Cell Stem Cell Supplemental Information

# The IsI1/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<sup>cre/+</sup>/Ldb1<sup>flox/flox</sup>***embryos.** *(A)* Left and right views of E9.5 control and Isl1<sup>cre/+/</sup>Ldb1<sup>flox/flox</sup> 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<sup>cre/+</sup>/Ldb1<sup>flox/flox</sup> embryos. Data are mean ± SEMs, n=3 for each genotype.



**Figure S2**, related to Figure 3. Ldb1 and IsI1 interact to regulate cardiogenesis. (*A*) Relative mRNA expression of *Ldb1* and *IsI1* in ES cells overexpressing either GFP alone (control) or together with IsI1, Ldb1 or a combination of IsI1/Ldb1. (*B*) Western blot analysis of total protein extracts of ES cells overexpressing either GFP alone (control) or together with IsI1, Ldb1 or Ldb1/IsI1, using IsI1 antibody. Tubulin served as loading control. (*C*) FACS analysis of *Flk-1* and *PdgfR-a* 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 *IsI1+/-* x *Ldb1+/-*. Total numbers (percentage) of recovered embryos or pups for all four different genotypes are shown. <sup>†††</sup>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*)<sup>s883</sup> 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*)<sup>s883</sup> 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 IsI1+ cells.** (*A*) Western blot analysis of total protein extracts of *Ldb1-/-* ES cells overexpressing either GFP alone (control) or together with IsI1, Ldb1, DN-Ldb1 or in different combinations. (*B*) Relative mRNA expression of *Ldb1* and *Isl1* in *Lbd1-/-* ES cells overexpressing GFP, IsI1, Ldb1, DN-Ldb1 or in different combinations. (*C*) IsI1 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.

Mef2c	-13Kb
	-13Kb F
mouse	
human	ATTCTTAGAACAGCATTTATTAT <mark>AATGT</mark> GATTTTT <mark>GATAATTGA</mark> CTAAATCAGTGGCTCTTAAGAACACCCTTGTCTCAGTCCCACTCA
Mef2c	-12Kb
mouse	GGGGACAATTAGTOCATTTGTTTTCATCAAATTACCTTTGGGGATACAAAAT <mark>CATAATTG</mark> TGATTTAATAGCCACCCAGTAGA
human	
Mef2a	Promoter -1 5Kh
HOLEC	-1 5Kh F
mouse g	g=gGGEAAGGATTGAAGGAGAAGAGAAGAGTATACTAACTTCCTGCTATTTCCCCCAAATACAAAGTTGAGTTG
human	-1 5Kb R
mouse	ATTOTT COT COACTTAAAACATAAGTTCATTTCATTCAATCACTCACTCCCCCGACCAAGACTGAAGCCAACTAAATAGCTA
human	ATICITITICITESTITAAATICAIGTAATCITETCICTITEASCICACUTECICACICITCAASCAAGCUTACASCAAATAAATTECCA
Mef2c	Promoter -200bp
mouse	TGATTAATCCCTCCTATGTCATAAGTTTTAACCTTCTAATATTTCTTTGGATTGAAAAAAGCAAATGAGCTGOCGCAAACAATGCC
human	TGAATAATCTCTCCCTATG <mark>TGATAA</mark> GTTAAATTTAACCTTCTAATATTTCTTAGAATCGAAAAGAACAAATGAGCTGCAACAAAGAATGGC
mouse	-2000p_5 ARATAACTACACTETTAACACACETTTAATAACCTGAAATGAAGCAAGTGTGTGCTATGTTTCCATTAAAAAGTTTCCAGCCACAATTAA
human	AAATAACTITTATGCTTAACACAATTTAATAACCTGAAATGAAGTAAGT
mouse	TTERACAAAAACTTETCTTETCCCAACATTATTCTTEGAAATETAATTTAACCCTETETEAAATEAGEAAACTTAACTT
	<mark>    </mark>
human	
Mef2c	+2,7Kb
mouse	TAAAAGATTACTTGAAATATATCTTCACAACA <mark>TAAAGA</mark> TAA-TTATTTCAGGGGTGGGAATTTAATCATTCTCCTGATTCAGAGAGCC
	- E THE FILE THE TE THEFTER E E THE THE THE THE THE THE THE THE THE
human	TTAAACATTATTTGCAAGTTATCTTCCTATCT <mark>TACAGAT</mark> AGCTTATTTACAGTGGAGAAAAATTGTTTTCCTGTTTCAGAAAGAC
mouse	ACTCAAAAGATTGTTTTGTTC-ATATTCTGTATTCATAAGAAAGCTGTTGATTTTTCTATTAATTA
h	
numan	ACHIGGAAAARCAIGI IAI HARCCHAIAI AHAALAGGGAAAAI CHGAI ICI CCCAGLAGI IAACH CAAL <mark>AAHA</mark> LAHHIAA
Mef2c	+150Кb
mo118.0	+150Kb F
1110036	
human	TATTTATGCAACCTCAAGTTCAAAAATAGAAACACACTTTCAAGAAAATTGAGCTGTTTCTTTGATGTAATAAAAAGCTCACTTTCTCA
mouse	A AGA TITITA ATGTAA AACCTA TITCTA GATCATAAA ATA TATAGA T-CCITTACACTITTAAA CAA AGCTAGTA <b>TAA TG</b> IQCTAA
human	GGATTGTTTAACATTTCTACATCATCAAAATATGTACATACCTTTATACTAGATTGTAGTTC <mark>TAATG</mark> AACTAG
mouse	CATTICA GGATCT ATTACT GTGGGATCTA GTGTGACATCCTTA-CTCAGA TGGATA ATA TTT TTTTTTTTTTTTTTA AATT CAAATTATC GG
human	AATTGCGGACTATGCTACTTCAGAGCATTCGTGGAATACTTATTTTTGGTTTATAGTATATTCCTTTTAAATTAGAA <mark>TTATC</mark> GC
Mef2c	3'UTR
mouse	115 TCA <b>FR</b> AGAGTGTCTGTCGTGCGTGCGTTTTTTTTTTTTTTTTTT
human	<i>TGA<mark>LE</mark>CAGIGICCGIGGIGCALTITITIAAA<mark>TIAAIG</mark>TITIGCIGITIAAATTIATICAATTITAITGIGITITITAAAAAAAGCAGCIT</i>
mouse	TTCCCCCAAGTACAAATGAGCTTTCTCAGTTTTCAGCTCAAAATGTATGGAAAGATGGGTATGTGTGGGAAGGT

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 IsI1 are shown in yellow and for GATA4 in light red. Primer sequences used for ChIP are shown in grey boxes. TTS, transcription termination site.

Hand2	Promoter ChIP Hand2Prom F
mouse	GTTTTCTGCCTCGGTTACCTACAAAACACTTGAAAAGCCATAATCTGCGTTCCTTTCACCCCCCCCCGTAATCAGAAAAGAAA
human	TTITCTTACCTOGATTACCTACAAAGCACTTOGAAAAGCAATACCTGOGTTTGGTCTTTCTTTCTTTCTTTCAACCAGAAAAGAGAGCA
mouse	AAATAATACTGTAOCTGGTTTAAGATTCTTCAGTATTGGGGGGGAAATATTTTAATAGOGAAGCATCTGTTAAACACTTG
human	GAST GATACT GTA OCT GGT TAG AGATTC TTC AAT ATT CAT GAG ACT TGG GGG GAA AAT ATT TTA ATA GT GAAG CAT CTG TTA AACACT TG
mouse	CATTAACCTATGGCTGATAAAAGGGTTTTTTTTTT-ACCCTGCCCCCCCCGTTT <mark>TAATTATCT</mark> TATGGTTATGAGTGGAC
human	CATTATCCTCTGACTGATAAAAAAGTTTTTTTTTTTTTT
mouse	AACCAAT OCT <mark>ADA AGA GGA CUT OGG CAATTA</mark> GCA ACGTGA ACATCA AA AAGT TIT ATT GOGGA GAGCGOGAGGOGCOGCOCCOGCOGCOC
human	AGCCAAT CCTGGAAGAGGACCTOGG <mark>CAATTA</mark> GCAACGTGAACATCAAAAAGTTTTATTGCCGAGAGCTOGAGGGCCCCCCCCCC
mouse	CGGGATTGGCGTGAGGAGCCTCTGACGACATATATTAACC <mark>GGAGCGGGCCCTAAAGATC</mark> TAGCTGTA <mark>CATG</mark> GA <mark>GATC</mark> TTGCTGGGAAAATC
human	CGGGATTGCCGTGAGGAGCCTCTGACGACATATATTAACCOGAGCGCCCTAAAGATGTAGCTGTACATGCAGATCTTGCTGGGAAAATC
mouse	GGCTTGCTCCCCCCACCCCCAACCCCAA
human	
Hand2	OFTRV
Hand2 mouse	OFTRV ChIP_Hand2OFTRV_F TACGGAAGTAAAATGTGTCAAAAGATGCTGAAAAAGAAAAAAAATGAACAATTGCAGTAAGTATAACTGAAGCCAGOC
Hand2 mouse human	OFTRV ChIP_Hand2OFTRV_F TACGGAAGTAAAATGTGTCAAAAAGATGCTAAAAAGAAAAAAAATGAACAATTGCAGTAAGTATA
Hand2 mouse human mouse	OFTRV ChIP_Hand2OFTRV_F TACGGAAGTAAAATGTGTCAAAAGATGCTAAAAAGAAAAAAAATGAACAATTGCAGTAAGTATAAAATGAACAATTGCAGTAAGTATAAAATGAACAATTGCAGTAAGTAAAAAAAGTGCAGTAGCAGTAGAGTCAACCAGTCAGGCCAGCCAAGTAAAACAGGCCAAAGGACAACTGCAGAGAAAAGTGGATGCGTTTGTGCATTTAGGGTAGAGCTCAACCAGTAGGCCAGCCAAGTAAGCATGGTAGATACCACCAAAATTTACCCACTGGTCCCCTCTC-TCCAGCACCTACAGAAAGGCTAAC
Hand2 mouse human mouse human	OFTRV ChIP_Hand2OFTRV_F TACGCAAGTAAAATGTGTCAAAAAGATGCTAAAAAGAAAAAAAATGAACAATTGCAGTAAGTATA
Hand2 mouse human mouse human	OFTRV ChIP_Hand2OFTRV, F TACGGAAGTAAAATGTGTCAAAAGATGCTGAAAAGAAAA
Hand2 mouse human mouse human mouse	OFTRV Chip_Hand2OFTRV_F TAGGGAAGTAAAATGTGTCAAAAGAAGTGCAAAGAAAAAAAATGAACAATTGCAGTAAGTAATAAGTCAGAAGCAGCCAGCCAGTCGGAACAAAACAGGGCCAAAGGACCACAGAGAAAAGTGGGATGGGATGGGATTGGCGATTTAGGGTAGAGACCACAGCAGGCCAGCCAACTAGTCATGGTAGATACCACCAAAAATTTACCCACTGGGTCCCTCC-TCCAGGCACCTACAGAAAGGCCAGCCTGGCCTCCCTGAGTCAGTTACTACAGTGGGGCACACCAACAAATTTACCCACTGGGTCCCTCC-TCCAGGCCAGCCAGCCAGCCAGCCAGCCAGCCAGCCAGCC
Hand2 mouse human mouse human mouse	OFTRV Chip_Hand2OFTRV, F TACGGAAGTAAAATGTGTCAAAAGATGCCTAAAAAGAAAAAAAATGACAATTGCAGTAAGTATAAAATGACAATTGCAGTAAGTATAAAATGACAATTGCAGTAAGTATAAAATGACAAGTGCAGTAGGACCAGCCAGCC ACCTGGAACAAAACAGGCCAAAGGACACTGCAAGAGAAAAGTGGATGCGTTTGTGCATTTAGGGTGAGGCTCAACCAGTAGGGCCAGCC ACCTGGAACAAAACAGGCCAAAGGACACTGCAAGAGAAAAGTGGATGCGTTTGTGCACTTAGGGTGAGGCCAACCAGTAGGGCCAGCC ACCTGGAACAAAACAGGCCAAAGGACACTGCAAGAGAAAAGTGGATGCGTTGGCCCTCTC-TCCAGGCAACCAGTACGGGCCAGCC ACCTGGAGTCAGTTACTACAGTGGGACAACCACCAGGAGTTCAGGCTGCCCTCCTCAAGGCAGCCTT NlaIII 3C Hand2OFTRV CTACCACTCTAGGTTTGGGTTTGGGTTTGGGCTTCGAGCCACGTGGACGCAAGGCAGGTTAATTGACGCTGGAC CTAAGGCAGTCTTGGGTTTGGGTTTGGGGTTGTGGCCACGCCAGGCAGG
Hand2 mouse human mouse human mouse human	OFTRV ChIP_Hand2OFTRV_F TAGGGAAGTAAAATGTGTGCAAAAGATGGCTAGGAGAGAAAAAAATGAACAATGGCGATGGAGGAGGCAAGCCAGCCAGCCAG
Hand2 mouse human mouse human mouse human mouse	OFTRV Chip_Hand2OFTRV_F TAGGGAAGTAAAATGTGTCAAAAGGACGCCAAGGGAAAAAGTGGCATGGCGAGGTAAGGAGGCCAAGGCAGGC
Hand2 mouse human mouse human mouse human mouse human	OFTRV ChIP_Hand2OFTRV_F TACGGAAGTAAAATGTGTCCAAAAGATGCCTAAAAAGAAAAAAATGAACAATGCGATGGTAGTAATAA-AATGAAGAAGAGAGAGGGAGGAGCCAGCCAGCCAAGCGAAAAACAGGCCAAAGGAGCAAAGGGCAAAGGGCAAAGGGCAAAGGGCAAAGGGCAAAGGGCAAAGGGCAAAGGGCAAAGGGCAAGGGCAGGCCAGCCAAGGGAAAAACGGGACAGGCAAAGGGCAAGGGCAAGGGCAAGGGCAAGCGCAAGCGAAAAACAGGGCAAAGGGCAAAGGGCAAGGGCAAGCGCAGGCCAGCCAAGGGGTGGGGGCCAGGCCAGGCCAGCCA
Hand2 mouse human mouse human mouse human mouse human	OFTRV Chip_Hand2OFTRV, F TACGGAAGTAAAAGGGCAAAGGACCTGCAAAAGGAGAAAAGTGGCATGGCGGGTGGGGGGGG
Hand2 mouse human mouse human mouse human mouse human	OFTRV ChIP_Hand2OFTRV,F TACGGAAGTAAAATGTGTCAAAAGAGGCAAGTGCAAAAAGTGGAGGAGGGAG

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. NIaIII 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 NIaIII 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 or *verexpressing either GFP alone (control)* or together with IsI1, Ldb1, DN-Ldb1, or in different

combinations (bottom). (B) ChIP of nuclear extracts from d4 (left) and d5 (right) EBs using anti-IsI1 and anti-Ldb1 antibodies or IgG as a control. PCRs were performed using primers flanking conserved IsI1 binding sites (red) or not containing IsI1 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-IsI1 antibodies. PCRs were performed using primers flanking the conserved IsI1 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  $\beta$ -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.

#### Caputo et al.

#### SUPPLEMENTAL EXPERIMENTAL PROCEDURES:

#### Plasmids

pcDNA3-IsI1, pcDNA3-IsI1ΔLIM1, pcDNA3-IsI1ΔLIM2, pcDNA3-IsI1HOMEO 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'
- Ldb1∆LID Ldb1\_F 5' ggatccatgtcagtgggctgtgcctgtcc 3' Ldb1∆LID\_R 5' ggatcctcagagagcgaaggtgctggctggcc 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' <del>→</del> 3'	Accession Number
qGAPDH_for	AACTTTGGCATTGTGGAAGG	XM_001476707
qGAPDH_rev	GGATGCAGGGATGATGTTCT	
q5'UTRIsI1_for	ACAGCACCAGCATCCTCTCT	NM_021459
q5'UTRIsI1_rev	TCCCATCCCTAACAAAGCAC	
qlsl1_for	GCGACATAGATCAGCCTGCT	NM_021459
qlsl1_rev	GTGTATCTGGGAGCTGCGAG	
qLdb1_for	GGGGGGTGGCAACACCAACAACA	NM 001113408
aLdb1_rev	CCCCCACCACCATCACATCAGGT	=
aNkx2.5 for	AAGCAACAGCGGTACCTGTC	NM 008700
aNkx2.5 rev	GCTGTCGCTTGCACTTGTAG	
aMef2c for	TCCATCAGCCATTTCAACAA	NM 001170537
gMef2c_rev		
aThy1 for		NM 011532
aTby1_rov		1111_011032
aThy20 for		NM 020406
	GUAGUAGAGAAUAUUATUAA	NW_020496
q1bx20_rev	GIGAGCAICCAGACICGICA	
q1bx5_for	AIGGICCGIAACIGGCAAAG	NM_011537
q1bx5_rev	ACAAGIIGICGCAICCAGIG	
qGATA4_for	TCTCACTATGGGCACAGCAG	NM_008092
qGATA4_rev	GCGATGTCTGAGTGACAGGA	
qHand1_for	GCGGAAAAGGGAGTTGCCTCAGC	NM_008213
qHand1_rev	GCTCCAGCGCCCAGACTTGC	
qHand2_for	CGGAGAGGCGGAGGCCTTCA	NM_010402
gHand2_rev	CAGGGCCCAGACGTGCTGTG	
aMlc2v for	CTGCCCTAGGACGAGTGAAC	NM 010861
aMic2v rev	CCTCTCTGCTTGTGTGGTCA	
gMlc2a for	CCCATCAACTTCACCGTCTT	NM 022879
gMic2a_rev	CGTGGGTGATGATGTAGCAG	
aTant2 for		NM 011619
aTantt2 rov		
aSM actin for		NM 007202
qSM actin_101		NIM_007592
uSM 222 for		NIM 044500
qSW-22a_tor	AACGACCAAGCCTTCTCTGCC	NIM_011526
qSM-22a_rev		
qSM-mhc_for	AGGAAACACCCAAGGTCAAGCA	NM_001161775
qSM-mhc_for	AGCCTCGTTTCCTCTCCTGA	
qBry_for	AGGGAGACCCCACCGAACGC	NM_009309
qBry_rev	CCGGGAACATCCTCCTGCCGTT	
qEoMes_for	CAGGGCAGGCGCATGTTTCCT	NM_010136
qEoMes_rev	TCCGCTTTGCCGCAGGTCAC	
gFlk1_for	GGGTTTGGTTTTGGAAGGTT	NM 010612
gFlk1_rev	AGGAGCAAGCTGCATCATTT	-
oPDGFra F	GGAACCTCAGAGAGAATCGGC	NM 001083316
oPDGFra R	CATAGCTCCTGAGACCCGC	·····
gVE-Cad for	TGAGGCAATCAACTGTGCTC	NM 009868
gVE-Cad_rev	TTCGTGGAGGAGCTGATCTT	1111_000000
drv1 for	CTTCTCCCAGATCCCCTCTC	NM 010573
		NW_010575
urx1_rev		NNA 001040504
	GIGGACCCICICGGAACTIG	NIM_001042504
dPitx2_rev	CICCATICCCGGITATCGGC	
qMyocd_for	GCTGAGACTCACCATGACAC	NM_145136
qMyocd_rev	TGGACCTTTCAGTGGCGGTA	
qFoxC1_for	CAACATCATGACGTCGCTGC	NM_008592
qFoxC1_rev	CTCTGGCCCGGAGAGTAGG	
qBmp2_for	ATCACGAAGAAGCCGTGGAG	NM_007553
gBmp2_rev	CTCGTCACTGGGGACAGAAC	-
gBmpr2 for	AGGTGGCCGAACAAATTCCA	NM 007561
aBmpr2 rev	TCTTGTGTTGACTCACCTATCTGT	
aFafr2 for	CACGACCAAGAAGCCAGACT	NM 010207
aFafr2 rev	CTCGGCCGAAACTGTTACCT	
asmad3 for		NM 016760
qomaus_ioi		ININI_010709
wCmaad? way		

Caputo et al.

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	ATGCAAGCACCTCTCACT	
Mef2c-200bp_for	GAATGGCAAATAACTACAGTGCT	
Mef2c-200bp_rev	TCCTCATTTCACACAGGCTT	
Mef2c_AHF_for	TCAGTGTCTGCTCCTGCTTC	
Mef2c_AHF_rev	TTCCCTCCACACCTTACTGG	
Mef2c13Kb_for	CTTGCAATTACTACCACTTCACA	
Mef2c13Kb_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	
Mef2c9,6Kb_for	AGTGAAGGAAGAAAAGGTGCA	
Mef2c9,6Kb_rev	GCTGGCGTTTGTGTTCTCTT	
Mef2c12Kb_for	ACCCAGAGACACAGGCATAA	
Mef2c12Kb_rev	TTCCCTTTGCGGTTCCAATG	
Mef2c14Kb_for	CTCAACTGGTGGTGTTAGC	
Mef2c14Kb_rev	GCTCAACTGGTGGTGTTAGC	
Mef2c6,5Kb_for	TGAGGTCCCATTTGTTGATGC	
Mef2c6,5Kb_rev	TGTCCTCCCACAGTTCTTCA	
Mef2c7,5Kb_for	TGTGTTCCATTCAGCAGAGG	
Mef2c7,5Kb_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 f	or 3C-qPCR	Analysis
----------------	------------	----------

Primer Name	Sequence 5ʻ→3ʻ
3C_Mef2cAHF	TTAATTTATTACTAACATTGGAGGATC
3C_Mef2c prom	GGGTCACACATCAAGGGTCT
3C_Mef2c-13Kb	CCTTGCCCAGAATGATCAGC
3C_Mef2c+2,7Kb	CCTTTGGCTCTCCCTATCCT
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_NIalli	AAGCTTTAGACCCCTGGATTG
3C_Hand2Negative1_NIaIII	CITCCCIGICACATCACCCI
3C_Hand2Negative2_NIaIII	GCATTTCCAGCAAGCATCCT
3C_Hand2Negative3_NIaIII	CIIGIIIGGGGIGAGAAGGG
3C_Hand2Negative4_Nlall	CACAGGGCAGTTAGGTCTCA
3C_Hand2 OFTRV_DpnII	
3C_Hand2Negative1_DpnII	CTAAGGGCTTCTGTTGACACC
3C_Hand2Negative2_DpnII	CCCATAGGCCTTGTTCTGGA
3C_Hand2Negative3_DpnII	CTAAGGTGGCTGGGACTAGG
3C_Hand2Negative4_DpnII	CGIGIGCIGICICICI
3C_Actin_F	
3C_Actin_R	CCCICIACACACACICAGAAIICAIC

#### **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  $\mu$ g/ml Streptomycin (Invitrogen) at 37 °C, 5% CO<sub>2</sub>. 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  $\mu$ l 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<sup>6</sup> 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-IsI1, pRRL.Sin18.PGK-GFP-IRES-Ldb1, pRRL.Sin18.PGK-GFP-IRES-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  $\mu$ m. The obtained sections were fixed in 2% formaldehyde, 0.1M PIPES, 1.0 mM MgSO<sub>4</sub>, 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-PDGFR $\alpha$  (e-Bioscience 12-1401-81) or control rat lgG2a 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<sup>6</sup> 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 0.5-1.0x10<sup>7</sup> 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, 500 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 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<sup>7</sup> 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 NIaIII (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 NIaIII (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.

#### **REFERENCES:**

Huang da, W., Sherman, B.T., and Lempicki, R.A. (2009a). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res *37*, 1-13.

Huang da, W., Sherman, B.T., and Lempicki, R.A. (2009b). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4, 44-57.

Stadhouders, R., Kolovos, P., Brouwer, R., Zuin, J., van den Heuvel, A., Kockx, C., Palstra, R.J., Wendt, K.S., Grosveld, F., van Ijcken, W., *et al.* (2013). Multiplexed chromosome conformation capture sequencing for rapid genome-scale high-resolution detection of long-range chromatin interactions. Nat Protoc *8*, 509-524.

Thongjuea, S., Stadhouders, R., Grosveld, F.G., Soler, E., and Lenhard, B. (2013). r3Cseq: an R/Bioconductor package for the discovery of long-range genomic interactions from chromosome conformation capture and next-generation sequencing data. Nucleic Acids Res *41*.