Hey target gene regulation in embryonic stem cells and cardiomyocytes

Regulation von Hey Zielgenen in
embryonalen Stammzellen und Kardiomyozyten

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David Weber

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Members of the Promotionskomitee:

Chairperson: Prof. Dr. Utz Fischer

Primary Supervisor: Prof. Dr. Manfred Gessler

Supervisor (Second): Prof. Dr. Martin Eilers

Supervisor (Third): Prof. Dr. Jörg Schultz

Date of Public Defence:

Date of Receipt of Certificates:

Abbreviations

aa	amino acid
bp	base pair
ASD	atrial septum defect
AVC	atrioventricular canal
BSA	bovine serum albumin
cDNA	complementary DNA
ChIP	Chromatin immuno precipitation
ChIPseq	Chromatin immuno precipitation and high throughput sequencing
CM	cardiomyocytes
CO2	carbon dioxid
ct-value	cycle threshold value
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside-triphosphate
dox	doxycycline
EB	embryoid bodies
EDTA	ethylenediaminetetraacetic acid
EMT	epithelial mesenchymal transition
ESC	embryonic stem cells
FBS	fetal bovine serum
GFP	green fluorescent protein
GO	gene ontology
Hdac	Histone deacetylase
IRES	internal ribosome entry site
kb	kilobase
KO	knockout
LIF	leukemia inhibitory factor
mRNA	messenger RNA
NEAA	non-essential amino acids
NICD	Notch intracellular domain
PAGE	poly acrylamide gel electrophoresis
PBS	phosphate buffered saline

PCR	polymerase chain reaction
qRT-PCR	quantitative realtime PCR
RNA	ribonucleic acid
RNAseq	high throughput sequencing of RNA
RPKM	reads per kilo base per million
SDS	sodium dodecyl sulfate
TF	transcription factor
TSS	transcription start site
VSD	ventricular septum defect

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1. Summary

1.1. Summary (English)

The Notch signaling pathway is crucial for mammalian heart development. It controls cell-fate decisions, coordinates patterning processes and regulates proliferation and differentiation. Critical Notch effectors are Hey bHLH transcription factors (TF) that are expressed in atrial (Hey1) and ventricular (Hey2) cardiomyocytes (CM) and in the developing endocardium (Hey1/2/L). The importance of Hey proteins for cardiac development is demonstrated by knockout (KO) mice, which suffer from lethal cardiac defects, such as ventricular septum defects (VSD), valve defects and cardiomyopathy. Despite this clear functional relevance, little is known about Hey downstream targets in the heart and the molecular mechanism by which they are regulated.

Here, I use a cell culture system with inducible *Hey1*, *Hey2* or *HeyL* expression to study Hey target gene regulation in HEK293 cells, in murine embryonic stem cells (ESC) and in ESC derived CM. In HEK293 cells, I could show that genome wide binding sites largely overlap between all three Hey proteins, but HeyL has many additional binding sites that are not bound by Hey1 or Hey2. Shared binding sites are located close to transcription start sites (TSS) where Hey proteins preferentially bind to canonical E-boxes, although more loosely defined modes of binding exist. Additional sites only bound by HeyL are more scattered across the genome. The ability of HeyL to bind these sites depends on the C-terminal part of the protein. Although there are genes which are differently regulated by HeyL, it is unclear whether this regulation results from binding of additional sites by HeyL.

Additionally, Hey target gene regulation was studied in ESC and differentiated CM, which are more relevant for the observed cardiac phenotypes. ESC derived CM contract in culture and are positive for typical cardiac markers by qRT-PCR and staining. According to these markers differentiation is unaffected by prolonged *Hey1* or *Hey2* overexpression. Regulated genes are largely redundant between Hey1 and Hey2. These are mainly other TF involved in e.g. developmental processes, apoptosis, cell migration and cell cycle. Many target genes are cell type specifically regulated causing a shift in Hey repression of genes involved in cell migration in ESC to repression of genes involved in cell cycle in CM.

The number of Hey binding sites is reduced in CM and HEK293 cells compared to ESC, most likely due to more regions of dense chromatin in differentiated cells. Binding sites are enriched at the proximal promoters of down-regulated genes, compared to up-or non-regulated genes. This indicates that up-regulation primarily results from indirect effects, while down-regulation is the direct results of Hey binding to target promoters. The extent of repression generally correlates with the amount of Hey

binding and subsequent recruitment of histone deacetylases (Hdac) to target promoters resulting in histone H3 deacetylation.

However, in CM the repressive effect of Hey binding on a subset of genes can be annulled, likely due to binding of cardiac specific activators like Srf, Nkx2-5 and Gata4. These factors seem not to interfere with Hey binding in CM, but they recruit histone acetylases such as p300 that may counteract Hey mediated histone H3 deacetylation. Such a scenario explains differential regulation of Hey target genes between ESC and CM resulting in gene and cell-type specific regulation.

1.2. Zusammenfassung (Deutsch)

Der Notch Signalweg ist essenziell für die Herzentwicklung in Säugetieren. Er kontrolliert Zell-differenzierung, koordiniert Musterbildungsprozesse und reguliert Proliferation und Differenzierung. Kritische Notch Effektoren sind Hey bHLH Transkriptionsfaktoren, welche im Herzen in atrialen (Hey1) und ventrikulären (Hey2) Kardiomyozyten und dem sich entwickelnden Endokardium (Hey1/2/L) exprimiert werden. Die Bedeutung von Hey Proteinen während der Herzentwicklung wird an Hand von verschiedenen KO Mäusen ersichtlich, welche letale Herzdefekte, wie ventrikuläre Septumdefekte, Herzklappendefekte und Kardiomyopathien, entwickeln. Trotz dieser klaren funktionalen Relevanz ist wenig über Hey Zielgene im Herzen und den molekularen Mechanismus bekannt, über den diese reguliert werden.

Hier wurde ein Zellkultursystem mit induzierbarer Expression von *Hey1*, *Hey2* oder *HeyL* verwendet, um Hey Zielgene in HEK293, murinen embryonalen Stammzellen und in differenzierten Kardiomyozyten zu studieren. In HEK293 Zellen konnte ich zeigen, dass die Bindestellen im Genom weitestgehend zwischen allen drei Hey Proteinen überlappen, HeyL jedoch viele zusätzliche Bindestellen aufweist, welche weder von Hey1 noch Hey2 gebunden werden. Gemeinsame Bindestellen befinden sich nahe Transkriptionsstartstellen, präferentiell an kanonische E-boxen. Die nur von HeyL gebunden Bindestellen sind mehr über das Genom verteilt. Dabei ist die Fähigkeit von HeyL diese Stellen zu binden vom C-terminalen Teil abhängig. Obwohl es Gene gibt, die unterschiedlich von HeyL reguliert werden, ist es auf Grund der sehr viel größeren Anzahl an HeyL Bindestellen unklar, ob diese Regulation das Resultat von zusätzlicher HeyL Bindung ist.

Zusätzlich wurde die Regulation von Hey Zielgenen in embryonalen Stammzellen und differenzierten Kardiomyozyten untersucht, da diese Zellen für die beobachteten kardialen Phänotypen relevanter sind. Differenzierte Kardiomyozyten kontrahieren in Kultur und sind positiv für typische kardiale Marker an Hand von qRT-PCR und Färbungen. Nach diesen Markern ist die Differenzierung durch kontinuierliche Überexpression von *Hey1* oder *Hey2* unverändert. Die Hey1 und Hey2 regulierten Gene sind weitestgehend redundant. Viele Zielgene sind andere Transkriptionsfaktoren, die zum Beispiel an Entwicklungsprozessen, Apoptose, Zellmigration und dem Zellzyklus beteiligt sind. Diese werden oft Zelltyp spezifisch reguliert, was zur Folge hat, dass in embryonalen Stammzellen auch an der Zellmigration beteiligte Gene reprimiert werden, während es in Kardiomyozyten vor allem Gene sind, die den Zellzyklus betreffen.

Die Zahl der Hey Bindestellen ist in Kardiomyozyten und HEK293 Zellen verglichen mit embryonalen Stammzellen reduziert, höchstwahrscheinlich da differenzierte Zellen weniger offenes Chromatin besitzen. Die Bindestellen sind in reprimierten Genen verglichen mit induzierten oder nicht regulierten

Genen angereichert. Dies deutet an, dass eine Induktion meist durch indirekte Effekte zu Stande kommt, während eine Repression das direkte Ergebnis der Hey Bindung an Zielpromotoren ist. Die Stärke der Repression korreliert dabei generell mit der Menge an Promoter gebundenem Hey Protein, welches Histon-Deacetylasen rekrutiert und zu einer Reduktion der Histon H3 Acetylierung führt.

In Kardiomoyzyten wird der repressive Effekt von Hey für bestimmte Gene unterbunden, wahrscheinlich durch Bindung herzspezifischer Aktivatoren, wie Srf, Nkx2-5 und Gata4. Diese Faktoren scheinen nicht die Bindung von Hey zu beeinflussen, aber sie rekrutieren Acetylasen wie p300, welche Hey vermittelter Histon H3 Deacetylierung entgegenwirken. Dieses Model erklärt die unterschiedliche Regulation von Hey Zielgenen zwischen embryonalen Stammzellen und Kardiomyozyten.

2. Introduction

Congenital heart defects affect 1 in 100 newborn children, which represent the main share among all defects seen in human live births (Hoffman and Kaplan, 2002). For several of these heart defects mutations of Notch signaling elements have been shown to be the underlying cause. Notch signaling regulates cell-fate specification, differentiation and patterning processes. It acts locally, specifying individual fates to single cells or a group of cells within a field of similar cells. It is crucial for the development of complex organs, such as the heart, which require the coordinated development of numerous individual elements. Several functional studies in mice have demonstrated the importance of Notch signaling for cardiovascular development (Xue et al., 1999, Krebs et al., 2000, McCright et al., 2001, Krebs et al., 2003, Duarte et al., 2004).

2.1. Hey bHLH transcription factors

Hairy/enhancer-of-split related with YRPW motif (Hey) proteins are integral members of the Notch signaling pathway. There are three mammalian Hey proteins Hey1, Hey2 and HeyL (also known as Hrt1/2/3, Hesr1/2/3, Herp2/1/3 or Chf2/1/3) (Kokubo et al., 1999, Leimeister et al., 1999, Sartorelli et al., 1999, Chin et al., 2000, Nakagawa et al., 2000, Iso et al., 2001a). They are conserved throughout vertebrate development (Winkler et al., 2003) and there is also a single Hey Drosophila homolog (Kokubo et al., 1999, Leimeister et al., 1999). In mammals Hey proteins are expressed in various tissues during embryonic development including heart and blood vessels and *Hey* KO mice show various defects in these tissues.

Hey proteins are bHLH factors with a conserved domain structure and they are closely related to the Hes family of proteins and the Drosophila hairy and Enhancer of split genes. Common features are a basic, a helix-loop-helix (HLH), and an Orange domain that are similar to those of Hes proteins and two conserved C-terminal motifs (figure 1).

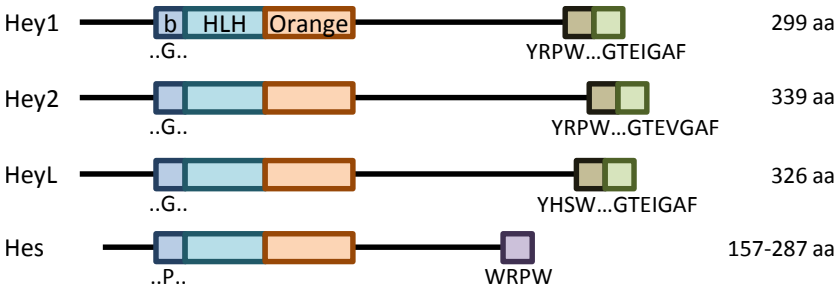


Figure 1: Domain structure of Hey and Hes proteins
b = basic; HLH = helix-loop-helix; aa = amino acid;

The basic domain has a high content of basic amino acids and directly contacts DNA, where it preferentially binds E-box sequences *in vitro* (CACGTG, CACGCG) (Iso et al., 2001b). This is similar to

Hes proteins, despite the exchange of a conserved and functionally relevant proline residue within the basic domain of Hes proteins to glycine in Hey proteins, which might affect DNA binding (Iso et al., 2001b, Popovic et al., 2014). The HLH domain serves as a platform for homo- and heterodimerization with Hey and Hes proteins as well as for other protein interactions (Iso et al., 2002, Leimeister et al., 2000a). These dimers form a scissor like structure (Murre et al., 1989, Shimizu et al., 1997), which binds to the two strands of DNA such that both basic domains are in direct contact with the DNA. Two further α-helices form the Orange domain, which functions as an additional interface for protein interactions. Characteristically for Hey proteins are the two conserved C-terminal YRPW (YXXW for HeyL) and GTE(I/V)GAF peptides. Contrary to the C-terminal WRPW peptide of Hes proteins, YRPW cannot bind TLE corepressors (Paroush et al., 1994, Fisher et al., 1996, Iso et al., 2001b). The function of both C-terminal Hey motifs is currently unknown.

2.2. Induction by Notch signaling and crosstalk with other pathways

Hey and *Hes* genes are the most prominent targets of canonical Notch signaling in vertebrates and they convey a significant part of the Notch signal. They are activated upon juxtacrine binding of Jagged or Delta-like ligands (Jag1, Jag2, Dll1, Dll3 and Dll4) to the extracellular domain of Notch receptors (Notch1-4) (figure 2). This interaction leads to two consecutive proteolytic cleavages releasing the Notch intracellular domain (NICD), which translocates into the nucleus and interacts with the DNA binding protein Rbp-Jκ. It displaces corepressors (e.g. N-CoR, Sharp, CtBP) and allows recruitment of coactivators (e.g. Mastermind/MAML, p300/CBP) converting the Rbp-Jκ complex from a repressor to an activator.

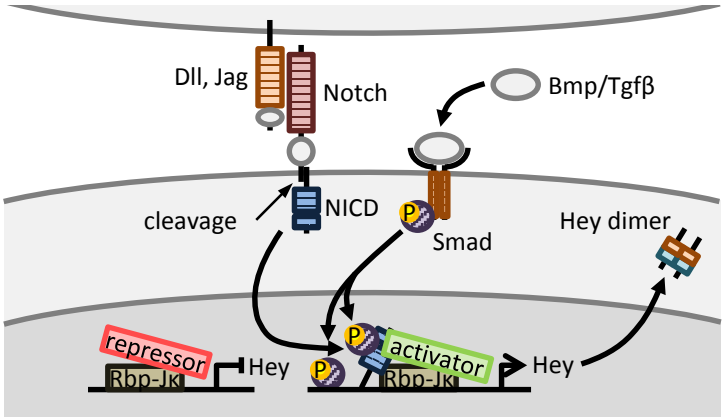


Figure 2: Hey proteins are activated by canonical Notch signalling
Hey proteins are induced by NICD-Rbp-Jκ activator complex. Bmp/Tgfβ can further induce *Hey* expression via Smad proteins and even activate Hey independent of Notch.

Hey genes are differentially expressed in several Notch pathway mutants: While *Notch1* deletion leads to a loss of *HeyL* expression in somites or the endocardium, overexpression of constitutively active *Notch1* (*N1ICD*) in the mesodermal lineage leads to induction of *Hey1* (Leimeister et al., 2000b, Firulli

et al., 2000, Watanabe et al., 2006). However, similar deletion and overexpression experiments with *Notch2* in the heart or *Notch3* in vascular smooth muscle cells did not identify obvious alterations in *Hey* gene expression suggesting a Notch2/3 independent expression in other instances (King et al., 2006, Kokubo et al., 2007). Reporter gene assays and mutation analysis of *Hey* promoters clearly supported the direct mode of activation via Rbp-Jκ (Maier and Gessler, 2000, Iso et al., 2001a, Iso et al., 2002). In these experiments there is little evidence for selectivity of certain Notch receptors towards an individual *Hey* gene.

In addition to activation by Notch, there is crosstalk with other signaling pathways to enhance *Hey* transcription: Jak2-Stat3 and Jak2-Erk1/2 signaling has been implicated in enhancing Notch activity in neuroepithelia and colon carcinoma (Kamakura et al., 2004, Neradugomma et al., 2013). Foxc1 and Foxc2 directly induce *Hey2* in endothelial cells via Foxc binding elements within the *Hey2* promoter and via interaction with NICD (Hayashi and Kume, 2008). In zebrafish the *Hey2* ortholog *gridlock* is induced by Sox7 and Sox18 during arteriovenous differentiation (Pendeville et al., 2008).

Tgfβ/Bmp and Notch signaling synergistically control osteoblast and myogenic differentiation (Dahlqvist et al., 2003, de Jong et al., 2004), sprouting angiogenesis (Itoh et al., 2004, Moya et al., 2012), and induce epithelial mesenchymal transition (EMT) (Zavadil et al., 2004). *Hey* activation by Tgfβ/Bmp seems to occur by two different mechanisms (figure 2): On the one hand induction can be independent of Notch, driven by direct binding of Smad proteins to the promoter (Zavadil et al., 2004, Sharff et al., 2009). On the other hand activation seems to depend on interaction of NICD with Smad1, 3 and 5, although one study was only able to confirm interaction with Smad3 (Blokzijl et al., 2003, Dahlqvist et al., 2003, Itoh et al., 2004, Zavadil et al., 2004).

CoupTF-II (also known as Nr2f2) is another important regulator of *Hey* expression in endothelia cells as well as in CM. CoupTF-II homodimers directly repress *Hey1* and *Hey2* in venous endothelia cells (Diez et al., 2007, Aranguren et al., 2013b, Korten et al., 2013). However, in lymphatic endothelia cells CoupTF-II forms heterodimers with Prox1, which lack this repressive capacity resulting in expression of *Hey1* and *Hey2* (Yoo et al., 2012, Korten et al., 2013). In CM CoupTF-II is also an important *Hey* regulator, it represses *Hey2* in atrial CM, but induces *Hey1* (Wu et al., 2013). The strong arterial expression of Hey proteins suggests that they are part of the hypoxia response. Hypoxia sensing Hif1 has been shown to induce *Hey* expression directly (Diez et al., 2007) and via induction of Vegf, Dll4 and Jag2 (Patel et al., 2005, Williams et al., 2006, Pietras et al., 2011).

In conclusion, while Hey proteins are classical downstream acting factors of canonical Notch signaling, multiple other signaling pathways control their expression, leading to a cell type specific expression in various tissues.

2.3. Interaction partners

Hey proteins form homo- and heterodimers via the HLH domain with other Hey and Hes proteins (Leimeister et al., 2000a, Iso et al., 2001b, Ross et al., 2006, Fischer et al., 2007). Via the HLH domain Hey proteins can also interact with numerous other TF (Fischer and Gessler, 2007).

Hey1 has been shown to directly interact with N-CoR and Sin3a *in vitro* (Iso et al., 2001b, Gould et al., 2009). Both proteins are part of the Sin3 corepressor complex, which recruits histone deacetylases (Hdac). Another study found also a direct interaction between Hey2 and Sirt1, which is another histone deacetylase, via the bHLH domain (Takata and Ishikawa, 2003). Further evidence comes from studies, which show that TSA treatment partially blocks Hey repression as it inhibits Hdacs but does not affect Sirt1 (Nakagawa et al., 2000, Takata and Ishikawa, 2003). These results indicate that Hey proteins might repress direct target genes via recruitment of histone deacetylases to target promoters.

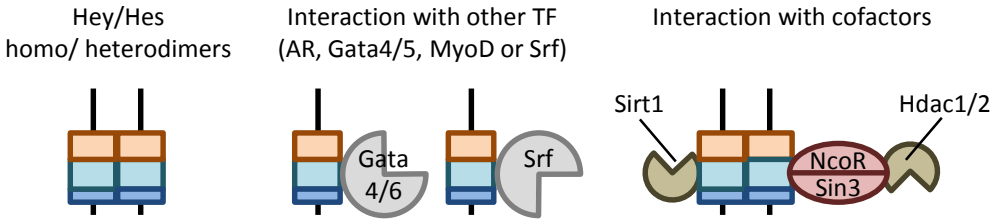


Figure 3: Hey interaction partner
Hey and Hes proteins form homo- and heterodimers, they interact with other TF such as e.g. androgen receptor (AR), Gata4/6, MyoD or Srf and recruit histone deacetylases (Hdac1/2, Sirt1).

All three Hey proteins were shown to interact with the androgen receptor and its coactivator Src1 and to repress transcription from androgen dependent promoters (e.g. *Dat1* promoter) (Kanno and Ishiura, 2012), possible via recruitment of trichostatin insensitive deacetylases like Sirt1 (Belandia et al., 2005, Lavery et al., 2011). Hey proteins also directly interact with Gata TF, this direct interaction prevents transcriptional activation of e.g. *Anf* promoter by Gata4/6 (Kathiriya et al., 2004, Fischer et al., 2005) and *Myh11* promoter by Gata6 (Shirvani et al., 2006). Hey1 also interacts with MyoD, thereby preventing MyoD target gene activation and inhibiting normal muscle cell differentiation (Sun et al., 2001). Another Hey interacting transcription factor is Srf: Hey1 binding interferes with Srf-CArG box binding, preventing Srf dependent target gene activation (Doi et al., 2005).

The above described interactions indicate that Hey proteins might affect gene expression not only directly by E-box binding but also indirectly by affecting other TF. In both cases regulation might be mediated by recruitment of histone deacetylases. These not only act on histones but also on other proteins including MyoD, Gata factors, and the androgen receptor, which require acetylation to reach their full activating potential (Mal et al., 2001, Ozawa et al., 2001, Fu et al., 2003, Hayakawa et al., 2004).

2.4. Known target genes

Early data for Hey regulated genes comes from individual target gene approaches. In the cardiovascular system, Hey proteins were shown to directly repress e.g. *Gata4/6* and *Tbx2* (Fischer et al., 2005, Kokubo et al., 2007). During smooth muscle cell differentiation Hey1 and Hey2 were shown to repress *Smooth muscle α-actin* (Tang et al., 2008) and several papers describe Hey repression of *Nfatc1* and *Runx2*, thereby inhibiting osteoblast differentiation (Jing et al., 2010, Salie et al., 2010, Tu et al., 2012).

There have been few genome and transcriptome wide screens to identify Hey target genes: A first screen was performed in endothelia cells overexpressing *Hey2* to see whether an arterial fate could be induced (Chi et al., 2003). Hey2 was found to activate expression of arterial specific genes, including *Adha1*, *Eva1*, and *keratin7*, while repressing *myosin I*, suggesting that Hey2 turns on features of artery specific gene expression program. However, a more recent publication found that *Hey2* overexpression is not sufficient to induce arterial cell fate (Aranguren et al., 2013a).

For another screen *Hey2* KO and wild-type mouse aortic smooth muscle cells were treated with platelet derived growth factor (PDGF), which induced proliferation and migration in wild-type but not in KO cells. Target genes were identified at different time points after PDGF induction and yielded a surprisingly large number of 9 827 transcripts with altered expression (Shirvani et al., 2007). Many identified genes have roles in vascular smooth muscle function, e. g. hyaluronan synthase 1, which was only up-regulated after PDGF treatment in *Hey2* KO cells, indicating repression by endogenous Hey2 (Shirvani et al., 2007). However, the large number of identified genes makes it difficult to identify meaningful groups.

Another screen used primary neonatal mouse CM from wild type and transgenic mice overexpressing *Hey2*, which are protected from phenylephrine induced hypertrophy. They found genes involved in water transport, adenylate cyclase activity, embryonic eye morphogenesis, gut development and fluid transport to be repressed by Hey2 (Yu et al., 2010).

2.5. Hey factors in development

The wide-ranging expression of *Hey* genes, controlled by Notch signaling, crosstalk with other signaling pathways, and a wide variety of downstream targets result in multiple functions of Hey proteins in development and tissues homeostasis.

The importance of Hey proteins for cardiovascular development was demonstrated by various *Hey* KO mice. While there are no developmental defects in *Hey1* and *HeyL* KO mice (Fischer et al., 2004b, Fischer et al., 2007), the *Hey2* KO is lethal within the first ten days after birth due to VSD and cardiac valve defects (Gessler et al., 2002, Donovan et al., 2002, Sakata et al., 2002, Kokubo et al., 2004).

Combined loss of *Hey1* and *HeyL* leads to similar deficiencies with membranous VSD and dysplastic atrioventricular and pulmonary valves (Fischer et al., 2007). The combined loss of *Hey1* and *Hey2* is embryonic lethal at around E9.5/E10.5 due to global lack of vascular remodeling and hemorrhaging and even more severe cardiac defects (Fischer et al., 2004b, Kokubo et al., 2005).

2.5.1. Hey factors in cardiac development

The heart is the first functional organ to develop during vertebrate embryogenesis and development starts in mice around embryonic day E7.5. A subset of mesodermal cells migrates antero-laterally to form the cardiac crescent and fuse into a single heart tube. Cardiogenic progenitors already express the earliest cardiac marker *Nkx2-5*, which induces further cardiac genes e.g. *Bmp2* and *Tbx2/3* that drive differentiation. Mesodermal cells differentiate into the endocardium lining heart chambers and valves and the myocardium forming the musculature of ventricles and atria. At E9.5, a third cardiac tissue, the epicardium develops from cells arising from mesodermal cells posterior to the inflow region, which migrate and cover the entire myocardial surface. At the same time the heart tube undergoes characteristic looping and the typical heart structure is established. Atrial and ventricular parts further expand by proliferation and recruitment of mesenchymal cells.

Hey1 is expressed in myocardial cells of the atrium, the developing inflow and outflow tract, whereas *Hey2* is expressed in myocardial cells of the ventricle, the outflow tract and the cushion mesenchyme of the outflow tract (figure 4) (Leimeister et al., 1999, Nakagawa et al., 1999, Donovan et al., 2002). Myocardial expression of *Hey1* and *Hey2* plays a role in establishing the atrioventricular canal (AVC) boundary by repressing *Tbx2* and *Bmp2* (Kokubo et al., 2007, Luna-Zurita et al., 2010).

At the same time the atrial and ventricular septa are formed, which consist of a muscular part and membranous parts that originate from endocardial cells of the AVC. All three Hey proteins are expressed in these endocardial cells (figure 4) (Fischer et al., 2007). These cells undergo EMT and mesenchymal cells subsequently proliferate and differentiate to develop the membranous septum as well as valvular leaflets. This process is impaired in *Hey1/L* and *Hey2* KO mice as demonstrated by atrioventricular explants that exhibited strongly reduced EMT (Fischer et al., 2007). In line with this, Notch1 induced myocardial expression of *Hey1* in the AVC leads to *Bmp2* repression, which subsequently also impairs endocardial EMT (Luna-Zurita et al., 2010). Due to these EMT defects, *Hey1/L* and *Hey2* KO mice at later stages develop a membranous VSD with overriding aorta and also atresia or abnormalities of the tricuspid valve and atrial septal defects (ASD) were observed (Donovan et al., 2002, Kokubo et al., 2004, Fischer et al., 2004a).

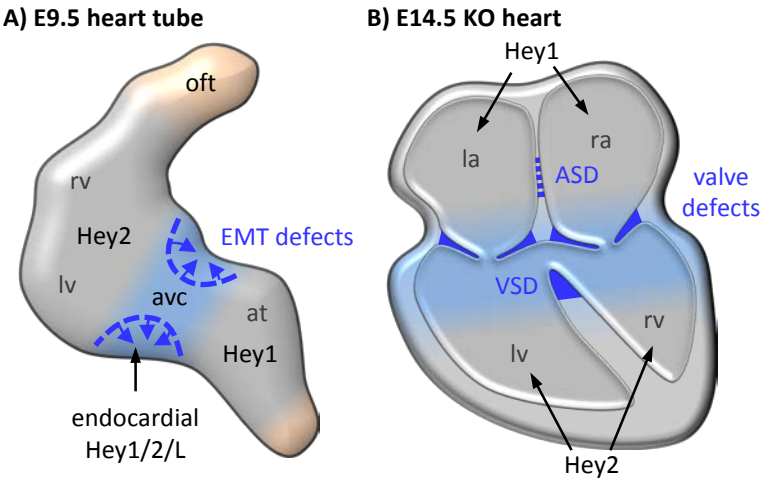


Figure 4: *Hey* expression in the developing heart and defects in KO mice
(A) At embryonic day E9.5 *Hey1* is expressed in CM of the developing atrium (at), while *Hey2* is expressed in CM of the future right and left ventricle (rv, lv). All three Hey proteins are expressed in the endocardium. *Hey2* and *Hey1/L* KO mice exhibit EMT defects in the endocardium of the AVC. Oft = outflow tract
(B) At embryonic day E14.5 *Hey1* and *Hey2* are expressed in CM of the left and right atrium (la, ra) and ventricles (lv, rv), respectively. *Hey2* KO mice have ventricular septum defects (VSD), valve defects and atrial septum defects (ASD). These are the result of the earlier EMT defect, as these structures develop from mesenchymal cells, which have undergone EMT.

The ventricles further mature through trabeculation and compaction of CM. Ventricular myocardial *Hey2* expression is important for suppressing atrial gene expression in the left ventricular myocardium, thereby allowing proper maturation and compaction (Koibuchi and Chin, 2007, Xin et al., 2007). In the atria, *Hey2* expression is suppressed by the transcription factor CoupTF-II (Wu et al., 2013). Consistently, CM specific *CoupTF-II* KO mice developed ventricularized atria. *Hey2* KO mice show an increase in heart size with enlarged ventricles and an abnormal pear-like shape (Gessler et al., 2002, Donovan et al., 2002, Sakata et al., 2002, Kokubo et al., 2004). Myocardial *Hey2* overexpression in *Hey2* KO mice could rescue myocardial and also septation defects, but not aortic and valve defects suggesting a combinatorial effect of Hey2 in different cell types (Sakata et al., 2006).

2.5.2. Hey factors in adult cardiac disease

In a mixed genetic mouse background *Hey2* KO mice reach adulthood and develop heart failures with cardiac hypertrophy being a possible reason (Sakata et al., 2006). Also heterozygous deletion of *Hey2* enhanced cardiac hypertrophy and progression to heart failure with increased apoptosis and elevated *Gata4* levels (Liu et al., 2010). Further studies implicated abnormalities in calcium cycling mediated by ryanodine receptor binding protein (Fkbp12.6), which was induced in myocardial specific *Hey2* KO mice after aortic banding (Liu et al., 2012). *Hey2* overexpression, on the other hand, is protective against phenylephrine-induced hypertrophy, possibly via suppression of hypertrophic genes like *Anf*, *Bnp*, and *Gata4* (Xiang et al., 2006). In a mouse model for pressure overload hypertrophy, *Hey2* overexpression

promoted physiological over pathological hypertrophy (Yu et al., 2009). Here, the authors implicated suppression of apoptosis and regulation of multiple transcriptional pathways.

Recently, a genome-wide association study described genetic polymorphisms in the *HEY2* gene as a regulator of cardiac electrical function involved in Brugada syndrome (Bezzina et al., 2013). In *Hey2* KO mice the expression of the Brugada syndrome associated cardiac sodium channel *Nav1.5* in the compact ventricular layer was higher compared to wild-type hearts and conduction velocity in the right ventricular outflow tract was increased. This suggests an additional regulatory role for Hey2 in repolarizing currents.

2.5.3. Hey factors in vascular development

During embryonic development new blood vessels are formed by two separate processes: (1) During vasculogenesis mesodermal progenitor cells differentiate into embryonic blood cells and endothelial cells, which aggregate into blood islands and build the primary vascular plexus (Flamme et al., 1997). The primary vascular plexus then forms the extra-embryonic yolk sac vasculature and intra-embryonic aorta and cardinal vein. (2) The complex vascular network is formed by angiogenesis, when endothelial cells become motile and form new capillaries by sprouting from pre-existing vessels (Risau, 1997).

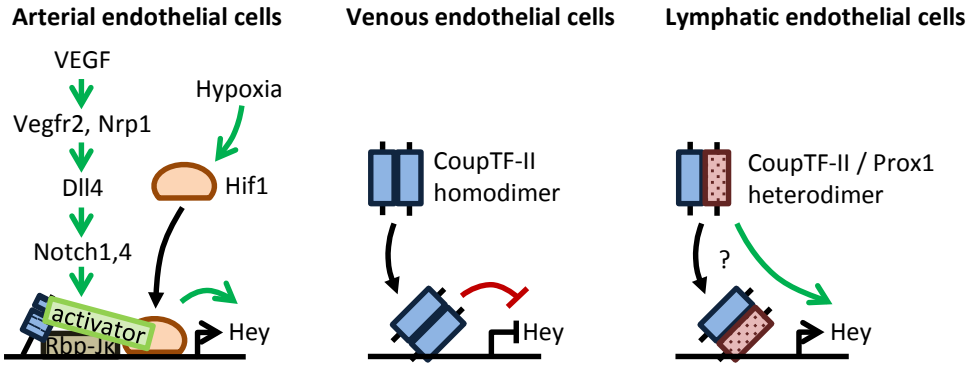


Figure 5: Regulation of *Hey* expression in endothelial cells
In arterial endothelial cells *Hey* is directly induced by hypoxia sensing Hif1 and by VEGF stimulation via the Delta-Notch pathway. In venous endothelial cells *Hey* transcription is repressed by CoupTF-II homodimers. In lymphatic endothelial cells repression by CoupTF-II is abolished by Prox1, which forms heterodimers with CoupTF-II and blocks its repressive capacity towards *Hey* genes.

Through extensive remodeling, arterial and venous blood vessels are formed as well as lymphatic vessels that arise from developing veins. All three vessel types are morphologically and functionally different. Hey proteins are confined to arteries through expression of the Hey antagonist CoupTF-II in veins (figure 5) (Nakagawa et al., 1999, Leimeister et al., 2000b). CoupTF-II was found to bind directly to the *Hey1* and *Hey2* promoter and overexpression in human arterial endothelial cells led to down-regulation of *Hey2* and other arterial markers (Aranguren et al., 2013b, Korten et al., 2013). However, repression of *CoupTF-II* by Hey remains controversial. On the one hand hypoxia-induced *Hey1* and *Hey2*

expression in embryonic endothelial cells was shown to repress *CoupTF-II* and to induce an arterial cell fate (Diez et al., 2007), on the other hand *Hey2* knockdown in human arterial or overexpression in venous endothelial cells did not influence *CoupTF-II* expression (Korten et al., 2013).

In zebrafish the *Hey2* ortholog *gridlock* is necessary for proliferation of vascular progenitor cells (Zhong et al., 2000, Chun et al., 2011). Diminished *gridlock* expression leads to arterial reduction and expansion of the vein region (Zhong et al., 2001). In the mouse neither *Hey1* nor *Hey2* are essential for embryonic vessel development on their own. However, *Hey1*/*Hey2* DKO mice die at embryonic day E9.5 due to global lack of vascular remodeling and hemorrhaging (Fischer et al., 2004b, Kokubo et al., 2005). Arterial endothelial markers like *EphB2* and smooth muscle cells markers like *smooth muscle actin* and *Sm22* are reduced indicating that Hey1 and Hey2 activity is required for arterial specification. However, despite the importance of Hey proteins for arterial differentiation, Hey2 was only able to partially restore arterial gene expression in cell culture experiments (Aranguren et al., 2013a). Only in combination with seven other TF it was possible to convert human venous endothelial cells robustly into arterial endothelial cells. In line with these findings, *Hey1* and *Hey2* expression in human umbilical vein endothelial cells has been shown to suppress the venous endothelial marker *EphB4*, but was unable to induce arterial *EphB2*. This suggests that Hey proteins repress venous rather than induce arterial fate (Iso et al., 2006).

2.5.4. Hey factors in other developmental processes

Apart from these cardiovascular functions, Hey proteins are also involved in myogenesis, bone formation and neuronal development (figure 6). *Hey1*/*HeyL* DKO myoblasts generate fewer quiescent satellite cells during development and these gradually decrease with age leading to severe impairment of adult muscle homeostasis (Fukada et al., 2011). Several studies provide evidence that all three Hey proteins, as well as Hes1, are at least partly responsible for controlling osteoblast differentiation. Mice deficient in *Hey1* and *HeyL*, exhibited high bone mass, due to higher numbers of osteoblasts (Tu et al., 2012). Contrary, transgenic *Hey1* overexpression leads to progressive osteopenia in adult mice (Salie et al., 2010). *Hey1* and *Hey2* misexpression by electroporation in the mouse brain at embryonic day E13.5 leads to an increase of neural precursor cells, however, at later stages it inhibits neurogenesis and promotes generation of astroglia (Sakamoto et al., 2003).

The main function of Hey proteins during these specification processes seems to be the maintenance of tissue specific progenitor cells and preventing exceeding differentiation.

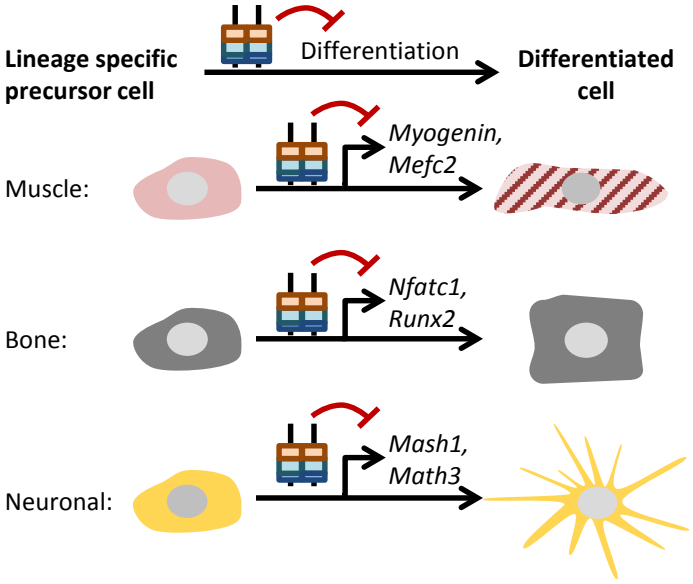


Figure 6: Hey proteins control lineage specific differentiation processes
Hey proteins repress target genes that drive differentiation within certain lineages and thereby affect myogenic, osteogenic, and neuronal differentiation.

2.6. Aim of the Thesis

The loss of either *Hey2* or *Hey1*/*HeyL* leads to a severe cardiovascular phenotype and early embryonic lethality *in vivo*. However, only few relevant Hey target genes are known and little is known about their regulation. The goal of my thesis was to identify new target genes involved in cardiovascular development and the regulatory mechanisms. Therefore, HEK293 cells, murine ESC and *in vitro* differentiated CM with inducible *Hey1*, *Hey2* or *HeyL* (only in HEK293 cells) expression were used. Gene expression was analyzed by microarray or RNAseq and DNA binding sites were identified by ChIPseq. Public available data for TF binding sites and histone modifications was used to investigate the regulatory mechanisms involved in Hey target gene regulation.

3. Materials

3.1. Equipment

Table 1: Equipment used

Berthold Tristar multimode reader	Berthold, Germany
Bioruptor Standard sonifier	Diagenode, USA
Illumina GAIIx Sequencer	Illumina, USA
Leica Fluoreszenz Mikroskop DMI 6000B	Leica, Germany
Mastercycler ep realplex	Eppendorf, Germany
NanoDrop ND-1000 Spektrophotometer	PeqLab, USA
Nikon Eclipse TS100 with Intenslight C-HGFI	Nikon, Japan

3.2. Disposables and chemicals

All used disposables, were either purchased from Sarstedt (Germany) or Eppendorf (Germany). Chemicals that are not listed separately were either purchases from ROTH (Germany), Sigma-Aldrich (Germany) or AppliChem (Germany). All enzymes used for cloning are either derived from Fermentas (Lithuania) or NEB (USA). Oligonucleotides listed in supplementary tables 13 – 18 were purchased from Sigma-Aldrich (Germany).

Table 2: Chemicals used

Beta-mercaptoethanol	PAN, Germany
CHIR99021	Axon, Netherlands
DMEM	Sigma-Aldrich, Germany
Fetal calf serum (FCS, lot was tested for efficient CM differentiation)	Sigma-Aldrich, Germany
His-taq polymerase	self-made
Leukaemia inhibitory factor (LIF)	self-made
MEM non-essential amino acids 100X	Sigma-Aldrich, Germany
Metafecten Pro	Biotex, Germany
PD0325901	Axon, Netherlands
Penicillin/Streptomycin 100X	PAA, Germany
peqGOLD TriFast	PeqLab, Germany
Polyethylenimin (PEI)	Eurogentec, Belgium
Roche Complete	Roche, Switzerland
Sera-Mag magnetic oligos	Thermo Scientific, USA
SybrGreen	Ambrex, USA

3.3. Buffers

Table 3: Used buffers

Standard buffers	
PBS	150 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.8 mM KH_2PO_4
TE	10 mM Tris pH 8.0, 1 mM EDTA
DNA work	
10X DNA loading dye	50 % glycerine, 15 % ficoll, 10 mM EDTA pH 8.0
10X PCR buffer	200 mM Tris pH 8.8, 100 mM ammonium sulphate, 100 mM KCl, 20 mM magnesium sulphate, 1 % TritonX-100, 1 % BSA-acetylated
20X SB	200 mM NaOH pH 8.0 with boric acid
50X TAE	50 mM EDTA, 2 M Tris pH 8.0 with acetate
Protein work	
RIPA buffer	50 mM Tris pH 8.0, 1 % Nonidet P40, 0.5 % Sodium deoxycholate, 0.1 % SDS, 150 mM NaCl, 1X Roche Complete protease inhibitor, 50 µg/ml PMSF
2X Protein loading buffer	100 mM Tris pH 6.8, 4 % SDS, 0.25 % Bromophenol blue, 25 % glycerine, 200 mM DTT
Detection buffer	100 mM Tris pH 8.0, 250 mM luminol, 90 mM coumaric acid, 0.01 % H_2O_2
SDS running buffer	25 mM Tris pH 8.3, 192 mM Glycine, 1 % SDS
Blotting buffer	25 mM Tris pH 8.3, 150 mM Glycine, 10 % Methanol
Luciferase assay	
Assay buffer	25 mM glycyl-glycine pH 7.8, 15 mM magnesium sulphate, 15 mM potassium phosphate, 4 mM EGTA, 1 mM DTT, 1 mM ATP and 1 µg/µl Luciferin
Cell lysis buffer	25 mM glycyl-glycine pH 7.8, 15 mM magnesium sulphate, 15 mM potassium phosphate, 4 mM EGTA, 1 mM DTT and 2 % TritonX-100
EMSA	
Binding buffer	20 mM Tris pH 7.6, 100 mM KCl, 0.5 mM EDTA, 0.1 % Nonidet P-40, 1 mM $MgCl_2$, 1 mM DTT, 10 % glycerol
ChIP buffer	
ChIP dilution buffer	0.01 % SDS, 1.1 % TritonX-100, 1.1 mM EDTA, 20 mM Tris pH 8.0, 167 mM NaCl, 1X Roche Complete protease inhibitor, 50 µg/ml PMSF

Elution buffer	50 mM Tris pH 8.0, 10 mM EDTA, 1 % SDS
High salt wash buffer	500 mM NaCl, 2 mM EDTA, 10 mM Tris-HCl pH 8.0, 1 % TritonX-100, 0.1 % SDS, 1X Roche Complete protease inhibitor, 50 µg/ml PMSF
LiCl wash buffer	250 mM LiCl, 1 mM EDTA, 10 mM Tris-HCl pH 8.0, 0.5 % Nonidet P-40, 0.5 % SDS, 1X Roche Complete protease inhibitor, 50 µg/ml PMSF
Low salt wash buffer	150 mM NaCl, 2 mM EDTA, 10 mM Tris-HCl pH 8.0, 1 % TritonX-100, 0.1 % SDS, 1X Roche Complete protease inhibitor, 50 µg/ml PMSF
Lysis buffer	50 mM Hepes-KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 % TritonX-100, 0.1 % Deoxycholate, 0.1 % SDS, 1X Roche Complete protease inhibitor, 50 µg/ml PMSF
Nuclei lysis buffer	50 mM Hepes-KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 % TritonX-100, 0.1 % Deoxycholate, 1 % SDS, 1X Roche Complete protease inhibitor, 50 µg/ml PMSF

3.4. Kits

Table 4: Used kits

Cycle pure kit	Omega Bio-Tek, USA
Experion DNA 1K analysis kit	Bio-Rad, USA
Experion RNA HighSense analysis kit	Bio-Rad, USA
Gel extraction kit	Omega Bio-Tek, USA
Gel extraction kit	Qiagen, Germany
NEBnext DNA library prep master mix set for Illumina	NEB, USA
NEBnext mRNA library prep master mix set for Illumina	NEB, USA
NEBnext multiplex oligosfor Illumina (Index Primer Set 1)	NEB, USA
PCR purification kit	Qiagen, Germany
PIERCE LightShift Chemiluminescent EMSA kit	PIERCE, USA
Plasmid midi kit	Omega Bio-Tek, USA
Plasmid mini kit	Omega Bio-Tek, USA
RNeasy kit	Qiagen, Germany

3.5. Antibodies

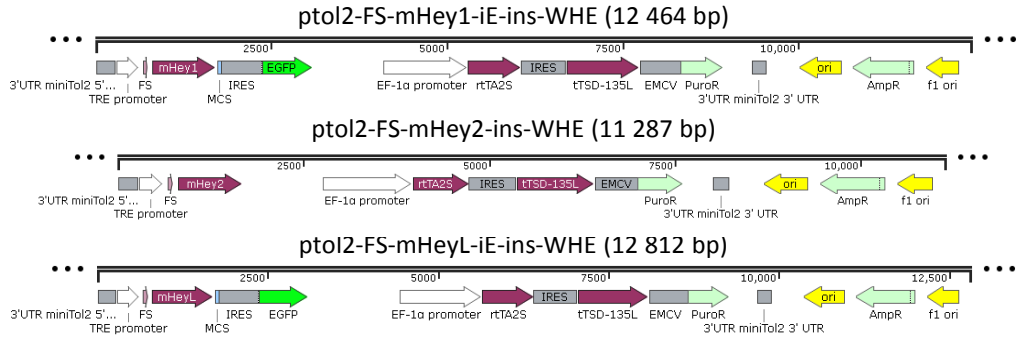
Table 5: Used antibodies

α-Cardiac Troponin I (TI-1)	Mouse		Developmental Studies Hybridoma Bank, USA
α-Flag-M2	Mouse	H9658	Sigma-Aldrich, Germany
α-H3ac	Rabbit	06-599	Upstate, USA
α-H3k27ac	Rabbit	39133	Active Motive, USA
α-H3k27me3	Mouse	Ab6002	Abcam, UK
α-H3K4me3	Rabbit	Ab8580	Abcam, UK
α-HA	Mouse	F3165	Sigma-Aldrich, Germany
α-Hdac2	Mouse	Ab51832	Abcam, UK
α-IgG	Rabbit	I5006	Sigma-Aldrich, Germany
α-Mouse-Alexa568	Goat	A11031	Molecular Probes, USA
α-Mouse-POD	Goat	AP124P	Chemicon International, USA
α-Myosin (MF20)	Mouse		Developmental Studies Hybridoma Bank, USA
α-Tubulin	Mouse	T6074	Sigma-Aldrich, Germany

3.6. Plasmids

To generate Hey inducible cell lines, expression constructs with minitol flanking sites or viral expression constructs were used (figure 7).

A) minitol expression constructs



B) Viral expression constructs

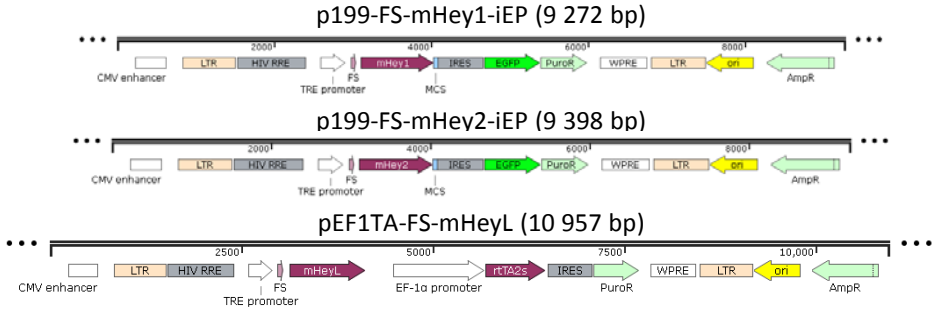


Figure 7: Doxycycline inducible Hey expression constructs
FS = Flag-Strep-tag;

For constitutive expression of Flag or HA tagged *Hey1*, *Hey2* or *HeyL* proteins under the control of the CMV promoter several pCS2p expression plasmids were used (Rupp et al., 1994): pCS2p-Flag-mHey1, pCS2p-Flag-mHey2, pCS2p-Flag-mHeyL, pCS2p-HA-mHey1, pCS2p-HA-mHey2 and pCS2p-HA-mHeyL (Fischer et al., 2005).

Two constructs pCS2p-Flag-mHeyL-1 and pCS2p-Flag-mHey1-L expressing either the C-terminal part of *Hey1* fused to the N-terminal part of *HeyL* or vice versa were generated. In both cases the junction between both protein parts is directly before or after the conserved region containing bHLH and Orange domain, which in both cases was derived from *HeyL* (see figure 8).

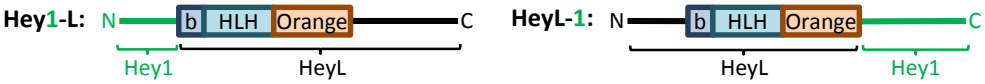


Figure 8: Hey1 and HeyL chimeric proteins
Both chimeric proteins Hey1-L and HeyL-1 contain the basic (b), helix-loop-helix (HLH) and Orange domains from HeyL. The adjacent N- or C-terminal regions are replaced with the matching parts from Hey1.

Additionally, a previously generated non-binding Hey1 mutant (pCS2p-Flag-mHey1-RK3) with three point mutations within the basic domain converting three arginine residues to lysine was used (Heisig et al., 2012). The same point mutations were introduced into the chimeric Hey1-L protein by PCR mediated mutation (pCS2p-Flag-mHey1-L-RK3). An activating Hey1 variant was generated by fusing the vp16 activator domain with the bHLH and Orange domains from Hey1 (pCS2p-vp16-mHey1-bHO).

For luciferase assay the following constructs containing the firefly luciferase gene under control of a promoter of interest were used: pluc-Hey1 (-2839/+87), pGL4.1-JAG1 (-573/0), pLuc-NEUROG3 (-1700/0), and pGL3-BMPR1A (-519/0). The Hey1 luciferase construct was previously generated by M. Maier (Maier and Gessler, 2000). The *JAG1* luciferase promoter construct was obtained from H. Kovar (Ban et al., 2008), the *NEUROG3* construct from M. S. German (Lee et al., 2001), and the *BMPR1A* construct from J. Howe (Calva-Cerqueira et al., 2010).

3.7. Cell lines

HEK293T cells containing the SV40 large T-antigen were used for transient transfection. For Hey overexpression experiments the previous by lentiviral transfection generated HEK293 cell lines HEK293-pWHE134-p199-FS-mHey1-iEP and -mHey2-iEP were used (Heisig et al., 2012). These express Hey proteins with Flag-Strep-tag under control of a doxycycline inducible promoter. For HeyL the inducible cell line HEK293-pTol2-FS-mHeyL-iEins-WHE generated by tol2-mediated transposition with pKate-N/Tol2 was used (Balciunas et al., 2006). These cell lines are called HEK293-Hey1, -Hey2 and -HeyL further down. Additionally a second doxycycline inducible HeyL cell line HEK293-EF1TA-FS-mHeyL was generated by lentiviral transfection.

Previously the murine embryonic stem cell line CM7/1 containing a neomycin resistance under the CM specific *myosin heavy chain* promoter (MHCneo) was used to generate ESC with inducible Hey expression (Zandstra et al., 2003). Two doxycycline inducible cell lines were generated by transfection with Metafecten Pro and tol2-mediated transposition: CM7/1-pTol2-FSmHey1-iE-ins-WHE and CM7/1-pTol2-FSmHey2-ins-WHE (Heisig, 2011). These are called ES-Hey1 and ES-Hey2 further down.

3.8. Mouse lines

Hey1, *Hey2* and *HeyL* KO mouse lines have been described before (Fischer and Gessler, 2007, Fischer et al., 2004b). The Act-*Hey1* transgenic line expressing *Hey1* under the control of the beta-actin promoter was obtained from M. Susa (Salie et al., 2010).

3.9. Data

Microarray expression and ChIPseq binding data for *Hey1* or *Hey2* overexpressing HEK293 cells and murine ESC was previously generated by Julia Heisig (Heisig et al., 2012, Heisig, 2011).

The HEK293T POLII ChIPseq data with the accession number GSE11892 was obtained from NCBI (Sultan et al., 2008). The HEK293 H3K4me3 and CTCF ChIPseq data was obtained from the ENCODE project (ENCODE, 2011). The Srf, Nkx2-5 and Gata4 binding data in HL1 cells was derived from He et al. (2011). ESC and heart H3K4me3 and H3K27ac ChIPseq data was also derived from the ENCODE project (ENCODE, 2011).

4. Methods

4.1. Cloning

All cloning was done using standard molecular methods. Cloned constructs were always verified by Sanger sequencing.

The non-binding HeyL mutant was generated with overlap extension PCR (Higuchi et al., 1988). Therefore, two complimentary primers containing the desired mutations were used in combination with a matching forward or reverse primer to amplify two separate PCR products, which overlap at the mutation. These PCR products were purified, mixed in stoichiometric ratio 1:1 and amplified in a 15 cycle PCR reaction without any primers. After that end primers were added and the sample was amplified for another 20 cycles. The final product was then size selected using gel electrophoresis and cloned in a pCS2p expression vector.

A mutated version of the *JAG1* construct lacking a Hey bound E-box binding motif within the promoter region was generated by cutting with MluI, removing the 5' overhangs with Mung Bean nuclease, and religation of the construct, thus removing 4 nucleotides within a Hey bound E-box motif.

4.2. Cell culture

HEK293 and HEK293T cells were cultured at 37 °C and 5 % CO_2 in DMEM medium containing 10 % FCS and penicillin/streptomycin (100 U/ml / 100 µg/ml).

CM7/1 murine ESC were grown at 37 °C and 5 % CO_2 in DMEM supplemented with 15 % FCS, glutamine (4 mM), MEM non-essential amino acids (1 %), beta-mercaptoethanol (100 mM), penicillin/streptomycin (100 U/ml / 100 µg/ml), leukaemia inhibitory factor (LIF, 1000 U/ml), glycogen synthase kinase 3 (GSK3) inhibitor CHIR99021 (3 µM), and the mitogen-activated protein kinase (MEK) inhibitor PD0325901 (1 µM) (Ying et al., 2008) on porcine skin gelatine (0.2 %) coated cell culture dishes.

For differentiation in embryoid bodies (EB), 10^6 cells were seeded in 10 ml DMEM supplemented with 10 % FCS, glutamine (4 mM), MEM non-essential amino acids (1 %), beta-mercaptoethanol (100 mM), and penicillin/streptomycin (100 U/ml / 100 µg/ml). Cells were kept on agitating petri-dishes (50 rpm) at 37 °C and 5 % CO_2. At day 3, 5, 7 and 9 the medium was changed. Therefore, EB were sedimented in 50 ml falcon tubes, old medium was removed, and after addition of new medium the suspension was transferred back on the original petri dishes. Starting from day 10 G418 (0.4 mg/ml) was added to the differentiation medium to select for CM (*myosin heavy chain* driven neomycin resistance, MHCneo). The medium was changed every day for 5 more days, in order to remove dead cells. At day

15 EB were washed twice with PBS and incubated for up to 15 min in 5 ml Trypsin (0.25 % in PBS) until they were completely dissociated into single cells. $5*10^7$ cells were than plated on porcine skin gelatine (0.2 %) coated 10 cm cell culture dishes.

4.3. Transfection

10^5 cells were seeded on 24-well-dish (5 x 10^5 cells on a 6-well-dish or 5 x 10^6 cells on a 10 cm-dish) and transfected with 0.5 µg plasmid DNA (3 µg for 6-well-dish or 8 µg for 10cm-dish). For transfection two times the DNA amount of either Polyethylenimine (PEI, for HEK293T) or Metafecten Pro (for ESC) was used. For PEI transfection DNA and PEI were mixed in 70 µl serum-free DMEM and vortexed. After 15 min incubation at RT the solution was added to the cells. Transfection with Metafecten Pro was done according to manufacturer's recommendations. The medium was changed after 6 to 8 h. Cells were harvested 48 h after transfection.

4.4. Immunofluorescence

Cells were grown on cover slips and fixed with methanol/acetone (7:3) at -20 °C for 10 min, washed twice with PBS at RT for 5 min and blocked in goat serum (10 %) in a humidified chamber at RT for 30 min. Cover slips were then incubated in primary antibody (undiluted hybridoma MF20 or TI-1) at 37 °C for 1 h, washed three times with PBS at RT for 5 min and incubated with secondary antibody (α-Mouse-Alexa568 1:500) in PBS with 0.5 % BSA in a humidified chamber at 37 °C for 45 min. After that nuclei were stained with Hoechst 33342 in PBS (0.5 µg/ml) at RT for 5 min to label nuclei, washed twice with PBS at RT for 5 min and embedded in mowiol.

4.5. Luciferase Assay

10^5 HEK293T cells were plated on 24-well-plates and transfected in triplicates with 250 ng of the luciferase reporter construct and 50 ng of the construct containing a potential effector. 48 h after transfection cells were washed twice with PBS and incubated for 15 min in 150 µl cell lysis buffer. The suspension was then transferred in tubes and spun down for 10 min at 4 °C at R_{max}. 50 µl of the supernatant were transferred on a black 96-well-microtiter plate. A Berthold Tristar multimode reader was used to inject 100 µl assay buffer and measure luminescence for 5 sec.

4.6. EMSA

EMSA was performed in binding buffer with 1 ng recombinant MBP-Hey1 protein, 1 mg poly-dAdT, 5 ng biotin labeled probe and either 0, 25, 75 or 250 ng competing unlabeled probe in a total volume of 10 ml. After incubation on ice for 30 min, samples were loaded on a 6 % polyacrylamide gel and later

blotted onto Amersham Hybond N+ membranes. Detection was done using the PIERCE LightShift Chemiluminescent EMSA kit according to the manufacturer's recommendations.

4.7. RNA Isolation

RNA was isolated either from cell culture or mouse heart tissue. For cell culture 500 µl peqGOLD TriFast (PEAQLAB, Germany) were added directly to a 10 cm cell culture dish. Tissue was homogenized in 1 ml peqGOLD TriFast with an Omni tissue homogenizer (Omni International, USA) for up to 2 min. After that RNA was isolated according to manufacturer's recommendations. The RNA concentration was determined by absorbance using a Nanodrop.

4.8. Quantitative realtime PCR

2 or 3 µg RNA were reverse transcribed using the Revert Aid First-Strand cDNA synthesis Kit (Thermo Scientific, USA) with oligo(dT)-primers according to manufacturer's recommendations. qRT-PCR reactions contained 1/100 of the cDNA reaction or for ChIP samples 1/25 of precipitated DNA (PCR reaction shown in table 6). PCR was performed with annealing at 60 °C and SybrGreen quantification (PCR program shown in table 7). PCR products were confirmed by melting curve analysis and SB agarose gel electrophoresis. The housekeeping gene HPRT was used to normalize expression levels. All measurements were done as technical duplicates of biological triplicates for cDNA samples and technical duplicates of biological duplicates in case of ChIP samples.

Table 6: qRT-PCR reaction

2.5 µl	10X PCR buffer
1.5 µl	Ethylenglycol
0.25 µl	dNTPs 100 mM
0.75 µl	SybrGreen 1:2000 in H_2O 0.5 % DMSO
0.25 µl	His-taq 15 u/µl
0.75 µl	5' primer 10 pmol
0.75 µl	3' primer 10 pmol
13.25 µl	H_2O
5 µl	cDNA / DNA Template

Table 7: qRT-PCR program

95°C	3 min	Initial denaturation	
95°C	15 sec	Denaturation	40 cycles
60°C	10 sec	Annealing	
72°C	20 sec	Elongation	
55 – 95°C	10 min	Melting curve (+4 °C/min)	
16°C		End of program	

4.9. Transcriptome sequencing

For transcriptome sequencing RNA was isolated using the Qiagen RNeasy Mini Kit according to manufacturer's recommendation. Quality was assessed with an Experion RNA HighSense analysis kit. mRNA was isolated from total RNA using Sera-Mag magnetic oligos according to the Illumina transcriptome sequencing library preparation protocol. The sequencing library was generated using the NEBnext mRNA library prep master mix set for Illumina. In short, mRNA was transcribed into cDNA and fragmented into 200 bp fragments. Fragments were end-polished and Illumina sequencing adapters were attached. In between these steps samples were purified using the Qiagen PCR purification kit. DNA fragments ranging from 200 – 300 bp were excised from an agarose gel using Qiagen gel extraction kit and subjected to 12 cycles of PCR amplification. The cDNA library was assessed with the Experion DNA 1K kit. After that samples were multiplexed and sequenced on an Illumina GAIIx platform.

33 bp sequences were generated and mapped to the hg19 or mm9 genome using bowtie 1.0.0 with standard parameters (Langmead et al., 2009). All genes with less than 10 mapped reads were removed from the analysis, after that RPKM values were calculated. The noise expression level was calculated from reads mapping to non-expressed regions. A gene was considered expressed, when the RPKM value was at least ten-fold higher than the noise level. Regulated genes were identified using the R-package DEseq (Anders and Huber, 2010). Heatmaps were generated using hierarchical clustering in Cluster 3.0 (Hoon, 2002, Eisen et al., 1998) and visualized using TreeView 1.1.6r2 (Saldanha, 2004).

4.10. Western blot analysis

Cells were washed twice in ice-cold PBS, scraped into a falcon tube and spun down at 2700 xg for 1 min at 4 °C. Cells were resuspended in ten-fold volume of RIPA buffer and incubated on an overhead wheel for 30 min at 4 °C. After centrifugation at R_{max} for 10 min at 4°C, the supernatant was mixed with protein loading buffer and denatured for 5 min at 100 °C.

Samples were separated on a 12 % SDS-polyacrylamide gel at 45 mA per gel for 1 – 2 h in SDS running buffer. After separation, proteins were transferred to a nitrocellulose membrane in blotting buffer using a semi-dry blotting chamber at 400 mA for 40 min at 4 °C.

The membrane was washed in PBS and blocked in 5 % milk powder in PBS for 1 h at RT. After that the first antibody was added for 1 h at RT or overnight at 4 °C (α-Flag-M2, α-HA and α-Tubulin 1:5000). Then the membrane was washed three times in PBS for 20 min at RT, incubated with the second antibody in 5 % milk powder in PBS (α-Mouse-POD 1:5000) and washed again three times in PBS for 20 min at RT. After that proteins were detected by incubation in detection buffer for 1 min and subsequent imaging.

4.11. Chromatin immunoprecipitation

5 x 10^6 cells either from stable cell lines with inducible *Hey* expression were induced with 50 ng/ml doxycycline for 48 h to obtain low level overexpression of Hey proteins or HEK293T cells were transiently transfected as described above. Non-induced or mock transfected cells were used as control. Chromatin immunoprecipitation was performed as described before (Wei et al., 2006). Briefly, the cells were fixed with 1 % paraformaldehyde for 10 min at RT. Fixation was stopped by adding glycine to 0.2 M for 5 min and cells were washed three times with ice-cold PBS and harvested. All subsequent steps were done at 4 °C. The cells were lysed in cell lysis buffer and spun down at 1000 xg for 5 min. The resulting pellet of nuclei was lysed in nuclei lysis buffer and sonicated using a Bioruptor Standard sonifier (for 20 min on “high” with 30 sec pulse and 30 sec break). Debris was removed by centrifugation. For immunoprecipitation, chromatin was diluted ten-fold with ChIP buffer. 1.5 ml of diluted chromatin was mixed with 40 µl 1:1 protein G agarose slurry (blocked for 4 h in ChIP buffer with 10 µg/ml BSA and 1 µg/ml Salomon sperm DNA), incubated for 1 h and spun down. The pre-cleared supernatant was than incubated with 4 µg antibody overnight. After that 60 µl 1:1 protein G agarose slurry (in ChIP buffer) was added and incubated for 2 h.

Then, the agarose beads were washed two times with low salt washing buffer, once with high salt washing buffer and four times with LiCl washing buffer. Elution was performed with 200 µl elution buffer at 68 °C for 30 min. The eluted chromatin was incubated with 0.8 mg/ml Proteinase K and PFA fixation was reversed by incubation at 68 °C overnight. The DNA was then purified using phenol-chloroform extraction and precipitation and subsequently quantified using PicoGreen.

For ReChIP the eluted chromatin was again diluted ten-fold with ChIP buffer and incubated with a second antibody, washed and eluted as described before.

4.12. Chromatin immunoprecipitation with high throughput sequencing

For ChIPseq the same protocol as for ChIP was used in principle, but 2.5 x 10^8 cells were used and 10 µg of antibody in order to isolate enough enriched DNA. The amount of DNA was determined by PicoGreen measurement. 7–12 ng of ChIP enriched DNA were subjected to library preparation using the NEBNext ChIPseq sample preparation kit according to the manufacturer's instruction. Briefly, DNA was end-polished with T4 DNA polymerase and kinase. After column purification, Illumina sequencing adaptors were ligated to the ChIP DNA fragments. DNA fragments ranging from 175 –225 bp were excised from an agarose gel using Qiagen gel extraction kit and subjected to 18 cycles of PCR amplification. The DNA fragments were assessed with the Experion DNA 1K kit and sequenced on an Illumina GAIIx platform.

33 bp sequences were generated and mapped to the hg19 or mm9 genome using bowtie 1.0.0 (Langmead et al., 2009) with standard parameters. Peaks were identified using MACS 1.4.1 (Zhang et al., 2008) using sequences from non-induced or mock transfected cells as control to identify the putative binding sites. All peaks with a maximum p-value of 10^{-5} and a minimum height of 10 overlapping sequences were included. The unique mapping locations for each factor were used to generate the genome-wide intensity profiles, which were visualized using the Integrative Genomics Viewer (Thorvaldsdottir et al., 2013). PeakAnalyzer (Salmon-Divon et al., 2010) was used to annotate peaks and to calculate overlaps between different factors. Heatmaps were generated using seqMiner 1.2.1 (Ye et al., 2011) with K-means raw clustering. The peak heights were normalized according to the 1000 highest peaks within each sample.

4.13. GO term analysis

GO term analysis was performed with DAVID 6.7 (Huang da et al., 2009) using the functional annotation clustering method and allowing only to enrich for biological processes. Clusters were named based on interpretation of enriched GO annotations.

4.14. Motif discovery

The R-package motifRG (Yao) and MEME (Bailey et al., 2009) were used to identify binding motifs, using sequences +/-100 bp around the summit of the top 300 highest ranking peaks. Unrelated sequences with a similar distance towards TSS of genes lacking ChIPseq peaks and with similar GC distribution were selected and used as control/background.

The software SMART (Veerla et al., 2010) was used to identify overrepresented binding motifs within sets of promoters.

5. Results

5.1. Hey target genes HEK293 cells

5.1.1. Expression of Hey proteins in HEK293 cells

HEK293 cells have low level expression of endogenous Hey proteins. According to RNAseq data endogenous Hey1 ranked at position 837 of all expressed gene within the HEK293 transcriptome, expression of Hey2 and HeyL, which were at position 11 818 and 8 442 was around 20 fold lower. HEK293 cells, therefore, are capable to respond appropriately to overexpression of Hey proteins. However, the endogenous expression should be low enough to detect regulatory effects on target genes.

A)

	Hey1	Hey2	HeyL
50 ng/ml dox	10	18	44
1000 ng/ml dox	223	1 677	27 713

B)

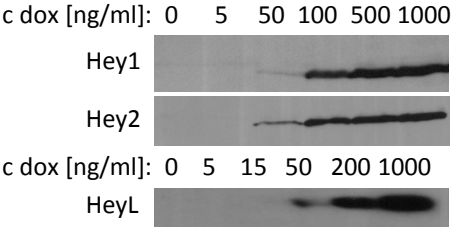


Figure 9: Induction of Hey proteins in stable HEK293 cell lines
Stable cell lines were induced with different doxycycline (dox) levels. After 48 h RNA and protein were harvested.
(A) qRT-PCR shows fold-induction compared to endogenous levels at different doxycycline levels.
(B) α-Flag Western blot shows Hey protein induction for different doxycycline levels.

As there were no reliable Hey antibodies available, HEK293 cells that overexpress Flag tagged Hey proteins under control of a doxycycline inducible Promoter were used. Low level induction with 50 ng/ml doxycycline resulted in a low overexpression in the range of 10 – 44 fold compared to endogenous level, whereas a high induction with 1 µg/ml doxycycline lead to a much higher overexpression (figure 9A). The higher fold change observed for Hey2 and HeyL is mainly due to the lower endogenous expression of HeyL. Doxycycline dependent induction could also be observed on protein level (figure 9B).

5.1.2. Genome-wide Hey binding in HEK293 cells

In order to identify genome-wide Hey binding sites, ChIPseq experiments were conducted. For each sample a comparable number of reads (15 – 16 million reads) was generated and on average 86 % of these could be mapped to the hg19 human genome (supplementary table 8). Using the before mentioned criteria (p-value ≤ 10^{-5} and peak height ≥ 10) 9 308 Hey1 and 9 128 Hey2 binding sites were identified within the HEK293 genome. For HeyL 60 922 binding sites were identified.

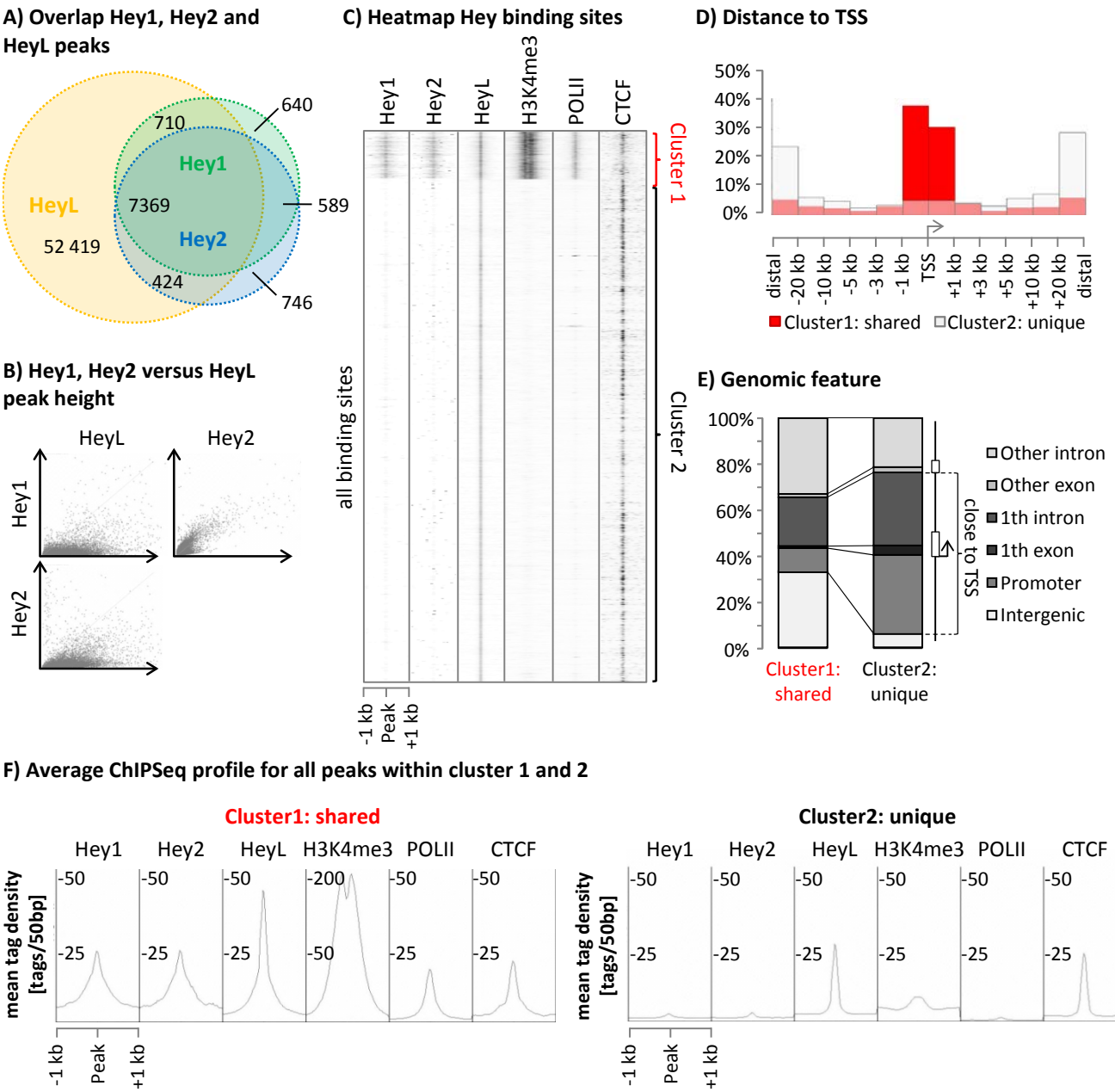


Figure 10: Hey1, Hey2 and HeyL have overlapping binding properties in HEK293 cells

ChIPseq data for Hey1, Hey2 and HeyL was generated by low level doxycycline induction (50 ng/ml for 48 h) of HEK293-Hey1, -Hey2 and HeyL cells, non-induced cells were used as control.

(A) Venn-diagram showing the overlap between all identified binding sites.

(B) Scatterplot showing the peak height in one sample plotted against the height in another sample.

(C) This heatmap represents all identified Hey binding sites as a stack. The ChIPseq signal is shown in a window of 1 kb up- and downstream of the peak summit, which is centered in the middle of each column. The shading indicates the signal intensities. Binding sites were clustered in one cluster of shared binding sites and one cluster of unique HeyL binding sites. Also shown are the signals for polymerase II (POLII), histone H3 lysine 4 tri-methylation (H3K4me3) and CTCF binding in HEK293 cells, which were derived from public databases.

(D) The histogram shows the percentage of peaks within a certain distance to the closest transcription start site (TSS).

(E) This diagram shows the percentage of peaks located within a certain genomic feature.

(F) Shown are the average ChIPSeq signals for all shared peaks and for all unique HeyL peaks.

There is a large overlap between DNA binding sites of different Hey proteins. About 85 % off all sites are shared between Hey1 and Hey2 and 92 % of those are also shared by HeyL. However, for HeyL

52 419 additional binding sites were identified (figure 10A). The peak height at shared binding sites is similar for Hey1 and Hey2, but higher for HeyL (figure 10B and F). The HeyL peak height is reduced at the HeyL uniquely bound sites. For these Hey1 and Hey2 show almost no enrichment.

There are two groups of Hey bound sites, one contains the shared binding sites and one the HeyL uniquely bound sites (figure 10C). The shared sites are within close proximity to TSS (figure 10D). They are enriched for polymerase II binding and histone H3 tri-methylation at lysine 4 (H3K4me3), both are markers found at active promoters, meaning these genes are either poised for transcription or actively transcribed (figure 10C and F). These markers are not found at the unique HeyL sites, which are located more distal to TSS (figure 10C and G). All binding sites show CTCF binding.

Shared binding sites are mainly located within proximal promoters or the first exon or intron of genes due to their close proximity to TSS (figure 10E). The relatively low percentage of peaks within the first exon is due to the much smaller size of exons compared to introns. HeyL unique sites are either in other introns further downstream or intergenic.

5.1.3. Identification of Hey binding motifs

Potential DNA binding motifs for Hey proteins were identified using the top 300 shared target sites and the top 300 unique HeyL bound sites. At shared sites, sequences near the peak summits are very GC-rich (around 85 %) due to their close proximity to TSS and the presence of CpG islands in their vicinity (figure 11A). At HeyL unique peaks GC-content is lower (around 62 %), but still above the average human GC-content. This is most likely due to the co-localisation with CTCF (figure 10C), which has a GC rich binding motif (CCGCGNGGNGGCAG) (Kim et al., 2007).

Therefore, sequences with similar GC-distribution were used as control for motif discovery. De novo motif discovery at shared peaks yielded a class B E-box motif (CACGTG/CACGCG) as the preferred binding site (figure 11B). From the unique HeyL bound sites GC-rich motifs (GCCGC/CGCCC) were recovered, despite the use of a matching control. These non-palindromic motifs are untypical for binding of HLH factors and the relevance is unclear. There is a positive correlation between occurrence rate of the identified E-box motifs and Hey peak height at the shared peaks (figure 11C). DNA binding sites with high Hey binding peaks have a high occurrence rate of these motifs (65 %/ 40 %).

Electrophoretic mobility shift assays with recombinant Hey1 protein expressed in E. coli demonstrated strong E-box binding (CACGTG) and efficient competition by the unlabeled oligonucleotide (figure 11D). The two related oligonucleotides CACGCG and CGCGCG were also able to compete CACGTG binding, but to a lower extent. However, as there is a large number of binding sites without E-box

sequence, *in vivo* binding might have a more relaxed consensus sequence or may depend on other factors, e.g. indirect binding by interaction with other TF.

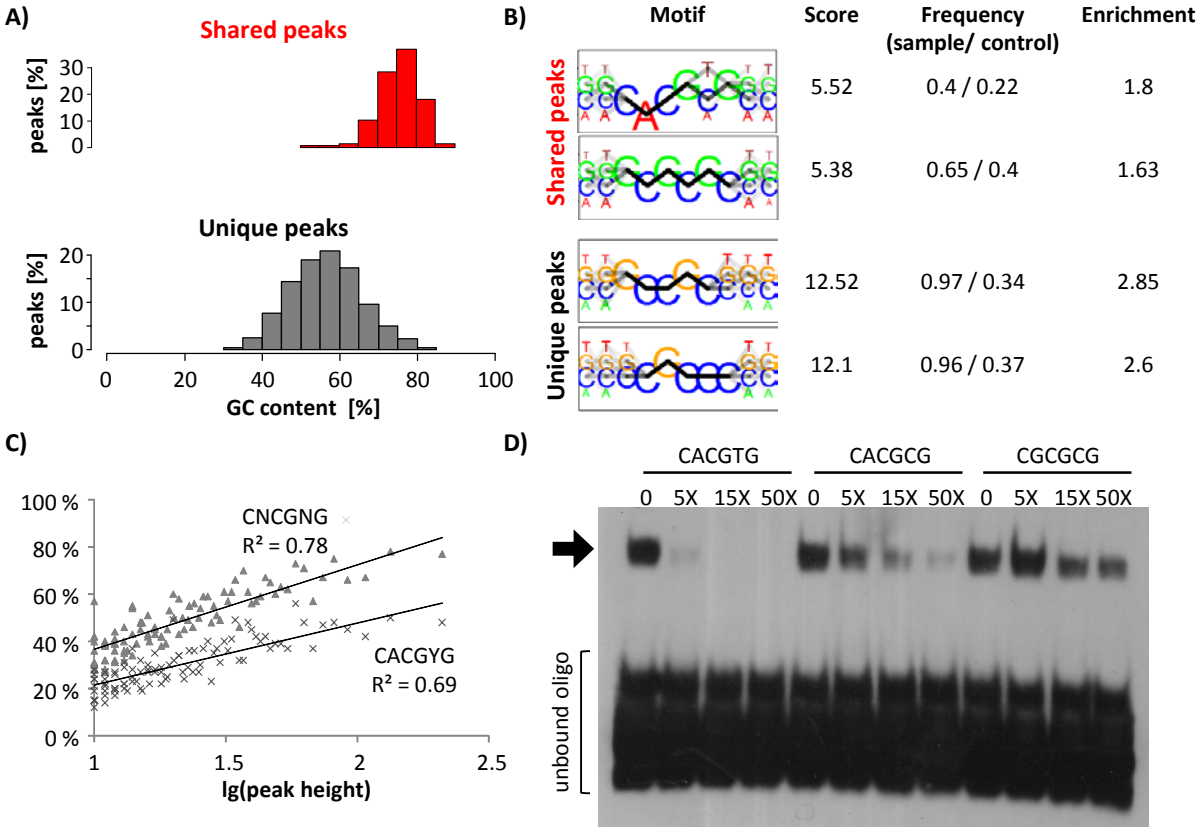


Figure 11: In silico identified Hey binding motifs are bound by recombinant Hey1 protein in EMSA
(A) Distribution of shared and unique HeyL DNA binding sites according to GC content.
(B) The top two motifs for DNA binding identified by de novo motif discovery for the 300 highest shared and unique HeyL binding peaks.
(C) Correlation between Hey1 peak height and motif occurrence rate at shared binding sites.
(D) Recombinant MBP-Hey1 protein interacts with CACGTG. Binding of the biotin labelled CACGTG probe is competed increasingly by 5-, 15- and 50-fold molar excess of unlabelled CACGTG, CACGCG and CGCGCG-probes. The latter were the least effective. Shifted oligonucleotides are indicated by an arrow.

5.1.4. Identification of Hey regulated genes

We previously identified Hey1 and Hey2 regulated genes in HEK293 cells by microarray analysis (Heisig, 2011, Heisig et al., 2012). To identify HeyL regulated genes in HEK293 we performed RNAseq. Around 10 million reads were generated for both the doxycycline induced sample and the non-induced control. In average 88 % of the reads were mapped to the human genome (supplementary table 9).

For all three Hey proteins we identified in total 661 genes, which are at least in one sample more than 1.8-fold regulated. The number of Hey1 regulated genes is considerably smaller when using these criteria, which might be due to the higher endogenous expression of Hey1 compared to Hey2 and HeyL (figure 12A). When clustering all regulated genes, Hey1 and Hey2 regulated genes cluster together, with HeyL being more different (figure 12B). Regulation by HeyL was also generally stronger, which might be due to lower endogenous expression and higher induction of HeyL (figure 12B). However, as

the HeyL expression data was obtained by RNAseq and Hey1 and Hey2 expression data by microarray, these effects might be due to the application of different techniques and not related to the additional identified HeyL binding sites. Previously, regulation of these genes was confirmed by qRT-PCR in *Hey* overexpressing and knockdown cells (Heisig, 2011, Heisig et al., 2012).

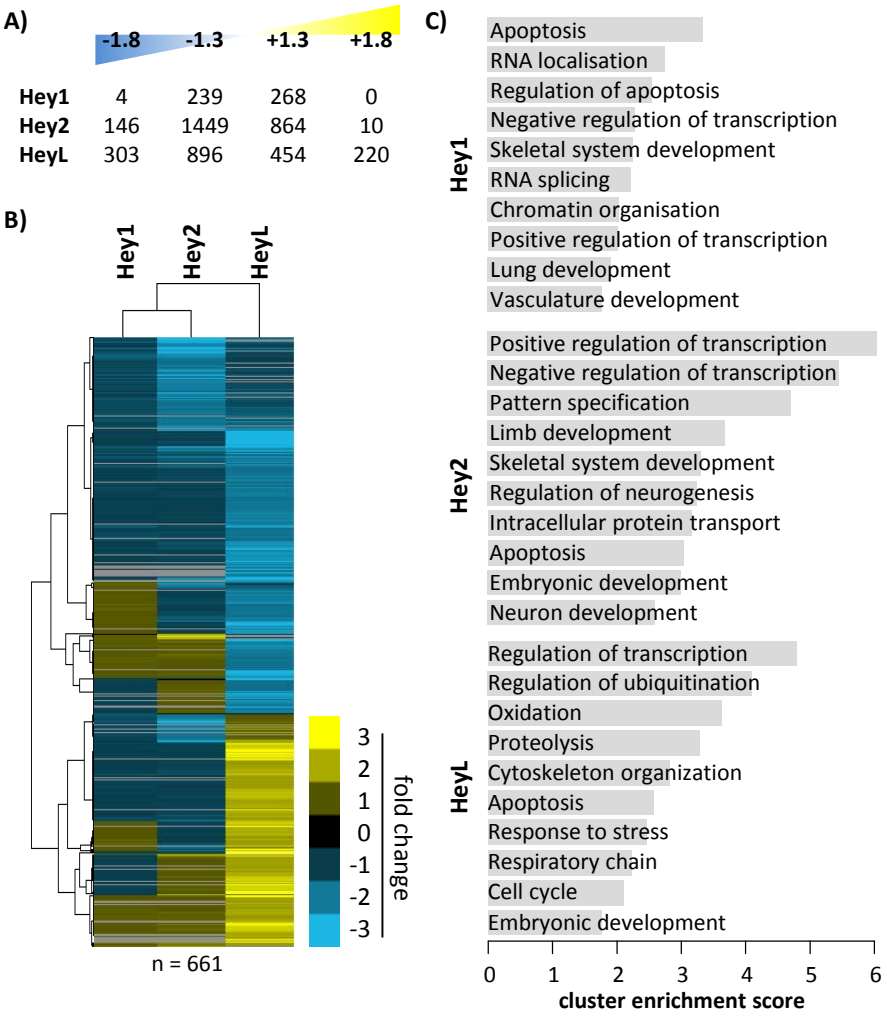


Figure 12: Hey target gene regulation in HEK293 cells
(A) Number of down- and up-regulated genes from expression analysis in HEK293 cells
(B) Hierarchical clustered heat map showing all genes which were more than 1.8-fold regulated in at least one sample.
(C) For all more than 1.3-fold regulated genes the most enriched GO terms are given (cluster enrichment score > 1.3 are significant).

By GO term analysis of regulated genes, we identified a striking overrepresentation of genes related to transcriptional control, developmental processes and apoptosis (figure 12C). The high occurrence of transcriptional control genes suggests that Hey proteins are higher up in the signaling hierarchy. Enriched developmental processes include limb and skeletal development, neurogenesis, and vascular development. These processes have previously been shown to be affected by Hey proteins and implicate them in a broad spectrum of developmental processes. HeyL regulated genes are also implicated in other processes including ubiquitination, oxidation, response to stress, and respiratory

chain. This also underlines that HeyL differs from Hey1 and Hey2 not only in its binding pattern but also in regulated target genes.

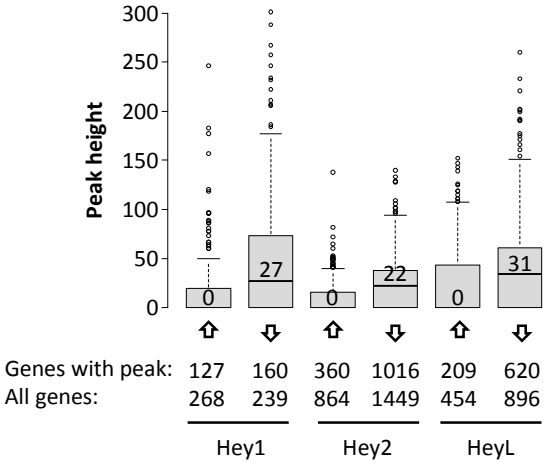


Figure 13: Hey binding in Hey up- and down-regulated genes
Boxplots depict the peak height at Hey1/2/L up- and down-regulated genes (≥ 1.3 fold) of binding sites near the TSS (-1 kb to +1 kb). Little arrows indicate up- or down-regulation. The median peak height, the total number of regulated, and the number of regulated genes with peak are given.

We further correlated data of Hey regulated target genes with Hey DNA binding sites (figure 13). Especially genes with repression on mRNA level frequently have higher binding peaks close to their TSS. This supports the concept of Hey proteins being repressors. The observed up-regulation is most likely the result of indirect effects.

5.1.5. Luciferase assays

We used luciferase reporter constructs to functionally validate promoters of Hey regulated genes. For this purpose, we have chosen promoters with high Hey binding peaks and an E-box motif near the peak summit (figure 14A). Four promoters (*Hey1*, *JAG1*, *BMPR1A* and *NEUROG3*) were efficiently repressed in transient co-transfections with Hey1 (figure 14B and C). Co-transfection with an activator construct encoding a fusion of the Hey1 bHLH-Orange sequence with the vp16 activation domain resulted in increased luciferase expression. The DNA non-binding Hey1-RK3 mutant (Heisig et al., 2012), was not able to efficiently repress target promoters. In case of *JAG1* a targeted mutation of the putative E-box motif (gggCACGCGtca to gggCAtca) abolished regulation by Hey1 and vp16-Hey1.

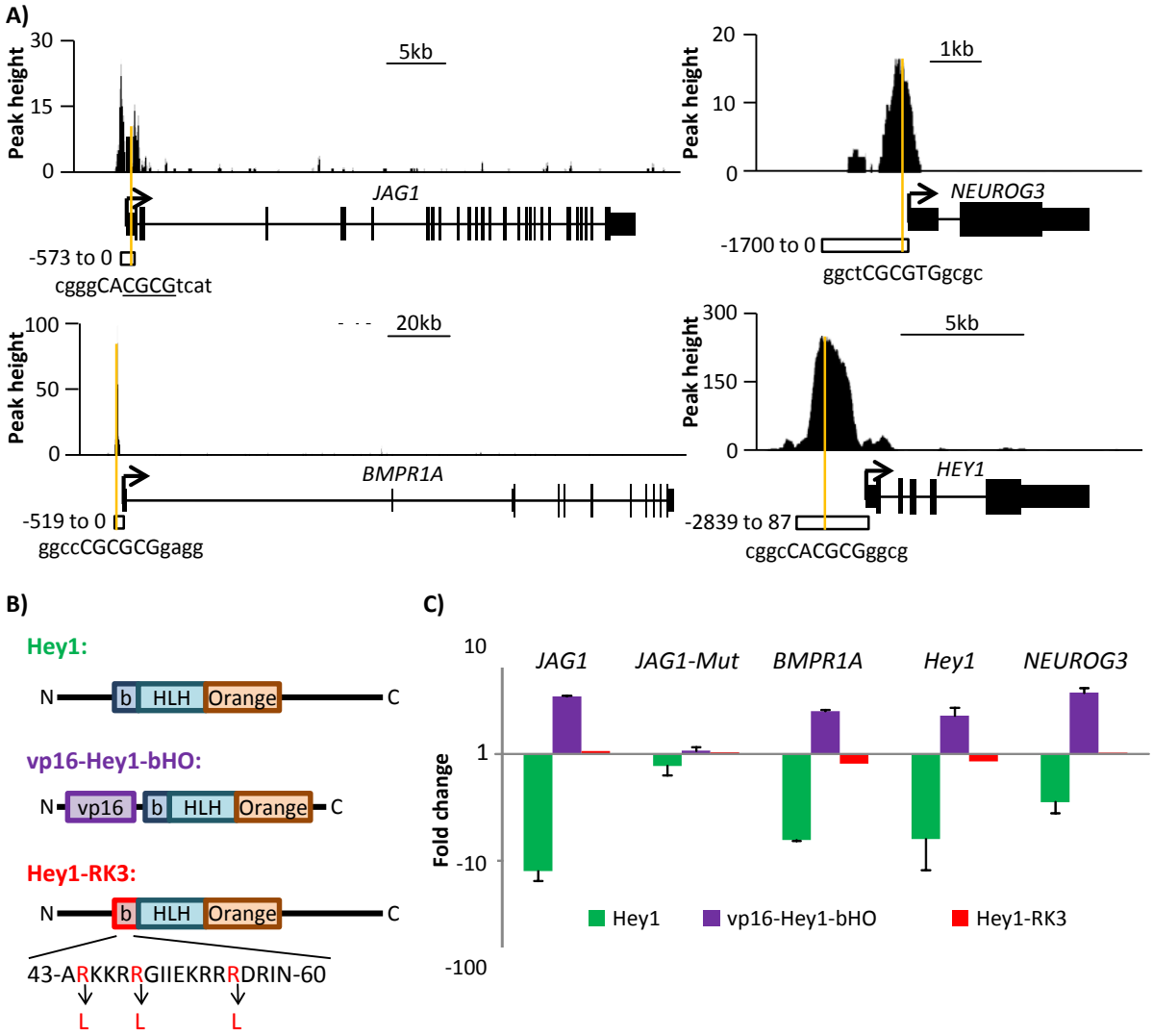


Figure 14: Functional analysis of Hey target promoters by luciferase assay

(A) Hey1 ChIPseq signal for the *JAG1*, *NEUROG3*, *BMPR1A* and *HEY1* genes. Orange lines indicate the closest potential Hey bond E-box motif under the peak summit. Open boxes indicate the fragment used in the luciferase assay and its position in bp relative to the TSS. For *JAG1* a second luciferase construct with a mutated binding site was used (*JAG1-Mut*, deleted nucleotides underlined).

(B) Schematic representation of different Hey1 variants used to determine the effect of Hey1 on luciferase-promoter constructs.

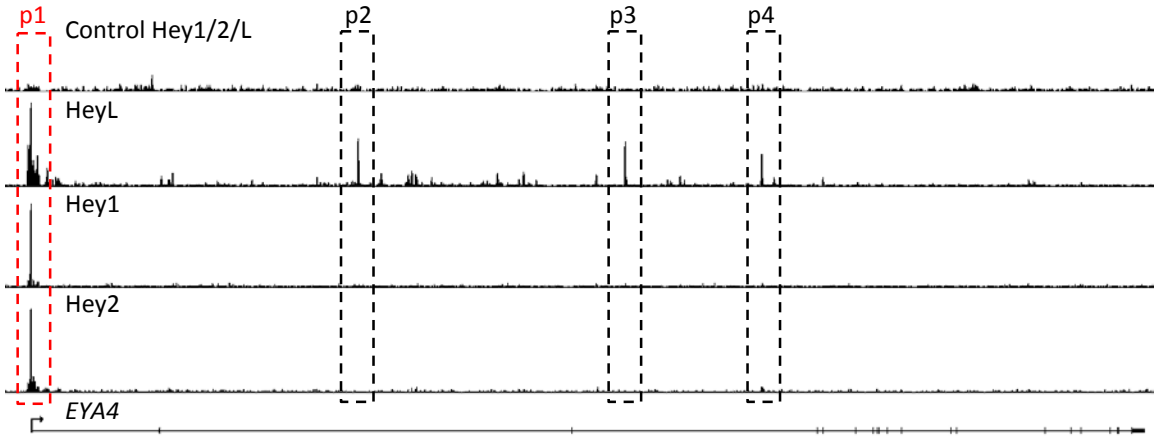
(C) Luciferase reporter analysis in HEK293T cells transiently transfected with reporter-constructs (JAG1, JAG1-Mut, BMPR1A, Hey1 and NEUROG3) and Hey expression plasmids or empty vector control (set at 1).

These data establishes Hey proteins as redundant repressors, which regulate other TF involved in various development processes. Hey proteins primarily bind to E-boxes close to TSS.

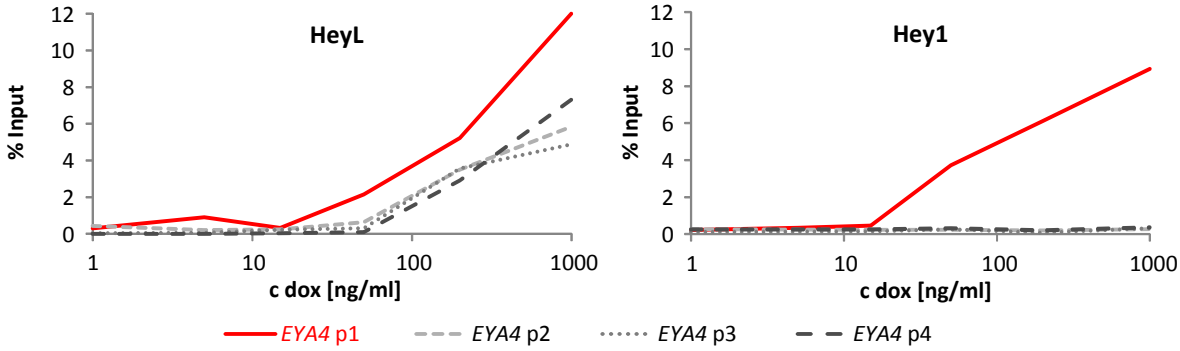
5.1.6. Additional identified HeyL binding sites are no artefact

The most striking difference in the ChIPseq data between the three Hey proteins are the many additional binding sites identified for HeyL. The HeyL overexpressing cell line was generated using a different construct and the fold-change induction was higher compared to Hey1 and Hey2. This raised the question whether the additional identified HeyL binding sites are just an artefact identified due to methodical differences.

A) Hey1, Hey2 and HeyL ChIPseq signal at *EYA4* locus



B) Additional HeyL peaks at *EYA4* locus are not dose dependent



C) Additional HeyL peaks at *EYA4* locus are not construct dependent

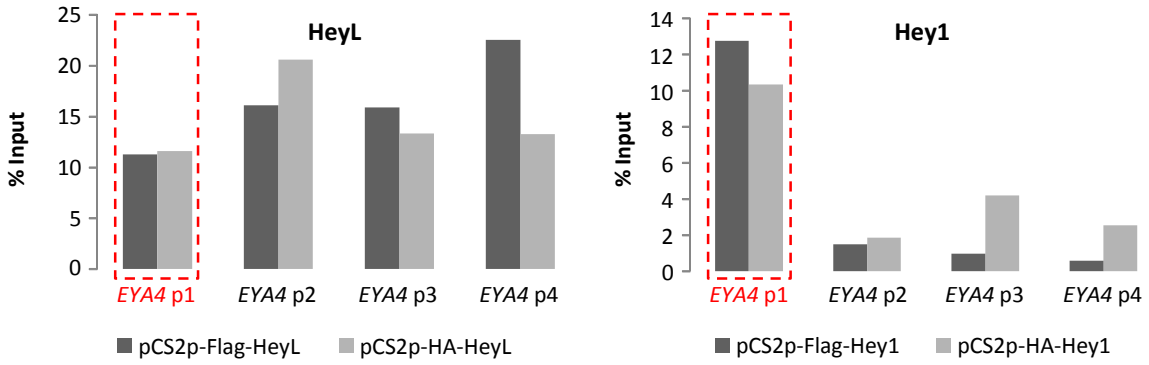


Figure 15: Additional HeyL binding at EYA4 locus
(A) Shown are the Hey1, Hey2 and HeyL ChIPseq signals for the EYA4 gene. All three Hey proteins bind to the proximal promoter region (peak 1). For HeyL there are three additional peaks further downstream (peak 2 – 4).
(B) For Hey1 and HeyL ChIP experiments after induction with different doxycycline concentration were carried out. Shown is the enrichment for the four binding peaks (p1 – p4), with the shared peak p1 in red.
(C) HEK293T cells were transiently transfected with pCS2p-Flag-Hey1 or -HeyL or pCS2p-HA-Hey1 or HeyL constructs. Enrichment was determined for the four binding peaks (p1 – p4). The shared peak p1 is indicated by a red box.

To test whether the additional sites were only enriched for HeyL, because of higher expression, we conducted ChIP experiments with different induction levels of doxycycline for genes with a shared binding site at the promoter that functions as control and one or more unique HeyL binding sites further downstream. This was true for e.g. *EYA4* (figure 15A), *HIVEP* and *PXT1*. For all three genes we

demonstrated that Hey enrichment is dose dependent: Higher induction correlates with higher enrichment at the binding site. However, although Hey1 binding could be observed at the shared sites, it could not be observed for the additional HeyL sites, even when Hey1 was highly induced with 1 µg/ml dox (figure 15B and supplementary figure 37A).

To exclude the possibility that the additional unique HeyL sites are just an artefact resulting from the different constructs used to generate the HeyL cell line (ptol2 vector backbone for HeyL instead of p199 lentivirus for Hey1 and Hey2), we also conducted ChIP experiments using transiently transfect HEK293T cells with pCS2p constructs, constitutively expressing either HA or Flag tagged Hey proteins. Here, we observed enrichment for HeyL and Hey1 at the shared promoter peak. For the additional peaks at the *EYA4* gene the enrichment for Hey1 was only low, whereas the enrichment for HeyL was about 15-fold stronger. This shows that HeyL binding of additional sites is not caused by use of different vector backbones (figure 15C). The same is true for the *HIVEP* and the *PXT1* genes (supplementary figure 37B).

These results show that the additional binding sites identified for HeyL are predominantly bound by HeyL and this is an actual difference to the other two Hey proteins and not just an experimental artefact.

5.1.7. Additional HeyL binding sites are not bound by Hey heterodimers

Hey proteins are bHLH factors which bind DNA as dimers. They are able to form homo- as well as heterodimers (Iso et al., 2001b, Leimeister et al., 2000a, Fischer et al., 2007). We did not observe enrichment for Hey1 at the additional HeyL bound sites when overexpressing Hey1 on its own. This indicates that either only HeyL homodimers bind these additional sites or that the expression of endogenous HeyL was not sufficient enough to observe an enrichment for Hey1-HeyL heterodimers. As the endogenous expression of HeyL was low, HEK293T cells were co-transfected with Flag tagged Hey1/2/L and HA tagged HeyL. Flag-ChIP was performed followed by another HA-ChIP (ReChIP) to identify bound dimers.

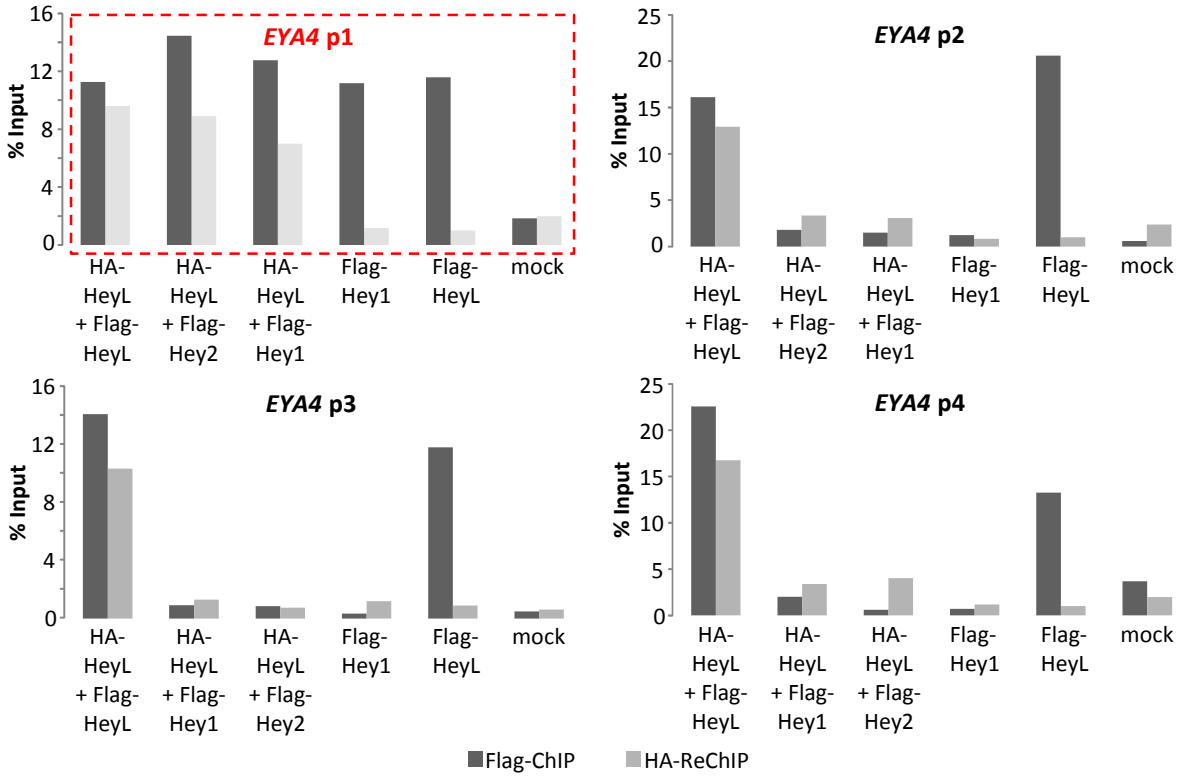


Figure 16: Heterodimer binding at *EYA4* gene
HEK293T cells were transiently co-transfected with pCS2p-HA-HeyL and pCS2p-Flag-Hey1, -Hey2 or -HeyL constructs. Control cells were either only transfected with pCS2p-Flag-Hey1 or -HeyL or empty pCS2p-Flag (mock). First a Flag-ChIP was carried out for all samples, after that half of the eluted chromatin was used for the HA-ReChIP. The enrichment was determined for the Flag-ChIP and the HA-ReChIP samples for the four binding peaks (p1 – p4). The shared peak p1 is indicated by a red box.

After the Flag-ChIP, enrichment for all three Hey proteins was observed at the shared site of the *EYA4* gene (figure 16 p1). For Hey1 or Hey2, however, no enrichment was observed at the additional unique HeyL bound sites (p2 – p4), even when HeyL was co-transfected. These results are very similar to those from the stable cell lines and indicate that the unique HeyL bound sites are neither bound by Hey1-HeyL nor by Hey2-HeyL heterodimers.

After the second ChIP against the HA-tagged HeyL (ReChIP), similar enrichment for HeyL was observed for all three co-transfections with Flag-Hey1/2/L at the shared locus (p1). That means HA-tagged HeyL was bound in combination with Flag-tagged Hey1, Hey2 or HeyL. This indicates that HeyL-HeyL homodimers, as well as Hey1-HeyL and Hey2-HeyL heterodimers bind to the shared locus (p1). For the additional peaks (p2 – p4) only the sample transfected with Flag-HeyL showed enrichment for HA-HeyL after HA-ReChIP. This suggests that only HeyL-HeyL homodimers bind to the additional HeyL binding sites (p2 – p4).

Control cells only transfected with Flag-tagged Hey1 or HeyL demonstrated enrichment after the Flag-ChIP, but not after HA-ReChIP and mock transfected cells showed no enrichment in either the Flag-

ChIP or the HA-ReChIP, as expected. Very similar results were obtained for *HIVEP* and *PXT1* (supplementary figure 38).

5.1.8. Binding of HeyL-Hey1 chimeric proteins

Having found that HeyL in contrast to Hey1 and Hey2 is able to bind additional sites, we cloned chimeric proteins. As the bHLH and the Orange domain are largely conserved between all three Hey proteins, it was unlikely that differences within that region were the cause for binding of additional sites. Therefore, the C- and N-terminal parts of the HeyL protein were replaced with the matching parts from Hey1 (figure 8 and 17B). The conserved region containing bHLH and Orange domain was in both cases derived from HeyL.

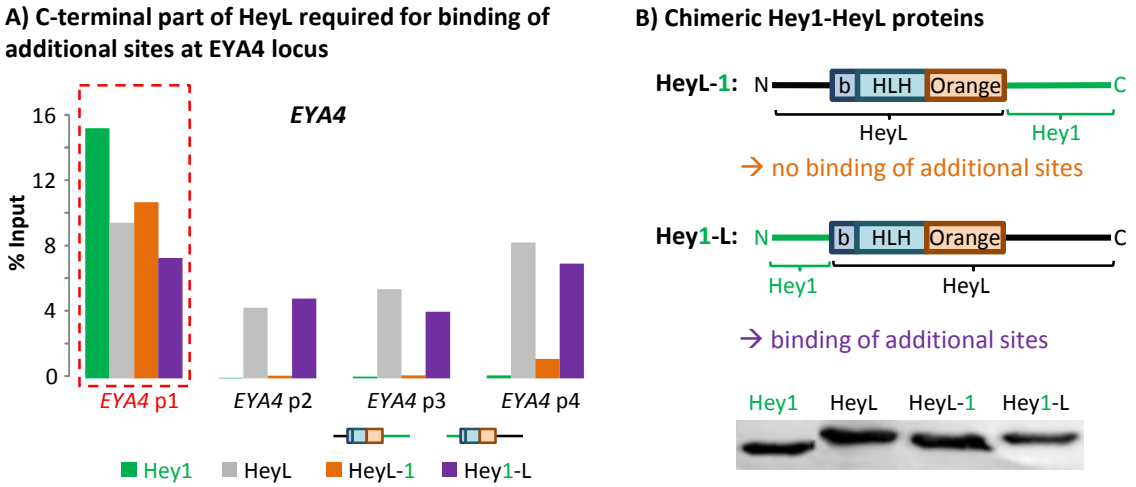


Figure 17: Binding of chimeric Hey1-HeyL proteins at EYA4 locus
(A) HEK293T cells were transfected with pCS2p-Flag-Hey constructs expressing either Hey1, HeyL or two chimeric proteins containing either the N-terminal part of HeyL and the C-terminal part of Hey1 (HeyL-1) or vice versa (Hey1-L). The enrichment was determined for the four binding peaks (p1 – p4) at the *EYA4* gene. The shared peak p1 is indicated by a red box.
(B) The two used chimeric proteins are depicted here. An α-Flag Western blot was performed. b = basic domain; HLH = helix loop helix domain;

ChIP experiments showed that only the chimeric protein containing the C-terminal part of HeyL was enriched at the additional sites at the *EYA4* gene (figure 17A). The same was true for the additional sites at *HIVEP* and *PXT* genes (see supplementary figure 39). The shared binding sites were bound by both chimeric variants, as well as by Hey1 and HeyL. This demonstrates that the ability of HeyL to bind the additional sites depends on the C-terminal region of the HeyL protein.

5.1.9. Binding behavior of the HeyL DNA non-binding mutant

Hey proteins can directly interact with DNA. With their basic domain they bind to an E-box motif. As the additional sites were only bound by HeyL and no E-box motif could be recovered, we were asking the question whether this is a direct interaction with DNA or whether HeyL binds to these sites via an

interaction partner. Therefore, we generated a HeyL binding mutant containing three point mutations within the basic domain converting three arginine residues that presumably interact with DNA to lysine (figure 18B). The same mutations were previously introduced into Hey1 and abolished direct DNA binding completely (Heisig et al., 2012). In case additional HeyL binding sites are only bound indirectly via an unknown interaction partner, this mutation should be without an effect on HeyL binding to these sites. Binding to the shared binding site, which most likely results from direct DNA binding, should be abolished.

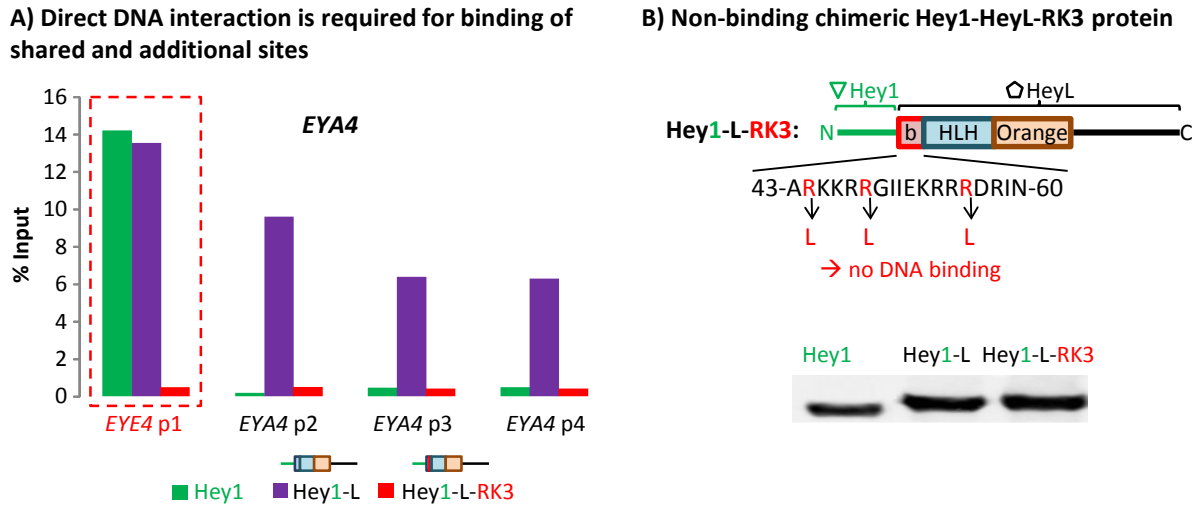


Figure 18: Binding of Hey1-L-RK3 nonbinding mutant at EYA4 locus
(A) HEK293T cells were transfected with pCS2p-Flag-Hey constructs expressing Hey1, the chimeric Hey1-L protein or the Hey1-L-RK3 mutant, with 3 point mutations in the binding sites, abolishing DNA binding. The enrichment was determined for the four binding peaks (p1 – p4) at the *EYA4* gene.
(B) The used binding mutant is depicted here. An α-Flag Western blot was performed. b = basic domain; HLH = helix loop helix domain;

ChIP experiments demonstrated that the binding mutant Hey1-L-RK3 is not able to bind to any of the analyzed DNA binding sites. This shows that a direct interaction of the DNA binding domain is required for all HeyL binding sites including the unique HeyL bound sites (figure 18A and supplementary figure 40). Binding of these sites may still depend on a direct interaction partner that interacts with the C-terminal part of HeyL but not with the C-terminal parts of Hey1 or Hey2. Nevertheless, no factors have been described so far, which only interact with HeyL but not Hey1 or Hey2.

5.1.10. Validation of HeyL differently regulated genes

In HEK293 cells we identified 661 genes with more than 1.8-fold regulation (figure 12B). 332 genes were differently regulated by HeyL compared to Hey1 and/or Hey2 (≥ 1.8-fold) and 329 were similarly regulated (< 1.8-fold).

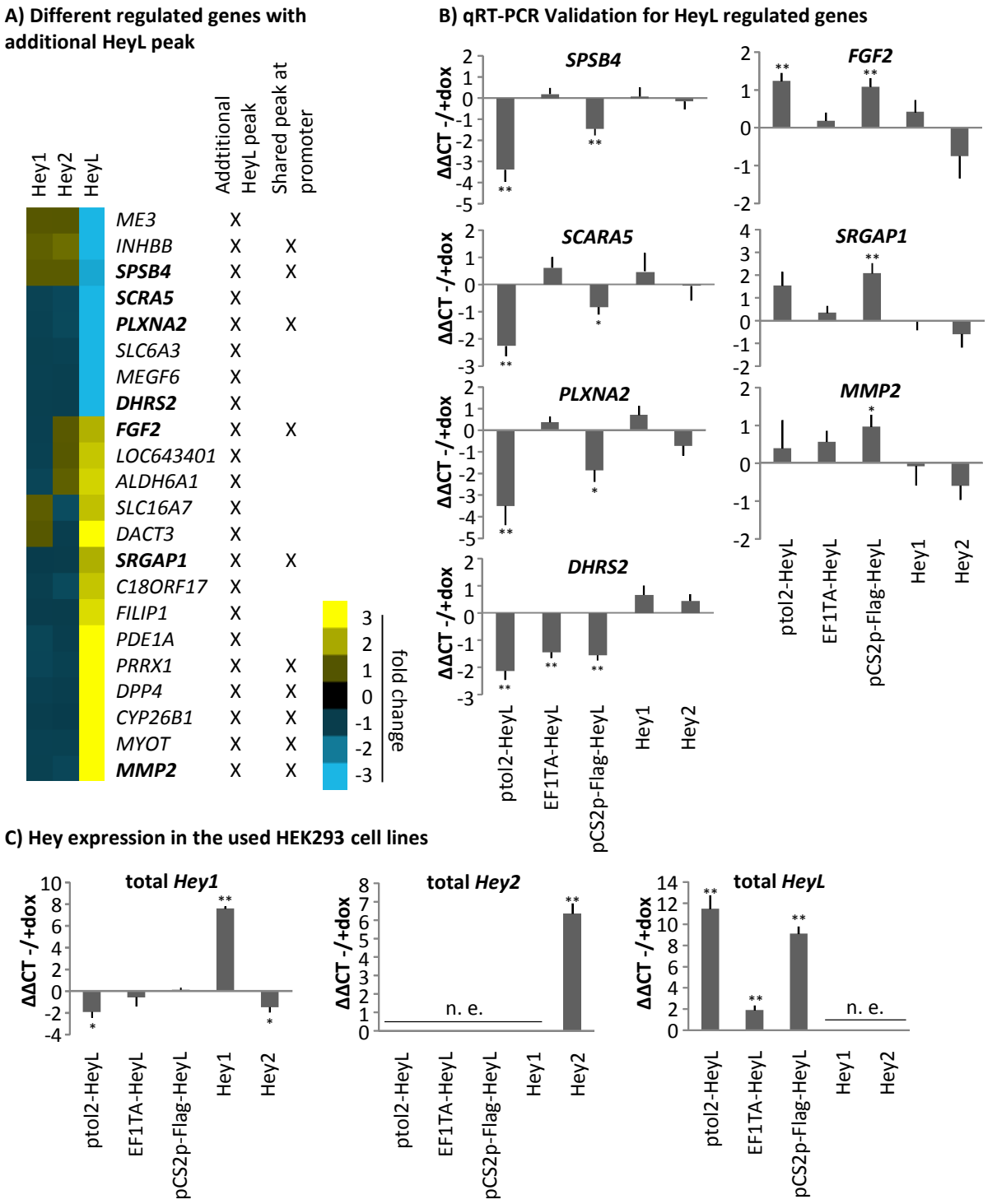


Figure 19: Validation of HeyL differently regulated genes

(A) Shown are differently regulated genes, which are highly expressed in HEK293 cells (> 20 RPKM) and have at least one additional HeyL binding site within their gene body. Indicated is also whether a gene has an additional shared peak at the promoter. Genes marked in bold were validated by qRT-PCR.

(B) qRT-PCR results for the in (A) indicated genes. Here an additional stable HeyL cell lines was used: the by lentiviral transfection generated EF1TA cell line. Cells were induced with 1 µg/ml doxycycline for 48 h and compared to non-induced control cells, transfected cells were compared to pCS2p-Flag mock transfected control cells.

(C) Shown is the overexpression of *Hey1*, *Hey2* and *HeyL* in the cell lines used.

These two sets of target genes were used to search for differences in HeyL DNA binding. However, no significant differences in binding were identified between the two groups, neither for proximal shared binding sites nor for distal unique HeyL binding sites (supplementary figure 41). To confirm that *HeyL* overexpression really results in additional regulation compared to *Hey1* and/or *Hey2*, regulation was confirmed by qRT-PCR for some of the differently regulated genes. Therefore, especially highly expressed genes (according to RNAseq data) and genes with at least one additional HeyL binding site were chosen (figure 19A).

Seven genes were analyzed by qRT-PCR, four HeyL down-regulated and three HeyL up-regulated genes. All down-regulated genes were significantly down-regulated in the same cell-line that was used for the RNAseq (HEK293-ptol2-HeyL), but only one was also significantly down-regulated in another stable HeyL cell line (HEK293-EF1TA-HeyL; figure 19B). However, this was most likely due to the fact that the induction of HeyL was much lower in the HEK293-EF1TA-HeyL cell line (figure 19C). Therefore, also transiently transfected HEK293T cells were used for validation. Here the induction of *HeyL* was much stronger (figure 19C) and it was possible to confirm the regulation for all four down-regulated genes (figure 19B). This down-regulation was not observed for Hey1 or Hey2. Up-regulation could also be confirmed, although regulation was not always significant.

These results show that there are indeed genes, which are differently regulated by HeyL, and it was possible to confirm this regulation also with transiently transfected cells. However, the large number of additional HeyL binding sites and the fact that there is no difference in HeyL binding between differently and similarly regulated genes raises doubt that the function of these additional HeyL DNA binding sites is additional transcriptional regulation. Therefore, the functional relevance of these unique HeyL bound sites remains unclear.

5.2. Hey target genes in murine ES cells and cardiomyocytes

5.2.1. Efficient differentiation of murine ES cells into cardiomyocytes

Besides HEK293 cells, ESC and *in vitro* differentiated CM were used to study Hey target genes and their regulation. This differentiation system is more relevant for the previously observed cardiac phenotype. Two murine ESC lines with a *MHC* promoter driven neomycin resistance and doxycycline inducible *Hey1* or *Hey2* expression were used.

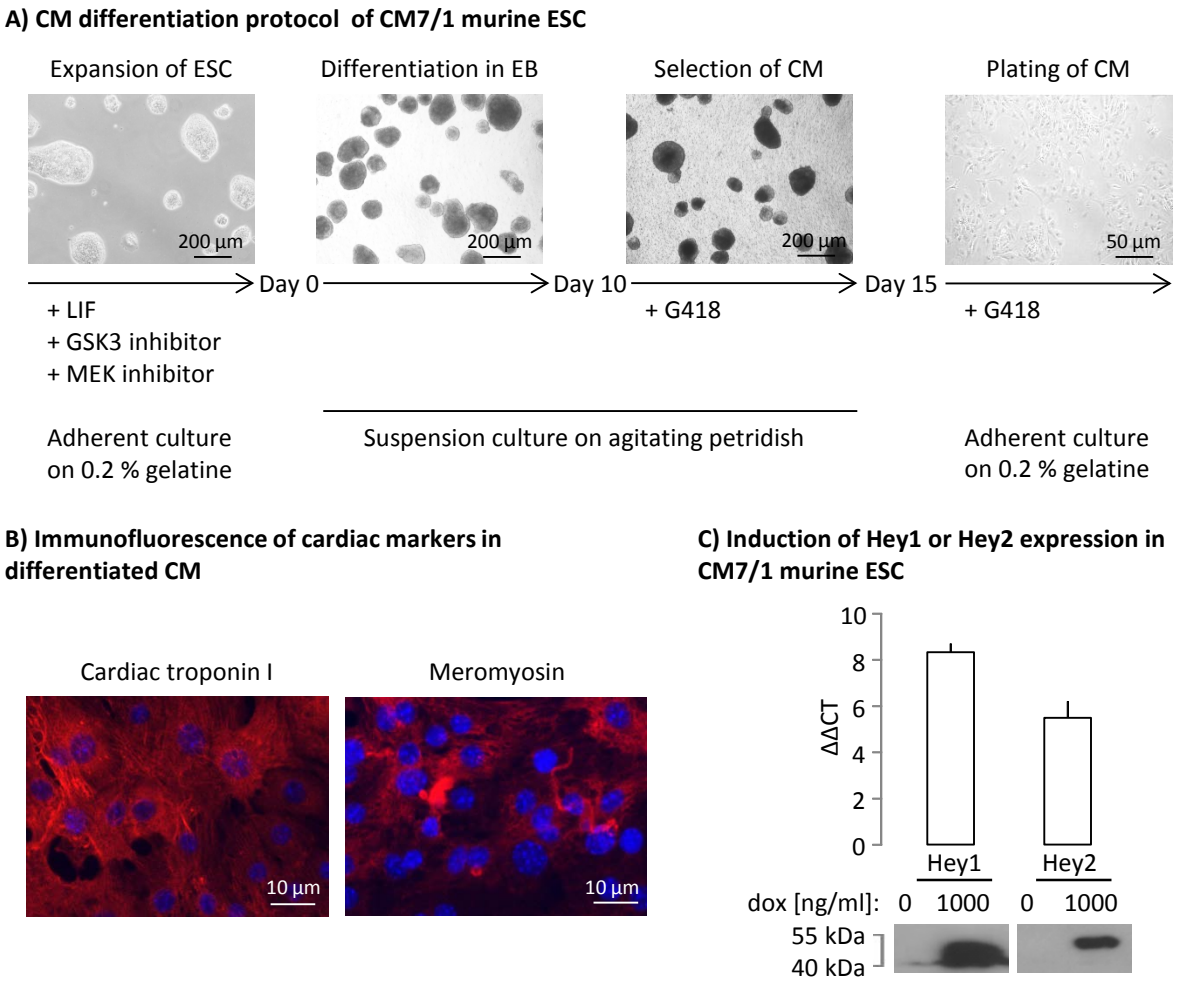


Figure 20: Efficient *in vitro* CM differentiation
(A) Schematic representation of the differentiation protocol of the murine ESC line CM7/1 into CM. Images were taking from the ES-Hey1 cell line. LIF = Leucemia inducing factor;
(B) Immunofluorescence shows positive staining for cardiac troponin I and meromyosin in *in vitro* differentiated CM.
(C) qRT-PCR results showing the induction of *Hey1* and *Hey2* on mRNA and α-flag Western blot demonstrating induction on protein level after induction with 1000 ng/µl doxycycline for 48 h in ESC.

ESC were maintained and expanded in ESC medium containing LIF, a GSK3 and a MEK inhibitor for at least 5 days before differentiation was started (figure 20A). During differentiation, cells were grown without these factors on shaking petri dishes. They formed EB and differentiated into all three germ layers. At day 10 the first EB started to contract spontaneously, indicating that CM were present. At

this stage, selection of CM was started by addition of G418 for 5 days. Many dead cells appeared in the medium and EB became roughly shaped due to dying cells without *MHC* promoter activity (figure 20A). At day 15 most EB were contracting. They were dissociated and plated as CM monolayer resulting in a very pure CM population. These CM stained positive for typical marker genes, like cardiac troponin I and meromyosin (figure 20B). It was possible to induce *Hey1* or *Hey2* expression in these cells via doxycycline (figure 20C).

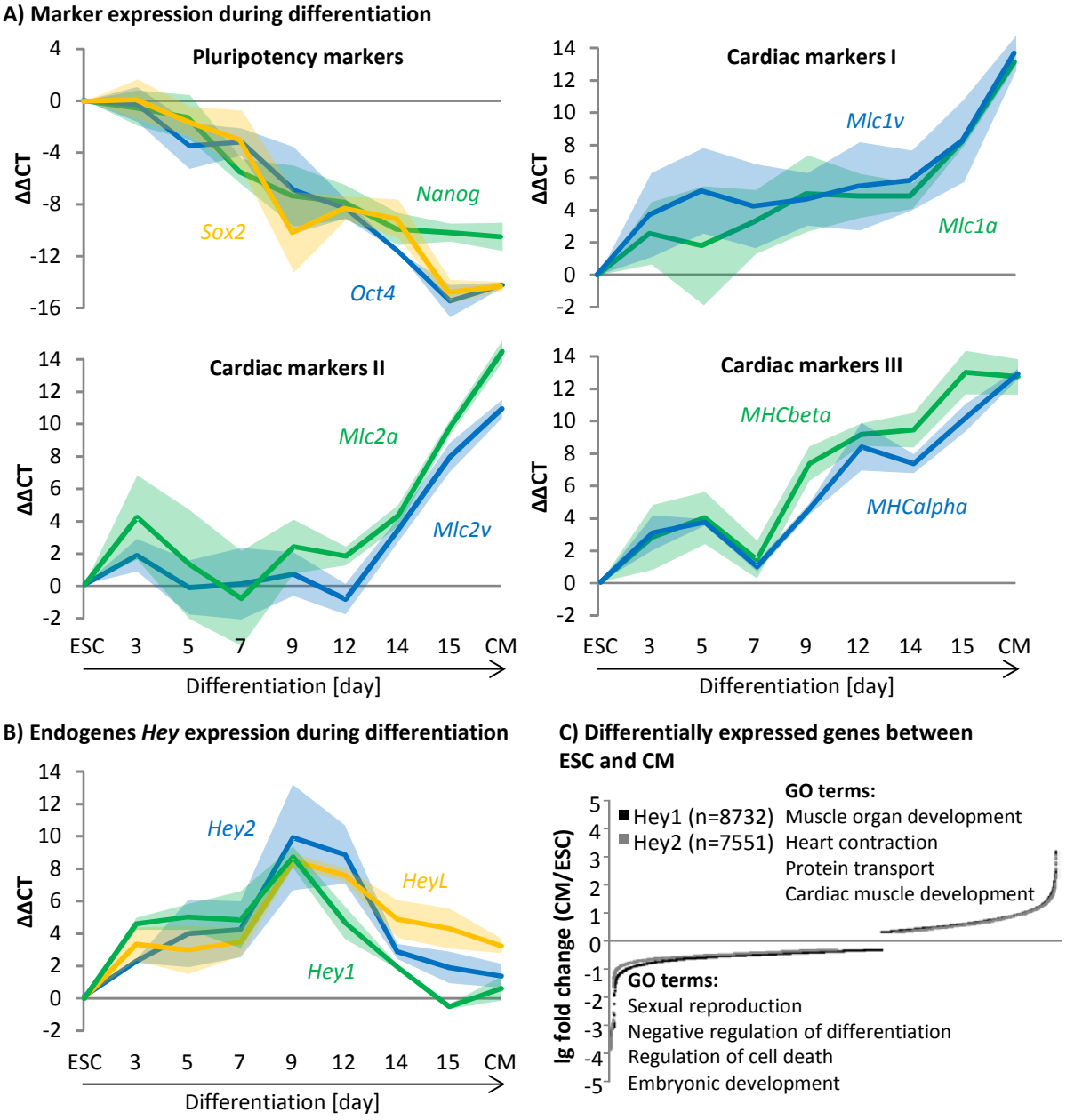


Figure 21: Expression of pluripotency, cardiac markers, and endogeneous Hey during differentiation
(A, B) RNA was isolated from non-induced ES-Hey1 cells before differentiation (ESC), at days 3, 5, 7, 9, 12, 14, 15 of differentiation and from differentiated CM from three independent differentiations. Lines show the mean change compared to ESC, standard deviations are shown as transparent fields surrounding the graphs.
C) lg fold change between non-induced *Hey1* or *Hey2* expressing ESC and differentiated CM for all more than two-fold regulated genes. They are sorted according to their regulation. Indicated are the four most enriched GO-terms for the 1000 most down- and up-regulated genes.

During differentiation, expression of the pluripotency markers *Sox2*, *Nanog* and *Oct4* was greatly reduced and typical cardiac markers were highly enriched like the ventricular and atrial variants of *myosin light chain 1* and *2* (*Mlc1*, *Mlc2*; figure 21A). A decline in stem cell markers was visible from day 3 onwards, the first time point measured after removement of LIF from the cell culture medium. Cardiac markers were highly enriched later during differentiation (after day 7/9; figure 21A), which coincides with the first contracting EB, observed at day 9. After that CM were enriched via selection with G418 and expression of cardiac markers was highly increased.

Endogenous *Hey* was expressed to a low, but detectable extend in the ESC (figure 21B). Expression increased during differentiation and peaked around day 9 and declined again. The decline coincided with the selection of CM via G418. In derived CM, *Hey* expression was only slightly elevated compared to ESC, with HeyL showing the highest elevation. All presented results were obtained from the *Hey1* expressing cell line (ES-Hey1), but similar results were also obtained for the *Hey2* expressing cell line (ES-Hey2; data not shown).

To further confirm the identity of the differentiated cells, gene expression between non-induced ESC and CM was compared. For the *Hey1* expressing cells, 8 732 genes were more than two-fold differently expressed between ESC and CM, while 7 551 genes were differently expressed for *Hey2* (figure 21C). Genes that were higher expressed in ESC are associated with GO terms like “negative regulation of differentiation” or “embryonic development”. Genes higher expressed in CM were associated with “muscle organ development” and “heart contraction”.

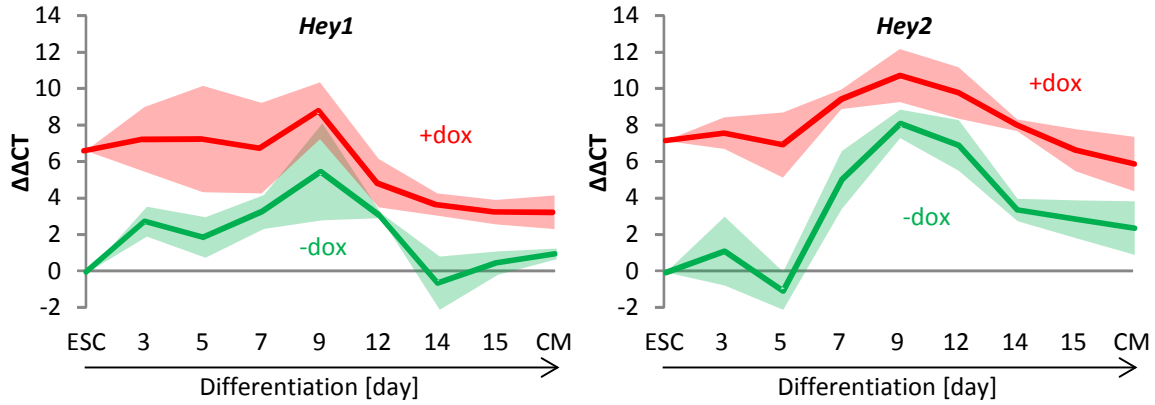


Figure 22: Total Hey level with and without induction during differentiation
RNA was isolated from non- and continuously induced (1 µg/ml dox from day -2 until cells were harvested) ES-Hey1 and ES-Hey2 cells before differentiation (ESC), at days 3, 5, 7, 9, 12, 14, 15 of differentiation and from differentiated CM from three independent differentiations. Lines show the mean change compared to non-induced ESC, standard deviations are shown as transparent fields surrounding the graphs.

It was also tested whether prolonged overexpression of *Hey1* and *Hey2* has an effect on differentiation. To this aim, cells were cultured for the entire time of differentiation with 1000 ng/µl doxycycline starting from two days before the differentiation (day -2). During the entire time *Hey1* or *Hey2* were

induced compared to endogenous levels (figure 22), but no differences in differentiation efficiency, cell morphology or marker expression (e.g. atrial vs. ventricular markers) were observed (supplementary figure 42).

5.2.2. Genome-wide Hey binding in murine ES cells and cardiomyocytes

The above presented *in vitro* differentiation system was used to identify Hey bound regions in ESC and differentiated CM by ChIPSeq experiments. Therefore, a comparable number of reads (13 – 19 million reads) was generated for each sample and on average 76 % of these could be mapped to the mm9 mouse genome (supplementary table 10 and 11). However, only 52 % of the CM Hey2 sample and 43 % of the CM Hey2 control reads could be mapped to the mm9 genome due to the presence of adapter dimers in the sequencing library.

54 613 and 31 014 DNA binding sites were identified for Hey1 and Hey2, respectively, within the ESC genome and a lower number of 17 874 and 20 498 binding sites within the CM genome (figure 23A). There is a large overlap between Hey1 and Hey2 binding sites in ESC (44 % compared to Hey1 and 78 % compared to Hey2) and in CM (73 % and 64 %). There is also a large overlap between ESC and CM, in total around 7 974 peaks are shared between Hey1 and Hey2 in both cell types (figure 23A).

The main difference between the samples is that for both Hey proteins more DNA binding sites were present in the ESC. This difference is also visible from scatterplots comparing the peak height of two samples (figure 23B). Here, the correlation between Hey1 and Hey2 in the same cell type is higher (0.92 and 0.93) than the correlation between ESC and CM (0.42 – 0.51). Focusing on Hey binding near TSS of annotated genes, revealed that Hey1 and Hey2 largely bind to the same genes, but that there are more Hey bound genes in ESC than in CM (figure 23C). Hey binding sites are mainly located within proximal promoters or the first exon or intron of genes due to their close proximity to TSS (figure 23D and E).

The observed redundancies between Hey1 and Hey2 and the genomic distribution of binding sites is also comparable to the results obtained for Hey1/2/L in HEK293 cells (figure 10A).

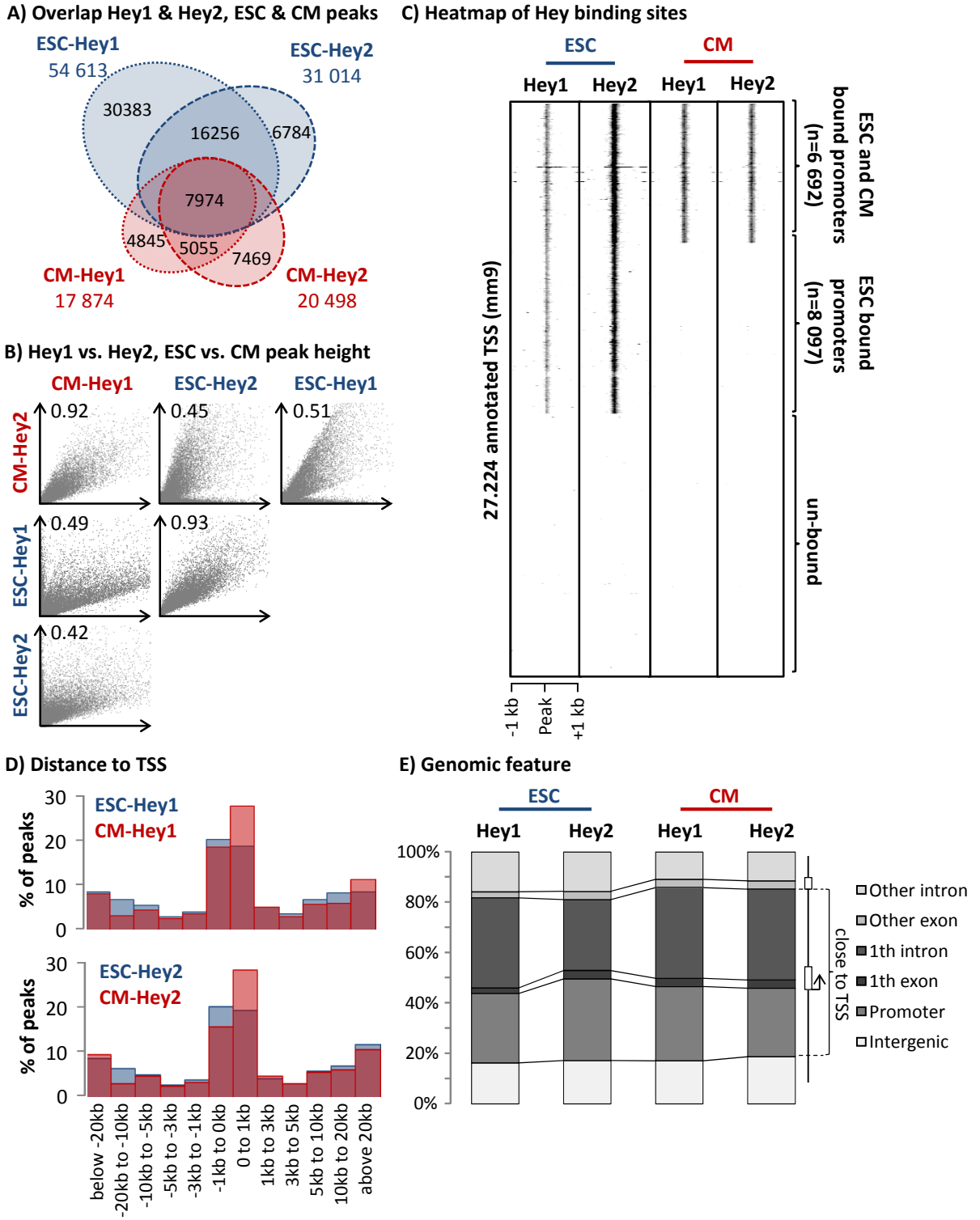


Figure 23: Hey1 and Hey2 have overlapping binding properties in murine ESC and CM

ChIPseq data for Hey1 and Hey2 was generated by low level doxycycline induction (50 ng/ml for 48 h) of ES-Hey1 and -Hey2 cells and differentiated CM, non-induced cells were used as control.

(A) Venn-diagram showing the overlap between all identified binding sites.

(B) Scatterplot showing the peak height in one sample plotted against the peak height in another sample at all TSS. The given numbers are the Pearson's product-moment coefficient.

(C) This heatmap represents all murine TSS as a stack. The ChIPseq signal is shown in a window of 1 kb up- and downstream of each TSS. The shading indicates the signal intensity. Promoters were clustered in three groups: ESC and CM bound, ESC bound, and unbound promoters.

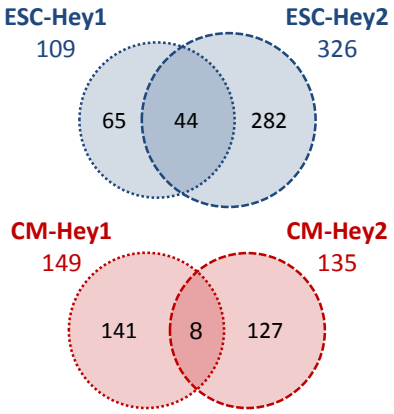
(D) The histograms show the percentage of peaks within a certain distance to the closest TSS.

(E) This diagram shows the percentage of peaks located within a certain genomic feature.

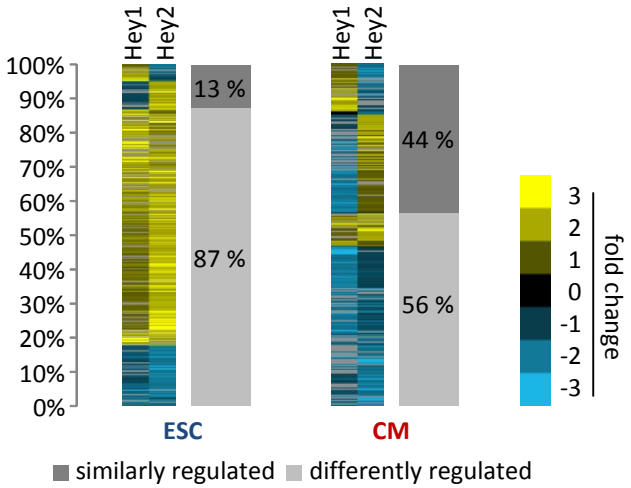
5.2.3. Hey gene regulation in ES cells and cardiomyocytes

In order to identify Hey regulated genes, microarray analysis was previously performed in ESC (Heisig, 2011) and RNAseq was performed in CM. In CM between 8 and 23 million reads were generated (supplementary table 12). In average 90 % of the reads were mapped to the mouse genome. We identified in total 660 genes, which are more than 1.8-fold regulated in at least one sample.

A) Overlap Hey regulation in ESC and CM



B) Percentage of similarly and differently regulated genes



C) Validation of Hey regulated genes

	ESC						CM				
	Microarray		qRT-PCR				RNAseq		qRT-PCR		
	Hey1	Hey2	Hey1	Hey2	Control		Hey1	Hey2	Hey1	Hey2	Control
Lefty1	-1.92	-3.35	**-2.52**	**-2.18**	1.22	*Mixl1*	-1.56	-2.72	**-1.85**	**-2.02**	-1.07
Zic2	-3.02	-2.08	**-2.02**	-1.23	1.17	*Sgcg*	-1.01	-2.48	**-2.47**	**-1.52**	-1.09
Id2	-1.57	-3.01	**-2.01**	**-1.53**	1.02	*Kcnip1*	-1.38	-2.25	-1.25	-1.42	1.12
Lefty2	-2.21	-2.84	**-3.15**	**-1.98**	1.11	*Agtrl1*	-1.58	-2.07	**-1.15**	**-1.98**	1.19
Tcf3	1.38	-2.67	1.09	1.20	1.06	*Enpp2*	-1.56	-2.10	**-2.09**	**-2.19**	1.19
Jag1	-1.94	-2.59	**-2.14**	**-1.86**	1.13	*Apln*		-2.01	1.30	-1.25	-1.25
Bcl11a	-2.17	-1.54	**-1.41**	**-1.44**	1.04	*Cxcr4*	-1.61	-2.00	**-1.50**	**-1.73**	-1.15
Sgcg	-1.37	-2.11	-1.23	-1.24	1.21	*Sema6d*	-1.52	-1.84	**-2.67**	**-2.83**	1.02
Zfhx3	-2.02	-2.06	-1.01	**-1.04**	-1.03	*CoupTF-II*	-1.11	2.22	1.39	1.20	-1.04
Calca	-1.47	-1.74	**-1.30**	**-1.76**	1.13	*Tgm1*		2.39	1.47	1.24	1.39
Sema3e	1.38	-2.00	-1.10	1.23	1.23	*Krt7*		2.45	1.45	**1.73**	-1.28
Plcb4	-1.07	3.29	1.17	1.27	1.24	*Rassf8*	1.13	3.33	-1.05	-1.06	-1.15
Dub1	14.59	1.43	1.23	1.26	-1.05						

Figure 24: Hey regulated genes in ESC and CM

ES-Hey1 and ES-Hey2 cells were induced with high levels of doxycycline (1 µg/ml for 48 h), and non-induced cells were used as control. In ESC gene expression was detected by microarray and in CM by RNAseq.
(A) Venn-diagram showing the overlap between Hey1 and Hey2 more than 1.8-fold regulated genes.
(B) Hierarchical clustered heatmap shows all genes that are more than 1.8-fold regulated in at least one sample. Given are the percentages of genes, which are similar or differently regulated by Hey1 and Hey2.
(C) Validation of selected target genes by qRT-PCR. Significantly regulated genes are printed in bold. As control cells without the inducible Hey constructs were also doxycycline treated and compared to untreated controls. All values are based on triplicates.

The number of Hey1 and Hey2 regulated genes in ESC and CM was comparable (figure 24A). Most genes were similarly regulated by Hey1 and Hey2 in ESC (87 %, figure 24B). In CM approximately half of the regulated genes were similarly regulated (56 %; figure 24B). This was despite the small overlap between the 1.8-fold Hey1 and Hey2 regulated genes per se (n = 44 in ESC and n = 8 in CM; figure 24A). However, many genes that were 1.8-fold regulated by only one Hey protein were similarly but to a lower extent regulated by the other Hey protein.

qRT-PCR confirmed most genes that were similarly regulated (14 out of 16 significant regulated), whereas confirmation of genes only regulated by either Hey1 or Hey2 was only possible to a much lower extent (2 out of 9; figure 24C). This was also true for the Hey up-regulated genes. Therefore, I focused my analysis on genes which were similarly regulated by Hey1 and Hey2 (n = 416), as they are more confident targets by using the results from Hey1 and Hey2 as "duplicates".

5.2.4. Differences in Hey gene regulation between ES cells and cardiomyocytes

My analysis focused on differences in Hey target gene regulation between ESC and CM. Therefore, I categorized all regulated genes in four main groups (figure 25): (1) genes up-regulated in both cell types, (2) genes down-regulated in CM, (3) genes down-regulated in ESC, and (4) genes down-regulated in both cell types. I used these four sets of genes in order to investigate, why some genes were only regulated in one cell type, despite most of the genes being similarly expressed in both cell types, whereas other genes were regulated in both cell types.

Among Hey regulated genes were many TF involved in various developmental processes, cell cycle, apoptosis, and proliferation (figure 25). The four clusters of regulated genes were quite similar in that regard. However, genes involved in cell migration were particular enriched within the cluster of ESC down-regulated genes, while genes involved in cell cycle were enriched within the cluster of CM down-regulated genes.

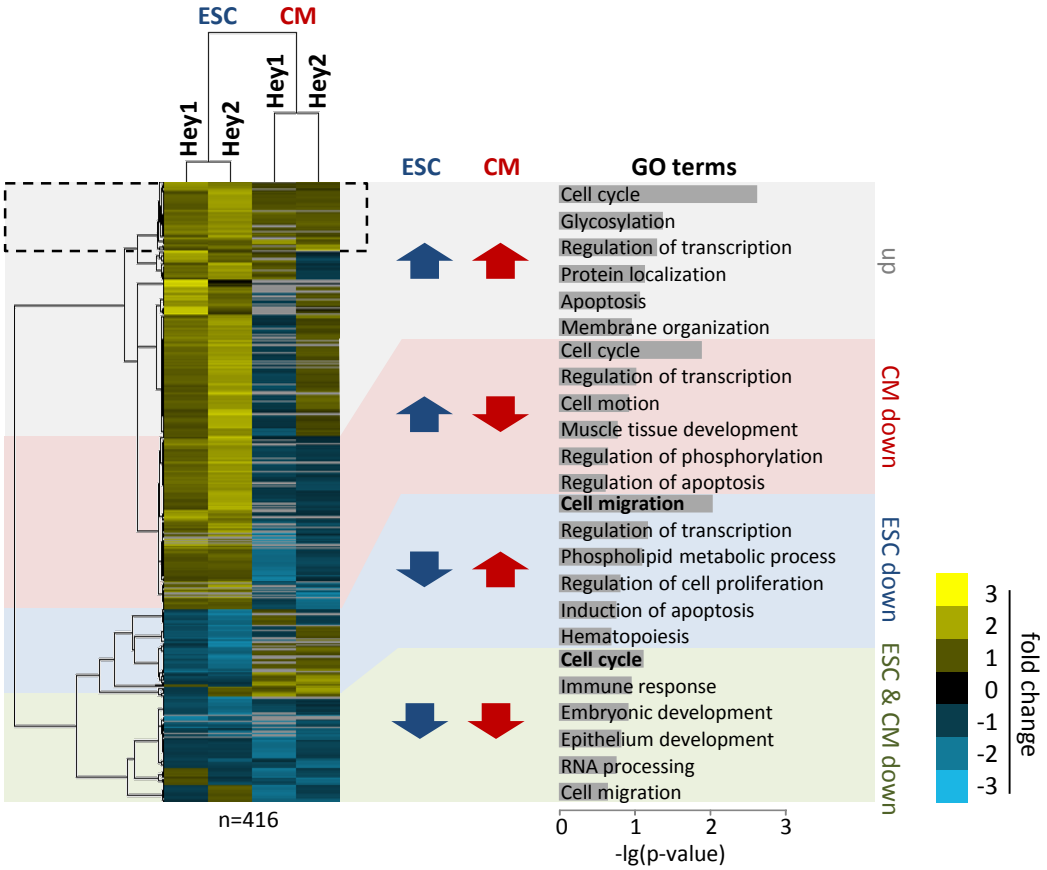


Figure 25: Differences between Hey regulated genes in ESC and CM
Hierarchical clustered heat map showing all genes, which are more than 1.8-fold regulated in at least one sample (ESC or CM) and similar regulated by Hey1 and Hey2. Genes are divided in four main groups. For each cluster the 6 most enriched GO terms are given (values > 1.3 are significant). The dashed box indicates a group of more concisely in CM up-regulated genes.

5.2.5. Correlation between gene regulation and Hey peak height

In order to see whether the observed regulations were the result of Hey promoter binding, I analyzed Hey binding sites within the regulated genes. For each cluster I calculated the average Hey binding peak height within the proximal promoter of the corresponding gene. In ESC the average Hey1 and even more so the Hey2 peak heights are higher in genes, which are down-regulated compared to up- or non-regulated genes (figure 26A). This was previously also observed for HEK293 cells, where up-regulated genes have less and lower Hey binding sites compared to down-regulated genes (figure 13). This indicates that there are less direct Hey targets among up-regulated genes.

When looking at CM, the same pattern can be observed as in ESC with up-regulated genes having lower peaks than down-regulated genes (figure 26A). Genes that are specifically down-regulated in CM have also increased Hey binding peaks within their promoters, which is a significant increase compared to ESC. This further supports the idea that whether a gene is down-regulated by Hey dependents on the Hey binding to its promoter. However, genes, which are only down-regulated in ESC, do not have

reduced binding peaks in CM. For these genes other mechanisms seem to prevent Hey repression in CM.

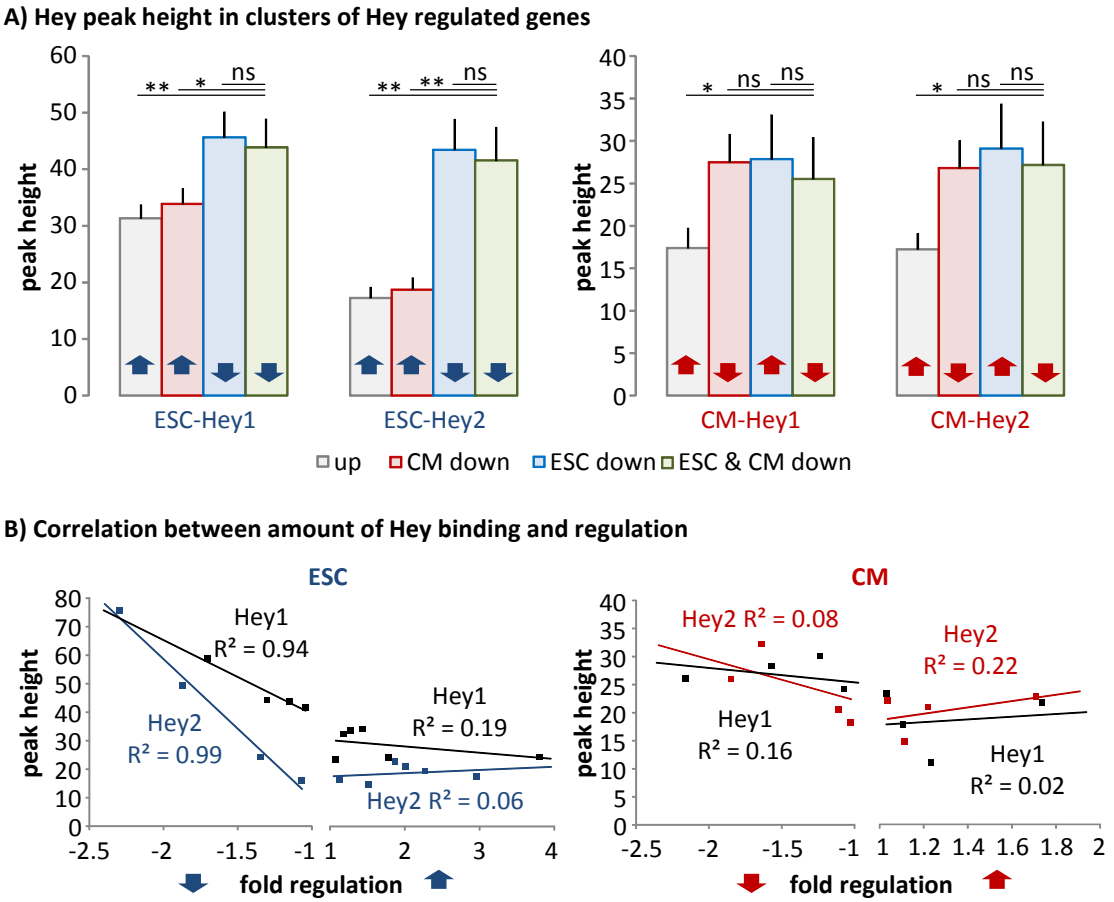


Figure 26: Average Hey binding signal in clusters of Hey regulated genes
A) For the four identified clusters of Hey regulated genes, the average Hey peak height at the proximal promoter (-1kb/+1kb) was calculated. For genes with several Hey binding peaks in their promoter the highest peak was considered. Peak height was normalized according to the 1000 highest peaks within each sample. A student's t-test was performed to identify significant differences (p-value ≤ 0.01 **; p-value ≤ 0.05 *; p-value > 0.05 ns).
B) The average Hey peak height and regulation was calculated for sets of 50 genes with similar regulation. The coefficient of determination (R^2) was calculated for a linear regression.

For the same set of genes the correlation between peak height and fold regulation was calculated, to find out if there is a quantitative relation between Hey binding and regulation. This is the case for the in ESC down-regulated genes (figure 26B). Here the correlation is extremely strong. The slope for the Hey2 regulated genes is steeper compared to Hey1, similar to the greater difference observed for Hey2 between the different groups of regulated genes (figure 26A). There is no robust correlation between Hey peak height and up-regulation.

In CM the correlation for both down- and up-regulated genes is poor (figure 26B). This indicates that the down-regulation in ESC is mainly the direct result of Hey binding, whereas in CM additional mechanisms influence down-regulation of target genes. Up-regulation is mainly the result of indirect effects in both cell types.

5.2.6. Investigation of possible Co-factors involved in Hey target gene regulation

In order to identify co-factors involved in the regulation of Hey target genes, over-represented binding motifs within the promoter regions of the regulated genes were identified. Then the expression data was used to identify binding factors, which are expressed in the used cellular system.

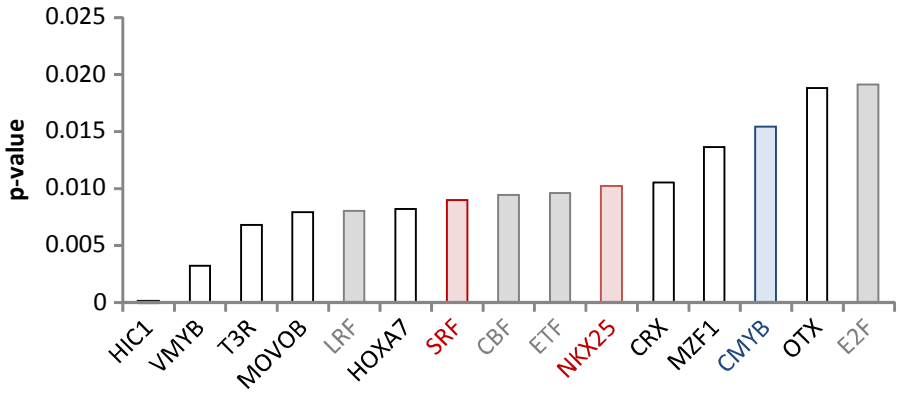


Figure 27: Enriched binding motifs in promoters of Hey regulated genes
Shown are the 15 most significantly enriched binding motifs within the promoters (-1kb/+1kb) of all Hey regulated genes. Colors indicate whether binding factors are expressed in CM (red), ESC (blue), both (grey) or not expressed (white).

Interestingly SRF and NKX25 binding motifs are overrepresented for all Hey regulated genes (figure 27). These motifs are bound by the serum response factor (Srf) and NK2 homeobox 5 (Nkx2-5). Both are cardiac specific TF, which are known to be involved in cardiac development, and are highly expressed in CM but not in ESC. In order to identify a possible correlation between Hey regulation and these factors, the average peak height for these TF in Hey regulated gene clusters was analyzed in a similar way as previously done for Hey binding. ChIPseq data for these factors and Gata4 previously generated in HL1 cells (murine CM cell line) was derived from publicly available data (He et al., 2011). There is no ChIPseq data for ESC available as these factors are not expressed in these cells.

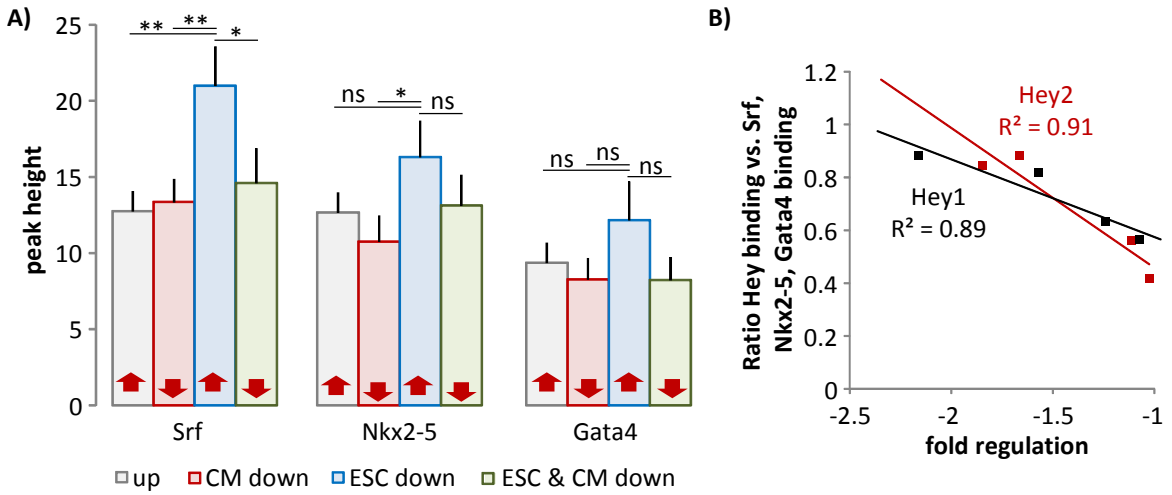


Figure 28: Average Srf, Nkx2-5 and Gata4 binding signal in clusters of Hey regulated genes
A) For the four identified clusters of Hey regulated genes, the average Srf, Nkx2-5 and Gata4 peak height in HL1 cells was calculated. For genes with several binding peaks the highest peak was considered. Peak height was

normalized according to the 1000 highest peaks within each sample. A student's t-test was performed to identify significant differences (p-value ≤ 0.01 **; p-value ≤ 0.05 *; p-value > 0.05 ns).
B) The average ratio between Hey peak height and the sum of Srf, Nkx2-5 and Gata4 peak heights was calculated for sets of 50 genes with similar regulation in CM. The coefficient of determination (R^2) was calculated for a linear regression.

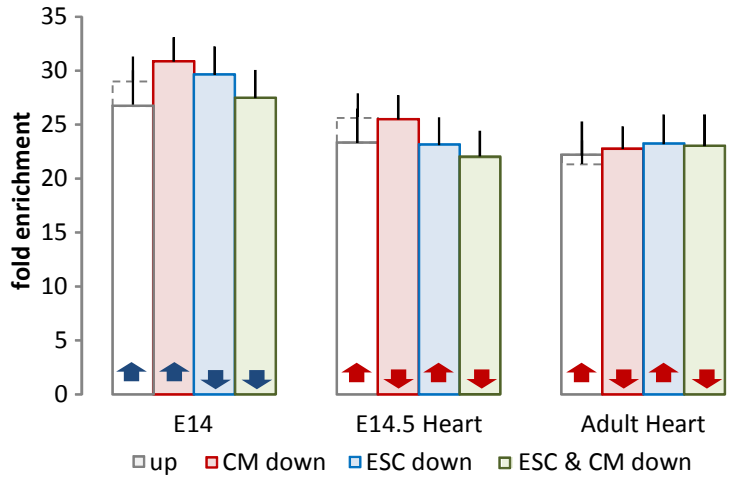
All three factors are enriched for genes that are down-regulated by Hey in ESC but not in CM: For Srf and Nkx2-5 this enrichment is significant (figure 28A). As these factors are not expressed in ESC, they cannot interfere with Hey regulation in these cells. However, as they are expressed in CM it appears as they could counteract possible down-regulation by Hey promoter binding. Genes that are repressed by Hey in CM are less bound by these factors.

There is a direct correlation between the ratio of Hey binding and binding of these factors with the observed down-regulation in CM (figure 28B). These results indicate that these three factors combined with Hey could play a role in achieving a cell type and gene-specific regulation of Hey target genes in a dose dependent manner.

5.2.7. Influence of Hey binding on histone acetylation

There are several histone markers known, which are associated with active promoters like histone methylation (H3K4me3) and histone acetylation (H3K27ac) (McLeay et al., 2012, Karlic et al., 2010). Therefore, public available histone modification data from the ENCODE project generated from murine ESC (E14 cell line), embryonic hearts (E14.5) and adult hearts was analyzed (ENCODE, 2011).

A) Average histone H3 lysine 4 methylation (H3K4me3) in clusters of Hey regulated genes



B) Average histone H3 lysine 27 acetylation (H3K27ac) in clusters of Hey regulated genes

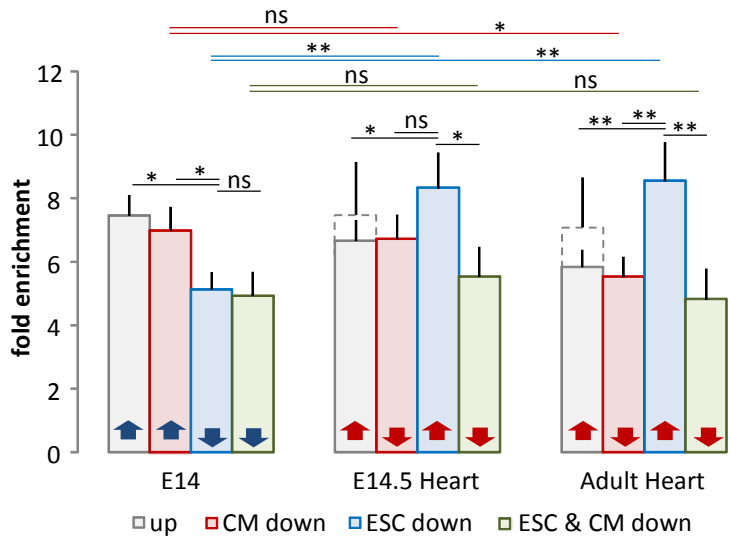


Figure 29: Average H3K4me3 and H3K27ac in clusters of Hey regulated genes
For the four identified clusters of Hey regulated genes, the average (A) H3K4me3 and (B) K3K27ac enrichment in E14 ESC, E14.5 embryonic hearts, and adult hearts at the promoter (-1 kb/+1kb) was calculated. In all cases the highest enrichment within the promoter was used. A student's t-test was performed to identify significant differences (p-value ≤ 0.01 **; p-value ≤ 0.05 *; p-value > 0.05 ns). For the up-regulated genes an additional more confident group including only genes which are up-regulated in CM was also analyzed. The results are indicated by dashed lines. For H3K4me3 no significant differences were observed.

For the H3K4me3 no significant differences could be identified between different clusters of Hey regulated genes or between cell types (figure 29A). However, for H3K27ac significant differences exist between the groups of Hey regulated genes: Genes which are down-regulated show a reduced promoter acetylation compared to up-regulated genes in ESC (figure 29B). This hints that Hey binding influences histone acetylation. Acetylation at promoters of genes, which are only down-regulated in ESC, is increased in murine hearts: in E14.5 embryonic hearts and even more in adult hearts. This higher acetylation correlates with elevated Srf, Gata4 and Nkx2-5 binding peaks for these genes (figure 28A).

Genes, which are only down-regulated in CM have a slightly lower acetylation in embryonic and adult hearts compared to ESC, but the effect is less pronounced. However, acetylation is much lower compared to the group of genes, which are only down-regulated in ESC. This is also true for genes, which are down-regulated in both cell types. For these genes there is no difference in acetylation between ESC and CM.

For the up-regulated genes acetylation is higher in ESC. In CM it appears similar to the down-regulated genes, which is not in agreement with the idea that Hey down-regulated genes have a reduced acetylation compared to up-regulated ones. However, among the cluster of up-regulated genes, there are many which are only consistently up-regulated in ESC, but not in CM (figure 25). This could cause a dilution of effects for this group in CM. To test whether this is the case, I reanalyzed the up-regulated genes, taking only genes into account that are consistently up-regulated in both cell types (dashed box in figure 25). For this group the average acetylation in ESC is nearly unchanged, but in embryonic and adult heart a higher average acetylation is observed (dashed box in figure 29B).

5.2.8. Diversity among Hey regulated genes

Hey down regulated genes usually have higher Hey binding peaks within their promoter regions and their promoters have less histone acetylation, which supports the idea, that Hey proteins recruit Histone deacetylases (Hdacs) to repress target genes. These affects ale may be overwritten in CM by binding of cardiac activators such as Srf, Nkx2-5 and Gata4, which have in average increased binding in genes only down-regulated in ESC. This would results in a higher level of histone acetylation in CM (as observed) and might prevent Hey mediated down-regulation. All these conclusions were drawn from focusing on average binding intensities for groups of regulated genes. However, there are many individual genes that behave differently from the average trend suggested. This can be seen in the heatmap of figure 30, showing regulation and binding signals for individual genes.

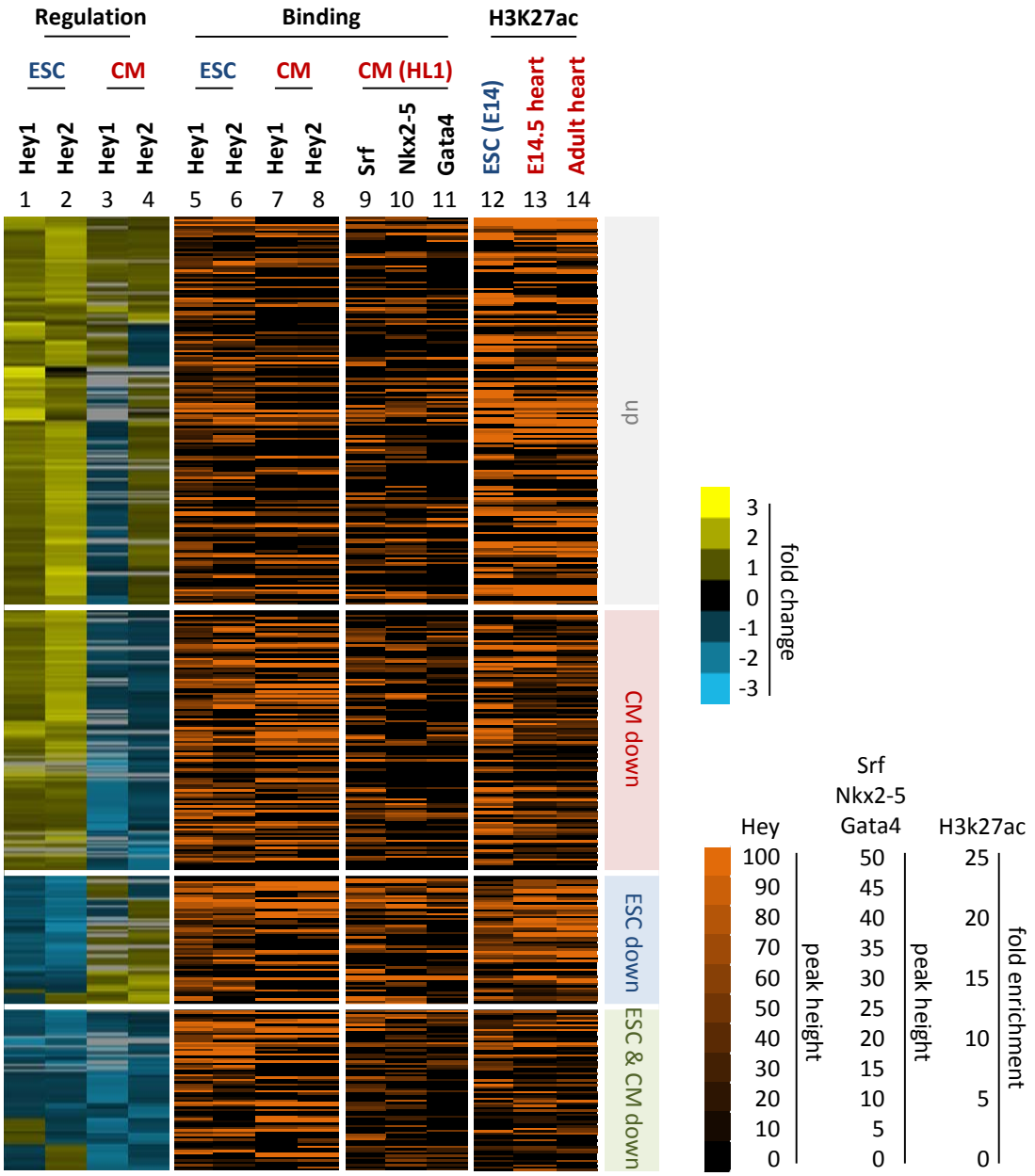


Figure 30: Heatmap showing Hey regulation, Hey, Srf and Nkx2-5 and Gata4 binding, and histone acetylation
Lanes 1 – 4 show the hierarchical clustered heat map of genes that were more than 1.8-fold regulated in at least one sample (ESC or CM) and similar regulated between Hey1 and Hey2. Genes are divided in four main groups. Lanes 5 – 8 show Hey binding to the promoters of these genes in ESC and CM. Lanes 9 – 11 show Srf, Nkx2-5 and Gata4 in HL1 CM and lanes 12 – 14 show H3K27ac in E14 ESC, in E14.5 murine embryonic hearts and in murine adult hearts.

Concentrating on the heatmap the same general trends as described previously can still be seen, but there is great diversity within the four groups for individual genes. Generally, higher and more Hey binding sites are coherent with down-regulation of genes, but there are many down-regulated genes, which do not have any Hey binding sites in their promoters (figure 30 lanes 5 – 8). Further examples would be the higher Srf, Nkx2-5 and Gata4 binding observed in CM (figure 30 lanes 9 – 11) as well as the histone acetylation in murine hearts (figure 30 lanes 13 – 14) for genes only down-regulated in ESC.

Apart from the cardiac activators analyzed here, we suppose additional factors and mechanism to be most likely involved, which also influence regulation of individual genes. This may result in a much more complex regulatory mechanism and gene and cell type specific regulation. However, the general trends remain visible, suggesting that the factors analyzed here might be the main contributor for the here observed regulation by Hey proteins.

5.2.9. Validation of Hey binding, promoter acetylation and target gene regulation

In order to confirm the previous findings, additional ChIP experiments for selected sample genes from each cluster were conducted. The selected genes are: *Krt7*, which is up-regulated, *Cxcr4*, which is down-regulated only in CM, *Zfhx3* and *Jag1*, which are down-regulated only in ESC and *Bcl11a*, which is down-regulated in both cell types.

5.2.9.1. *Krt7* - a Hey up-regulated gene

The profile for the up-regulated *Krt7* gene does not give much information, it does not reveal any Hey binding site and is therefore, like many up-regulated genes, no direct Hey target (figure 31A).

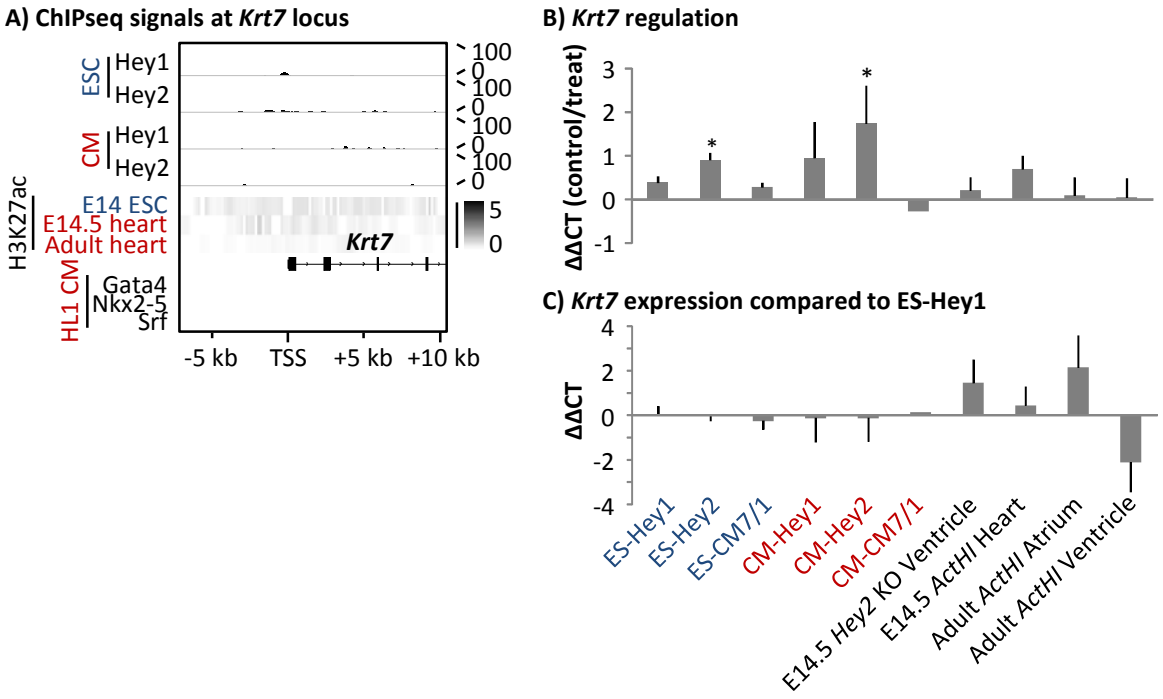


Figure 31: Validation of the Hey up-regulated *Krt7* gene
A) Given are the ChIPSeq profiles for ESC and CM. The peak height is normalized to the 1000 highest peaks within each sample. H3K27ac is shown as heatmap for E14 ESC, mouse E14.5 embryonic and adult hearts. Values scale from 0 (white) to 5 (black)
B) qRT-PCR results are derived from three independent biological samples (except CM-CM7/1 control – only one sample). The delta delta CT value to a non-induced or wild-type control is given. Error bars indicate the standard deviation. A student's t-test was performed to identify significant differences (p-value ≤ 0.01 **; p-value ≤ 0.05 *).
C) delta delta CT value of the non-induced or wild-type controls compared to ES-Hey1.

The promoter shows low levels of acetylation in ESC and CM, which are comparably. The up-regulation was confirmed by qRT-PCR in ESC and CM (figure 31B). It was not possible to confirm the observed regulation in murine hearts of *Hey2* KO or *Hey1* overexpressing mice. Only in E14.5 embryonic hearts a slight up-regulation was observed when *Hey1* was overexpressed (figure 31B).

5.2.9.2. Cxcr4 – a Hey cardiomyocyte specific down-regulated gene

The *Cxcr4* gene, which is down-regulated in CM, shows Hey binding peaks in ESC and CM (figure 32A). The promoter is highly acetylated especially in ESC around the Hey binding peaks, but acetylation is reduced in CM.

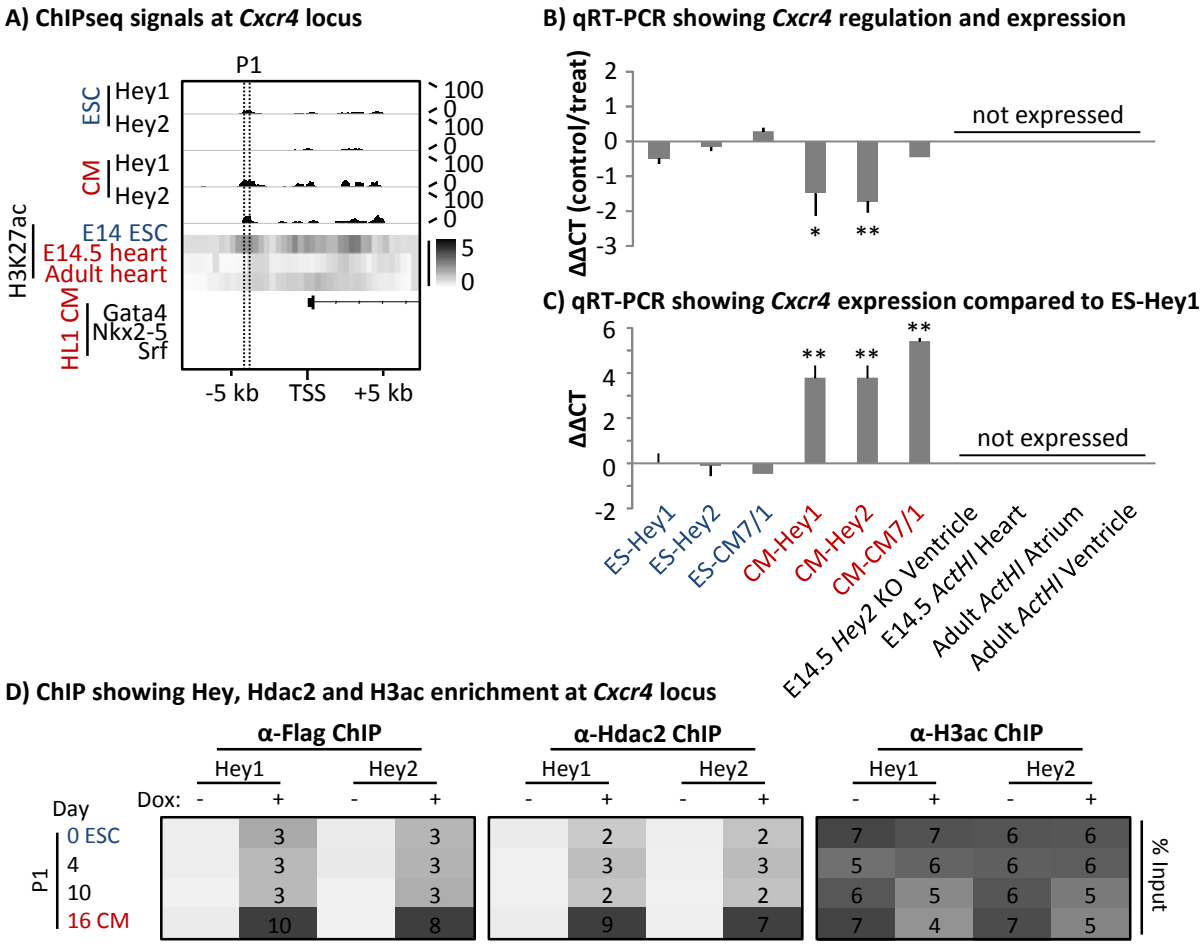


Figure 32: Validation of the in CM by Hey down-regulated gene *Cxcr4*

A) Given are the ChIPSeq profiles for ESC and CM. The peak height is normalized to the 1000 highest peaks within each sample. H3K27ac is shown as heatmap for E14 ESC, mouse E14.5 embryonic and adult hearts. Values scale from 0 (white) to 5 (black)

B) qRT-PCR results are derived from three independent biological samples (except CM-CM7/1 control – only one sample). The delta delta CT value to a non-induced or wild-type control is given. Error bars indicate the standard deviation. A student's t-test was performed to identify significant differences (p-value ≤ 0.01 **; p-value ≤ 0.05 *).

C) delta delta CT value of the non-induced or wild-type controls compared to ES-Hey1.

D) ChIP results from two replicates at four time points during CM differentiation for Hey1 and Hey2 over-expressing cells. Flag-ChIP was performed after 48 h of low level doxycycline induction (50 ng/µl). ChIP against Hdac2 and H3ac was performed after 48 h of high level induction at which regulation was observed (1 µg/ml). Values below 1 % are not shown.

The observed down-regulation was confirmed in CM (figure 32B). Here, base-line expression of *Cxcr4* is higher compared to ESC (figure 32C). Hey binding was proven by independent ChIP experiments. Hey is bound to the *Cxcr4* promoter in ESC and during differentiation and is increased in differentiated CM (figure 32D). Histone deacetylases (Hdacs) were previously shown to be implicated in Hey gene regulation (Iso et al., 2001b, Gould et al., 2009, Nakagawa et al., 2000, Takata and Ishikawa, 2003). Here, Hdac2 binding was assessed, because of its high expression in the used cells (rank 1 710 in ESC and 846 in CM). Other Hdacs were also expressed, but to a lower extent with *Hdac1* being the second highest expressed Hdac after *Hdac2* (rank 2 203 in ESC and in 3 265 in CM). At the *Cxcr4* locus, Hdac2 binding correlates with Hey1 binding and is also increased in CM (figure 32D).

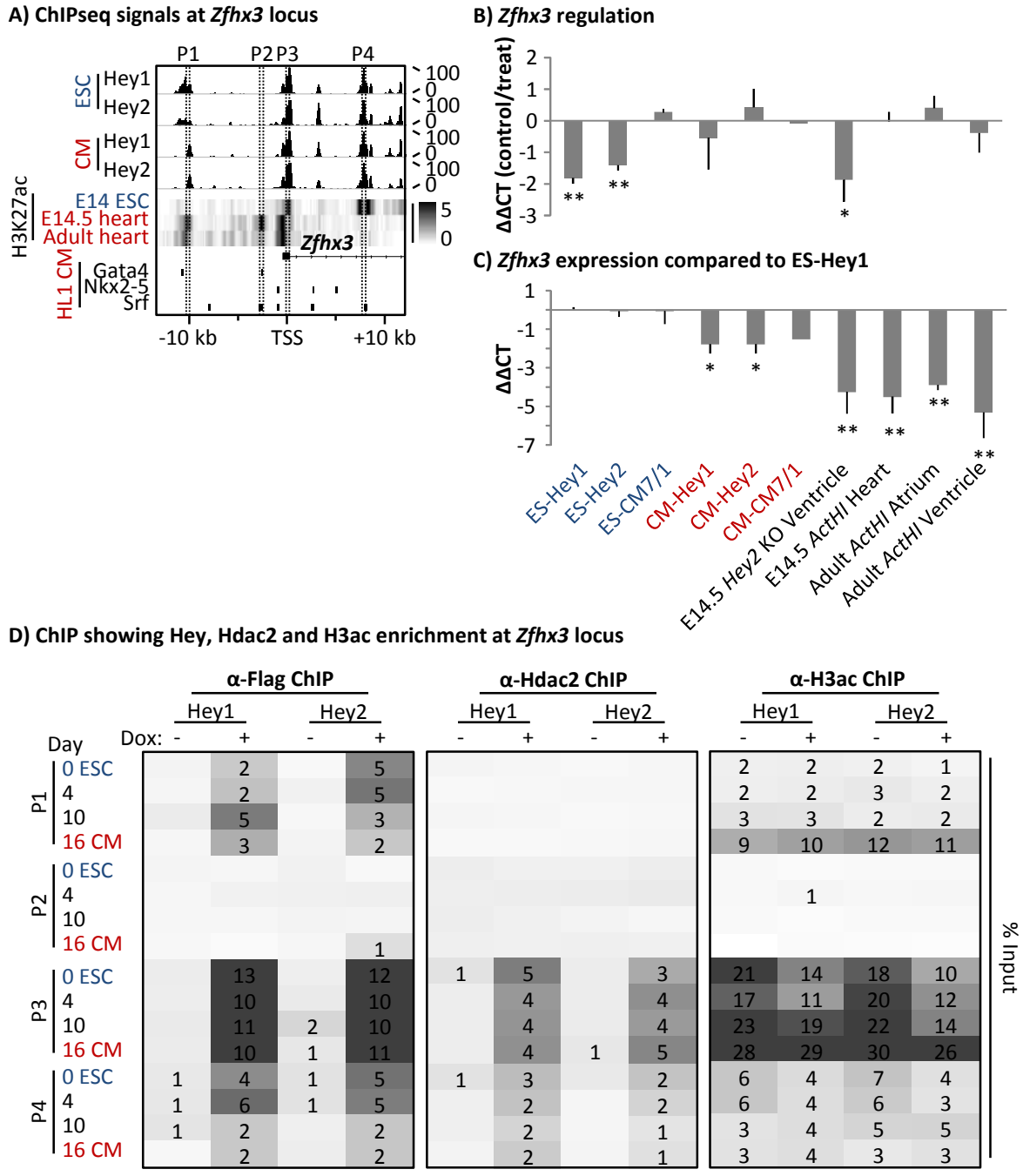
The overexpression of Hey has no effect on histone acetylation in ESC, but there is a small reduction in CM (figure 32D), which might be a direct result of more Hey binding and Hdac recruitment. The increased Hey binding in CM might be caused by the higher baseline expression of *Cxcr4* (figure 32C), as this might lead to more open chromatin, making the *Cxcr4* promoter more accessible for binding of TF. However, genes, which are only repressed in CM, shown no mean increase in expression.

5.2.9.3. Zfhx3 and Jag1 – Hey ES cell specific down-regulated genes

Zfhx3 and *Jag1* are both examples for genes, which are only down-regulated in ESC. Nevertheless both genes display very different Hey binding patterns. *Zfhx3* shows a very similar Hey binding profile in ESC and CM (figure 33A), whereas *Jag1* binding is nearly abolished in CM (figure 34A).

Zfhx3 down-regulation was confirmed for ESC, but it was also possible to confirm this regulation in embryonic hearts of *Hey1* overexpressing mice (figure 33B). However, *Zfhx3* is not down-regulated in the differentiated CM. Base-line expression is reduced in CM and even stronger reduced in murine hearts (figure 33C).

The ChIPseq results show no differences in Hey binding between ESC and CM (figure 33A). The extent of acetylation, however, is lower in ESC (max enrichment 4.4) compared to CM (max enrichment E14.5 7.7/ adult 8.0). Here, acetylation correlates with down-regulation, in contrast to Hey binding. This is likely the case for most genes of this cluster. For *Zfhx3* the acetylation sites seem to change, whereas they correlate with the Hey binding sites near the TSS and downstream of it in ESC, they are located near Hey binding sites upstream of the promoter that are also occupied by Gata4, Srf and Nkx2-5 in CM (figure 33A). This indicates that these factors might counteract the deacetylating effects of Hey via acetylation at specific sites.



D) ChIP showing Hey, Hdac2 and H3ac enrichment at *Zfhx3* locus

		α-Flag ChIP				α-Hdac2 ChIP				α-H3ac ChIP			
		Hey1		Hey2		Hey1		Hey2		Hey1		Hey2	
Day	Dox:	-	+	-	+	-	+	-	+	-	+	-	+
P1	0 ESC		2		5					2	2	2	1
	4		2		5					2	2	3	2
	10		5		3					3	3	2	2
	16 CM		3		2					9	10	12	11
P2	0 ESC												
	4										1		
	10												
	16 CM				1								
P3	0 ESC		13		12	1	5		3	21	14	18	10
	4		10		10		4		4	17	11	20	12
	10		11	2	10		4		4	23	19	22	14
	16 CM		10	1	11		4	1	5	28	29	30	26
P4	0 ESC	1	4	1	5	1	3		2	6	4	7	4
	4	1	6	1	5		2		2	6	4	6	3
	10	1	2		2		2		1	3	4	5	5
	16 CM		2		2		2		1	3	4	3	3

% Input

Figure 33: Validation of the in ESC by Hey down-regulated gene *Zfhx3*

A) Given are the ChIPSeq profiles for ESC and CM. The peak height is normalized to the 1000 highest peaks within each sample. H3K27ac is shown as heatmap for E14 ESC, mouse E14.5 embryonic and adult hearts. Values scale from 0 (white) to 5 (black)

B) qRT-PCR results are derived from three independent biological samples (except CM-CM7/1 control – only one sample). The delta delta CT value to a non-induced or wild-type control is given. Error bars indicate the standard deviation. A student's t-test was performed to identify significant differences (p-value ≤ 0.01 **; p-value ≤ 0.05 *).

C) delta delta CT value of the non-induced or wild-type controls compared to ES-Hey1.

D) ChIP results from two replicates at four time points during CM differentiation for Hey1 and Hey2 over-expressing cells. Flag-ChIP was performed after 48 h of low level doxycycline induction (50 ng/µl). ChIP against Hdac2 and H3ac was performed after 48 h of high level induction at which regulation was observed (1 µg/ml). Values below 1 % are not shown.

The binding site P1 shows a low level of Hey and Hdac2 binding independent of the differentiation status (figure 33D). Acetylation at this site is not affected by *Hey* overexpression, but is increased in CM, possibly as a result of Gata4, Srf, and/or Nkx2-5 binding. P2 shows no Hey or Hdac2 binding or acetylation and functions as a negative control. Biding sites P3 displays a high level of Hey and Hdac2 binding during all stages of differentiation. Induction of *Hey* results in a reduced acetylation at this site in ESC. This Hey dependent reduction is gradually abolished starting from day 10 of differentiation. The site becomes strongly acetylated in CM independent of *Hey* overexpression, possibly again as a result of Gata4, Srf, and/or Nkx2-5 binding. P4 shows a reduction in Hey and Hdac2 binding during differentiation. Here, Hey dependent deacetylation was observed in ESC and at day 4 of differentiation, but not at later time points.

Another example for a gene only down-regulated in ESC is *Jag1* (figure 34B). However, here the ChIPseq data shows a dramatic difference in Hey binding between ESC and CM. Hey binding appears to be completely abolished in CM (figure 34A), although the gene remains comparably strong expressed (figure 34C). Therefore, the loss of Hey binding is not simply a result of the promoter becoming inaccessible due to condensed chromatin. Loss of Hey binding was confirmed by individual ChIP experiments. Hdac2 binding correlates with Hey binding and the reduction in acetylation (figure 34D). Like *Zfhx3* the *Jag1* promoter has several previously identified Gata4, Srf and/or Nkx2-5 binding sites (figure 34A).

Jag1 serves as an example for a gene, which behaves different from most genes within the group of genes only repressed in ESC. This points towards the existence of several different mechanisms for different genes, which may involve additional factors, that prevent Hey binding in CM e.g. at the *Jag1* promoter.

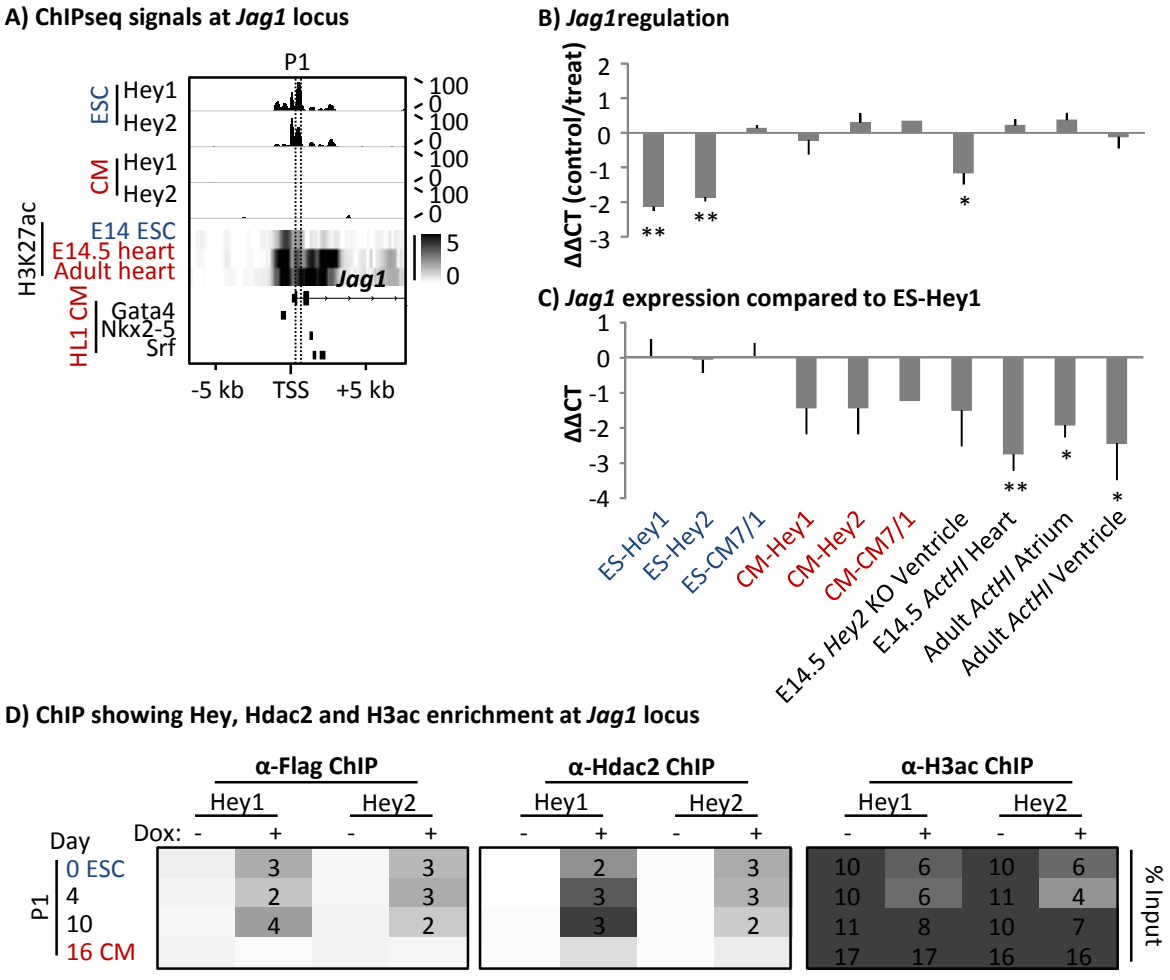


Figure 34: Validation of the in ESC by Hey down-regulated gene *Jag1*

A) Given are the ChIPSeq profiles for ESC and CM. The peak height is normalized to the 1000 highest peaks within each sample. H3K27ac is shown as heatmap for E14 ESC, mouse E14.5 embryonic and adult hearts. Values scale from 0 (white) to 5 (black)

B) qRT-PCR results are derived from three independent biological samples (except CM-CM7/1 control – only one sample). The delta delta CT value to a non-induced or wild-type control is given. Error bars indicate the standard deviation. A student's t-test was performed to identify significant differences (p-value ≤ 0.01 **; p-value ≤ 0.05 *).

C) delta delta CT value of the non-induced or wild-type controls compared to ES-Hey1.

D) ChIP results from two replicates at four time points during CM differentiation for Hey1 and Hey2 over-expressing cells. Flag-ChIP was performed after 48 h of low level doxycycline induction (50 ng/µl). ChIP against Hdac2 and H3ac was performed after 48 h of high level induction at which regulation was observed (1 µg/ml). Values below 1 % are not shown.

5.2.9.4. Bcl11a – a Hey down-regulated genes in ES cells and cardiomyocytes

Bcl11a is an example for a gene, which is down-regulated by Hey in ESC and CM. However, this gene shows no down-regulation in the analyzed murine hearts (figure 35B), indicating that *in vivo* regulation might be different or limited to a subset of cells.

For all time points during differentiation Hey binding is observed, as well as Hdac2 binding and histone deacetylation when Hey is overexpressed (figure 35D). The promoter has no previously identified Gata4, Srf or Nkx2-5 binding sites (figure 35A).

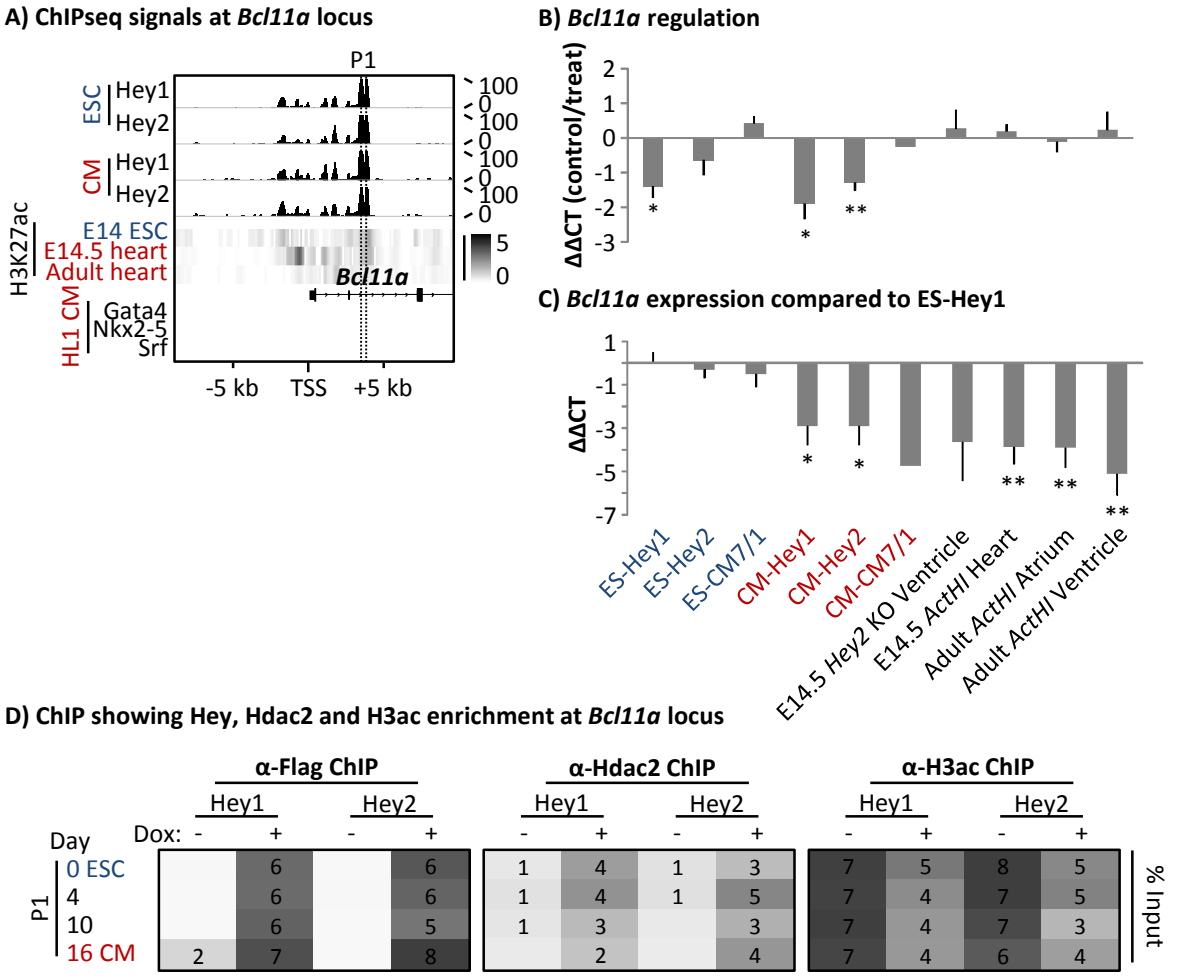


Figure 35: Validation of the Hey down-regulated gene *Bcl11a*

A) Given are the ChIPSeq profiles for ESC and CM. The peak height is normalized to the 1000 highest peaks within each sample. H3K27ac is shown as heatmap for E14 ESC, mouse E14.5 embryonic and adult hearts. Values scale from 0 (white) to 5 (black)

B) qRT-PCR results are derived from three independent biological samples (except CM-CM7/1 control – only one sample). The delta delta CT value to a non-induced or wild-type control is given. Error bars indicate the standard deviation. A student's t-test was performed to identify significant differences (p-value ≤ 0.01 **; p-value ≤ 0.05 *).

C) delta delta CT value of the non-induced or wild-type controls compared to ES-Hey1.

D) ChIP results from two replicates at four time points during CM differentiation for Hey1 and Hey2 over-expressing cells. Flag-ChIP was performed after 48 h of low level doxycycline induction (50 ng/µl). ChIP against Hdac2 and H3ac was performed after 48 h of high level induction at which regulation was observed (1 µg/ml). Values below 1 % are not shown.

6. Discussion

The strong phenotypes of *Hey* KO mice raise the question whether these are due to misregulation of Hey target genes that mediate the effects in various cell types. To gain insight into the regulatory potential of Hey proteins we employed an *in vitro* system with inducible expression of Hey proteins. Initially, HEK293 cells were used as a cellular model system as they are easy to manipulate and express endogenous *Hey* proteins suggesting that they can react appropriately to altered Hey protein levels. Additionally, ESC, which can be differentiated into CM, were used as these cells are more relevant especially for the observed cardiac phenotypes that result partially from loss of *Hey1* and/or *Hey2* expression in atrial and ventricular CM.

6.1. Hey proteins are redundant modulators of gene expression

Previously, Hey proteins have been described mainly as repressors for a small number of individual target genes identified coincidentally by separate approaches (Fischer and Gessler, 2007). To identify a wider spectrum of regulated genes we used HEK293, ESC and CM with *Hey* overexpression. In all three cell types regulated genes overlap between Hey1 and Hey2, although with varying extend of regulation. For the more divergent family member HeyL more differences in target gene regulation were observed than between Hey1 and Hey2, but this might be partially due to methodical differences (microarray and RNAseq). However, it was possible to confirm divergent regulation by HeyL for some genes by qRT-PCR in HEK293 cells. Despite these differences, there is still a large overlap in regulation between HeyL and the other two Hey proteins. This redundancy is likely the result of dimerization between different Hey proteins (Iso et al., 2001, Leimeister et al., 2000, Fischer et al., 2007), when overexpressed tagged Hey1, Hey2 or HeyL proteins interact with endogenous Hey proteins.

Surprisingly, the observed level of regulation of Hey proteins was rather limited in all analyzed cell types. Among the most down-regulated genes are *Lefty1* and *Lefty2* in ESC and *Sema6d* in CM (up to 3 fold). In HEK293 cells the most down-regulated gene is the endogenous *Hey1* (up to 5 fold), indicative of a negative feedback loop. Also for *Hey2* and *HeyL* repression was seen, but to a lesser extent. The negative feedback on the *Hey1* promoter was also confirmed by luciferase reporter assay. Similar negative feedback loops for *Hes1* and *Hes7* have previously been shown to be essential in somitogenesis and neural stem cell biology (Kageyama et al., 2010). During somite formation several components of the Notch signaling system are expressed in a cyclical fashion (Jouve et al., 2000, Bessho et al., 2001). This cycling is the result of expression and subsequent auto-repression. However, whether Hey proteins are also expressed in a cycling fashion is unclear.

The generally modest expression changes suggest that Hey proteins modulate expression of already active target genes rather than completely switch expression states. This is also reflected in the

chromatin signature of corresponding promoters. There is a striking overlap in Hey binding sites with the presence of polymerase II and the active chromatin mark H3K4me3, arguing that Hey proteins modulate expression of active genes rather than switching expression on or off.

However, low endogenous expression of Hey proteins may already affect target genes that can only be enhanced to a limited extent by further overexpression. Recently, it was shown that *Hey2* overexpression did not affect cardiac differentiation, whereas knockdown of *Hey2* led to a modest effect on *Anf* expression (Hartman et al., 2014). However, the authors only analyzed few individual target genes, so the entire effect of *Hey2* overexpression and knockdown remains unclear.

6.2. Hey proteins bind redundantly to target sites

We found a similar number of up- and down-regulated genes upon induction of Hey proteins, which was surprising as Hey proteins have previously been described as repressors. The observed regulation could be either due to direct or indirect effects on target promoters. To distinguish these, we conducted ChIP experiments to identify Hey binding sites within the genomes of the analyzed cell types. For these experiments we relied on a rather limited overexpression of Hey proteins in order to mimic a physiological situation. Nonetheless, a very large number of binding sites was identified for Hey1, Hey2 and even more so for HeyL.

In differentiated cells such as the analyzed HEK293 and CM, the number of Hey1 and Hey2 binding sites is lower compared to undifferentiated ESC. ESC contain larger stretches of open chromatin, therefore more binding sites are accessible for TF. Differences between Hey1 and Hey2 are predominantly restricted to less enriched target sites. This translates to a Pearson's correlation of r = 0.75 in HEK293 cells and r = 0.93/0.92 in ESC and CM, which is in the same range of values obtained for biological replicas (r = 0.83) in other studies (Ho et al., 2011). This shows that Hey1 and Hey2 largely bind the same targets independent of the analyzed cell type.

For HeyL a much larger number of binding sites was identified in HEK293 cells. This finding was surprising considering that despite HeyL being more divergent than Hey1 and Hey2, DNA binding domains are largely conserved between all three proteins. However, the vast majority of Hey1 and Hey2 binding sites is also bound by HeyL in HEK293 cells.

There is a huge discrepancy between the large number of ChIP peaks and the much smaller number of regulated genes for all three Hey proteins. This indicates that the vast majority of binding sites may not contribute to gene regulation, although endogenous Hey proteins may have already exhausted the regulatory potential of some of these sites. On the other hand, an overabundance of bound DNA sequences has also been observed for other TF e.g. Gata proteins and other HLH factors e.g. MyoD (He

et al., 2011, Cao et al., 2010). For functionally active binding sites additional modifications or concomitant binding of additional factors might be required.

6.3. Hey proteins act as direct repressors on target promoters

Binding sites shared between all three Hey proteins in HEK293 cells as well as Hey1 and Hey2 binding sites in ESC and CM are located near TSS. Considering these sites, the majority of Hey repressed genes are Hey targets, whereas up-regulation is mainly due to indirect effects as these genes do not have more Hey binding sites than other expressed but non-regulated genes. There is even a quantitative correlation between down-regulation and the amount of Hey binding in ESC and also in CM, when considering additional factors. Such a correlation was not found for up-regulated genes, which suggests that Hey expression leads to a repression of transcriptional activators for these genes.

Direct repression of target promoters could also be verified *in vitro* by luciferase reporter assays. Up to 10-fold repression of target promoters by Hey1/2/L was previously demonstrated for e.g. Gata4/6 promoters (Fischer et al., 2005). The here presented experiments provide additional proof for Hey function as direct repressors. The conserved bHLH and Orange domains can be turned into an activator of transcription when fused to the strong vp16 activation domain. Furthermore, mutation of three putative DNA binding arginine residues into lysine completely abolished promoter repression in luciferase assays, which clearly shows that direct binding of Hey proteins is required to affect target gene expression. This data clearly establishes Hey proteins as direct DNA binding transcriptional repressors.

6.4. Hey proteins preferentially bind to E-box motifs

Previously, a putative DNA binding motif tggCACGYGcca for Hey proteins has been identified by *in vitro* oligonucleotide selection (Fischer et al., 2002). However, the consensus E-box site CACGYG was either not present in target promoters analyzed previously, or deletion of related E-box sites did not alter expression of luciferase reporter constructs (Fischer and Gessler, 2007). Therefore, it was proposed that Hey functions indirectly, not via direct DNA binding.

When applying a de novo motif discovery algorithm, it was possible to extract a corresponding E-box motif CACGYG from the most highly enriched shared Hey binding sites. The second most enriched motif GCGCGC does not resemble typical E-box properties. There is a positive correlation between motif occurrence rate of these motifs and Hey peak height, which indicates that the presence of an E-box binding motif aids in Hey binding.

Our *in vitro* data from EMSA assays shows that CACGTG is the preferred binding site of recombinant Hey1 protein. Related sequences are also bound but to lesser extent. Additionally, mutation of an E-box site within the *Jag1* promoter abolished Hey regulation indicating that *in vitro* this site is required for Hey binding. However, even when considering only the most highly enriched binding sites identified in HEK293 cells, less than half of these contain CACGYG motifs. This suggests that Hey proteins may either use less stringent criteria for DNA binding *in vivo* or they might also bind in a manner that does not fully rely on sequence specificity as suggested for other HLH factors (Perna et al., 2011). Another explanation would be that certain sites are not directly bound by Hey proteins themselves but rather via TF that interact with Hey proteins via the HLH domain.

6.5. Additional HeyL binding sites

The most striking difference between all three Hey proteins in the here analyzed data sets are the many additional binding sites identified for HeyL. We considered that factors like the more efficient overexpression of HeyL in HEK293 cells and differences in methodology might be the cause. However, we could show at least for some of the additional bound sites that even much higher induction of *Hey1* or *Hey2* does not lead to a comparable enrichment as observed for HeyL at lower induction. Additionally, using different Hey1-HeyL fusion constructs, we could show that binding to these additional sites is lost when the C-terminal domain of HeyL is replaced with the matching part of the Hey1 proteins. These findings argue against methodical reasons.

The additional only by HeyL bound sites do not localize to TSS and are more scattered across the genome. HeyL binds these sites with a lower affinity compared to shared sites. The reason might be that sites are not enriched for an E-box motif, for which a positive correlation in peak height and motif concurrency was observed for the shared binding sites. Additional HeyL sites are also bound by CTCF and it was possible to recover GC rich motifs similar to the CTCF binding motif from these. Due to these differences in binding motif, we considered that HeyL does not directly bind to these sites, but rather interacts via its C-terminal domain with other TF that do not interact with Hey1 or Hey2. However, when we introduced the same mutation, which abolished target gene regulation and DNA binding for Hey1, not only binding to the shared sites, but also binding to the additional sites was lost. This demonstrates that they are also directly bound by HeyL. However, binding of additional sites might still depend on other factors that do not interact with Hey1 or Hey2.

Although there are genes which are differently regulated by HeyL, it is unclear whether this is related to HeyL binding of additional sites. Due to the lower number of regulated genes and scattering of numerous additional HeyL sites, it is not obvious to assign these to certain genes. Nonetheless, when assigning them to the closest TSS there are no differences in peak height between genes that are

differently regulated by HeyL and genes with similar regulation by all three Hey proteins. Therefore, the functional role of these sites remains largely elusive. In order to follow these up, it would be interesting to study whether there are additional interaction partners, which HeyL does not share with Hey1 or Hey2, as up to now none are known. Additionally, the striking overlap to CTCF binding sites still remains to be elucidated. CTCF was shown to direct the 3D organization of the genome within the nucleus and to insulate enhancer elements (Phillips and Corces, 2009). However, Hey proteins have not been implicated in these processes so far.

6.6. Hey factors regulate various developmental factors

Overrepresented groups of GO terms for Hey regulated and bound genes include many other TF that are involved in various developmental processes including mesodermal and neuronal development in HEK293 cells, embryonic and cardiovascular development in ESC and CM and other developmental terms. This fits to the cardiovascular phenotypes observed in *Hey* KO mice and is also in line with the prototypic function of Enhancer of split genes in Drosophila that act on downstream neurogenic TF to control cell fate decision and differentiation (Fisher and Caudy, 1998). Additionally target genes are also involved in regulation of cell cycle and apoptosis.

There are cell type specific differences. Regulation of neuronal genes in HEK293 cells might be related to the neuronal character of these cells and there is a shift away from repression of genes involved in cell migration in ESC to genes involved in cell cycle control in CM during differentiation. Repression of genes involved in cell migration (e.g. *Nrp2*) might be more relevant in other differentiation processes and other cell types, such as endocardial or endothelial cells, where misregulation of these genes might contribute to the observed EMT and vascular defects in *Hey* KO mice. In vessels *Nrp2* expression is mostly confined to veins and lymphatic vessels, maybe due to repression by Hey2 that is present in arteries (Yuan et al., 2002).

Genes repressed in CM are involved in cell cycle progression, differentiation and proliferation. They could potentially mediate anti-hypertrophic effects seen in *Hey2* overexpressing mice and cause hypertrophy observed in mice with heterozygous *Hey2* deletion upon pressure overload and even more so in *Hey2* KO mice (Gessler et al., 2002, Donovan et al., 2002, Sakata et al., 2002, Kokubo et al., 2004, Xiang et al., 2006, Liu et al., 2010). Transcripts regulated in CM are also enriched for genes involved in response to retinoic acid, which was previously shown to restrict the developmental potential of the heart field and to subsequently reduce cardiac proliferation. Reduced RA signaling leads to hypoplasia especially of the compact ventricular layer, where *Hey2* is preferentially expressed and to a shift from ventricular to atrial differentiation, similar to *Hey2* KO mice (Xin et al., 2007, Koibuchi and Chin, 2007, Lin et al., 2010, Zhang et al., 2011).

These data establishes Hey proteins near the top of a transcriptional hierarchy, with Notch and Bmp signaling acting directly upstream of Hey factors and many other TF downstream of them. The conducted experiments do not provide enough functional evidence to draw further conclusions about the regulation of specific genes and the functional implications during cardiovascular developmental. However, the provided data can be a vital source for further *in vivo* experiments.

6.7. Hey target gene repression by Hdac recruitment and histone deacetylation

In HEK293 cells we have found that Hey proteins bind primarily to active chromatin with H3K4me3 and in ESC and CM a similar observation was made. However, no differences in H3K4me3 were observed for differently regulated groups of target genes. Previously, it was reported that Hey proteins interact with the Sin3/N-CoR repressor complex, which recruits Hdac proteins, as well as with Sirt1 another histone deacetylase (Iso et al., 2001, Gould et al., 2009). Indeed, a reduction in H3K27ac was observed for Hey repressed genes. These findings suggest that Hey proteins might act on histone acetylation via recruitment of these deacetylases. However, as repression was also observed *in vitro* on luciferase promoter constructs, which are not embedded in chromatin, Hey proteins might also affect expression of target genes in other ways.

In our analysis of individual Hey target promoters Hdac2 enrichment correlates largely with Hey enrichment. Additionally a reduction in histone H3 acetylation was observed. Previous studies could show that treatment with the Hdac inhibitor TSA can partially abolish Hey repression of target genes, as TSA does not inhibit Sirt1 another Hey interacting histone deacetylase (Takata and Ishikawa, 2003, Nakagawa et al., 2000). Furthermore, CM specific *Hdac1* and *Hdac2* KO mice exhibit strong cardiac phenotypes, indicating their relevance as important co-factors during cardiac development (Montgomery et al., 2007). These findings support the idea that Hey proteins indeed repress target promoters trough recruitment of histone deacetlyases.

6.8. Cardiac activators can overwrite Hey down-regulation

In CM, for a subset of genes binding of Hey proteins and recruitment of Hdac2 similar to ESC was observed, even though these genes were only repressed in ESC. However, when we analyzed histone acetylation, a reduction was only observed for genes that are actually down-regulated. Therefore, Hey binding and Hdac recruitment does not always have an impact on histone acetylation. Previous studies found that a few histone modifications can explain most of the observed gene expression, but data from many more TF can only explain a smaller fraction (McLeay et al., 2012). H3K27ac was shown to be among the most predictive histone modifications for gene expression (Karlic et al., 2010). This indicates that numerous TF act on a few histone modifications, leading to certain expression levels.

I identified cardiac activators, such as Gata proteins, Nkx2-5 and Srf as other important factors in the regulation of Hey target genes. These three factors co-localize on cardiac enhancers (He et al., 2011). They were shown to recruit histone acetylases such as p300 and activate the cardiac expression program. It appears that for a set of Hey bound genes, the acetylating effects of these factors overpower Hey mediated deacetylation. This leads to hyperacetylation of target promoters and prevents Hey mediated repression. However, cardiac activators do not interfere with Hey binding as recruitment to target promoters is in most cases unchanged.

A recent investigation on target gene regulation during AVC specification also implicated combinatory effects between Hey and Gata factors on target genes (Stefanovic S., in press). Here the authors could show that Hey proteins expressed in atrial and ventricular CM prevent Gata dependent gene activation outside the AVC, thereby defining the borders of that region. On one hand, it appears that activation of some genes by Gata4 is overwritten by Hey in atrial and ventricular CM. On the other hand, high binding of cardiac activators including Gata4 is able to overpower repression by Hey proteins for other genes in CM. These combined findings indicate that whether a gene is down-regulated depends on the ratio of Hey binding and binding of these cardiac activators.

Previous studies found also that Hey proteins directly interact with Gata4 and Srf. In such complexes Hey proteins act as co-repressors and abolish target gene activation (Elagib et al., 2004, Kathiriya et al., 2004, Fischer et al., 2005, Doi et al., 2005) indicating even more complex regulatory mechanism.

6.9. Model for Hey target gene regulation

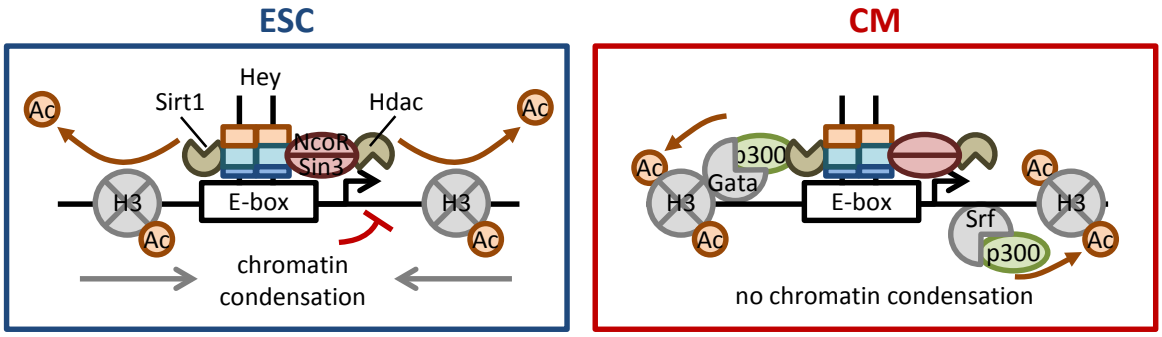


Figure 36: Model of Hey target gene regulation in ESC and CM

Hey proteins directly repress target genes via binding close to TSS, preferentially to E-box motfis. They recruit histone deacetylases (Hdac), which deacetylate histones, leading to chromatin condensation and reduced expression of target genes. In CM this can be counteracted by binding of cardiac activators (Srf, Gata4, Nkx2-5), which recruit histone acetylases (p300).

Here we could show, that Hey repression of target genes correlates with Hdac recruitment and histone deacetylation, which likely results in chromatin condensation. This renders target promoters less accessible and results in a down-regulation of target genes. However, histone deacetylation can be overwritten for a subset of genes in CM by cardiac activators (Srf, Gata4, Nkx2-5). This is achieved by counteracting Hey mediated histone deacetylation by recruitment of histone acetylases such as p300. This mechanism leads to cell type and gene specific regulation of Hey target genes.

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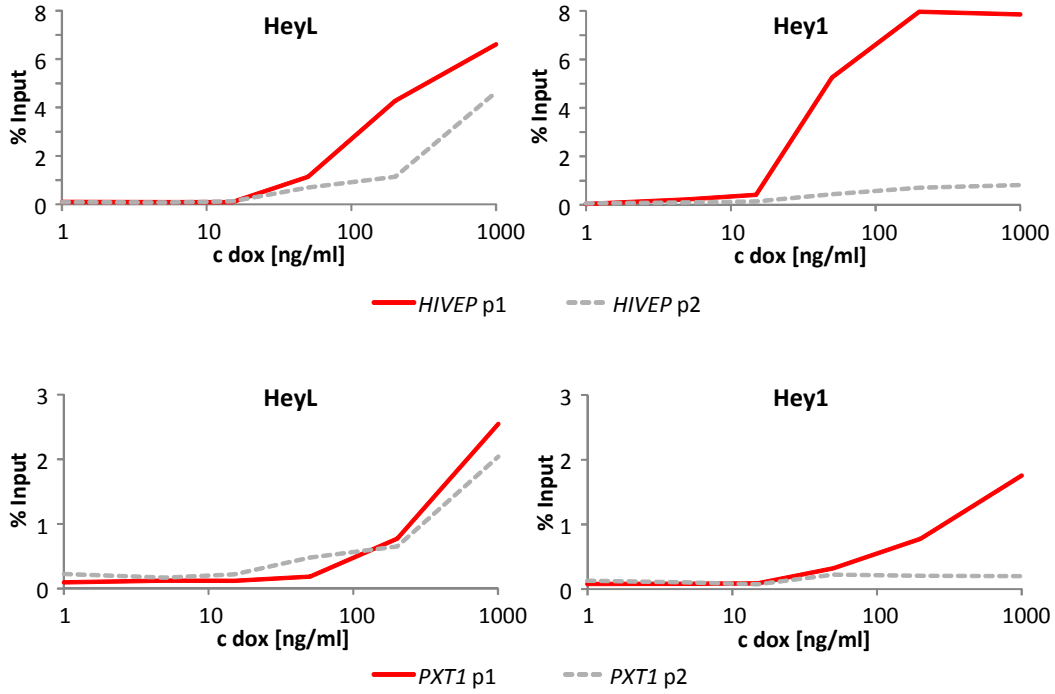
8. Supplement

8.1. Supplementary Information

A) Additional HeyL peaks at *HIVEP* and *PXT1* loci are not dose dependent



B) Additional HeyL peaks at *HIVEP* and *PXT1* loci are not construct dependent

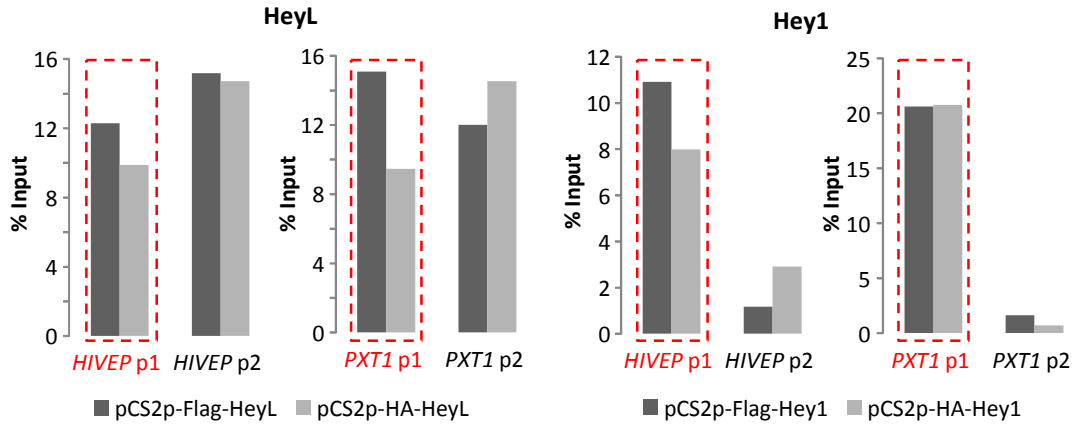


Figure 37: Additional HeyL binding sites at *HIVEP* and *PXT1* loci are no artefact
(A) For Hey1 and HeyL ChIP experiments after induction with different doxycycline concentration were carried out. Shown is the enrichment for the two binding peaks (p1 and p2) for the *HIVEP* and *PXT1* loci, with the shared peaks p1 in red.
(B) HEK293T cells were transiently transfected with pCS2p-Flag/HA-Hey1/L constructs. Enrichment was determined for the two binding peaks (p1 and p2). The shared peak p1 is indicated by a red box.

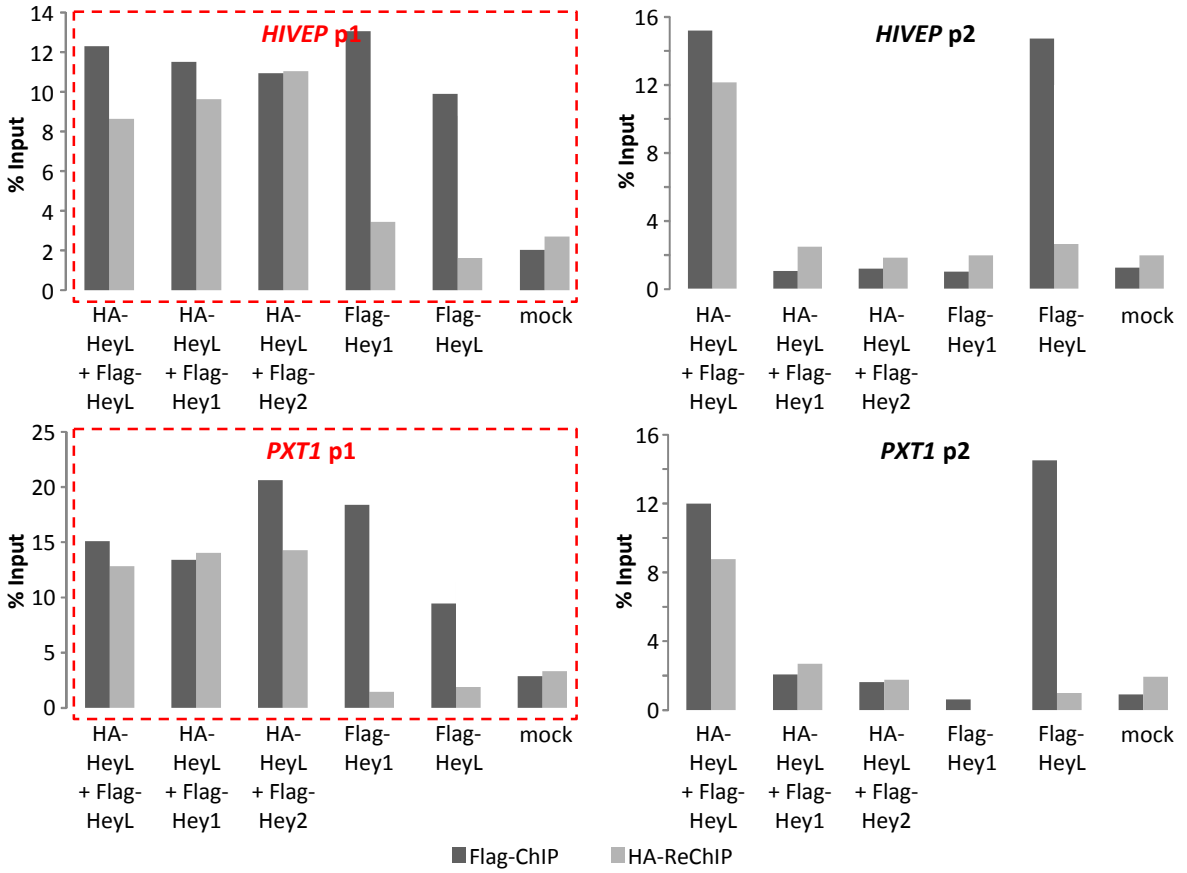


Figure 38: Heterodimer binding at *HIVEP* and *PXT1* loci
HEK293T cells were transiently co-transfected with pCS2p-HA-HeyL and pCS2p-Flag-Hey1, -Hey2 or -HeyL constructs. As control cells were either only transfected with pCS2p-Flag-Hey1 or –HeyL or empty pCS2p-Flag (mock). First a Flag-ChIP was carried out for all samples, after that half of the eluted chromatin was used for the HA-ReChIP. The enrichment was determined for the Flag-ChIP and the HA-ReChIP samples for the two binding peaks (p1 – p2) at the HIVEP and PXT1 loci. The shared peak p1 is indicated by a red box.

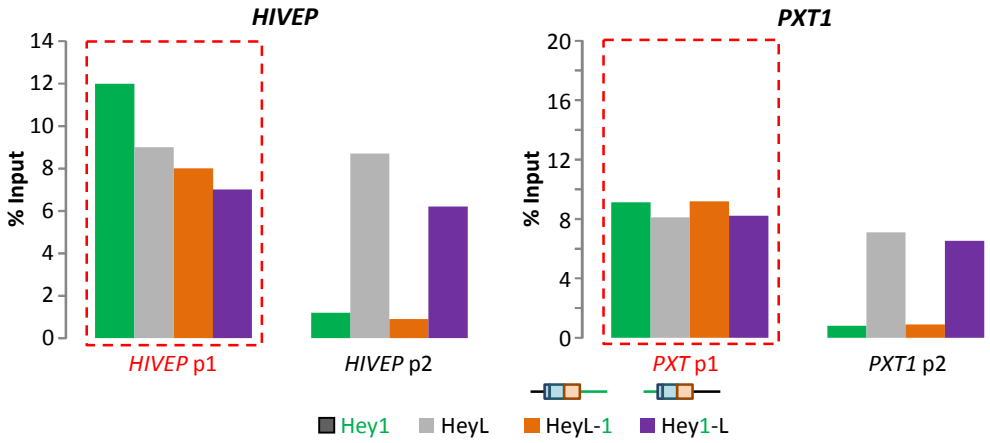


Figure 39: Binding of chimeric Hey1-HeyL proteins at HIVEP and PXT1 loci
HEK293T cells were transfected with pCS2p-Flag-Hey constructs expressing either Hey1, HeyL or two chimeric proteins containing either the c-terminal part of Hey1 and the n-terminal part of HeyL (HeyL-1) or vice versa (Hey1-L). The enrichment was determined the two binding peaks (p1 – p2) at the HIVEP and PXT1 loci. The shared peak p1 is indicated by a red box.

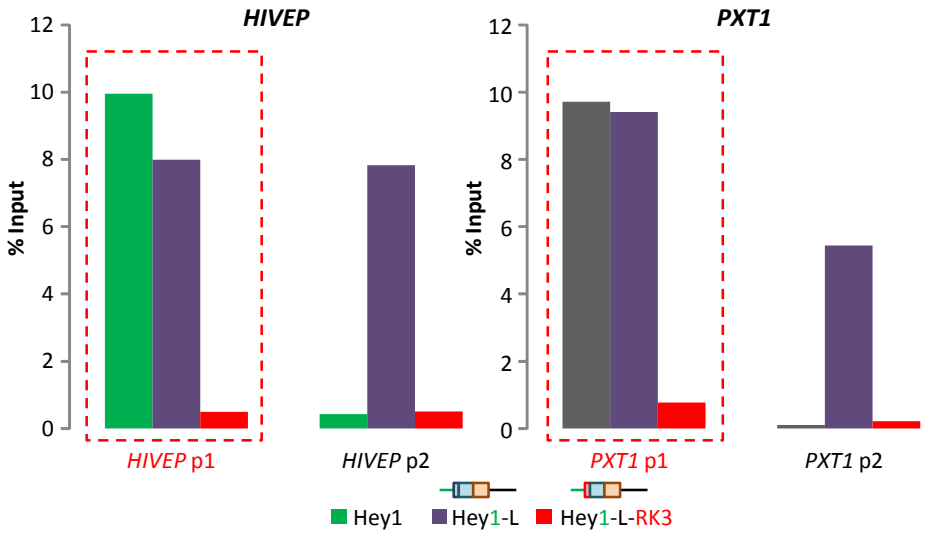
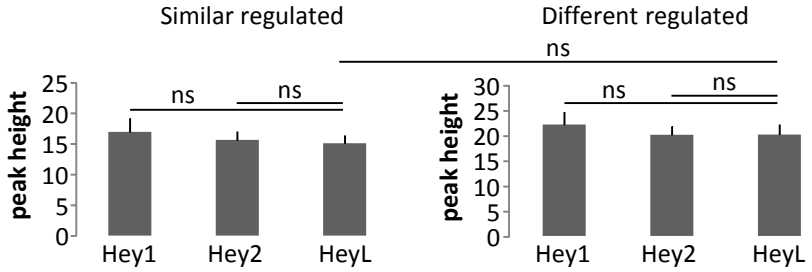


Figure 40: Binding of Hey1-L-RK3 nonbinding mutant at HIVEP and PXT1 locus
(A) HEK293T cells were transfected with pCS2p-Flag-Hey constructs expressing either Hey1, the chimeric Hey1-L protein containing the c-terminal part of Hey1 and the n-terminal part of HeyL and the Hey1-L-RK3 mutant, with 3 point mutations in the binding sites, which abolishes DNA binding. The enrichment was determined for the two binding peaks (p1 – p2) at the HIVEP and PXT1 loci. The shared peak p1 is indicated by a red box.

A) Normalized average peak height near TSS



B) Normalized average distal peak height

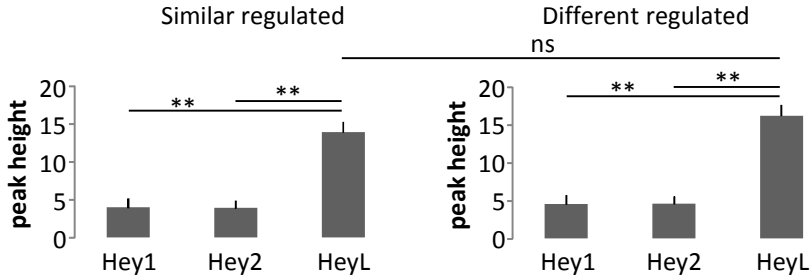


Figure 41: Average ChIPSeq peak height in Hey regulated genes in HEK293 cells
(A) For the two groups of differently and similarly regulated genes the average peak height of peaks located in the promoter region (-1 kb to +1 kb) was determined. Peaks were normalized according to the average peak height of the 1000 highest peaks, within each sample. Stars indicate significant and highly significant differences according to a student's t-test.
(B) The same was done for all distal peaks (more than 1 kb away from the closest TSS).

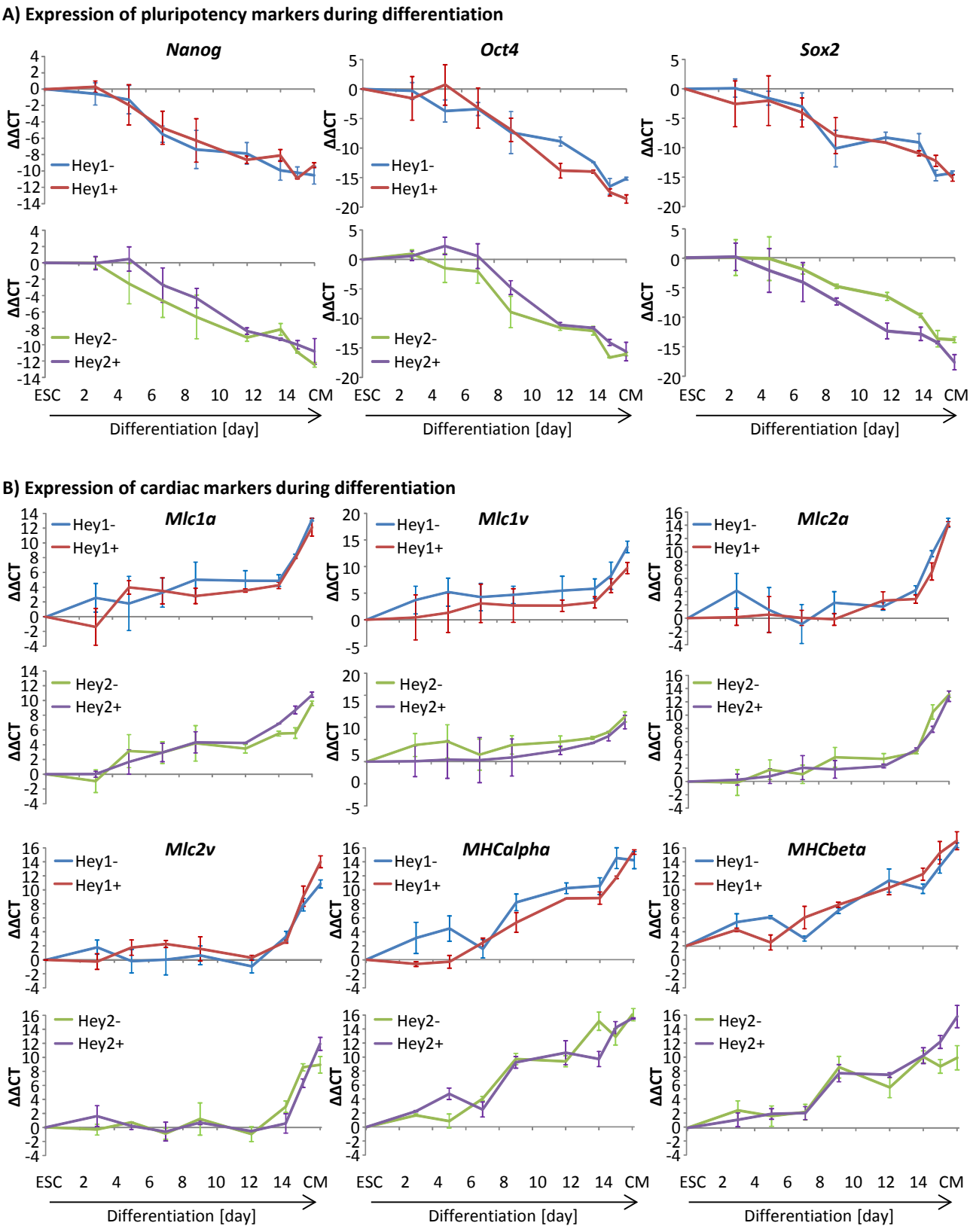


Figure 42: Marker expression during differentiation with prolonged Hey1 or Hey2 overexpression
RNA was isolated from non- and continuously induced (1 µg/ml dox from day -2 until cells were harvested) ES-Hey1 and ES-Hey2 cells before differentiation (ESC), at days 3, 5, 7, 9, 12, 14, 15 of differentiation and differentiated CM from three independent differentiations. Lines show the mean change compared to non-induced ESC, error bars indicate the standard deviation.

Table 8: ChIPseq total reads for HEK293 cells

	Hey1 +dox	Hey2 -dox	Hey2 +dox	HeyL -dox	HeyL +dox
total reads	13073816	13525913	14040194	16142238	15142197
mapped reads	12250465	9047086	12386941	15137928	13839026
mapped reads %	93.7	66.8	88.2	93.8	91.4

Table 9: RNAseq total reads for HEK293-ptol2-mHeyL

	HeyL -dox	HeyL +dox
total reads	4645098	4471381
mapped reads	4217498	3831625
mapped reads %	90.8	85.7

Table 10: ChIPseq total reads for ESC

	Hey1 -dox	Hey1 +dox	Hey2 -dox	Hey2 +dox
total reads	13212856	19776762	13318035	17967960
mapped reads	11773144	17849853	11732914	16456164
mapped reads %	89.1	90.3	88.1	91.6

Table 11: ChIPseq total reads for CM

	Hey1 -dox	Hey1 +dox	Hey2 -dox	Hey2 +dox
total reads	18200688	18360337	15328934	16704134
mapped reads	14324108	15684074	7979212	7119052
mapped reads %	78.7	85.4	52.1	42.6

Table 12: RNAseq total reads for CM

	Hey1 -dox	Hey1 +dox	Hey2 -dox	Hey2 +dox
total reads	8347129	8547469	12738990	22848834
mapped reads	7381914	7549578	11526903	20751945
mapped reads %	88.4	88.3	90.5	90.8

Table 13: EMSA oligonucleotides

ChIP-Ebox-bio1	[BTN]GTGTTATGGCACGTGCCATACTG
ChIP-Ebox-bio2	[BTN]GCAGTATGGCACGTGCCATAACA
ChIP-Ebox1	GTGTTATGGCACGTGCCATACTG
ChIP-Ebox2	GCAGTATGGCACGTGCCATAACA
ChIP-Ebox1-CGCGCG	GTGTTATGGCGCGCGCCATACTG
ChIP-Ebox2-CGCGCG	GCAGTATGGCGCGCGCCATAACA
ChIP-Ebox1-CACGCG	GTGTTATGGCACGCGCCATACTG
ChIP-Ebox2-CACGCG	GCAGTATGGCGCGTGCCATAACA

Table 14: Oligonucleotides for cloning

Hey1ArgLysint_5	GATTTTGGCCAAAAAAAGACGGAAAGGAATAATTGAGAAG
Hey1ArgLysint_3	GATCCGGTCTTTTCGGCGCTTCTCAATTATTCC
mHeyL_ArgLys_int_rev	TTTGCGCCGTTTCTCTATGATCCCTTTGCGCTTCTTTTTCGCCAAAACCTG
mHeyL_ArgLys_int_for	AAAAAGAAGCGCAAAGGGATCATAGAGAAACGGCGCAAAGACCGCAT
Hey1_PauI_rev	GCGGCGCGCCAAAACCTGGGACGATG
HeyL_PauI_for	GCGGCGCGCAAGAAGCGCAGAGGGA
HeyL_AlfII_rev	GCGCTTAAGGTGGGAGAGGAGGCGA
Hey1_AlfII_for	GCGCTTAAGAACTACGCATCCCAGC

Table 15: Human oligonucleotides for qRT-PCR

hDhrs2-real-for	ACCAGTGAGCAGATCTGGGA
hDhrs2-real-rev	TCCATGTAGGGCAGCAACTG
hFGF2-real-f	CCTGGCTATGAAGGAAGATGG
hFGF2-real-r	TCTGCCCAGGTCCTGTTTT
hHey1 total (Clikseq5)	ACCCCAAACTCCGATAGTCC
hHey1 total (huclik-3)	TGAGCTGAGAAGGCTGGTAC
hHey1-real3-ex2 (endogenous)	GGGGACATGGAACCTAGAGC
hHey1-real5-ex1 (endogenous)	CCAGCATGAAGCGAGCTCAC
hHey2-realex1-f (endogenous)	GGAAAGTTGTGACGGTCGAG
hHey2-realex3-r (endogenous)	CCGACGCCTTTTCTCTATAATCC
hHeyL-realex1-f (endogenous)	CCGACTGGGAGCCTTAGC
hHeyL-realex2-r (endogenous)	GTTTCTTCCTGGCTTGCATCT
hHPRT_3'real_neu2	GTCAAGGGCATATCCTACAACAA
hHPRT_5'real_neu2	AAGATGGTCAAGGTCGCAAG
hMMP2-real1	GAATGCCATCCCCGATAAC
hMMP2-real2	TTGGTTCTCCAGCTTCAGGT
hPlxnA2-real3'	AATCACGTTCACCCAGAAGC
hPlxnA2-real5'	TCCTAGATGAGCAGGCAGACA
hScara5-real-for	CTGTCCTGGGGCTCTACCTG
hScara5-real-rev	CCCGGAAGCTCTCATTCAGC
hSlc16a7-real-for	GCAATGGTTCACAAGGAAACT
hSlc16a7-real-rev	TCTGCTCCTCTAGTGGAAATTCA
hSpsb4-real-for	ACCAGTGAGCAGATCTGGGA
hSpsb4-real-rev	TCCATGTAGGGCAGCAACTG
hSrgap1-real-for	CACTTGAAGCCACCAATGCC
hSrgap1-real-rev	AGGGCTCTGTTCAGACTTGC

Table 16: Human oligonucleotides for ChIP

hEYA4-p1-for	CTCACGAGCCCGCAGTAG
hEYA4-p1-rev	CTTCACGTGGACAGGATGG
hEYA4-p2-for	CACAGGCCTTTCTTCCTCTG
hEYA4-p2-rev	ATGGCAATCTGCCCATTAAG
hEYA4-p3-for	CAGGTGGAATGCACACACTC
hEYA4-p3-rev	TTTCAGGTTTGGACAGCACA
hEYA4-p4-for	CATGCTAGAGCCTGGGAAAA
hEYA4-p4-rev	ATTTTGTCTCCCGTTCGATG
hHivep2_p1_for	GGTGCACGTCGCTCATTAGT
hHivep2_p1_rev	GCGGATCTATGCAGATGAGG
hHivep2_p2_for	CACCACACATCAAAGCCAGA
hHivep2_p2_rev	CCACGTCCCAGTTTGCTATT
hPxt1_p1_for	GCAGAGTGGACTGGAGGAAA
hPxt1_p1_rev	GCGCTTCACGTTAAGAGTCC
hPxt1_p2_for	CAGGTTAGGCGATAGAGGTCA
hPxt1_p2_rev	AACCCGGCCTCCCTAACT

Table 17: Mouse oligonucleotides for qRT-PCR

mAgtrl1-real3'	ATACAGCCACCTGCTGGTTC
mAgtrl2-real5'	CCCTTCCCCTCAAACCTTCC
mApln-real3'	GGTAGCGCATGCTTCCTTCT
mApln-real5'	ATGAATCTGAGGCTCTGCGT
mBcl11a-real3'	GCTTCCATCCGAAAACTGCC
mBcl11a-real5'	GCACGCCCCATATTAGTGGT
mCalca-real3'	AGTGTTGCAGGATCTCTTCTGA
mCalca-real5'	CACTGGTGCAGGACTATATGC
mCxcr4-real3'	AGGTGCAGGTAGCAGTGACC
mCxcr4-real5'	ACTCACACTGATCGGTTCCA
mDub1-real3'	CAGCTAGAGGTGGTGTGTGTGT
mDub1-real5'	CTCTTTCCTTCCCAGAAGCAG
mEnpp2-real3'	TCGAGGGCGAGAGAAGTTTA
mEnpp2-real5'	AAAAGAATGTCCCGGCTCTC
mHey1 endogenous (Clik-race)	ATTCTCGTCCGCGCTCTCCTTTTCC
mHey1 endogenous (mHey1-5'UTR)	CTGCAGTTAACTCCTCCTTGC
mHey1 total (clikseq5)	CTGGCCAAAACCTGGGAC
mHey1 total (huclik3)	TGAGCTGAGAAGGCTGGTAC
mHey2 endogenous (mHey2-real-e2rev)	GTCGGTGAATTGGACCTCAT
mHey2 endogenous (mHey2-real-e1for)	AGTAGCTGCTCCTCCTTCGTC
mHey2 total (mHey2-real-e3for)	TGAGAAGACTAGTGCCAACAGC
mHey2 total (mHey2-real-e5rev)	TGGGCATCAAAGTAGCCTTTA
mHeyL-real-ex2/3	GAAGCGCAGAGGGATCATAG
mHeyL-real-ex4rev	GGCATGGAGCATCTTCAAGT
mHPRT-real-ex8	TGTTGTTGGATATGCCCTTG
mHPRT-real-ex9	ACTGGCAACATCAACAGGACT
mId2-real3'	CTCCTGGTGAAATGGCTGAT
mId2-real5'	GGACATCAGCATCCTGTCCT
mJag1-real3'	CTCAGCAGAGGAACCAGGAA

mJag1-real5’	CTCGTTAGTAAACGGGATGGAA
mKcnip1-real3'	CTGTCTGGGTGGTGTCGAAG
mKcnip1-real5'	CGCTCAGTTTTTCCCTCACG
mKrt7-real3'	CCATTCCGTCTCCAGACAAC
mKrt7-real5'	ATCGCAGAGGCTGAGGAAC
mLefty1-real3’	GACACCAGGAACCTGCCTGCCACCTCTC
mLefty1-real5’	GGCTCTGCTGGGCACTCTGGGCACT
mLefty2-real3'	CGAGGCCCCAGAAATGGCCACCCGA
mLefty2-real5'	ACGCCGGACGGCAAGGGGCAG
mMHC-alpha-3'	CGCGAATTCGCAGAGTCGAACGTTTATGTTT
mMHC-alpha-5'	CGCGGATCCACGACGAGGAATAACC
mMHC-beta-3'	CGCGAATTCCTTTATTCTGCTTCCACCTA
mMHC-beta-5'	CGCGGATCCTGAATGAGGAGTAGATCTTG
mMixl1_real3'	TGCTACCCGAGTCCAGGAT
mMixl1_real5'	CCTTGAGGATAAGGGCTGAA
mMLC-1A-3'	CGCGAATTCAAGCTGGGGCTCTTTATTTC
mMLC-1A-5'	CGCGGATCCGGGTAAAGCACGTTTCTC
mMLC-1V-3'	CGCGAATTCTAAGGCCACAGGGTGGATAC
mMLC-1V-5'	CGCGGATCCGAGCTGAGCCTCTCAGGAAG
mMLC-2a-3'	CGCGAATTCAGGCACAGAGTTTATTGAGG
mMLC-2a-5'	CGCGGATCCGAGGAAGCCATCCTGAGT
mMLC-2v-3'	CGCGAATTCCTGTTTATTTGCGCACAGC
mMLC-2v-5'	CGCGGATCCTTAAAGGGGCTGATCCTGAA
mNanog-real3'	GAGGAAGGGCGAGGAGAG
mNanog-real5'	TTGCCTAGTTCTGAGGAAGCA
mNr2f2-real3'	CAGGTACGAGTGGCAGTTGA
mNr2f2-real5'	AGTACTGCCGCCTCAAAAAG
mOct4-real3'	GAAGCGACAGATGGTGGTCT
mOct4-real5'	CCGTGAAGTTGGAGAAGGTG
mPlcb4-real3'	CATCGCCAGTCTCTTTCTTTCT
mPlcb4-real5'	GAAAATAGCAAGGCCATCAGTC
mRassf8_real3'	GATGGGGTTTTCATGCGGTG
mRassf8_real5'	ACGGAAGTCACAACTTGCCA
mSema3e-real3'	GAGGAAGAGCAAGCCCAAG
mSema3e-real5'	CGTTGGACAGGACTGAAGAGA
mSema6D-real3'	GACCACCTTTCTGTCCTCCTC
mSema6D-real5'	CTGAAGCTGGCGTGGTACTT
mSgcg-real3'	GGGGCATCCATGCTTAGACT
mSgcg-real5'	CGTAACAGGGCCAGAAGGAG
mSox2_fwd_mus	AAGGGTTCTTGCTGGGTTTT
mSox2_rev_mus	AGACCACGAAAACGGTCTTG
mTcf3-real3'	GTAGCTGTCACCTGAGCTGG
mTcf3-real5'	GTGGCCTGGCAGATACTCAG
mTgm1-real3'	GTGCACTTGGGAAAGCTGTG
mTgm1-real5'	ACAGAGACCCAAGGTCCTCA
mZfhx3-real3'	ATTGCACCCAGTACGGATCG
mZfhx3-real5'	CTGGGGTGGGTAAGTTCGG
mZic2-real3'	GCGCCGGTCACAGCCCTCGAACTCA

mZic2-real5'	GCACGTCGGCGGCCCGGAACA

Table 18: Mouse oligonucleotides for ChIP

mBcl11a-p1-for	ACTTTTTCCACCCCCTTCC
mBcl11a-p1-rev	TTGTTGTGATTTCGGGCTTC
mCxcr4-p1-for	GGAGAAAAGGGTGGGGTCAG
mCxcr4-p1-rev	GGAGAAAAGGGTGGGGTCAG
mJag1-p1-for	CCTCTCGGCTTTCTTTCCTT
mJag1-p1-rev	CACGCGTCATTGTGTTACCT
mZfhx3-p1-for	GCTGCACCCTGGACACTAG
mZfhx3-p1-rev	GTCCTCCGATCCGTTGTCTG
mZfhx3-p2-for	TCTGGCTCGGAGAATGGGTA
mZfhx3-p2-rev	CTTTCCTTCCCTGGCCTCAG
mZfhx3-p3-for	ACTGGAATCTCCCGTTGTCG
mZfhx3-p3-rev	CACAAAGCCTCCGTACACCT
mZfhx3-p4-for	CTAAGTGAGCAGCCCACTCC
mZfhx3-p4-rev	ACAAGCCAGGACAAGTCAGG

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8.4. Affidavit

I hereby confirm that my thesis entitled “Hey target gene regulation in embryonic stem cells and cardiomyocytes” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Regulation von Hey Zielgenen in embryonalen Stammzellen und Kardiomyozyten“ eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum Unterschrift

8.5. Publications

HEISIG, J.*, **WEBER, D.***, ENGLBERGER, E., WINKLER, A., KNEITZ, S., SUNG, W.-K., WOLF, E., EILERS, M., WEI, C.-L. & GESSLER, M. 2012. Target Gene Analysis by Microarrays and Chromatin Immunoprecipitation Identifies HEY Proteins as Highly Redundant bHLH Repressors. PLoS Genetics, 8, e1002728.

* contributed equally

BEZZINA, C. R., BARC, J., MIZUSAWA, Y., REMME, C. A., GOURRAUD, J. B., SIMONET, F., VERKERK, A. O., SCHWARTZ, P. J., CROTTI, L., DAGRADI, F., GUICHENEY, P., FRESSART, V., LEENHARDT, A., ANTZELEVITCH, C., BARTKOWIAK, S., BORGGREFE, M., SCHIMPF, R., SCHULZE-BAHR, E., ZUMHAGEN, S., BEHR, E. R., BASTIAENEN, R., TFELT-HANSEN, J., OLESEN, M. S., KAAB, S., BECKMANN, B. M., WEEKE, P., WATANABE, H., ENDO, N., MINAMINO, T., HORIE, M., OHNO, S., HASEGAWA, K., MAKITA, N., NOGAMI, A., SHIMIZU, W., AIBA, T., FROGUEL, P., BALKAU, B., LANTIERI, O., TORCHIO, M., WIESE, C., **WEBER, D.,** WOLSWINKEL, R., CORONEL, R., BOUKENS, B. J., BEZIEAU, S., CHARPENTIER, E., CHATEL, S., DESPRES, A., GROS, F., KYNDT, F., LECOINTE, S., LINDENBAUM, P., PORTERO, V., VIOLLEAU, J., GESSLER, M., TAN, H. L., RODEN, D. M., CHRISTOFFELS, V. M., LE MAREC, H., WILDE, A. A., PROBST, V., SCHOTT, J. J., DINA, C. & REDON, R. 2013. Common variants at SCN5A-SCN10A and HEY2 are associated with Brugada syndrome, a rare disease with high risk of sudden cardiac death. Nat Genet, 45, 1044-9.

STEFANOVIC, S., BARNETT P., VAN DUIJVENBODEN, K., **WEBER, D.**, GESSLER, M. & CHRISTOFFELS, V. M. 2014. GATA-dependent regulatory switches establish atrioventricular canal specificity during heart development. Nat Communications, in press.

WEBER, D., WIESE, C. & GESSLER, M. 2014. Review: Hey bHLH transcription factors. Current Topics in Developmental Biology, submitted.

8.6. Oral presentations and posters

03/2011 Joint Meeting of the German and Japanese societies for Developmental Biology (GfE)
Poster: “Transcriptional regulation by Hey and Hes bHLH factors from the Notch pathway”

01/2012 Joint Group Meeting, Biocenter Wuerzburg
Talk: “Target gene identification for Hey/Hes transcription factors”

06/2012 17th International Vascular Biology Meeting
Poster: “Hey bHLH factors are redundant repressors in cardiovascular development”

10/2012 SFB688 Retreat
Poster: “Functional analysis of the Hey bHLH factors in cardiovascular development”

10/2012 7th International Student Symposium of the Graduate School of Life Sciences, EPOS, Everything’s Part Of Science
Poster: “Wanted – Hey target genes”

03/2013 International Joint Meeting of the German Society for Cell Biology (DGZ) and the German Society for Developmental Biology (GfE)
Poster: “Targets of Hey bHLH factors in cardiac development”

10/2013 8th International Student Symposium of the Graduate School of Life Sciences, SCI, Scientific Crosstalk
Poster: “Dynamics of Hey target gene regulation during cardiomyocyte differentiation”

04/2014 SFB688 Progress Symposium
Talk: “Hey target genes in cardiac development: Repression by Hdac recruitment and histone deacetylation may be overwritten by cardiac activators”

8.7. Curriculum Vitae

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