Cell Stem Cell

Supplemental Information

The Isl1/Ldb1 Complex Orchestrates Genome-wide Chromatin Organization to Instruct Differentiation of Multipotent Cardiac Progenitors

Luca Caputo, Hagen R. Witzel, Petros Kolovos, Sirisha Cheedipudi, Mario Looso, Athina Mylona, Wilfred F.J. van IJcken, Karl-Ludwig Laugwitz, Sylvia M. Evans, Thomas Braun, Eric Soler, Frank Grosveld, and Gergana Dobreva

SUPPLEMENTAL FIGURES

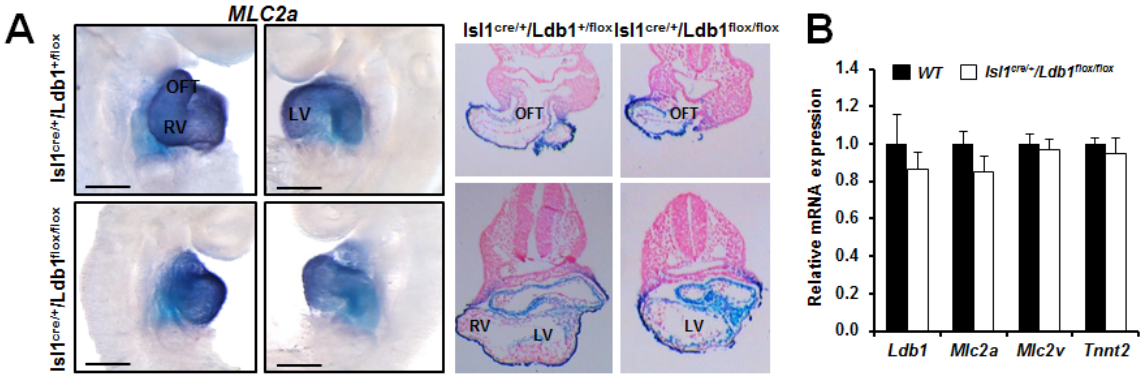
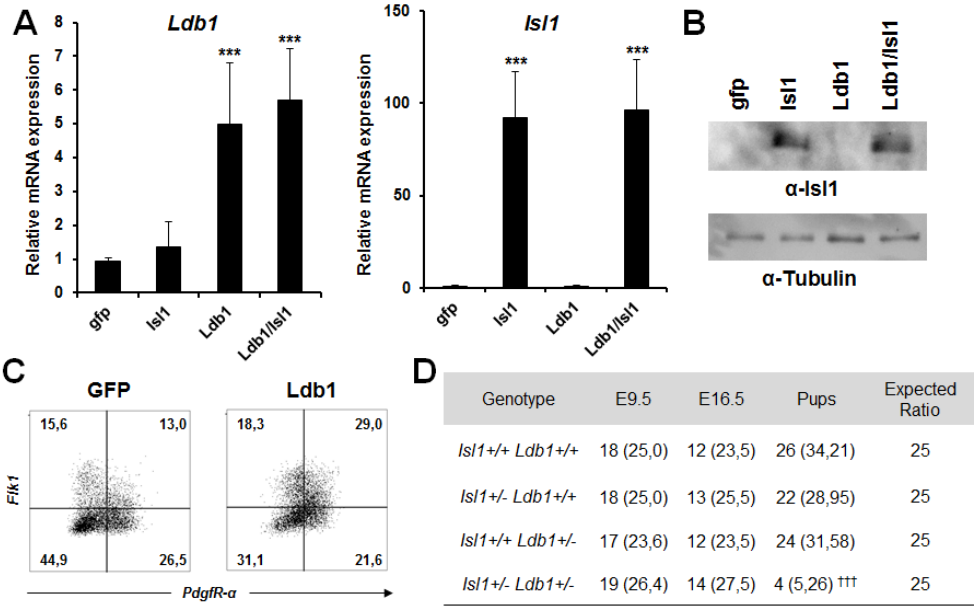


Figure S1, related to Figure 1. Aberrant cardiac morphology in *Isl1$^{cre/+}$/Ldb1$^{flox/flox}$* embryos. ***(A)*** Left and right views of E9.5 control and Isl1$^{cre/+/}$Ldb1$^{flox/flox}$ embryos after in situ hybridization with an Mlc2a riboprobe and corresponding sections, demonstrating aberrant cardiac morphology. Mlc2a in situ hybridization was used to stain the cardiac tissue to help with the visualization of the heart and was not performed in a quantitative manner. Scale bars, 500 μm. Abbreviations: OFT, outflow tract; RV, right ventricle; LV, left ventricle. ***(B)*** Relative mRNA expression analysis of cardiomyocyte genes in dissected left ventricles of E9.25 control and Isl1$^{cre/+}$/Ldb1$^{flox/flox}$ embryos. Data are mean ± SEMs, n=3 for each genotype.



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Genotype	E9.5	E16.5	Pups	Expected Ratio
Isl1+/+ Ldb1+/+	18 (25,0)	12 (23,5)	26 (34,21)	25
Isl1+/- Ldb1+/+	18 (25,0)	13 (25,5)	22 (28,95)	25
Isl1+/+ Ldb1+/-	17 (23,6)	12 (23,5)	24 (31,58)	25
Isl1+/- Ldb1+/-	19 (26,4)	14 (27,5)	4 (5,26) †††	25

Figure S2, related to Figure 3. Ldb1 and Isl1 interact to regulate cardiogenesis. ***(A)*** Relative mRNA expression of *Ldb1* and *Isl1* in ES cells overexpressing either GFP alone (control) or together with Isl1, Ldb1 or a combination of Isl1/Ldb1. ***(B)*** Western blot analysis of total protein extracts of ES cells overexpressing either GFP alone (control) or together with Isl1, Ldb1 or Ldb1/Isl1, using Isl1 antibody. Tubulin served as loading control. ***(C)*** FACS analysis of *Flk-1* and *PdgfR-α* expression in d3.75 EBs differentiated from ES cells overexpressing either GFP alone (control) or together with Ldb1. ***(D)*** Analysis of the genotype of animals born from the cross *Isl1+/-* x *Ldb1+/-*. Total numbers (percentage) of recovered embryos or pups for all four different genotypes are shown. †††p<0.0001 Chi squared test.

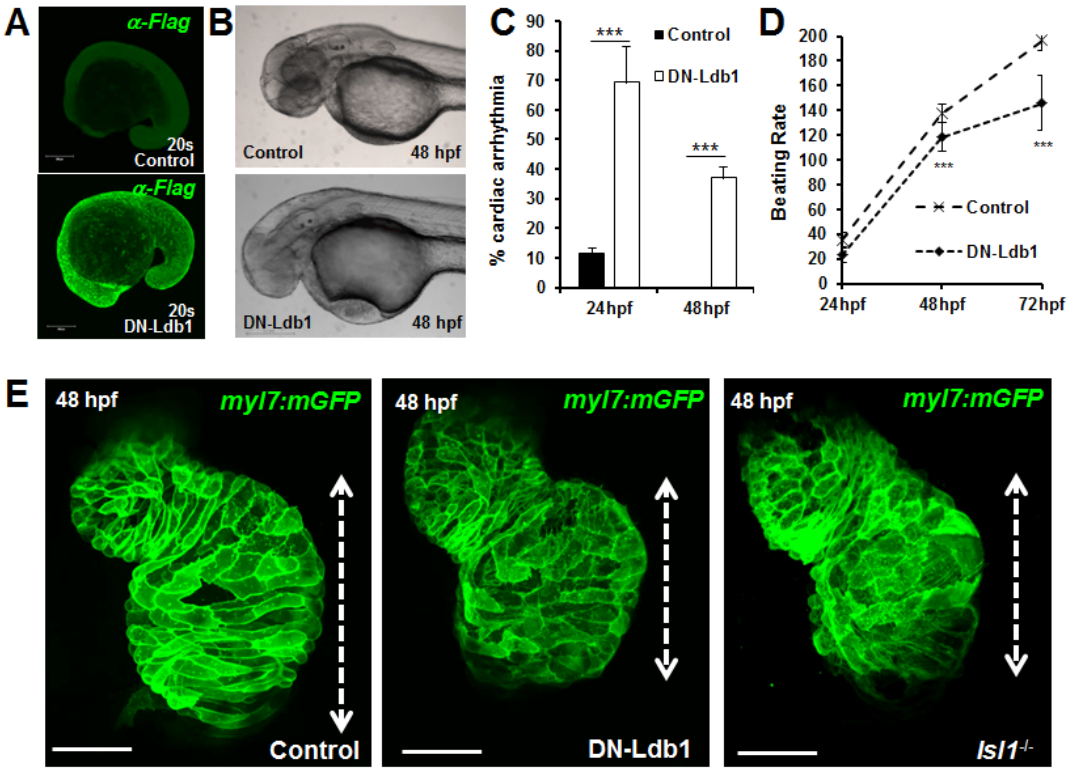

Figure S3, related to Figure 4. Cardiac morphogenesis defects in zebrafish embryos overexpressing DN-Ldb1. ***(A)*** Confocal images of control and *FLAG-HA-DN-Ldb1* mRNA injected zebrafish embryos, stained with anti-FLAG antibody at 20 somites. ***(B)*** Control or *DN-Ldb1* mRNA injected Tg(*myl7:EGFP-HsHRAS*)^{s883} embryos at 48 hpf. Lateral view, anterior to

the left. ***(C-D)*** Percentage of embryos with cardiac arrhythmia ***(C)*** and analysis of the number of heart beats per minute ***(D)*** measured at 24, 48 and 72 hpf in control and DN-Ldb1 overexpressing zebrafish embryos. ***(E)*** Confocal images of control, DN-Ldb1 overexpressing and *Isl1* mutant Tg(*myl7:EGFP-HsHRAS*)^{s883} hearts, showing shortening of the atrium (dotted lines) in DN-Ldb1 overexpressing and *isl1* mutant embryos.

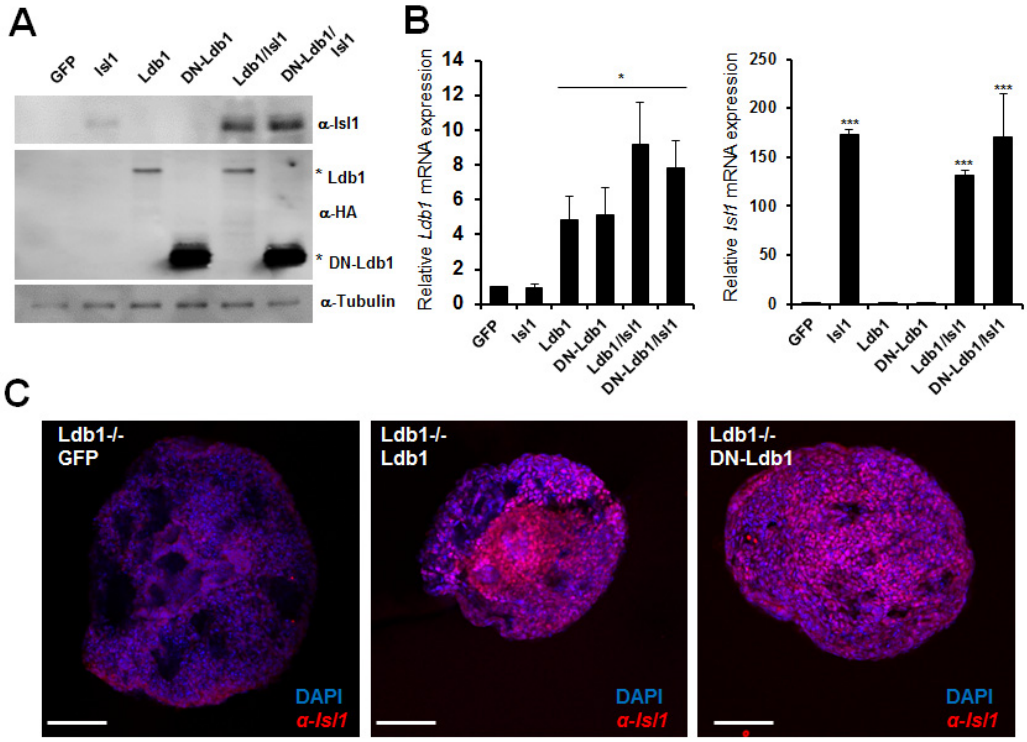

Figure S4, related to Figure 4. Overexpression of Ldb1 and DN-Ldb1 restores Isl1+ cells. ***(A)*** Western blot analysis of total protein extracts of *Ldb1-/-* ES cells overexpressing either GFP alone (control) or together with Isl1, Ldb1, DN-Ldb1 or in different combinations. ***(B)*** Relative mRNA expression of *Ldb1* and *Isl1* in *Lbd1-/-* ES cells overexpressing GFP, Isl1, Ldb1, DN-Ldb1 or in different combinations. ***(C)*** Isl1 immunostaining on vibratome sections of d5 EBs differentiated from *Ldb1-/-* ES cells overexpressing either GFP alone (control) or together with Ldb1 and DN-Ldb1. Scale bars, 100 µm.

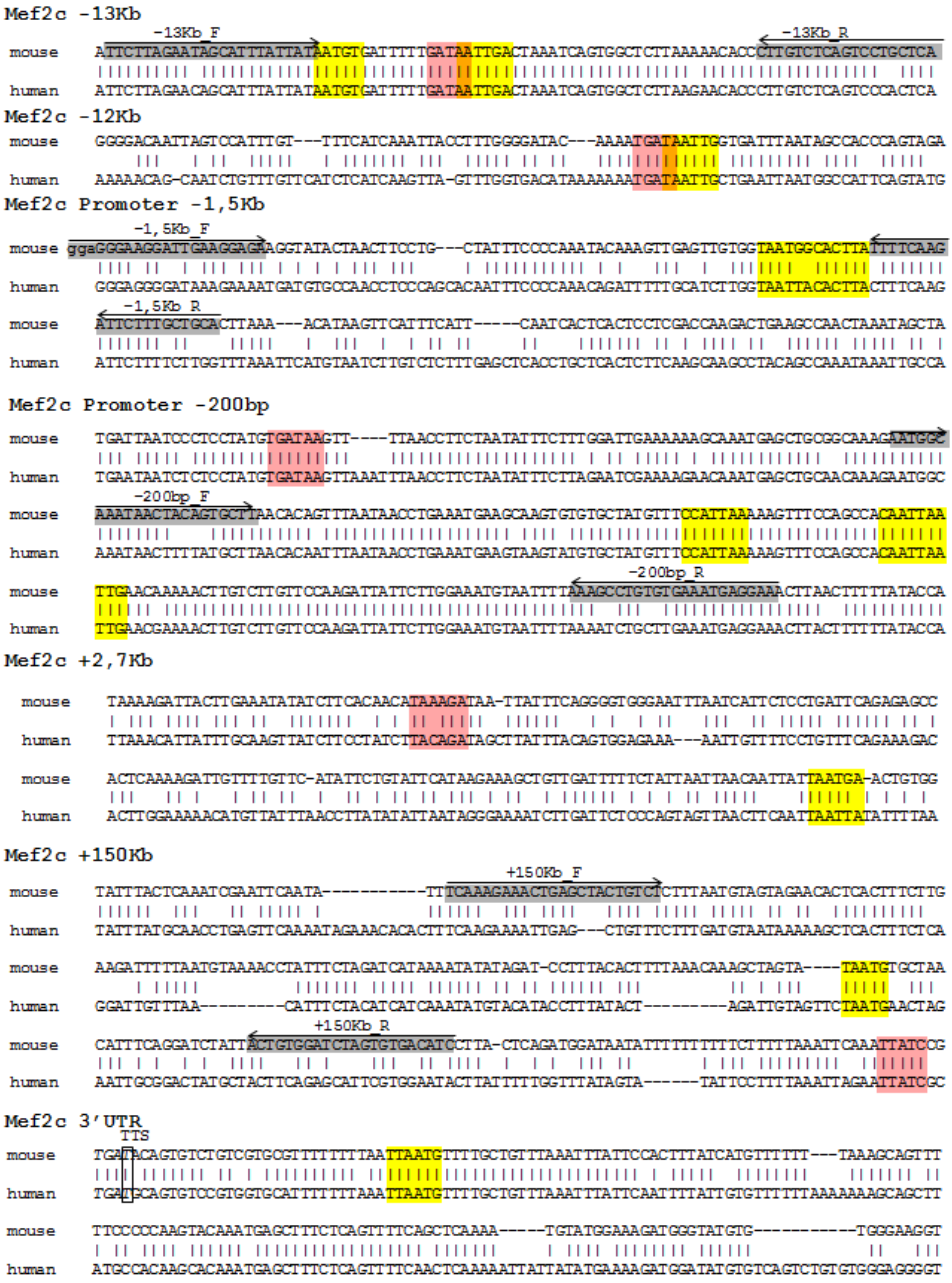

Figure S5, related to Figure 4 and 5. Alignment of conserved mouse and human sequences in the *Mef2c* locus, associated with the AHF enhancer. Consensus sites for Isl1 are shown in yellow and for GATA4 in light red. Primer sequences used for ChIP are shown in grey boxes. TTS, transcription termination site.

Figure S6, related to Figure 4 and 5. Alignment of conserved mouse and human sequences in the *Hand2* locus. Isl1 consensus sites are shown in yellow and GATA4 consensus sites in light red. Primer sequences used for ChIP or 3C analysis are shown in grey boxes. NlaIII and DpnII restriction sites are shown in green and orange respectively. TSS, transcription start site.

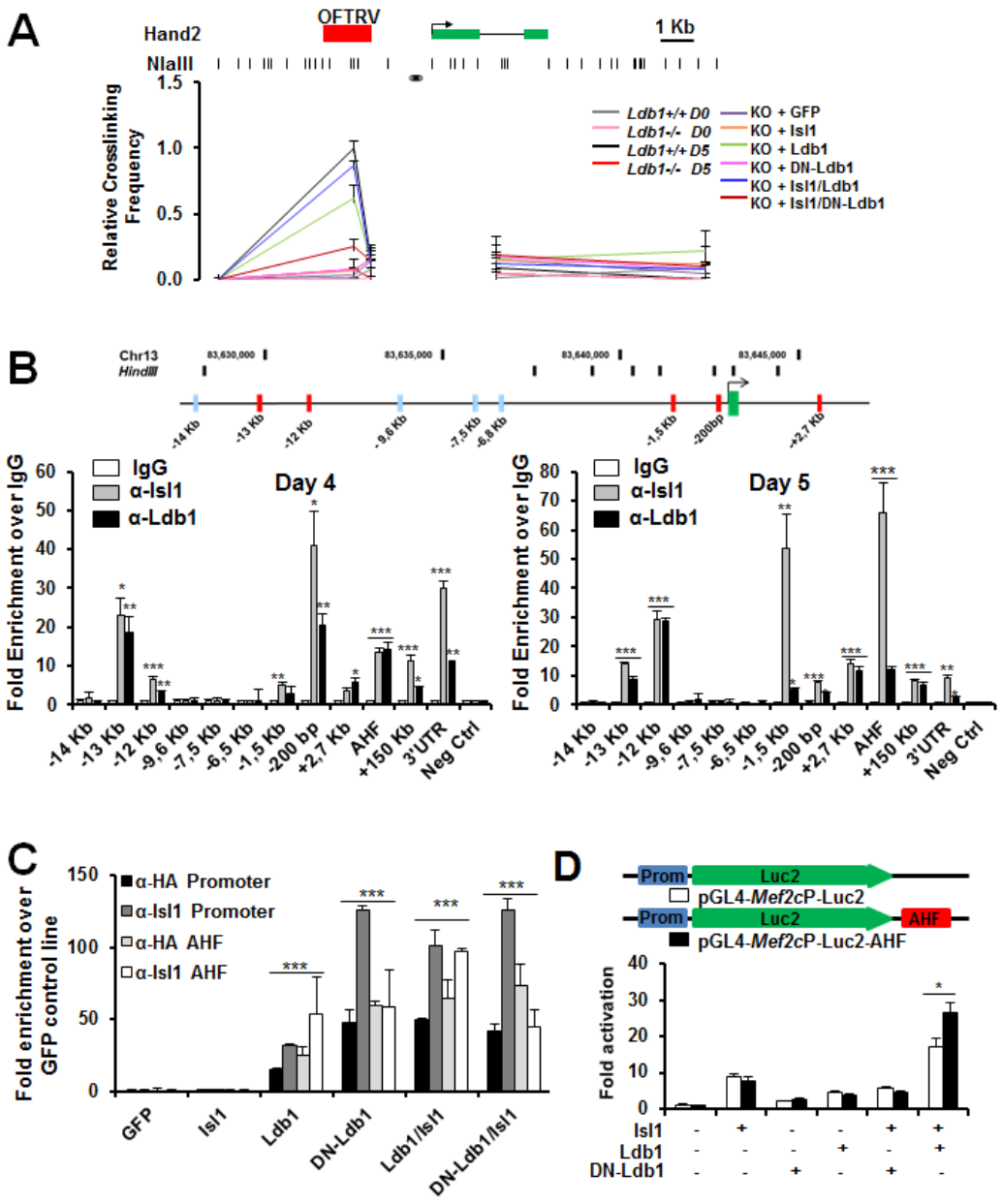

Figure S7, related to Figure 5. Ldb1 facilitates enhancer-promoter interactions within the *Hand2* and *Mef2c* loci. ***(A)*** Schematic representation of the *Hand2* genomic locus and the position of the NlaIII restriction sites, used in the 3C assay (top). 3C-qPCR analysis of WT, *Ldb1-/-* ES cells and d5 EBs derived from WT and *Ldb1-/-* ES cells or *Ldb1-/-* ES cells overexpressing either GFP alone (control) or together with Isl1, Ldb1, DN-Ldb1, or in different

combinations (bottom). ***(B)*** ChIP of nuclear extracts from d4 (left) and d5 (right) EBs using anti-Isl1 and anti-Ldb1 antibodies or IgG as a control. PCRs were performed using primers flanking conserved Isl1 binding sites (red) or not containing Isl1 consensus sites (light blue) in the -14 to -5.5 kb region within the *Mef2c* locus found by the 3C-seq in close proximity to the *Mef2c*-AHF. ***(C)*** ChIP of FLAG-HA-Ldb1 and FLAG-HA-DN-Ldb1 or Isl1 of nuclear extracts from d4 EBs using anti-HA and anti-Isl1 antibodies. PCRs were performed using primers flanking the conserved Isl1 binding sites in the Mef2c promoter and the AHF enhancer. Fold enrichment values were calculated relative to the GFP control. Data are mean ± SEM, n=3. ***(D)*** COS7 cells were transiently transfected with a 100 ng luciferase reporter construct containing the Mef2c promoter alone or in combination with the AHF enhancer, located downstream of the luciferase gene (Dodou et al., 2004), together with pcDNA (400 ng), Isl1 (400 ng), Ldb1 (400 ng), DN-Ldb1 (400 ng) or in combinations. The luciferase levels were normalized for the β-galactosidase activity of a cotransfected RSV-lacZ reporter (10 ng) and presented as fold activation relative to the luciferase levels of the reporter construct alone. All transfections were performed at least three times in triplicates, and representative experiments with the standard deviations are shown. *p≤0.05, **p≤0.01, ***p≤0.005.

Table S1, related to Figure 5 and 6. Peak coordinates of sequences interacting with the Mef2c-AHF and the Mef2c-promoter in d5 EBs.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES:

Plasmids

pcDNA3-Isl1, pcDNA3-Isl1ΔLIM1, pcDNA3-Isl1ΔLIM2, pcDNA3-Isl1HOMEO are described elsewhere (Witzel et al. 2012).

Ldb1 and Ldb1 truncated proteins were amplified from mouse cDNA and cloned into the BamHI site of pcDNA3-Flag-HA vector. The following primers were used for amplification:

Ldb1	Ldb1_F 5' gatccatgtcagtgggctgtgcctgtcc 3'
	Ldb1_R 5' ggatcctcactgggaagcctgtgacgtgg 3'
Ldb1ΔLID	Ldb1_F 5' ggatccatgtcagtgggctgtgcctgtcc 3'
	Ldb1ΔLID_R 5' ggatcctcagagagcgaaggtgctggctgggc 3'
DN-Ldb1	DN-Ldb1_F 5' ggatccatggagcccgcacgacagcagcccag 3'
	Ldb1_R 5' ggatcctcactgggaagcctgtgacgtgg 3'

The pCS2+Flag-HA-DN-Ldb1 plasmid was generated by subcloning the Flag-HA-DN-Ldb1 HindIII - EcoRV insert of pcDNA3-Flag-HA-DN-Ldb1 in the blunted BamHI site of pCS2+. Lentiviral constructs were created by subcloning of the Flag-HA-Ldb1 or the Flag-HA-DN-Ldb1 HindIII - EcoRV insert from pcDNA3 plasmids into the blunted BamHI site of pRRL.sin18-IRES-GFP.

The *Mef2c* promoter and AHF enhancer were cloned from wild type mouse (C57BL/6) genomic DNA in pJet1.2 (Fermentas) and subsequently subcloned in pGL4-luciferase plasmid (Promega).

The following primers were used for amplification:

Mef2cpromoter_F	5' gagctctcatactgaaagtgatttgac 3'
Mef2cpromoter_R	5' agatcttctccccaccccaagcctct 3'
Mef2cAHF_F	5' ggatcccattaaaatagtactctgca 3'
Mef2cAHF_R	5' gtcgacgggccattaactttcgaatc 3'

Synthetic oligonucleotides used in the study

Primers used for RT-PCR analysis:

Primer Name	Sequence 5'→3'	Accession Number
qGAPDH_for	AACTTTGGCATTGTGGAAGG	XM_001476707
qGAPDH_rev	GGATGCAGGGATGATGTTCT	
q5'UTRIsl1_for	ACAGCACCAGCATCCTCTCT	NM_021459
q5'UTRIsl1_rev	TCCCATCCCTAACAAAGCAC	
qIsl1_for	GCGACATAGATCAGCCTGCT	NM_021459
qIsl1_rev	GTGTATCTGGGAGCTGCGAG	
qLdb1_for	GGGGGGTGGCAACACCAACAACA	NM_001113408
qLdb1_rev	CCCCCACCACCATCACATCAGGT	
qNkx2.5_for	AAGCAACAGCGGTACCTGTC	NM_008700
qNkx2.5_rev	GCTGTCGCTTGCACTTGTAG	
qMef2c_for	TCCATCAGCCATTTCAACAA	NM_001170537
qMef2c_rev	AGTTACAGAGCCGAGGTGGA	
qTbx1_for	CGACAAGCTGAAACTGACCA	NM_011532
qTbx1_rev	AATCGGGGCTGATATCTGTG	
qTbx20_for	GCAGCAGAGAACACCATCAA	NM_020496
qTbx20_rev	GTGAGCATCCAGACTCGTCA	
qTbx5_for	ATGGTCCGTAACTGGCAAAG	NM_011537
qTbx5_rev	ACAAGTTGTCGCATCCAGTG	
qGATA4_for	TCTCACTATGGGCACAGCAG	NM_008092
qGATA4_rev	GCGATGTCTGAGTGACAGGA	
qHand1_for	GCGGAAAAGGGAGTTGCCTCAGC	NM_008213
qHand1_rev	GCTCCAGCGCCCAGACTTGC	
qHand2_for	CGGAGAGGCGGAGGCCTTCA	NM_010402
qHand2_rev	CAGGGCCCAGACGTGCTGTG	
qMlc2v_for	CTGCCCTAGGACGAGTGAAC	NM_010861
qMlc2v_rev	CCTCTCTGCTTGTGTGGTCA	
qMlc2a_for	CCCATCAACTTCACCGTCTT	NM_022879
qMlc2a_rev	CGTGGGTGATGATGTAGCAG	
qTnnt2_for	ATCCCCGATGGAGAGAGAGT	NM_011619
qTnntt2_rev	CTGTTCTCCTCCTCCTCACG	
qSM-actin_for	CTGACAGAGGCACCACTGAA	NM_007392
qSM-actin_rev	AGAGGCATAGAGGGACAGCA	
qSM-22a_for	AACGACCAAGCCTTCTCTGCC	NM_011526
qSM-22a_rev	TCGCTCCTCCAGCTCCTCGT	
qSM-mhc_for	AGGAAACACCAAGGTCAAGCA	NM_001161775
qSM-mhc_for	AGCCTCGTTTCCTCTCCTGA	
qBry_for	AGGGAGACCCCACCGAACGC	NM_009309
qBry_rev	CCGGGAACATCCTCCTGCCGTT	
qEoMes_for	CAGGGCAGGCGCATGTTTCCT	NM_010136
qEoMes_rev	TCCGCTTTGCCGCAGGTCAC	
qFlk1_for	GGGTTTGGTTTTGGAAGGTT	NM_010612
qFlk1_rev	AGGAGCAAGCTGCATCATTT	
qPDGFra_F	GGAACCTCAGAGAGAATCGGC	NM_001083316
qPDGFra_R	CATAGCTCCTGAGACCCGC	
qVE-Cad_for	TGAGGCAATCAACTGTGCTC	NM_009868
qVE-Cad_rev	TTCGTGGAGGAGCTGATCTT	
qIrx1_for	CTTCTCGCAGATGGGCTCTC	NM_010573
qIrx1_rev	TTCGTTGAGCCAGGCTTTCA	
qPitx2_for	GTGGACCCTCTCGGAACTTG	NM_001042504
qPitx2_rev	CTCCATTCCCGGTTATCGGC	
qMyocd_for	GCTGAGACTCACCATGACAC	NM_145136
qMyocd_rev	TGGACCTTTCAGTGGCGGTA	
qFoxC1_for	CAACATCATGACGTCGCTGC	NM_008592
qFoxC1_rev	CTCTGGCCCGGAGAGTAGG	
qBmp2_for	ATCACGAAGAAGCCGTGGAG	NM_007553
qBmp2_rev	CTCGTCACTGGGGACAGAAC	
qBmpr2_for	AGGTGGCCGAACAAATTCCA	NM_007561
qBmpr2_rev	TCTTGTGTTGACTCACCTATCTGT	
qFgfr2_for	CACGACCAAGAAGCCAGACT	NM_010207
qFgfr2_rev	CTCGGCCGAAACTGTTACCT	
qSmad3_for	AAGAAGCTCAAGAAGACGGGG	NM_016769
qSmad3_rev	CAGTGACCTGGGGATGGTAAT	

qAcvr2a_for	TCCTACTCAAGACCCAGGACC	NM_007396
qAcvr2a_rev	TCTGCCAGGACTGTTTGTCC	
qRyr2_for	GACTGAGGAAGGATCAGGGGA	NM_023868
qRyr2_rev	TTGTTGCCGGTCTGAGTTCT	
qKcnq1_for	ACTTCACCGTCTTCCTCATTGT	NM_008434
qKcnq1_rev	AGAGGCGGACCACATATTCTG	
qKcnj2_for	TCTCACTTGCTTCGGCTCAT	NM_008425
qKcnj2_rev	ACTTGTCCTGTTGCTGGTACA	
qFgf10_for	TGCGGAGCTACAATCACCTC	NM_00800
qFgf10_rev	GTTATCTCCAGGACACTGTACG	
qFgf8_for	GCTGAGCTGCCTGCTGTT	NM_010205
qFgf8_rev	GAGAGTGTCAGCTGGGTTCC	
qHDAC2_for	CCCGTCAGCCCTCTTGTC	NM_008229
qHDAC2_rev	TGCCAATATCACCATCATAGTAGT	
qRai_for	CTTGGTGACAGCAGCGACAG	NM_198409
qRai2_rev	CCACGTGGCCTCGGGAT	
qXrcc4_for	GCAAACCACGGTATTAGCGG	NM_028012
qXrcc4_rev	TGGCTACCTCTCAGTACTCCA	

Primers used for Mef2c isoforms absolute quantification

Primer Name	**Sequence 5'→3'**
Mef2cTotal_for	ACGAGGATAATGGATGAGCGT
Mef2cTotal _rev	CAGCTTGTTGGTGCTGTTGAA
Mef2cRefSeq_for	GGCAAAGCTTCGGTGTTCAT
Mef2cRefSeq _rev	CTGCTGAGGGCTTTGTTGTC
AK0077603_for	GGTCAGCCTGTCCAAAAGGA
AK0077603_rev	ACAATGGATGTCAGTTGACCCA

Primers used for ChIP analysis

Primer Name	**Sequence 5'→3'**
Mef2c-1,5Kb_for	CTGATGGAGAGGTTGGGACT
Mef2c-1,5Kb_rev	ATGCAAGCACCTCTCTCACT
Mef2c-1Kb_for	CTGATGGAGAGGTTGGGACT
Mef2c-1Kb_rev	ATGCAAGCACCTCTCTCACT
Mef2c-200bp_for	GAATGGCAAATAACTACAGTGCT
Mef2c-200bp_rev	TCCTCATTTCACACAGGCTT
Mef2c_AHF_for	TCAGTGTCTGCTCCTGCTTC
Mef2c_AHF_rev	TTCCCTCCACACCTTACTGG
Mef2c_-13Kb_for	CTTGCAATTACTACCACTTCACA
Mef2c_-13Kb_rev	CCTTGTCTCAGTCCTGCTCA
Mef2c+2,7Kb_for	GGGGTGGGAATTTAATCA
Mef2c+2,7Kb_rev	GTCTGGTCAATGAGGAGGT
Mef2c_+150Kb_for	TCAAAGAAACTGAGCTACTGTCT
Mef2c_+150Kb_rev	GATGTCACACTAGATCCACAGT
Mef2c_3'UTR_for	CAGTGTCTGTCGTGCGTTTT
Mef2c_3'UTR_rev	ACCCAATTCACACCTTCCCA
Mef2c_-9,6Kb_for	AGTGAAGGAAGAAAAGGTGCA
Mef2c_-9,6Kb_rev	GCTGGCGTTTGTGTTCTCTT
Mef2c_-12Kb_for	ACCCAGAGACACAGGCATAA
Mef2c_-12Kb_rev	TTCCCTTTGCGGTTCCAATG
Mef2c_-14Kb_for	CTCAACTGGTGGTGTTAGC
Mef2c_-14Kb_rev	GCTCAACTGGTGGTGTTAGC
Mef2c_-6,5Kb_for	TGAGGTCCCATTTGTTGATGC
Mef2c_-6,5Kb_rev	TGTCCTCCCACAGTTCTTCA
Mef2c_-7,5Kb_for	TGTGTTCCATTCAGCAGAGG
Mef2c_-7,5Kb_rev	CCCCAAAGAACATGCATGGT
Hand2_promoter_for	TTCACCCCACCCCTGTAATC
Hand2_promoter_rev	AATTGCCGAGGTCCTCTTCT
Hand2_OFTRV_for	CTCAGAGCCAGCCAACTACT
Hand2_OFTRV_rev	TCACTCCTCACTGACAGCAC
Actin_for	GGAGCGGACACTGGCACAGC
Actin_rev	ATGCCCACACCGCGACCCTA
Intergenic_for	AAACCTCAAAGCCCAGGACACA
Intergenic_rev	ACTTGGTCCCGAGTTGATGGAA

Primers used for 3C-seq Analysis

Mef2cpromoter_F	ACACTTGTGCAGAGGGATC
Mef2cpromoter_R	AAGCTTTCTAATTTGGGAGC

Mef2cAHF_F	TTAATTTATTACTAACATTGGAGGATC
Mef2cAHF_R	AAGCTTGTGCTCTGTGACA

Primers used for 3C-qPCR Analysis

Primer Name	Sequence 5'→3'
3C_Mef2cAHF	TTAATTTATTACTAACATTGGAGGATC
3C_Mef2c prom	GGGTCACACATCAAGGGTCT
3C_Mef2c-13Kb	CCTTGCCCAGAATGATCAGC
3C_Mef2c+2,7Kb	CCTTTGGCTCTCTCCTATCCT
3C_Mef2c +150	GCAGAGATTAGCCAGTCTATGC
3C_Mef2c 3'UTR	CCAAGCCGCATATCTACTGC
3C_Mef2c Negative	TGTCTGACTCAGCTGTGGAG
3C_Mef2c Negative2	ACCCAAGAAATTTTGAGAACCAA
3C_Mef2c Negative3	AACTGCAGCTTGTTTCACGT
3C_Mef2C_Negative4	TAGGGGTGGCTTCTGGTTTT
3C_Mef2c Negative5	TGCTTTCCCACATTACTGAAGA
3C_Ryr2	CAAATGTAGTGGTGGGTGCC
3C_FoxC1	CAGCCCAAAGATGTTTCAGGT
3C_Bmpr2	TGGATGAGTGGATGGGTAGA
3C_Bmp2	CACACGCCATCACTTAGCAG
3C_Fgf10	AGTGTTAGGATGCAGGGCTT
3C_Acvr2a	ACTCTGAAGGCTGGGAGTTC
3C_Isl1	GCTTAAAGAGGCAGGCTCC
3C_Smad3	AATATGTCCCAAATGTTTCACAGAA
3C_Myocd	CCACCATGGTCACTCTGTCC
3C_Kcnq1	AGGAACCACTCTCCCAAAGG
3C_Kcnj2	ACCGGTTAGCATGGTTTTAGC
3C_Rai2	GAGAGGCTGGAGGGAAGAAA
3C_Xrcc4	GGGTCCATGATTTGCCAAAGA
3C_Hand2prom	CGAGCGGCCCTAAAGATGTA
3C_Hand2 OFTRV_NlaIII	AAGCTTTAGACCCCTGGATTG
3C_Hand2Negative1_NlaIII	CTTCCCTGTCACATCACCCT
3C_Hand2Negative2_NlaIII	GCATTTCCAGCAAGCATCCT
3C_Hand2Negative3_NlaIII	CTTGTTTGGGGTGAGAAGGG
3C_Hand2Negative4_NlaIII	CACAGGGCAGTTAGGTCTCA
3C_Hand2 OFTRV_DpnII	TGTTGTTGTTGGTGGTGGTG
3C_Hand2Negative1_DpnII	CTAAGGGCTTCTGTTGACACC
3C_Hand2Negative2_DpnII	CCCATAGGCCTTGTTCTGGA
3C_Hand2Negative3_DpnII	CTAAGGTGGCTGGGACTAGG
3C_Hand2Negative4_DpnII	CGTGTGCTGTGTCTTCTCTT
3C_Actin_F	CTTCTGACCTAGAACTCTTGATCCC
3C_Actin_R	CCCTCTACACACACTCAGAATTCATC

Cell Culture and Transfection

HEK293T and COS7 cells were grown in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen), 2mM L-Glutamine, 100 U/ml Penicillin and 100 μg/ml Streptomycin (Invitrogen) at 37 °C, 5% CO_2. Undifferentiated embryonic stem (ES) cells were maintained on mouse embryonic fibroblast (MEFs) feeder cells in DMEM supplemented with 15% fetal bovine serum (FBS, Invitrogen), 2 mM L-Glutamine, 0.1 mM 2-mercaptoethanol (Sigma), 0.1 mM non-essential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 4.5 mg/ml D-glucose, and 1000 U/ml of leukemia inhibitory factor (LIF ESGRO, Millipore). To induce EB formation, dissociated ES cells were cultured in hanging drops of 500 cells per 15 µl of ES cell medium, in the absence

of LIF. After 2 days in the hanging drop culture, the resulting EBs were transferred to bacterial culture dishes. For the transfection of HEK293T, cells were seeded at a density of $2x10^6$ cells/10cm dish and transfected with 10-20µg DNA using calcium phosphate precipitation. COS7 were transfected using FuGENE HD Transfection Reagent (Roche), according to the manufacturer instructions. For stable expression, ES cells were transduced with pRRL.Sin18.PGK-GFP-IRES (control construct) and pRRL.Sin18.PGK-GFP-IRES-Isl1, pRRL.Sin18.PGK-GFP-IRES-Ldb1, pRRL.Sin18.PGK-GFP-IRES-DN-Ldb1 or in combinations. Transduced cells were FACS sorted for GFP expression and used for EB differentiation.

Luciferase Assay

For Luciferase Assays, $3x10^4$ COS7 cells were seeded in 24 well plates (details in Figure 6). 48h after transfection, cells were lysed in 100 µl lysis buffer (Promega, Luciferase Assay System) and luciferase activity was measured on Mithras LB 940 (Berthold Technologies) according to the Luciferase Assay System Manual (Promega). β-galactosidase assays were performed using CPRG as substrate (Sigma).

RNA Isolation, RT-PCR, and Real-Time PCR

Embryos were dissected, and after removal of the pericardium, the SHF region and the heart were separately collected in cold PBS. RNA from EBs and embryos was isolated using Trizol (Invitrogen). cDNA was synthesized with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and real-time PCR was performed using the SYBR GREEN PCR master mix (Applied Biosystems) on Applied Biosystems StepOnePlus real-time PCR detection system. The cycle numbers were normalized to GAPDH (ES/EBs and embryos). Primer pairs are described in *Synthetic oligonucleotides used in the study* section.

Immunofluorescence of Embryoid Bodies (EBs)

EBs were collected, washed with PBS, embedded in 17% gelatin and fixed overnight at room temperature in 4% PFA. Next day the EBs were sectioned with vibratome at 70 µm. The obtained sections were fixed in 2% formaldehyde, 0.1M PIPES, 1.0 mM $MgSO_4$, 2.0 mM EGTA

O/N at 4 °C, followed by 1 hour blocking (4% BSA + 0.4% Triton X-100) and incubated with primary and secondary antibodies diluted in blocking solution.

Flow Cytometry

For FACS analysis the EBs were dissociated, $1x10^6$ cells were washed with 1 ml PBS and blocked in 100 µl FACS buffer (10% FBS in PBS) for 1 hour at room temperature. After blocking, the cells were stained with 0.5 µg each APC-conjugated anti-Flk1 (e-Bioscience 17-5821-81) and PE-conjugated anti-PDGFRα (e-Bioscience 12-1401-81) or control rat IgG2a K isotype APC- (e-Bioscience 17-4321) or PE-conjugated (e-Bioscience 12-4321) antibodies. After PBS washes, cells were fixed for 10 minutes at room temperature in 2% PFA. Data were acquired on an LSRII flow cytometer (BD) and analyzed using FlowJo software.

Chromatin immunoprecipitation

Embryoid bodies at day 4 or 5 of differentiation were dissociated with trypsin to obtain single cells suspension and resuspended with complete differentiation medium to obtain a concentration of 10^6 cells/ml. Cardiogenic region of 30 E8-9 embryos was dissociated with trypsin to obtain single cells, and resuspended in PBS containing 10% FBS. For chromatin immunoprecipitation $0.5\text{-}1.0x10^7$ cells were fixed with 1% formaldehyde for 10 min. Formaldehyde was quenched with glycine at a final concentration of 125 mM and washed three times with PBS. Cells were lysed in L1 lysis buffer (50 mM Tris pH8, 2 mM EDTA pH8, 0.1% NP-40, 10% glycerol) for 5 min, the nuclei were spun down and resuspended in L2 nuclear resuspension buffer (1% SDS, 5 mM EDTA pH8, 50 mM Tris pH8), followed by sonication to fragment the chromatin. The samples were centrifuged, diluted 1:10 with DB-dilution buffer (0.5% NP-40, 200 mM NaCl, 5 mM EDTA, 50 mM Tris pH8) and incubated with primary antibody overnight at 4°C, followed by 3 h incubation with Protein-A/G Sepharose beads (GE Healthcare). Immunoprecipitates were washed two times with NaCl-washing buffer (0.1% SDS, NP-40 1%, 2 mM EDTA, 500 mM NaCl, 20 mM Tris pH8), followed by two washes with LiCl-washing buffer (0.1% SDS, 1% NP-40, 2 mM EDTA, 500 mM LiCl, 20mM Tris pH8) and eluted with EB-

extraction buffer (TE pH8, 2% SDS). Cross-linking was reverted by overnight incubation at 65°C, DNA was purified and subjected to qPCR analysis. Primer pairs are described in *Synthetic oligonucleotides used in the study* section.

Chromosome Conformation Capture Assays - 3C-seq and 3C-qPCR

3C-seq and 3C-qPCR were performed as described in (Stadhouders et al., 2013). In brief, 10^7 cells or dissected SHF regions or tails of 20 E8-9 embryos were crosslinked with 2% formaldehyde at room temperature for 10 min, followed by glycine quenching, cell lysis, HindIII (for 3C-seq and 3C-qPCR of the Mef2c locus) or NlaIII (or DpnII) digestion (3C-qPCR of the Hand2 locus), and T4 ligation. As a positive control genomic DNA or bacterial artificial chromosomes (BAC) containing the entire *Mef2c* and *Hand2* loci (Invitrogen) digested with HindIII or NlaIII (or DpnII) respectively were used, and religated to generate random ligation products. Primers sequences are listed in the Supplemental Experimental Procedures. R3Cseq, a R/Bioconductor package, was used for the discovery of long-range genomic interactions in the 3C-seq datasets (Thongjuea et al., 2013).

GO Analysis

GO analysis was performed using DAVID software (Huang da et al., 2009a, b).

Histological analysis

Embryos were dissected in ice cold PBS, fixed in 4% PFA overnight at 4°C, dehydrated in Ethanol and stored at -20°C. For histological analysis, the tissues were incubated in 100% xylol and embedded in paraffin for further processing. Embedded organs were sectioned using an RM2245 microtome (Leica) and Hematoxilyn-Eosin (H&E) staining was performed.

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