3 Materials and Methods

3.1 Materials

3.1.1 Chemicals and Enzymes

If not specified otherwise, all chemicals have been purchased from Amersham Bioscience (GE Healthcare), Fluka, GibcoBRL, Merck, Roth, and Sigma-Aldrich. Enzymes, nucleic acids (plasmids and kb ladders), and nucleoside triphosphates were ordered from Amersham Bioscience / GE Healthcare , Fermentas GmbH, Invitek, Invitrogen, Roche, and Stratagene.

3.1.1.1 Oligonucleotides

Oligonucleotides for genotyping and sequencing were synthesized by BioTez GmbH. Primer and probes for RT-PCR analysis were ordered from Applied Biosystems as complete gene expression assay or synthesized by BioTez GmbH.
Sequences of primers are provided in 5' → 3' orientation:

cre800	GCTGCCACGACCAAGTGACAGCAATG
cre1200	GTAGTTATTCGGATCATCAGCTACAC
PL1	GTGTCTGGCACTGCTTCCTTGGAAGTG
PL2	ACCGCTCCCATGCCTTCGAGAGTCTTG
PL4	ATTCCGGGCTAGAGCCACTAGACCTG
T7	TAATACGACTCACTATAGG
Sp6	ATTTAGGTGACACTATAGAATA

Sequences of primers and probes for RT-PCR analysis are provided in 5' → 3' orientation:

Titin amplicons

Z1/2 for	CGATGGCCGCGCTAGA
Z1/2 rev	CTCAGGGAGTATCGTCCACTGTT
Z1/2 probe	6-FAM-TGATGATCCCCGCCGTGACTAAAGC-TAMRA
N2B for	ACAGTGGGAAAGCAAAGACATC
N2B rev	AGGTGGCCCAGAGCTACTTC
N2B probe	6-FAM-GAAAGAGCTGCCCCTGTGATCA-TAMRA
Mex6 for	GCCTTGTGTGGTAGTTCTAAATTCAA
Mex6 rev	TTTGCTGTGGCTCATTGCTT
Mex6 probe	6-FAM-TTTCACCGGGAACTGGGCAA-TAMRA

Titin binding proteins

Calm for	GCTCGCACCATGGCTGAT
Calm rev	CCCCAGTTCCTTGGTTGTG
Calm probe	6-FAM-AGCAGATTGCTGAATTCAAGGAAGC-TAMRA
FHL2 for	CTTCTGTGACTTGTACG
FHL2 rev	ATTGGCGTTCCTCGAAAG
FHL2 probe	6-FAM-CCCATTAGTGGTCTGGGTGGCACAA-TAMRA
MuRF-1 for	CCGAGTGCAGACGATCATCTC
MuRF-1 rev	CCTTCACCTGGTGGCTATTCTC
MuRF-1 probe	6-FAM-TGCACAGCCACACTAACTGTGACAGTGC-TAMRA
MuRF-2 for	TGGAGAACGTATCCAAGTTGGT
MuRF-2 rev	CCTTTGATGCTTCCACGATCT
MuRF-2 probe	6-FAM-CATGGATGAGCCCGAAATGGCA-TAMRA
Myomesin for	GCGTAGTCATCACTCCTG
Myomesin rev	GTGGCTGCACCCATTTTG
Myomesin probe	6-FAM-ATAGAAATGGAGCACCTGTTTCTCC-TAMRA
Nbr1 for	TCGTACAAGGCCCTGTTT
Nbr1 rev	CAGGGCTGTGGTCTGATC
Nbr1 probe	6-FAM-ACTGCACAGCCCATCGTTTCTGAAG-TAMRA
Sqstm1 for	CCACCAGAAGATCCCAAT
Sqstm1 rev	CTTCTCTTCCCTCCATGC
Sqstm1 probe	6-FAM-CCCTCAGCCCTCTAGGCATTGAGGT-TAMRA

T-cap for	GGTCTCATGCCTCTGTGC
T-cap rev	GCTGAGTGGAAAGACCTG
T-cap probe	6-FAM-CGTGCAAGGAGCATCCCTCTTCCGG-TAMRA

Signaling proteins

ANP for	TTCTAGGCGCAGCCCCT
ANP rev	GCAGAGCCCTCAGTTTGCTT
ANP probe	6-FAM-ACCCCTCCGATAGATCTGCCCTCTTGAA-TAMRA
MAPKAP for	GTGTGGGTATCCCCCCTTCT
MAPKAP rev	TACGAGTCTTCATGCCCGG
MAPKAP probe	6-FAM-TCCAATCACGGCCTTGCCATCTC-TAMRA
Mef2C for	GGCTCTGTAACTGGCTGGCA
Mef2C rev	TCCCAACTGACTGAGGGCAG
Mef2C probe	6-FAM-CAGCAGCACCTACATAACATGCCGCC-TAMRA
MEK5 for	AGAGCCGCTGCAGAT
MEK5 rev	TCACCTTCAGGCCAT
MEK5 probe	6-FAM-ATTTCCAAGAGCCTGCAAGCCTCCC-TAMRA
TGF-β for	CCCCTGCTACTGCAAGTCAG
TGF-β rev	GTCCTCAGGTCCTGCCTCCT
TGF-β probe	6-FAM-CTTCTTGGCACTGCGCTGTCTCGC-TAMRA

Housekeeper

GAPDH for	GGCAAATTCAACGGCACAGT
GAPDH rev	AGATGGTGATGGGCTTCCC
GAPDH probe	6-FAM-AGGCCGAGAATGGGAAGCTTGTCATC-TAMRA
18S RNA for	CGCCGCTAGAGGTGAAATTC
18S RNA rev	TGGGCAAATGCTTTCGCTC
18S RNA probe	6-FAM-TGGACCGGCGCAAGACGGAC-TAMRA

3.1.2 Solutions and Buffers

Agarose loading buffer	50% (v/v) glycerol, 1 mM Na_2EDTA, 0.1% (v/v) Xylencyanol, 0.1% (v/v) orange G
Blocking solution	5% (w/v) skim milk, 0.1% (v/v) Tween 20 in PBS
5x coupling buffer	250 mM Tris-HCl, 25 mM EDTA, pH 8,5
Embryo lysis buffer	50 mM KCl, 3 mM $MgCl_2$, 10 mM Tris/HCl, pH 8.9, 0.01% (w/v) gelatine, 0.45% (v/v) NP-40, 0.45% (v/v) Tween 20
10x FA gel buffer	200 mM 3-[N-morpholino] propanesulfonic acid (MOPS), 50 mM sodium acetate, 10 mM EDTA, pH 7.0
1x FA running buffer	1/10 volume 10x FA gel buffer, 250 mM formaldehyde
Fixation solution	4% (w/v) paraformaldehyde in PBS, pH 7.4
HAB lysis buffer	6 M urea, 2% (w/v) CHAPS, 1 mM DTT
HBSS	5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.2 mM NaHCO, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM $MgSO_4$, 137 mM NaCl, 5.6 mM glucose, pH 7.4
HS-PBS	637 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4
Low TE	10 mM Tris/HCl, 0.1 mM Na_2EDTA, pH 8.0
Laemmli-buffer	50 mM Tris, 1% (v/v) ß-mercaptoethanol, 2% (w/v) SDS, 10% (v/v) glycerine, 0.1% (w/v) bromphenolblue, pH 6.8
PBS	137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4
PBST	PBS with 0.1% (v/v) Tween 20
Solution 1	50% (w/v) formamide, 5x SSC, 0.15% (v/v) Tween 20
Solution 2	10 mM Tris, pH 7.5, 50 mM NaCl, 0.15% (v/v) Tween 20
Solution 3	50% (w/v) formamide, 2x SSC, 0.15% (v/v) Tween 20
20x SSC	3 M NaCl, 0,3 M sodiumcitrat pH 7,0
Stripping solution	200 mM glycine, 0.1% (w/v) SDS, 0.1% (v/v) Tween 20, pH 2.6
1x TAE	10 mM sodium acetate, 1 mM Na_2EDTA, 40 mM Tris/HCl, pH 8.0
TAB lysis buffer	8 M urea, 2 M thiourea, 2% (w/v) SDS, 75 mM DTT, 0.05 M Tris, pH 6.8
Tail buffer	20 mM Tris/HCl, pH 8.0, 5 mM EDTA, pH 8.0, 0.4 M NaCl 0.2% (w/v) SDS,
TBS	137 mM NaCl, 2.7 mM KCl, 50 mM Tris, pH 7.4
TBST	TBS with 0.05% (v/v) NP-40

1x TE	10 mM Tris/HCl, 1 mM EDTA, pH 8.0
TFB	30 mM potassium acetate, 50 mM $MnCl_2$, 100 mM KCl, 10 mM $CaCl_2$, 15% (v/v) glycerine, pH 6.3
Transfer buffer	192 mM Tris, 25 mM glycine, 20% (v/v) methanol, pH 8.3
Trypsin-EDTA (1x)	0.05% (v/v) trypsin, 0.53 mM EDTA
Washing buffer	PBS with 0.02% (v/v) sodium azide

3.1.3 Media

LB-Medium	10 g/l bacto-tryptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.0
LB/Amp-Medium	LB-media with 100 μg/l ampicillin
SOB-Medium	20 g/l bacto tryptone, 5 g/l yeast extract, 8.6 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$
SOC-Medium	20 g/l bacto-peptone, 5 g/l yeast extract, 0.5 g/l NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose, pH 7.0
LB-Agar	LB-medium with 15 g/l agar
LB/Amp-Agar	LB-agar with 150 μg/l ampicillin
Feeder-Medium	DMEM, 10% (v/v) FCS, 100 U/ml penicillin, 1% (v/v) 100 x non-essential amino acids, 2 mM L-glutamine, 100 μg/ml streptomycin

3.1.4 Bacterial strain

Following *E.coli* strain has been used:

DH5α	supE44 ΔlacU169 (ϕ80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1

3.1.5 Vectors

pBluescript®II KS +/-	T3 and T7 promoters for *in vitro* transcription
pBluescript®II SK +/-	T3 and T7 promoters for *in vitro* transcription
pGem® T-easy	for cloning of PCR products

3.1.6 Antibodies

Polyclonal antisera from rabbit (rab) and goat (g) or monoclonal antibodies from mouse (m) have been applied as primary antibodies for immunofluorescence staining (IF) and western blotting (WB). Name of the antibodies or antisera, origin, and dilutions are indicated below:

		IF	WB	
α-Actinin (EA53)	m	1:500	-	Sigma-Aldrich
Actin (A2066)	rab	-	1:2000	Sigma-Aldrich
Calmodulin (N-19)	rab	-	1:50	Santa Cruz Biotechnology, Inc.
FHL2 (BL455)	m	-	1:200	MBL
M8/M9 Titin	rab	1:500	1:375	Dr. Labeit, University of Mannheim
Myomesin EH	rab	1:5000	1:10000	Dr. Perriard, ETH Zürich
MuRF-1	rab	-	1:500	Regeneron Pharmaceuticals, Inc.
MuRF-2	m	-	1:200	Dr. Labeit, University of Mannheim
Sequestosome 1 (P-15)	g	-	1:50	Santa Cruz Biotechnology, Inc.
Titin-N2B	rab	1:200	-	Dr. Labeit, University of Mannheim
T-cap	rab	-	1:500	Eurogentec GmbH

Horse radish peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich), goat anti-mouse IgG (Calbiochem), and donkey anti-goat (Santa Cruz Biotechnology, Inc.) were used as secondary antibodies. Fluorescence conjugated secondary antibodies (Alexa Fluor 488 goat anti-rabbit, Molecular Probes; Cy3 goat anti-mouse, Jackson Immuno Research Inc.) were applied for immunostaining experiments.

3.1.7 Kits

Easy Pure DNA Purification Kit	Biozym GmbH
In situ cell death detection kit (TUNEL assay)	Roche
High pure PCR product purification kit	Roche
pGem®-T Easy Vector Systems	Promega
Plasmid Mini Kit	Peqlab
Plasmid Maxi Kit	Qiagen
Protein Assay	Bio-Rad
qPCR MasterMix Plus	Eurogentec GmbH
RNase-Free DNase Set	Qiagen
RNeasy Mini Kit	Qiagen
SulfoLink® Kit	Pierce
TAKARA ligation kit	Panvera

TaqMan® Gene Expression Assays	Applied Biosystems
Thermoscript First-Strand synthesis system	Invitrogen

3.1.8 Software

If not specified otherwise, software as indicated below was used for detecting, analyzing, and editing data:

ABI Prism Big Dye Terminator Kit v3.1	Applied Biosystems
Adobe Acrobat 5.0	Adope Systems Inc.
Aida version 3.51.042	Raytest GmbH
Corel Draw 12.0	Corel Corp.
Excel 10.0	Microsoft
LSM 5 Image Browser	Carl Zeiss GmbH
Metamorph 6.2r2	Universal Imaging Corp.
Photoshop 7.0	Adope Systems Inc.
SeqMan II 4.03	DNAStar Inc.
Sequence Detection System 2.1 software	Applied Biosystems
WinEdt 5.2	MiKTex
Word 10.0	Microsoft

3.2 Methods

3.2.1 Molecular Biology Methods

3.2.1.1 DNA preparation for genotyping

Total genomic DNA for genotyping was extracted from tissue of the ear by o/n digest with 5 μl of 20 μg/ml Proteinase K solution in 200 μl embryo lysis buffer at 56°C. Proteinase K was inactivated by heat shock at 95°C for 5 min. Digested tissue was diluted and utilized directly for polymerase chain reaction (PCR).

3.2.1.2 Polymerase chain reaction

If not mentioned otherwise, all PCR amplification reactions were conducted with the thermostable *Taq* DNA Polymerase. The enzyme has a highly processive 5'-3' polymerase activity with optimum activity achieved at 74°C but lacks 3'-5' exonuclease activity. All reaction were carried out in the PCR-Thermocycler Px2 (Thermo Hybaid) or PTC-200 (MJ Research). The following PCR reaction mix was applied to PCR softstrips (Biozym GmbH):

2.5 μl	NH_4- reaction buffer (10x)
0.75 μl	$MgCl_2$ (50 mM)
0.5 μl	dNTP Mix (10 mM)
0.5 μl	Primer Mix (10 μM)
20-50 ng	DNA Template
0.2 μl	*Taq* Polymerase (5 U/μl)
- μl	to a final of 25 μl with dH_2O

The following amplification conditions were chosen:

	Step	Temperature	Time	
1.	Denaturing	95°C	2 min	
2.	Denaturing	95°C	15 sec	⎫
3.	Annealing	55°C	15 sec	⎬ 38x
4.	Extension	72°C	60 sec/ 1kB	⎭
5.	Extension	72°C	8 min	
6.	Storage	10°C	forever	

3.2.1.3 Agarose gel electrophoresis

After amplification, the PCR reaction mix was loaded with 1/10 volume agarose loading buffer. Depending on the expected product size it was applied onto a 1.0 to 2.0% (w/v) agarose gel to separate PCR products. Agarose gels were prepared with high melt agarose (Invitrogen), 0.5x TAE, and 0.5 μg/ml ethidiumbromide solution. A 1 kB DNA ladder was used to determine the size of the PCR product. The separation took place in 0.5x TAE running buffer for 20 min at 100 volts in an agarose gel chamber Mupid®-ex (Eurogentec GmbH). PCR fragments were visualized on a UV unit (U-RFL-T; Olympus) at 312 nm. DNA fragments that were utilized for cloning experiments were cut out with a scalpel. The purification of PCR products was performed with the Easy Pure DNA Purification Kit (Biozym GmbH) according to manufacturer's instructions.

3.2.1.4 Preparation of plasmid DNA

The isolation of purified plasmid DNA from 3 ml overnight cultures (mini culture) was performed using the Plasmid Mini Kit according to manufacturer's instruction. The yield was about 15-25 μg plasmid DNA. To gain a larger amount of DNA, maxi DNA preparation of 200 ml overnight bacteria cultures was conducted using a plasmid maxi kit as indicated. 500 μg DNA was obtained when using the Qiagen-tips 500.

3.2.1.5 Determination of nucleic acid concentration

The concentration of DNA solubilized in water was determined with a photometer (Amersham Bioscience / GE Healthcare) using a silica cuvette. It was measured at a wavelength of 260 nm the absorption maximum of single-stranded as well double-stranded DNA. Absorption of 1 at 260 nm corresponds to a concentration of 50 μg/ml for double-stranded DNA, 20 μg/ml for single-stranded DNA and 40 μg/ml for RNA. The ratio A260 to A280 is used to estimate the purity of nucleic acid, since proteins absorb at 280 nm. A ratio of 1.7 to 2.0 for the solubilized DNA was declared as pure.

3.2.1.6 Enzymatic modifications of DNA

3.2.1.6.1 Restriction digest of DNA The restriction or linearization of plasmid DNA was performed according to standard protocols (Sambrook and Russell, 2000). Amounts of enzyme, buffer, and conditions of the reaction were set up as recommended by manufacturer's information. Restriction digests were conducted for 2-3 h or o/n at 37°C in a heating block. The restriction reaction was stopped by adding DNA loading buffer for agarose gel electrophoresis.

3.2.1.6.2 Dephosphorylation of DNA-fragments If DNA fragments were linearized with a single restriction enzyme (generating either blunt or overhanging ends), dephosphorylation of the vector is a prerequisite to reduce religated vector background. Therefore after 2 hours of restriction digest alkaline phosphatase (CIP) (1 U /pmol 5´- ends) was added and incubated for 30 min at 37°C. The restriction reaction was stopped by adding DNA loading buffer for agarose gel electrophoresis subsequently.

3.2.1.6.3 Ligation of DNA-fragments DNA-fragments were ligated using the TAKARA ligation kit according to the protocol provided by the manufacturer. The molar ratio of vector and insert was generally chosen 1:3. The reaction took place for 2 h at 16°C or o/n at 4°C.

3.2.1.7 DNA Sequencing

DNA sequencing is a linear extension reaction initiated at a specific site on the template DNA by using a complementary oligonucleotide. All sequencing reactions were performed according to the chain termination method from Sanger (Sanger et al., 1977) using the standard T7 or Sp6 sequencing primers and the ABI Prism Big Dye Terminator Kit v3.1 (Applied Biosystems). These reagents are suitable for performing fluorescence-based cycle sequencing reactions on single-stranded or double-stranded DNA templates and on polymerase chain reaction fragments.
The following PCR sequencing reaction mix was used in softstrips (Biozym GmbH) on the PCR-Thermocycler Px2 (Thermo Hybaid) or PTC-200 (MJ Research):

2.0 μl	BigDye Terminator v1.1/3.1 Seq. Buffer (5X)
0.25 μl	Primer (10 μM)
5 ng/ 100 bp	DNA Template
1 μl	Big Dye
- μl	to a final of 10 μl with dH_2O

PCR cycling conditions:

	Step	Temperature	Time	
1.	Denaturing	95°C	10 sec	30x
2.	Annealing	50°C	5 sec	
3.	Extension	60°C	4 min	
4.	Storage	10°C	forever	

The ABI PRISM® 377 DNA Sequencer (Applied Biosystems) automatically analyzed DNA molecules labelled with fluorescent dyes. After samples were loaded onto the system's vertical gel, they underwent electrophoresis, laser detection, and computer analysis. The interpretation of the sequences was completed with SeqMan II version 4.03 (DNASTAR Inc.).

3.2.1.8 In-vitro transcription

When designing a transcription template, it must be decided whether sense or antisense transcripts are needed. If the RNA is to be used as a probe for *in situ* hybridization complementary so called antisense transcripts are required. Therefore the DNA to be transcribed was cloned antisense into the polylinker of an appropriate transcription vector which contains a suitable promoter (T7) adjacent to the polylinker. As negative control the DNA was also cloned in sense orientation. For the synthesis of transcripts, the plasmid was linearized by the appropriate enzyme, purified using the high pure PCR product purification kit, and solubilized in DEPC treated water. Proper linearization was confirmed by agarose gel electrophoresis. DIG-labelled, single-stranded RNA probes for *in-situ* hybridization were then generated by *in-vitro* transcription. DIG-11-UTP (Roche) was incorporated by T7 RNA polymerase (USB) at approximately every 20-25th nucleotide of the transcript. The *in-vitro* RNA synthesis was performed for 2 hours at 37°C as indicated below:

1 μg	linearized plasmid
2 μl	transcription buffer (10x) (USB)
2 μl	DIG RNA labelling Mix (10x) (Roche)
1 μl	Protector RNase Inhibitor (40 U/μl) (Roche)
2 μl	T7 RNA polymerase (20 U/μl) (USB)
- μl	to a final of 20 μl with RNase free dH_2O

2 μl of RNase-free, DNase I (Roche) were added and incubated for 45 min at 37°C to remove template DNA. The RNA was purified using the RNeasy Mini Kit in accordance to manufacturer's instructions. RNA concentration was determined photometrically. To control quality and possible degradation purified RNA was applied to a formaldehyde agarose gel (see section 3.2.1.10).

3.2.1.9 Isolation of total RNA

To avoid RNase contamination all experiments involving RNA were conducted with filtertips (Starlab). Hearts were dissected in DEPC treated PBS on ice, quick-frozen, and

stored at -80°C. For isolation of total RNA the hearts of 5 to 10 mouse embryos of different litters were pooled (depending on the size and age of the embryo) in the lysis buffer provided within the RNeasy Mini Kit. To reduce viscosity of lysate and shear genomic DNA, samples were homogenized with an Ultra-Turrax T-8 (IKA®-Werk). RNA was isolated according to manufacturer's recommendations including an on-column DNase digest with the RNase-free DNase Set to remove contaminating genomic DNA. The ratio of the readings at 260 nm and 280 nm in a spectrophotometer provided an estimate of the purity of the RNA with respect to contaminants that absorb in the UV, such as protein. Isolated RNA had in 10 mM Tris-HCl, pH 7.5 a ratio of 1.9 - 2.1.

3.2.1.10 Formaldehyde agarose gel electrophoresis

100-500 μg of total RNA were separated on denaturing formaldehyde agarose gels to control quality and possible degradation. RNA was loaded with 5x RNA loading buffer onto a 1.2% (w/v) FA gel prepared with 1/10 volume of 10x FA gel buffer, high melt agarose, and 0.5 μg/ml ethidiumbromide solution. The separation took place in 1x FA gel running buffer for 40 min at 100 volts in an agarose gel chamber Mupid®-ex (Eurogentec GmbH). Ribosomal RNA was visualized on a UV unit (U-RFL-T; Olympus) at 312 nm. The 28S ribosomal RNA from mouse tissue was present with an intensity approximately twice that of the 18S RNA band. Diffuse bands would have indicated degradation during preparation.

3.2.1.11 cDNA Synthesis

For quantitative real-time polymerase chain reaction (RT-PCR) it was necessary to generate cDNA. Therefore total RNA was reverse transcribed using the two-step Thermoscript first-strand synthesis system according to the manufacturer's guidelines:

2 μl	dNTP Mix (10 mM)
1 μl	Random Primer (50 ng/μl)
1 μg	RNA Template
- μl	to a final of 12 μl with RNase free dH_2O

RNA and primer were denatured by incubating at 65°C for 5 min. A mastermix was prepared and added to the denatured primer/RNA mix:

4 μl	cDNA Synthesis Buffer (5x)
1 μl	DTT (0.1 M)
1 μl	RNase OUT (40 U/μl)
1 μl	DEPC-treated water
1 μl	Thermoscript RT (15 U/μl)

The cDNA synthesis was performed in the PCR-Thermocycler PTC-200 (MJ Research) at 65°C for 60 min and terminated by incubation at 85°C for 5 min.

3.2.1.12 Real-time PCR

Quantitative RT-PCR provides an accurate method to determine levels of specific DNA and RNA in tissue samples. It is based on detection of a fluorescent signal produced proportionally during amplification of a PCR product. It was carried out using the TaqMan® probe-based chemistry (Applied Biosystems) on an ABI Prism 7000 Sequence Detection System and 7900 HT Fast Real-Time PCR System. Primer and probes were designed using Primer Express 1.5 (Applied Biosystems) and ordered from BioTez GmbH (18S RNA, ANP, GAPDH, MAPKAP, Mef2C, Titin's M-line (MEx6), MuRF-1, MuRF-2, Titin's N2B, TGF-β, Titin's Z1/2) or ordered as TaqMan® Gene Expression Assays from Applied Biosystems (Calm, FHL2, MEK5, Myomesin, Nbr1, Sqstm1, T-cap). Real-time PCR amplification reactions were performed using the qPCR MasterMix Plus (Eurogentec GmbH) according to manufacturer's specifications with 2x TaqMan universal PCR master mix, 900 nM primer, and 250 nM probe. Thermal cycling conditions were as followed:

	Step	Temperature	Time	
1.	Annealing	50°C	2 min	
2.	Denaturing	95°C	10 min	
3.	Denaturing	95°C	15 sec	) 50x
4.	Extension	60°C	1 min	)
5.	Storage	4°C	forever	

Data were collected and analyzed with the Sequence Detection System 1.2 or 2.2 software (Applied Biosystems). The Comparative CT Method (Δ CT Method) was used as described in the User Bulletin 2: ABI PRISM 7700 Sequence Detection System.

3.2.2 Microbiological Methods

3.2.2.1 Generation of chemically competent DH5α bacteria cells

From a -80°C glycerol stock, bacteria were plated on a freshly prepared LB-agar plate without antibiotics. 5 single colonies were used to inoculate 50 ml SOB medium in a 1000 ml Erlenmeyer flask. The cells were grown at 37°C and 230 rpm until optical cell density measured at 600 nm (OD_{600}) reached 0.15 and then sedimented in a Beckmann JA-10-Rotor centrifuge (2500 rpm, 20 min, 4°C). Supernatant was discarded and cells washed once with 1/3 volume TFB by gentle mixing. After incubation on ice for 15 min and centrifugation with the same conditions as above, the cells were resuspended in 1/12 volume TFB and 7 μl DMSO / 200 μl suspension. Cells were shaken gently and chilled for 10 min before adding additionally 7 μl DMSO / 200 μl suspension. Chemically competent cells were aliquoted in 100 μl fractions into cold tubes and stored at -80°C.

3.2.2.2 Transformation of bacteria

Competent cells were thawed on ice and 5 μl of the ligated plasmid was gently mixed with the cells. After incubation on ice for 10 min, the transformation of the bacteria was performed using the heat shock method at 42°C for 40 seconds. The cells were incubated in 0.8 ml SOC-medium for 50 min at 37°C, sedimented and resuspended in 100 μl of SOC-medium. The cells were plated on appropriate LB-antibiotic plates and incubated o/n at 37°C.

3.2.2.3 Cultivation and storage of transformed bacterial cells

Bacterial cells carrying the transformed plasmid were antibiotic resistant and grew as single colonies o/n at 37°C. They were picked with a sterile tooth stick. 3 ml of LB-medium with the appropriate antibiotic in falcon tubes that were permeable to air were inoculated. Bacteria were cultivated at 37°C and shaking at 230 rpm. Cells could be harvested on the following day by centrifugation at 14,000 rpm for 1 min (Centrifuge 5417 R, Eppendorf). For long term storage, cells were resuspended in LB-medium and mixed with $\frac{1}{2}$ volume sterile glycerine, quick-frozen, and stored at -80°C.

3.2.3 Cellbiological Methods

3.2.3.1 Preparation and cultivation of primary cells

Primary cell culture was prepared from E8.5, E9.5, and E10.5 knockout, heterozygous, and wildtype whole mount embryos. Pregnant mice were sacrificed according to the day of the

vaginal plug. The uterus was removed and transferred into a petri dish containing cold PBS. The embryos were dissected of the uterus tissue and head and if possible internal organs were removed. Embryos were kept in 100 μl trypsin-EDTA solution o/n at 4°C to allow trypsin to penetrate the tissue. Embryos were digested at 37°C for 10 min after removing excessive trypsin solution. Digestion was stopped by adding 5 volumes of feeder medium. Cells were separated by pipetting up and down and plated on cell culture dishes and incubated at 37°C and 5% CO_2 in an with water vapor saturated incubator (Binder). Cells were monitored every day and pictures were taken with the camera system from Visitron System GmbH (Model: 2.2.1). Medium was changed every other day.

3.2.3.2 Cell Viability assay

Isolated murine fibroblasts were applied 1:10 to a trypan blue solution (Sigma-Aldrich) and incubated for 5 min at RT. Cells were observed in Neubauer cell-counter chamber (Roth). Viable cells exclude trypan blue, while dead cells stain blue due to trypan blue uptake.

3.2.4 Biochemical Methods

3.2.4.1 Generation of polyclonal antisera

Antibodies were generated using the service from Eurogentec GmbH. The peptide H2N -CRRTLSRSMSQEAQRG representing the C-terminal 16 AA of murine T-cap was synthesized and conjugated to the carrier protein keyhole limpet hemocyanin (KLH). 250 μg of this peptide were injected 3 times every two weeks subcutaneously in to 2 female rabbits. Ten and 30 days after the last injection, the first blood samples were taken. 87 days after the last immunization, the animals were sacrificed and the final bleed was taken.

3.2.4.2 Purification of polyclonal antisera

The antiserum was first tested by western blot analysis and subsequently purified with affinity chromatography. For this purpose, the peptide was coupled via the cysteine residue to SulfoLink gel (Pierce) an activated agarose-gel according to the manufacturer's instructions. The antiserum was diluted 1:4 with 5x coupling buffer and incubated with the equilibrated agarose gel for 2 h at RT. Nonspecific binding sites on the gel were blocked with 0.05 M L-Cysteine for 45 min at RT. The gel was washed once with HS-PBS, two times with PBS and with 0.1x PBS before elution of the antibody with 5x 400 μl 100 mM glycine that was immediately neutralized by 40 μl of 1 M Tris and 2 M NaCl, pH 8,5. To stabilize the purified antibody, 100 μg/ml BSA and 0.01% sodium azide were added.

3.2.4.3 Preparation of protein lysate from heart tissue

Hearts from adult and embryonic mice were dissected in PBS on ice. If possible the atria were removed and depending on the age left ventricles of embryos with the same genotype were pooled. Embryonic tissue until E15.5 was dispersed in 0.5 M Tris-HCl, pH 7.5 (25 μl/ 10 mg tissue), homogenized using a micro mortar followed by a DNase digest with 50 U for 30 min at 37°C. HAB lysis buffer (25 μl/ 10 mg tissue) with the serine- and cysteine-protease inhibitors 1 mM PMSF and 2 μg/ml leupeptine was added. Tissue of older embryos and adult hearts were directly added to HAB lysis buffer and homogenized with an Ultra-Turrax T-8 (IKA®-Werk) for 30 sec. All samples were treated with a sonopuls sonicator SH70G (BANDELIN electronic GmbH) at 70% intensity on ice until the lysis was completed. Cell debris and nuclei were separated by centrifugation for 10 min at 14,000 rpm and 4°C (Centrifuge 5417 R, Eppendorf). An aliquot of the solubilized protein solution was removed for protein quantification and the remaining solution was quick-frozen in liquid nitrogen and stored in aliquots at -80°C.

3.2.4.4 Protein quantification

For protein quantification different methods were utilized. Samples that were prepared for SDS agarose gels were quantified using the amido black method. If the samples were treated without SDS for western blot analysis the protein concentration could be determined with a protein assay according to manufacturer's information. The protein assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when protein binding occurs (Bradford, 1976). Bovine serum albumin (BSA) diluted in the appropriate lysis buffer was used for each method separately to generate a protein standard curve. Protein concentration was quantified using plastic cuvettes in a spectrophotometer (Amersham Bioscience / GE Healthcare) at a wavelength of 595 nm. To improve accuracy each sample was measured in duplicates. The respective lysis buffer was used as the blank value.

3.2.4.5 SDS-polyacrylamide electrophoresis (SDS-PAGE)

The separation of proteins by molecular mass was carried out according to Laemmli's method (Laemmli, 1970). The protein solution was reduced and denatured by applying 2x Laemmli-buffer, 0.2% (v/v) ß-mercapoethanol, and incubation at 95°C for 5 min. Depending on the molecular mass of the protein, the electrophoresis was performed with 6-12% acrylamide gels at 60-120 volts in a mini gel chamber SE 250 (Hoefer^{TM}) in 1x electrophoresis buffer. A prestained protein ladder was applied to estimate the size of the proteins.

3.2.4.6 Western Blot

The SDS protein gel was transferred to a hybond-C extra nitrocellulose-membrane (Amersham Bioscience / GE Healthcare). Whatman 3MM filter paper was used without air bubbles. All elements of the blot were equilibrated in transfer buffer. The transfer was performed in a Trans-Blot Cell (Bio-Rad) at 70 V for 1 h and 40 V for 2 hours while cooling or o/n at 20 V and 4°C. The membrane was stained with Ponceau S to confirm the transfer. For immunodetection the membrane was incubated in blocking solution for 60 min and with the specific first antibody diluted in PBS for 2 h at RT or o/n at 4°C. The membrane was washed with PBST, PBS, and PSBT for 15 min each. Horse radish peroxidase-conjugated goat anti-rabbit IgG (Amersham), goat anti-mouse IgG (Calbiochem), and donkey anti-goat IgG (Santa Cruz Biotechnology, Inc.) were used as the secondary antibodies diluted 1:2000 in PBS and incubated with the membrane for 60 min at RT. The membrane was washed again and developed by chemiluminescence staining using ECL (Supersignal West Pico Chemiluminescent Substrate, Pierce) according to manufacturer's instructions. The chemiluminescence was detected in an Intelligent Dark Box and a LAS-1000 CCD camera (both FUJIFILM). The membrane was stripped if it was reused for incubation with a different antibody. Therefore the membrane was washed with PBST, incubated for 20 min at 80°C with stripping solution, washed again with PBST, and developed by chemiluminescence staining using ECL to confirm complete absence of the first used antibody. The membrane was blocked again for 60 min before incubating with the first antibody.

3.2.4.7 Preparation of protein lysate from heart tissue for VAGE

The preparation of protein lysates for SDS agarose gel electrophoresis was conducted as described before (see section 3.2.4.3). Except for the use of TAB buffer instead of HAB lysis buffer and dispense the sonication step that would have disrupted the large Titin molecule.

3.2.4.8 Vertical SDS-agarose gel electrophoresis (VAGE)

The electrophoretic separation of high-molecular-weight proteins (> 500 kDa) using polyacrylamide is difficult because gels with a sufficient pore size for adequate protein mobility are mechanically unstable. A 1% vertical sodium dodecyl sulfate agarose gel electrophoresis (VAGE) system has been developed that allows Titin (a protein with the largest known SDS subunit size of 3000-4000 kDa) to migrate and gain reproducible separation of Titin isoforms. The VAGE system was performed as described in the literature (Warren et al., 2003) using a Hoefer SE 600 apparatus. Following electrophoretic separation gels were fixed as described and dried o/n in a forced air oven set at 37°C. This is important to keep background staining low. For SYPRO® Ruby staining (Molecular Probes) gels were

shortly washed with water and stained for 6 h at RT according to manufacturer's instructions. Images were taken with the Camilla Imaging system and analyzed with Camilla version 1.00.070 (both Raytest GmbH).

3.2.5 Animal Procedures

3.2.5.1 Setting up timed matings and dissection

Timed matings were set up in the afternoon and the morning of detecting a vaginal plug was regarded as day 0.5 post conception. Pregnant mice were sacrificed following institutional guidelines and the German animal protection law. Embryos were harvested at different stages of embryonic development. The exact developmental stage of embryos was determined by the number of somites. Whole mount embryos for immunohistology were fixed o/n in freshly prepared 4% PFA in PBS, pH 7.4 at 4°C. In order to prepare protein lysates for expression analysis hearts were dissected in PBS, quick-frozen, and stored at -80°C. For RNA studies, hearts were dissected in RNase free PBS and treated as described previously.

3.2.5.2 Preparation of embryonic tissue for histology

Histology is the study of tissue sectioned as thin slices. The tissue is fixed to prevent decay. The most common technique to investigate the microscopic anatomy of tissue is wax embedding. Alternative technique are cryosections. Paraffin sections have an advantage over cryosections because structural preservation is improved. The preparation of tissue for cryosections is less time consuming, less toxic, and preserves the expression of proteins much better. This makes cryosection a suitable tool for localization studies *in situ*.

3.2.5.3 Paraffin embedding

The preservation of the structure using paraffin sectioning compared to cryostat histology is much better but required an extensive treatment. If the tissues are to be embedded in paraffin, all traces of water must be removed because water and paraffin are immiscible. Whole mount embryos were processed through a series of increasing graded alcohol concentrations. The embryos were transferred sequentially to 70%, 80%, 90%, 96%, and 100% ethanol for two hours each. They were then placed in a second 100% ethanol solution to ensure that all water was removed. Before paraffin embedding, the tissue has to be cleared. Clearing refers to the use of an intermediate fluid that is miscible with ethanol and paraffin, since these two compounds are immiscible. Xylol was used as clearing agent two times for 20 min. Embryos were embedded transversally and 5 μm sections were cut on the Mi-

crotome RM2155 (Leica). Up to 6 sections were placed on 3-aminopropyl-triethoxysilane (APTEX) coated slides and stored at RT.

3.2.5.4 H & E staining

H & E staining is a staining method in histology. It is the most widely used stain in medical diagnosis. Before staining of the sections, all traces of wax had to be removed with two incubation steps in xylol for 5 min. The sections were rehydrated sequentially in 100%, 96%, 90%, 80%, 70% ethanol, and H_2O for 5 min. The staining method involves application of the basic dye haematoxylin, which colors basophilic structures with blue-purple after rinsing in tap water, and alcohol-based acidic eosin, which colors eosinophilic structures bright pink. The basophilic structures are usually the ones containing nucleic acids. The eosinophilic structures are generally composed of intracellular or extracellular protein and most of the cytoplasm is eosinophilic. However, the staining of the nuclei was performed with 20% Mayer's haemalaun solution (Merck) for 1 min. The cytoplasm and connective tissue were counterstained pink with 1% eosin solution for 2 min. Stained sections were dehydrated through a series of increasing graded alcohol concentrations (70%, 80%, 90%, 96%, and 100%) and two times xylol for 5 sec before mounting with canada balsam (Merck) and cover slips. Multiple serial sections were analyzed for each developmental stage and genotype on the BX51 Microscope (Olympus).

3.2.5.5 Cryostat histology

Dissected and fixed embryos were equilibrated with 30% sucrose in PBS for 6 h at RT. For cryosections, embryos were embedded sagittally in Tissue Tek® (OCT Compound, Vogel) and 5 μm sections were cut using the Cryocut 3000 (Leica) at -20 to -25°C and placed on 3-aminopropyl-triethoxysilane (APTEX) coated slides. Slides were immediately used for immunostaining or stored at -20°C.

3.2.5.6 Immunostaining of cryosections

An advantage of immunofluorescence staining of embryonic cryosections over detection with enzyme-coupled secondary antibodies is that it is possible to work with antibodies generated in different species. It makes it possible to co-localize proteins. Prior to staining, sections were airdried, re-fixed with 4% PFA for 15 min, rinsed with PBS, blocked and permeabilized with 10% (v/v) goat serum, 0.3% (v/v) Triton X-100, 0.2% (w/v) BSA in PBS for 60 minutes. Cryosections were incubated o/n with primary antibodies diluted in washing buffer at 4°C in a wet chamber, followed by rinsing with washing buffer. The fluorescent-conjugated secondary antibodies (Alexa Fluor 488 goat anti-rabbit, Molecular

Probes; Cy3 goat anti-mouse, Jackson Immuno Research, Inc.; Alexa Fluor 488 donkey anti-goat, Molecular Probes) were applied 1:1000 in PBS and incubated for 2 h at RT. Nuclei were stained for 30 min with DAPI (4´,6-diamidino-2-phenylindole) diluted 1:2000 in PBS. Immunofluorescence stained tissue was washed with PBS, rinsed with water and mounted with fluorescence protecting medium (DakoCytomation). Protein localization was documented using a confocal laser scanning microscope (Carl Zeiss Laser Scanning Microscope LSM 5 Pascal Version 3.0 SP2) with a Carl-Zeiss PLAN-NEOFLUAR 100X lens (1.3 NA). Images were processed with the LSM 5 Image Browser and Photoshop 7.0. Confocal laser microscopy is a valuable tool to obtain high resolution images. The big advantage of confocal microscopy is the possibility to collect light exclusively from a single plane. A pinhole sitting conjugated to the focal plane keeps the light from the detector that is reflected/emitted from others than the focal plane. The laser scanning microscope scans the sample sequentially point by point and line by line and assembles the pixel information to one image. That way optical slices of the specimen are imaged with high contrast and high resolution in x, y, and z.

3.2.5.7 Electron microscopy

Electron microscopes use a beam of highly energetic electrons to examine specimens on a very fine scale. Embryos for ultrastructural analysis of the sarcomere assembly were fixed during dissection with 3% formaldehyde in 200 mM Hepes, pH 7.4, for 30 min, followed by immersion in 8% formaldehyde/ 0.1% glutaraldehyde in 200 mM Hepes, pH 7.4, o/n at 4°C. Embryos were postfixed with 1% OsO_4 for 2 h, dehydrated in a graded ethanol series (30%, 50%, 70%, 90%, and 100%) and propylene oxide for 30 min, and embedded in Poly/Bed® 812 (Polysciences, Inc., Eppelheim, Germany). Ultrathin sections (70 nm) were contrasted with uranyl acetate and lead citrate and examined with a Zeiss 910 electron microscope. Digital images were taken with a 1k x 1k high speed slow scan CCD camera (Proscan). The diameter of about 25 sarcomeres per embryo was measured on a range of ultrathin sections taken from 2 embryos per genotype and developmental stage using the analySIS 3.2 software (Soft Imaging System, Münster, Germany).

3.2.5.8 In-situ cell death assay

Apoptosis is a genetically programmed process for cells to commit suicide under certain circumstances. During apoptosis, DNase activity introduces strand breaks (so called nicks) into the high-molecular-weight DNA. This process can be visualized by labelling the free 3'-OH termini with terminal transferase (TdT), which attaches labelled nucleotides to all 3´OH-ends (TUNEL reaction; TdT-mediated dUTP nick end labelling). Cryosections of mouse embryos were processed using the *In-situ* cell death detection kit (Roche Diagnostics, Germany) according to the protocol supplied by the manufacturer. In brief, sections

were fixed with 4% PFA and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. After washing, slides were incubated with TdT terminal transferase and fluorescein-dUTP for nick end labelling. Sections were counter-stained with α-Actinin (Sigma-Aldrich) diluted 1:500 in PBS. Stainings were analyzed using Olympus BX51 Microscope, CCD camera Visitron Systems Model 7.4 Slider from Diagnostic Instruments, and Metamorph software Version 6.2r2 (Universal Imaging Corp.).

3.2.5.9 In-situ hybridization

In-situ hybridization is a method to determine the spatial expression pattern of specific genes by using labelled RNA riboprobes (see section 3.2.1.8). Fixed embryos were washed in PBST, dehydrated sequentially in 25%, 50%, 75% in PBST and 2x 100% methanol for about 15 min, and bleached with 25% H_2O_2 in MeOH. After washing in 100% methanol embryos were incubated in a descending series of methanol concentrations. The tissue was permeabilized with 20 μg/ml Proteinase K and re-fixed with 4% PFA and 0.2% glutaraldehyde for 20 minutes at RT. Embryos were pre-hybridized in 50% formamide, 5x SSC, 0.1 mg/ml ssDNA (Sigma-Aldrich), 40 μg/ml Heparin, 50 μg/ml tRNA, 0.15% Tween 20, and 60 mM citric acid. Whole-mount RNA *in-situ* hybridization was performed using digoxigenin-labelled antisense riboprobes o/n at 65°C. Embryos were washed in solution 1 at 65°C for 2x 40 min, in solution 2 3x for 15 min, and 3 x in solution 3 for 60 min. Whole mounts were incubated with 0.75 U Anti-Digoxigenin-Antibody, Fab fragments (Roche) in 5% goat serum in TBST o/n at 4°C followed by washing with TBST for $>$ 8 h at RT. Embryos were stained with NBT/BCIP (nitroblue tetrazolium chloride / 5-bromo-4-chloro-3-indolyl-phosphate) solution (Roche, 350 μg/ml; 175 μg/ml, respectively). Images were taken after re-fixing with 4% PFA and clearance with 80% glycerol.

3.2.5.10 Statistical analysis

Results were expressed as means. A 2-tailed t-test with heteroscedastic variance was performed to assess differences between 2 groups. A p-value of < 0.05 was considered significant.