia Heisig, David Weber, **Eva Englberger**, Anja Winkler, Susanne Kneitz, Wing-Kin Sung, Elmar Wolf, Martin Eilers, Chia-Lin Wei, and Manfred Gessler

PLoS Genet 8(5), epub ahead

Meetings

- October 2010: **SFB-Retreat**, Bad Brückenau
- **March 2011:** Joint Meeting of the German and Japanese Societies of Developmental Biologists**, Dresden**
  poster presentation

Eidesstattliche Erklärung

Hiermit erkläre ich, Eva Englberger, dass ich die Dissertation „Gene regulation in hearts of Hey-mutant mouse embryos and monitoring of sub-cellular Hey1 distribution“ selbstständig angefertigt habe und nur die angegebenen Hilfsmittel und Quellen benutzt habe.

Ich erkläre außerdem, dass ich die Dissertation weder in dieser, noch anderer Form bereits zuvor einem anderen Prüfungsausschuss vorgelegt habe.

Weiterhin habe ich nicht versucht, weitere akademische Titel als die angegeben zu erwerben.

Würzburg, den 31.05.2012

Eva Englberger

Danksagung

Allen voran gilt mein Dank meinem Doktorvater Prof. Manfred Gessler, der es mir ermöglicht hat, in seinem Labor zu promovieren, und mir die Möglichkeit gegeben hat, mich weiterzuentwickeln. Vielen Dank dafür.

Als 2. möchte ich Herrn Prof. Ricardo Benavente für seine Rolle als Zweitbetreuer und für seine Hilfe in Rat und Tat danken.

Ich danke allen Mitgliedern des Lehrstuhls Entwicklungsbiochemie, ganz speziell aber meinen Kollegen im „Gessler-Lab“. Besonders danken möchte ich dabei Andreas, Conny und Katharina für die Hilfe bei einigen Mausgewebepräparationen, Sabrina und Melanie für die guten Gespräche, Sabrina auch für die Hilfe beim Anfertigen von Gewebeschnitten und *in situ*-Färbungen und Daniela für unsere Zusammenarbeit am „Kerntransport“-Projekt. Bettina gilt mein Dank für einen hervorragenden Job als Sekretärin – die Wirren der Uni-Bürokratie wurden durch ihre Mithilfe deutlich einfacher. Mein besonderer Dank geht auch an Christina Kober, unsere Tierpflegerin, die mir die „Mausarbeit“ erheblich erleichtert hat. Am Ende möchte ich noch Christian Danke sagen, der nicht nur für uns alle einen Großteil der nervigen Bestellungen übernommen hat und tolle Sommerfeste in seinem wunderschönen Garten veranstaltet hat, sondern der auch immer ein offenes Ohr für mich hatte; danke dir dafür.

Ich danke PD Dr. Svenja Meierjohann, zu der ich jederzeit mit Fragen und der Bitte um Rat gehen konnte und immer wusste, dass sie mir bestmöglich helfen wird, und für die Bereitstellung einiger Reagentien in den Zellkulturversuchen.

Mein Dank geht auch an alle anderen Mitarbeiter des Lehrstuhls Physiologische Chemie I, ganz besonders an die Teilnehmer der „Mittagsrunde in der Küche“ Hannes und Katja – war immer sehr lustig mit euch beiden.

Danke an Katrin Wiese aus der AG Eilers, die mir bei der FACS-Methodik äußert nett mit Rat und Tat und v.a. VIEL Geduld geholfen hat.

Thanks to Shannon for proof reading – and for the good talks in the last months.

Vielen Dank an Sandra, Bernd und Mona Schulte – die besten Nachbarn, die man sich wünschen kann. Und Sandra, trotz deiner Befürchtungen hast du nie eine Blume von mir vertrocknen lassen, wenn ich abwesend war. Daher danke noch mal für's jahrelange Blümchengießen – aber auch dafür, dass du mir in den letzten 4 Jahren als Freundin ans Herz gewachsen bist. Unsere spontanen Gespräche im Hausflur oder im Sommer beim Wäscheaufhängen im Hof waren immer eine willkommene Abwechslung.

Caro Haller danke ich für ihre Freundschaft – grade auch in schweren Zeiten konnte ich mich immer auf sie verlassen – v.a. dass sie mich von der Wissenschaft abgelenkt hat – Gespräche über Klamotten, Schuhe und Männer sowie deine bevorstehende Hochzeit müssen manchmal auch sein, genauso wie gemeinsame Kinobesuche und verquatsche Abende bzw. Nächte.

Danke an Katrin, Kathrin, Susi, Patrick, Philipp, Katja und Ulli – die Freundschaften zu euch waren für mich immer wichtige Ankerpunkte außerhalb der Wissenschaft.

Mein Dank geht auch an Andreas Berning – für die wunderbaren Momente beim Laufen, grade bei unserem 1. Halbmarathon, aber auch für alles andere – du hast immer einen „großer Bruder-Rat" für mich und warst auch nicht abgeneigt, Teile dieser Arbeit Korrektur zu lesen. Ich danke dir für deine ehrliche Freundschaft – und für die Tatsache, dass ich in downtown FFM immer einen kostenlosen Parkplatz bekommen kann.

Danke auch an Otmar – geteiltes Fußballleid mit dem FCB ist eben manchmal auch doppelte Freude mit dem FCA.

Ich glaube, es gibt gar keine wirklich passenden und v.a. ausreichenden Worte, um meinen Dank an meine Eltern gebührend auszudrücken – daher belasse ich es einfach bei einem „Danke für alles“, für eure wunderbare Hilfe und Unterstützung, auf die ich immer zählen kann. Und danke für euer Verständnis und dass ihr mir die Möglichkeit, aber vor allem auch die Freiheit gegeben habt, meinen eigenen Weg zu gehen.

Mein größter Dank gilt Björn, der die letzten beiden Jahre meiner Doktorarbeit im wahrsten Sinne des Wortes „miterlebt“ hat – lieber Björn, als du in mein Leben getreten bist, ging die Sonne auf und die Welt wurde bunt. Und plötzlich war alles so einfach, auch in schwierigen Phasen. Durch deine Liebe, dein Verständnis, dein Vertrauen, deine Unterstützung und deine Geduld hast du mir so viel Stärke gegeben, dass ich wusste, dass ich alles schaffen kann, wenn ich es will. Du hast mir so viele neue Dinge gezeigt: deine Heimat das Rheinland, die „Freuden“ einiger BWL-Vorlesungen am Wochenende, in denen ich Teile dieser Arbeit geschrieben habe (ich werde nie den Rechnungswesen-Dozenten vergessen), die Wies’n, wir haben eine unvergessliche Nacht im Casino Monte Carlo verbracht und nicht zuletzt kann ich dank dir jetzt anhand der Anzahl und Anordnung der Auspuffrohre eines BMWs erkennen, welche Motorisierung er hat…; ich habe aber auch so viel über mich selber gelernt – und du bringst mich immer mit den kleinsten Kleinigkeiten zum Lachen.

Du bist ein ganz wunderbarer Mann, Björn Junglas, ein ganz besonderer Mensch und das tollste Geschenk, das ich jemals bekommen habe. Ich liebe dich… it’s the way you make me feel, it’s better than I’ve ever known it, better than it’s ever been, I can’t seem to control it, no, that’s the way you make me feel, like the sun coming up in the morning, like holdin’ the world in your hands, in a way I could never imagine, that’s the way you make me feel…[1]