2 Materials and Methods

2.1 Chemicals, reagents and equipment

1 kb marker DNA Ladder	New England BioLabs, USA
100 bp marker DNA Ladder	New England BioLabs, USA
30 % Hydrogen peroxide	Sigma, USA
384 clear well optical reaction plates	Applied Biosystems, USA
Agarose	Invitrogen, USA
Ampicillin	Sigma, USA
AMV reverse transcriptase	Promega, USA
Benzonase	Roche, Germany
BSA	Sigma, USA
Calf thymus histones	Worthington, USA
Cell culture flasks with filter (T75, T300)	Biochrom, Germany
Cell culture tubes	Biochrom, Germany
Cell scraper	Biochrom, Germany
Chloramphenicol	Sigma, USA
Claycomb Medium	JRH Biosciences, USA
DEPC water	Invitrogen, USA
DMSO	Sigma, USA
DNase	Promega, USA
dNTPs	Amersham, UK
DPX Mountant for histology	Fluka, Germany
DTT 0.1 M, RNase free	Promega, USA
ECL Advanced detection system	Amersham, UK
Eosin	Sigma, USA
Ethidium bromide solution	Sigma, USA
FBS Lot #0055H (C2C12 cells)	Biochrom, Germany
Fetal calf serum Lot #3J0229 (HL-1 cells)	JRH Biosciences, USA
Glutathione Sepharose 4B	GE Health Care
l-Ascorbic acid Na-salt	Sigma, USA,
l-Glutamine	Gibco, USA,
Lipofectamine 2000	Invitrogen, USA
MagneGST Magnetic Beads	Promega, USA
Norephinephrine	Sigma, USA,
Optical adhesive covers	Applied Biosystems, USA
PBS, cell culture grade	Sigma, USA
Penicillin-Streptomycin solution, cell culture grade	Gibco, USA
Protein Marker: Precision Plus Protein Standard	Biorad, USA

Proteinase K	Sigma, USA
RNAse A	Sigma, USA
RNase Away	Roth, Germany
RNAsin Ribonuclease inhibitor	Promega, USA
Superfrost plus slides	Menzel-Glaeser, Germany
SybrGreen Master Mix	ABgene
T4-DNA Ligase	Promega
T4-DNA Ligation Buffer	Promega
Transfer membrane for Western Blots	Millipore
Trizol Reagent	Invitrogen, USA
Trypsin, cell culture grade	Gibco, USA
Trypsin soybean inhibitor	Invitrogen, USA,
Water, cell culture grade	Invitrogen, USA
X-Gal	Roth, Germany
X-ray films Fuji Super RX 100 NIF	Fuji, Japan
XtremeGene	Roche, Germany
Xylene	Roth, Germany

2.2 Consumables and Machines

Phase lock Gel (Heavy) 1.5/2 ml tubes	Eppendorf, Germany
Neubauer Counting Chamber	Carl Roth, Germany
Agarose gel electrophoresis equipment	Amersham, UK
SDS-PAGE gel electrophoresis equipment	Biorad, Germany
Nanodrop Spectrophotometer	Nanodrop technologies, USA
Thermocycler	PTC100, MJ Research Inc, USA
Thermomixer	Eppendorf, Germany
ABI Prism 7700	Applied Biosystems, USA
Microscopes	Carl Zeiss AG, Germany
Bioanalyzer	Agilent
Luminometer Centro LB	Berthold
Sonifier	Kinematika, Switzerland
Gel documentation system	Biorad, USA
UV cross linker	Stratagene, USA
Microtome	Leica, Germany

2.3 Software

Primer Express 2.0	Applied Biosystems, USA

Axio Vision	Carl Zeiss, Germany
SDS 2.1 software	Applied Biosystems, USA
Vector NTI	Invitrogen, USA

2.4 Kits

Dual-Luciferase Assay	Promega, USA
Wizard SV Gel- and PCR Clean-Up System	Promega, USA
DIG-Gel-Shift-Kit, 2nd generation	Roche, Germany
TNT coupled transcription/ translation system	Promega, USA
Quik Change site directed mutagenesis kit	Stratagene, USA
TOPO-TA Dual promoter cloning kit	Invitrogen, USA
Fast plasmid purification Kit mini	Eppendorf, Germany

2.5 Bioinformatic analyses

Bioinformatic sequence analysis was performed using the UCSC Genome Browser (http://genome.ucsc.edu/) and VISTA plots with default settings (http://genome.lbl.gov/vista/servers.shtml). VISTA aligns two or more sequences and creates a visual output that depicts similarities and differences among sequences. Transcription factor binding sites were predicted using TRANSFAC Professional 11.4 (http://www.biobase.de/cgi-bin/biobase/transfac/start.cgi).

2.6 Cell lines

2.6.1 C2C12 Cells

C2C12 mouse myoblast cells were obtained from Prof. Jakob Schmidt (Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, New York) and cultivated at 5 % CO_2 and 37 ºC in Dulbecco's modified Eagle's medium supplemented with 1 % Penicillin/Streptomycin and 10 % fetal calf serum.

2.6.2 HL-1 Cells

HL-1 cells were provided by Prof. William C. Claycomb (Departments of Biochemistry and Molecular Biology and Cell Biology and Anatomy, Louisiana State University Medical Center, New Orleans) and cultured as described (Claycomb *et al.*, 1998).

2.6.3 HEK293T cells

HEK293T human kidney cells were cultivated at 5 % CO_2 and 37 ºC in Dulbecco's modified Eagle's medium supplemented with 1 % Penicillin/Streptomycin and 10 % fetal calf serum. Cells were subcultured at confluency and split 1:5 for the next passage.

2.6.4 H9c2 cells

H9c2 rat cardiomyocytes cells were cultivated at 5% CO_2 and 37 ºC in Dulbecco's modified Eagle's medium supplemented with 1 % Penicillin/Streptomycin and 10 % fetal calf serum. Cells were subcultured as described (Kimes and Brandt, 1976).

2.7 Molecular Biology techniques

2.7.1 DNA gel electrophoresis

Agarose gel electrophoresis was performed for the isolation of DNA fragments and analytical purposes. 1-2 % agarose was dissolved in 0.5x TBE buffer by boiling and 1 µg/ml ethidiumbromide was added to the liquid agarose. Ethidiumbromide intercalates into DNA and emits light when subjected to UV light of 254 nm, allowing for the visualization of the DNA. DNA samples were mixed with 6x DNA Loading Dye (Fermentas) prior to gel electrophoresis. DNA size markers (100 bp DNA Ladder or 1 kb DNA Ladder, NEB) were used to estimate the size of the analyzed DNA fragments. Gel extraction of DNA fragments was performed using a clean razor blade and the Wizard SV Gel- and PCR Clean-Up System (Promega) according to manufacturer's instructions.

2.7.2 Enzymatic DNA digestion

DNA fragments were treated with restriction endonucleases for analytical purposes or subsequent DNA cloning. In general, 1 µg of DNA was incubated with 5 units of restriction enzyme in the appropriate buffer supplied by the manufacturer for 1 hour at 37 ºC. Digestion of plasmid DNA and genomic DNA was controlled by agarose electrophoresis. Plasmid DNA and PCR products were purified using the Wizard SV Gel- and PCR Clean-Up System (Promega). Genomic DNA was purified by ethanol precipitation. For self-ligation of genomic DNA used for inverse PCR, 4 µg of genomic DNA were digested with appropriate restriction enzymes for at least 6 hours and 250 ng DNA was run on a gel together with undigested

DNA to check for completion of the digest. Suitable enzymes that cut frequently in the genome and also have a recognition site within the region analyzed were selected.

2.7.3 Ethanol precipitation of DNA

Unwanted salts and enzymes can be removed from DNA by ethanol precipitation. DNA samples were precipitated by adding 2.5 volumes of 100 % EtOH and 1/10 volume 3 M NaAc and incubated at –20 ºC for 1 hour. To collect the DNA, samples were centrifuged for 30 min at 14,000 g. The supernatant was removed and 750 µl of 70 % EtOH added to wash out salts that were co-precipitated. Samples were again centrifuged at 14,000 g for 10 min, the supernatant removed and the DNA was left to dry at room temperature for 10-15 min. The DNA pellet was resuspended in an appropriate volume of TE-buffer.

2.7.4 DNA ligation

T4-DNA ligase (Promega) was used to ligate DNA fragments with complementary overhangs or blunt ends. For cloning of DNA fragments into plasmid vectors, 50 ng of digested vector was added to 10x T4-DNA Ligation Buffer (Promega) diluted in H_2O, the appropriate amount of DNA insert and 1 µl of T4-DNA ligase. A molar insert:vector ratio of 3:1 was calculated as follows: ((50 ng vector * kb of insert) / kb of vector)) * 3 = ng insert used.

2.7.5 Genomic DNA isolation

Genomic DNA was isolated from tail biopsies for genotyping and isolated liver samples of mice. Tails were incubated in tail-buffer (100 mM Tris/HCl, pH 8.5; 5 mM EDTA; 0.2 % (w/v) SDS; 200 mM NaCl; 1 µg/µl Proteinase K) over night at 37 ºC. Liver samples were pulverized in liquid nitrogen prior to incubation in tail-buffer. Proteinase K was inactivated by a 5-minute incubation at 95 ºC. RNase A was diluted to 20 µg/ml in TE, 750 µl added to the digested tails and incubated for 10 min at room temperature. The samples were subsequently centrifuged for 10 min at 10,000 g and 4 ºC. 600 µl of the supernatant containing the genomic DNA were transferred into a 2 ml reaction tube and precipitated by adding 60 µl 3 M NaAc and 1200 µl 100 % EtOH as described above.

2.7.6 Isolation of RNA

Total RNA from zebrafish embryos was isolated using TRIzol reagent (Invitrogen) followed by DNase digest (Promega) according to manufacturer's instructions. Trizol is a mono-phasic solution of phenol and guanidine and is based on the single-step RNA isolation method

developed by Chomczynski and Sacchi (1987). 1 ml of TRIzol reagent was added to 30 zebrafish embryos in a 2 ml cryo-tube and embryos were homogenized two times for two min using a Polytron homogenizer. The homogenate was centrifuged in a pre-spun Phase-lock tube (Eppendorf) for 1 min and RNA quality was analyzed using the RNA 6000 Nano assay on the 2100 Bioanalyzer (Agilent).

2.7.7 Reverse transcription of RNA – cDNA synthesis

1 µg of total RNA was reverse transcribed to cDNA synthesis using and AMV-Reverse Transcriptase (Promega) according to manufacturer's instructions.

2.7.8 Polymerase chain reaction (PCR)

Polymerase chain reactions were performed using Taq Polymerase produced at the Max Planck Institute for Molecular Genetics. The standard PCR setup was as follows: 5 µl 10x Leos PCR buffer; 2 µl dNTPs (10 mM); 1 µl fwd primer (10 µM); 1 µl rev primer (10 µM); 39.5 µl H_2O; 1 µl DNA template (50 ng/µl); 0.5 µl Taq Polymerase. Cycling conditions were as follows: 95 ºC, 2 min; 95 ºC, 30 sec; 58 ºC, 30 sec; 72 ºC, 1 min/kb; 35 cycles; 72 ºC, 5 min; 4 ºC, hold.

2.7.9 Site-directed mutagenesis

Site-directed mutagenesis of DNA was carried out using the QuikChange site-directed mutagenesis kit (Stratagene) according to manufacturer's instructions. Oligonucleotides for mutagenesis were designed to introduce amino acid changes in the protein sequence of coding regions or deletions in potential transcription factor binding sites of non-coding regions. Mutagenesis was confirmed by plasmid sequencing carried out at MWG Biotech.

2.7.10 Inverse PCR

To identify unknown DNA sequence adjacent to a known stretch of sequence, inverse PCR can be applied. Therefore, genomic DNA was isolated from the liver of transgenic mice and digested for at least 6 hours with restriction enzymes that cut at the ends of the transgene and presumably within the host genome near the integration site. The enzymes chosen were RsaI, HinfI, HpaII, SacI and EcoRV. Following heat-inactivation of the enzymes, DNA was analyzed for complete digestion by agarose gel electrophoresis and compared to untreated genomic DNA. The DNA was diluted to 30 ng; 10 ng; 5 ng; 2 ng/µl and 10 µl each added to the ligation reaction to form circularized DNA fragments. (2 µl 10x T4-Buffer, 1 µl

PEG (50 %), 6.5 µl H_2O, 10 µl DNA, 10 U T4 DNA-Ligase). The reaction was incubated at 16 ºC over night and 2 µl each was used in a PCR using primers located within the transgene and facing outwards, complementary to conventional primers. The PCR products were analyzed by gel electrophoresis, cut out from the gel and cloned into TOPO-TA vectors (Invitrogen). In case no PCR product was obtained, a second round PCR using nested PCR primers located within the transgene were used with 1 µl of first round PCR as template. The resulting PCR products were treated as described above.

2.7.11 TOPO cloning

TOPO-TA Dual promoter vectors (Invitrogen) were used to subclone PCR fragments intended for *in vitro* transcription according to manufacturer's instructions and fragment orientation was analyzed by DNA sequencing.

2.7.12 Plasmid transformation into bacteria

Plasmid DNA was introduced into *E.coli* cells by heat shock transformation. 50 µl chemically competent DH10b cells were incubated on ice with 50 ng of plasmid DNA for 30 min followed by a heat shock at 42 ºC for 45 sec. The cells were incubated on ice for 1 min and 300 µl LB medium was added. Cells were then grown at 37 ºC in a thermomixer and plated on LB-agar plates containing the appropriate antibiotic. The plates were incubated over night at 37 ºC and single colonies of cells were used for plasmid preparations.

2.7.13 Reportergene assays

Potential gene regulatory elements were tested by Dual-Luciferase reportergene assay. Promoter elements were cloned into the Firefly reportergene vectors pGL3 basic or pGL3 promoter (Promega) and transfected into mammalian cells together with an internal control plasmid, which expressed *Renilla* luciferase. 48 hours after transfection, cells were harvested, lysed in 30 µl Passive lysis buffer and analyzed using a 96-well plate Luminometer (Berthold). Firefly activity was normalized to *Renilla* control activity. Assays were carried out in triplicates and three biological replicates.

2.7.14 Electromobility shift assays

The DIG Gel Shift Kit, 2nd generation (Roche) was used according to manufacturer's instructions. DIG-labeled oligonucleotides were incubated with HEK293 nuclear extracts from cells previously transfected with expression vectors for the respective transcription

factor and subjected to gel electrophoresis. The ability of the transcription factor to bind and thus alter the migratory behavior of the oligonucleotides was analyzed by blotting. Specificity of the reaction was tested by addition of 100-fold excess unlabeled oligonucleotides to the reaction.

2.7.15 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) is a method widely used for the absolute or relative quantification of gene expression. Quantification is based on the measurement of fluorescence of the dye SybrGreen I, whose fluorescence increases ≈ 200 fold when intercalated into double stranded DNA. Primers were designed using Primer Express software to amplify 100-150 bp fragments. Primer amplification efficiency was calculated according to (Swillens *et al.*, 2008) and was found to be comparable for all primers. All qPCRs were measured on ABI Prism 7700 in a 10 µl reaction volume with 2 x SybrGreen I master mix and 100 nM primer in triplicate. Ct values were determined using the integrated SDS 2.1 software. Fold changes were calculated using the relative quantification method of ΔΔCt as described in the manufacturer's manual.

2.7.16 Microarray analysis of *dpf3* morphant zebrafish embryos

Affymetrix GeneChip Zebrafish Genome Arrays were hybridized with labeled cDNAs obtained from embryos injected with morpholino-modified antisense oligonucleotide against *dpf3* (MO-^{dpf3}) and unspecific morpholino-modified antisense oligonucleotide (MO-^{control}) at 72 hpf (see chapter 2.8.5). 4 chips were hybridized (2x MO-^{control}, 2x MO-^{dpf3}, 30 embryos each) (www.ebi.ac.uk/arrayexpress/, E-TABM-354). Array analysis was performed with the Bioconductor 2.0 software package (Gentleman *et al.*, 2004). Data was normalized via qspline after MAS background correction using the affy-package and the zebrafish annotation package (Appendix E). Differentially expressed genes were calculated via the limma package (Smyth, 2004). P-values were adjusted for multiple testing using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). Transcripts with an adjusted p-value < 0.1 were defined as differentially expressed. With these sets of transcripts, a Gene Ontology (GO) analysis was carried out, looking for overrepresented GO terms in the Ontologies biological process (abbreviated as BP) and cellular component (abbreviated as CC) in the differentially upregulated, differentially down-regulated and all differentially expressed transcripts. The resulting tables contain the p-value, odds ratio, expected gene count, and actual gene count for each term tested along with the total number of transcripts associated with this term in the

"universe" of transcripts. All transcripts with an interquartile range bigger or equal to 0.5 were used for the universe of transcripts resulting in 1760 transcripts.

2.8 Animal studies

2.8.1 Generation of transgenic mice

All transgene techniques were done according to (Hogan, 1994), if not otherwise stated. Transgenic mice were generated with the help of Ingo Voigt at the animal facility of the Max Planck Institute for Molecular Genetics. The mouse strain *Mus musculus* C57/BL6 was used. DNA for microinjection of oocytes was prepared by excision of the transgene from the vector backbone followed by agarose gel-purification. The transgene was resuspended and diluted to 2 ng/µl in microinjection buffer (8 mM Tris-HCl pH 7.4; 0.15 mM EDTA, sterile-filtered). In order to obtain a large number of oocytes and to have better control of ovulation, donor females were treated with gonadotropins for three days prior to mating with stud males. Mating was controlled by vaginal-plug control in the morning and used for oocyte isolation. Superovulated ooctye donors with a vaginal plug were sacrificed by cervical dislocation and uteri were dissected from the animals to collect oocytes. Transgenic DNA was injected into oocytes using two micromanipulators, a stereomicroscope (Axiovert35, Zeiss) and a microinjector. One day before oocyte injection, adult female mice were mated with vasoectomized males to induce pseudopregnancy. Foster mothers were anaesthetized and oocytes were injected into the oviduct. Potentially transgenic offspring of injected foster mothers was analyzed by genotyping-PCR of tail biopsies.

2.8.2 X-gal staining of mouse embryos

C57/BL6 female mice were mated with transgenic male animals and matings were controlled by vaginal-plug control in the morning. Pregnant females were sacrificed and embryos dissected in cold PBS, transferred to fixing solution (4 % Paraformaldehyde in PBS) and incubated for 1 hour at 4 ºC on a shaker. Embryos were washed three times for 15 min in rinse buffer (5 mM EGTA; 0.01% Deoxycholate; 0.02% NP40; 2 mM $MgCl_2$ in PBS) at room temperature to remove the fixative followed by incubation in staining buffer (20 mM $K_3Fe(CN)_6$; 20 mM $K_4Fe(CN)_6$; 5 mM EGTA; 0.01 % Deoxycholate; 0.02 % NP40; 2 mM $MgCl_2$; 1 mg/ml X-gal) at 37 ºC for 30 min to over night with frequent control of the staining reaction. After completion of the staining reaction, embryos were washed two times in PBS and subjected to post-fixation in fixing solution over night at 4 ºC.

2.8.3 Embedding and sectioning of mouse embryos

For the preparation of histological sections, embryos were dehydrated through a graded EtOH series (PBS; 50 % EtOH/H_2O; 70 % EtOH/H_2O; 90 % EtOH/H_2O; 96 % EtOH/H_2O; 3x 100 % EtOH, 30 min each) followed by 3x 10 min incubations in Ultraclear, 30 min in Ultraclear: Paraffin (1:1) and kept in Paraffin until embedding. After embedding, 8 µm sections were prepared on a microtome. Sections were transferred onto cover slips and left to dry over night.

2.8.4 Counter-staining of histological sections after X-gal staining

For better visibility of anatomical structures, histological sections of X-gal stained embryos were counter-stained with Eosin (Sigma). Eosin is acidic and stains cytoplasmic proteins. Staining procedure was as follows: 10 min Xylene; 10 min Xylene; 5 min 100 % EtOH; 5 min 100 % EtOH; 5 min 70 % EtOH; 10 min H_2O; 1 min H_2O; 1 min Eosin; 2 min 70 % EtOH 2 min 100 % EtOH; 2 min 100 % EtOH; 10 min Xylene; 10 min Xylene.
After staining, sections were covered with mounting medium, a glass lid was added and left to solidify over night at room temperature.

2.8.5 Morpholino antisense-oligonucleotide knockdown in zebrafish embryos

Morpholinos (Gene Tools) were injected at a concentration of 100 µmol/l. The morpholino sequence was directed against the exon4-intron4 boundary of *dpf3*:
MO^{dpf3}: 5-GCTCATCACTCACCCTGCCTTTGTT-3.

2.8.6 mRNA rescue of zebrafish embryos

Full-length zebrafish *dpf3* (NM_001111169) was cloned into the pCS2+expression vector and used as rescue construct. Constructs were transcribed using the SP6 MessageMachine kit (Ambion). For rescue experiments, WT or *Tg*(*cmlc2:GFP*) embryos were injected with approximately 75 pg of mRNA.

2.8.7 *in situ* hybridization

Zebrafish whole-mount *in situ* hybridization was performed as previously described (Jowett and Lettice, 1994). Digoxigenin-UTP-labeled riboprobes were synthesized according to manufacturer's instructions (Boehringer Mannheim). The *dpf3* probe was amplified from cDNA and subcloned into pCS2+. Probes for *amhc* and *vmhc* were a gift from D. Yelon.

Embryos were mounted in Permount (Fisher Scientific) and documented using an Axioplan 2 microscope (Zeiss).

Sea urchin embryo whole mount *in situ* hybridization was carried out as described with modifications in (Ransick *et al.*, 1993). First, proteinase K treatment was omitted in our experiments. Second, hybridization and posthybridization washes were carried out at 65 °C. The third posthybridization wash was performed using 0.1x SSC instead of 1x SSC. Finally, probe concentration was increased to 1 ng/µl, whereas the anti-digoxygenin (DIG)-AP antibody concentration was reduced to a dilution of 1:3500. Staining was carried out using the BCIP/NBT system (Vector Laboratories Inc., Burlingame, VT, USA) according to the manufacturer's instructions. Antisense DIG-labeled RNA probes were synthesized from linearized plasmid DNA using a DIG-RNA labeling kit (Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturer's instructions. DNase treatment was omitted because it was observed that including the unlabeled linearized DNA in the hybridization significantly reduced background.

2.9 Protein biochemistry

2.9.1 Recombinant protein expression and purification

GST fusion proteins were created using the pGEX3x vector, expressed in *Escherichia coli* BL21 DE3 pRARE3 for 3 h at room temperature and purified using Glutathione-Sepharose matrix (Amersham) according to manufacturer's instructions.

2.9.2 GST pulldown assays

GST fusion proteins were coupled to MagneGST beads (Promega) and subsequently incubated with *in vitro* translated Flag-Baf60c for 1 h at room temperature or HEK293T nuclear extracts overnight at 4 °C in binding buffer (4.2 mM Na_2HPO_4, 2 mM KH_2PO_4, 250 mM NaCl, 10mM KCl, 0.1 % NP40, 0.5 %BSA, pH 7.2, complete protease inhibitor (Roche)). Beads were washed three times in binding buffer, resuspended in 6x SDS sample buffer, denatured at 95 °C for 5 min and subjected to Western analysis using anti-GST antibody (Zymed) (1:2000) in 3 % milk/PBS.

2.9.3 Histone Peptide Binding Assays

For histone peptide binding assays, 1 µg of biotinylated histone peptide (Upstate, and kind gifts of D. Patel and D. Allis) was incubated with 1 µg of purified GST fusion protein in

binding buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.1 % NP-40, 50 μM ZnAc) overnight at 4 °C with rotation. Streptavidin beads (Dynabeads) were added and incubated for 1 h at 4 °C with rotation followed by four rounds of washing in binding buffer. Beads were subsequently resuspended in 4x LDS sample buffer (Invitrogen). Bound proteins were analyzed by LDS-PAGE gels (Invitrogen) and subjected to immunoblotting analysis using anti-GST antibody (Zymed) (1:2000) in 3% milk/PBS.