The Isl1/Ldb1 complex orchestrates

heart-specific chromatin organization and transcriptional regulation

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SUMMARY

Cardiac stem/progenitor cells hold great potential for regenerative therapies, however, the mechanisms regulating their expansion and differentiation remain insufficiently defined. Here we show that the multi-adaptor protein Ldb1 is a central regulator of cardiac progenitor cell differentiation and second heart field (SHF) development. Mechanistically, we demonstrate that Ldb1 binds to the key regulator of SHF progenitors Isl1 and protects it from proteasomal degradation. Furthermore, the Isl1/Ldb1 complex promotes long-range promoter-enhancer interactions at the loci of the core cardiac transcription factors *Mef2c* and *Hand2*. Chromosome conformation capture followed by sequencing identified surprisingly specific, Ldb1-mediated interactions of the Isl1/Ldb1 responsive *Mef2c* anterior heart field enhancer with genes which play key roles in cardiac progenitor cell function and cardiovascular development. Importantly, the expression of these genes was downregulated upon Ldb1 depletion and Isl1/Ldb1 haplodeficiency. In conclusion, the Isl1/Ldb1 complex orchestrates a network for heart-specific transcriptional regulation and coordination in three-dimensional space during cardiogenesis.

INTRODUCTION

Heart failure is the leading cause of morbidity and mortality worldwide. Due to the limited regenerative capacity of the human heart, cardiomyocytes lost during heart injury are substituted by a non-contractile fibrotic scar tissue, resulting in decreased cardiac function and ultimately heart failure. A number of cardiac regenerative strategies have been proposed of which stem/ progenitor cells hold great promise for heart repair (Aguirre et al., 2013; Hansson et al., 2009; Laflamme and Murry, 2011). Knowledge accumulated from developmental studies have significantly improved the methods for *in vitro* cardiac differentiation from embryonic stem (ES) cells and studies utilizing ES differentiation have brought further insights in the regulatory networks integrating multiple transcription factors and signaling molecules, which strictly control the distinct steps of cardiogenesis. However, the current limitations for the use of stem/ progenitor cells in regenerative medicine, e.g. linked to their expansion, differentiation efficiency and functional integration, call for a more complete understanding of the mechanisms driving cardiovascular lineage commitment, expansion and differentiation.

During embryogenesis the heart is generated by a common progenitor at gastrulation that segregates into two distinct populations, termed first and second heart fields. The first heart field (FHF), fuses at the midline and differentiates into the myocardium of the heart tube. After the initial heart tube formation, the heart tube grows by addition of Isl1-positive second heart field (SHF) progenitor cells to its anterior and venous poles (Cai et al., 2003; Evans et al., 2010; Vincent and Buckingham, 2010). Studies in different model systems revealed the crucial function of the LIM-homeodomain transcription factor Isl1 in heart morphogenesis (Cai et al., 2003; de Pater et al., 2009; Witzel et al., 2012). Isl1-deficient mouse embryos lack the right ventricle and the outflow tract, both structures derived from the SHF, as Isl1 is required for the proliferation, survival, and migration of these cells into the forming heart (Cai et al., 2003). Importantly, the Isl1-positive cardiovascular progenitors are multipotent and can differentiate into all three cardiovascular lineages: cardiomyocytes, smooth muscle cells and endothelial cells (Moretti et

al., 2006). Moreover, Isl1 is required for the differentiation of these cells into the cardiomyocyte and smooth muscle lineage (Kwon et al., 2009), but the mechanisms underlying its function are poorly understood.

The acquisition of cellular identity involves genome reorganization and a coordinated series of large-scale transcriptional changes (Dixon et al., 2015; Dixon et al., 2012; Gorkin et al., 2014; Peric-Hupkes et al., 2010). Using chromosome conformation capture (3C) assays and 3C-based technologies (de Wit and de Laat, 2012; Dekker et al., 2002) recent studies have suggested that CTCF together with Cohesin might be involved in general formation of chromatin structures by promoting constitutive long-range DNA interactions, whereas specific transcription factors, their co-factors together with CTCF, Cohesin and the Mediator complex might be involved in controlling locus-specific looping interactions and lineage-specific transcription (Kagey et al., 2010; Phillips-Cremins et al., 2013; Shen et al., 2012). In ES cells for example the binding of the key pluripotency transcription factors Oct4, Sox2, and Nanog was shown to be a key determinant of genome organization, as regions with a high density of binding sites for these factors tend to colocalize in nuclear space (de Wit et al., 2013; Denholtz et al., 2013). Another example for a cofactor involved in promoting locus-specific and cell type-specific interactions is Ldb1 (Deng et al., 2012; Morcillo et al., 1997; Soler et al., 2010; Song et al., 2007). Ldb1 directly binds to LIM domain- and Otx proteins and is found in large complexes with bHLH and GATA transcription factors (Bach et al., 1997; Jurata et al., 1998; Meier et al., 2006; Tripic et al., 2009). By bringing together distinct transcription factors and their coregulators, associated with different transcriptional control elements, Ldb1 facilitates long range promoter-enhancer interactions and regulates cell-type specific expression patterns (Deng et al., 2012; Morcillo et al., 1997; Soler et al., 2010; Song et al., 2007).

Here we show that Ldb1 is a multifunctional regulator of SHF development and cardiac differentiation. We show that the importance of Ldb1 for SHF development is two-fold. On the one hand, Ldb1 binds to Isl1 and protects it from proteasomal degradation, as a consequence of

which Ldb1-deficiency leads to an almost complete loss of Isl1+ cardiovascular progenitor cells. On the other hand, the Isl1/Ldb1 complex promotes long-range promoter enhancer interactions at the loci of the key developmental regulators of cardiogenesis *Mef2c* and *Hand2*. Using 3C-seq we identified specific Ldb1-mediated interactions of the *Mef2c* AHF enhancer with genes which play key roles in cardiovascular development as well as cell fate commitment. Moreover, Ldb1-deficient cells and Isl1/Ldb1 haplodeficient embryos, which show various cardiac anomalies, show a dramatically decreased expression of genes associated with the AHF enhancer and overexpression of Isl1 and Ldb1 strongly promoted their expression. Thus, the Isl1/Ldb1 complex plays a central role in transcriptional regulation and chromatin organization in three-dimensional space during cardiac differentiation and SHF development.

RESULTS

Ldb1 regulates cardiac progenitor cell differentiation and second heart field development

The LIM domain transcription factor Isl1 is required for the proliferation, survival, and differentiation of SHF cardiac progenitor cells, as a consequence of which Isl1-deficient mouse embryos lack the right ventricle and the outflow tract, both structures derived from the SHF (Cai et al., 2003). Similarly, Ldb1 deficiency results in early embryonic lethality with a series of developmental defects, including lack of heart formation (Mukhopadhyay et al., 2003). Therefore, we reasoned that Isl1 and Ldb1 might work in concert to regulate cardiac progenitor cell function. To elucidate the functional role of Ldb1 in cardiogenesis we first utilized wild-type and *Ldb1* knockout (*Ldb1*-/-) ES cells and differentiated them in embryoid bodies (EBs) to facilitate the generation and differentiation of cardiac progenitor cells (Figure 1A). Importantly, in contrast to control EBs, which started beating already at day 6 (d6), the EBs differentiated from *Ldb1*-/- cells showed no beating foci (Figure 1B). Consistent with this, the expression of cardiomyocyte marker genes (*Mlc2a*, *Mlc2v*, *Tnnt2*) was dramatically reduced (Figure 1C). Additionally, the expression of endothelial markers (*Flk1*, *CD31*) was also downregulated (Figure

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1C). By contrast, the expression of smooth muscle genes (*SM-actin*, *SM-22α*) was upregulated (Figure 1C), suggesting that Ldb1 is important for proper differentiation into cardiomyocyte and endothelial cell lineages. Next, we analyzed whether Ldb1-deficiency affects early developmental decisions, e.g. mesoderm induction, which could subsequently affect cardiac progenitor cell differentiation. Real-time PCR analysis for mesoderm (*Eomes*, *Bry*) and cardiac mesoderm markers (*Mesp1, Mesp2*) showed no significant difference in the peak expression level between control and Ldb1-deficient EBs, however the peak expression of these markers was delayed by one day (Figure 1D). Next, we analyzed the expression of pan-cardiac genes and genes specifically expressed in the FHF and the SHF (Figure 1E). Interestingly, while the expression of *Isl1*, *Tbx1*, *Hand2* and *Fgf10*, which play important roles in the SHF, and the pan-cardiac genes *Nkx2-5* and *Mef2c* was significantly downregulated at d4, d5 and d6, genes expressed in the FHF (*Tbx5*, *Hand1*, *Gata4*) showed no significant change (Figure 1E, data not shown), suggesting a role of Ldb1 in the regulation of SHF progenitor cells. To further investigate the role of Ldb1 in SHF development, we ablated *Ldb1* using an *Isl1-Cre* driver line that results in Cre recombination in SHF progenitor cells (Cai et al., 2003). *Isl1-Cre:Ldb1 flox/flox* embryos died by E10.5 with shortened outflow tract and small right ventricle (Figure 1F-G, Figure S1A). Furthermore, we observed significant downregulation of cardiomyocyte marker genes in the right ventricle and the outflow tract, both structures derived from the SHF, whereas no change in these genes was observed in the left ventricle, derived mainly from the first heart field (Figure 1H, Figure S1B). Taken together, these results confirm that Ldb1 is essential for SHF progenitor cell differentiation and cardiac development.

Ldb1 protects Isl1 from proteasomal degradation

To gain a better understanding of the mechanisms underlying Ldb1 function in cardiogenesis, we analyzed cardiovascular progenitors in more detail. FACS analysis for Flk-1 and PdgfR-α demonstrated no significant differences in early cardiovascular progenitor numbers (Figure 2A). Interestingly, although *Isl1* mRNA levels were only slightly reduced in EBs differentiated from

Ldb1-/- ES cells at all time-points that we analyzed (Figure 1E, 2B), Isl1+ cells were virtually absent in *Ldb1*-/- EBs (Figure 2C). Western blot analysis confirmed the dramatically reduced levels of Isl1 in *Ldb1*-deficient EBs (Figure 2D). The absence of Isl1 protein, without pronounced changes of *Isl1* mRNA levels, suggested that Isl1 is regulated at the protein level. Pull down of Isl1 detected slower-migrating ubiquitinated forms of Isl1, that were increased in the presence of the proteasomal inhibitor MG-132, indicating that Isl1 is targeted for proteasomal degradation (Figure 2E). To analyze this in more detail, we transfected HEK293T cells with Isl1 deletion constructs lacking the LIM1 and/or LIM2 domain and treated them with MG-132. The levels of the truncated proteins lacking LIM2 or containing only the homeodomain were relatively unchanged, but the levels of the Isl1 protein lacking LIM1 were significantly increased upon MG-132 treatment, suggesting that LIM2 is subjected to ubiquitination (Figure 2F). Next we analyzed whether binding of Ldb1 to Isl1 might protect it from proteasomal degradation, similarly to Lhx3 (Gungor et al., 2007). To assess this possibility we transfected HEK293T cells with an Isl1 plasmid alone or together with increasing amounts of Ldb1 or Ldb1 deletion constructs and constant amounts of GFP, which served as a control for transfection efficiency (Figure 2G). Importantly, increasing levels of Ldb1 and truncated Ldb1 protein lacking the dimerization domain (DN-Ldb1) led to a significant increase of Isl1 protein levels but did not change the levels of truncated Isl1 proteins lacking either the LIM1 or LIM2 domain, or harboring only the Isl1 homeodomain (Figure 2G, 2H). Furthermore, a truncated Ldb1 protein, lacking the LIM-interaction domain (LID), responsible for the interaction of Isl1 with Ldb1, did not have an effect on Isl1 protein levels (Figure 2G). Co-immunoprecipitations using Isl1 deletion constructs revealed a critical role of the LIM1 domain of Isl1 in mediating the interaction with Ldb1 (Figure 2I), corroborating previous findings (Jurata et al., 1996). Together these data suggest that the binding of Ldb1 to the LIM1 domain of Isl1 protects it from ubiquitination at the LIM2 domain and subsequently from proteasomal degradation (Figure 7E).

Ldb1 and Isl1 physically, functionally and genetically interact to regulate cardiac progenitor cell differentiation and heart development

Ldb1 plays fundamental roles in development and cell differentiation as a cofactor for LIM-domain proteins, suggesting that Ldb1 and Isl1 might functionally interact with each other during cardiogenesis. To test this hypothesis, we first confirmed that Ldb1 and Isl1 interact in cardiac progenitor cells expressing endogenous levels of each protein by performing immunoprecipitation of Ldb1 from nuclear extracts of embryoid bodies (EBs) differentiated for 5 days, a stage enriched in cardiac progenitors. We found that Ldb1 was efficiently co-immunoprecipitated together with Isl1 (Figure 3A). Next, we transfected mouse ES cells with an expression plasmid carrying GFP alone or together with Isl1, Ldb1 or both Isl1 and Ldb1 (Figure S2A, S2B). GFP-expressing cells, isolated by FACS, were subjected to differentiation in EBs. Overexpression of Isl1 or Ldb1 alone significantly increased the percentage of beating EBs. Importantly, EBs differentiated from Isl1/Ldb1 overexpressing cells showed a higher number of beating foci compared to control EBs or EBs overexpressing Isl1 or Ldb1 alone, which had similar levels of Isl1 and Ldb1 compared to Isl1/Ldb1 overexpressing cells (Figure 3B, S2A, S2B). Consistently, the expression of the cardiomyocyte marker genes *Mlc2a* and *Mlc2v* was markedly increased and we observed a significant synergistic effect of Isl1 and Ldb1 on *Mlc2a* expression (Figure 3C). Furthermore, the expression of endothelial (*Flk1*, *VE-Cad*) and smooth muscle markers (*SM-actin*, *SM-Mhc*) was also increased (Figure 3D, 3E). Moreover, cardiac progenitor genes, which play key roles in SHF development (endogenous *Isl1*, *Mef2c*, *Hand2* and *Fgf10*) were significantly up-regulated (Figure 3F). Interestingly, overexpression of Ldb1 significantly increased Flk-1$^+$PDGFR-α$^+$ cardiovascular progenitor numbers and the expression of Flk-1 and PDGFR-α (Figure 3G, Figure S2C), which may account for the increased expression of markers of all major cardiovascular lineages in Ldb1 and Isl1/Ldb1 overexpressing cells. Finally, we tested whether *Isl1* and *Ldb1* genetically interact during heart development. Crossing of *Isl1* heterozygous and *Ldb1* heterozygous mice revealed that only 5% of pups were

compound heterozygotes at the weaning stage, despite an expected ratio of 25%, whereas the other genotypes were recovered at similar percentages (Figure S2D). Timed mating revealed expected ratios of compound heterozygotes during mid to late gestation (Figure S2D), suggesting that the compound heterozygotes die after birth. Further histological analysis revealed that *Isl1/Ldb1* double haplodeficient embryos had various heart abnormalities, including a small and thin right ventricle, ventricular septal defect (VSD), overriding aorta (OA) and double outlet right ventricle (DORV) (Figure 3H-K). To analyze the primary cause of the observed heart defects we microdissected the heart and the SHF from wt, *Isl1+/-*, *Ldb1+/-* and *Isl1+/−Ldb1+/-* E9.25 embryos. Importantly, real-time PCR analysis revealed significant downregulation of *Hand2*, *Mef2c*, *Fgf10* and *Mlc2v* expression in *Isl1/Ldb1* double haplodeficient embryos, supporting a key role of Isl1/Ldb1 complex in cardiomyocyte differentiation and *Mef2c*, *Hand2* and *Fgf10* expression (Figure 3L).

The dimerization domain of Ldb1 is required for SHF development and cardiac progenitor cell differentiation

To further investigate the functions of Ldb1 during heart development, we ectopically overexpressed a truncated form of Ldb1 lacking the dimerization domain (DN-Ldb1, Figure 2G, Figure S3A), by injecting mRNA into one-cell stage embryos of the zebrafish transgenic line Tg(*myl7:EGFP-HsHRAS*)[s883], which allows the easy monitoring of the cardiac morphology, through heart-specific expression of membrane-bound GFP (D'Amico et al., 2007). DN-Ldb1 contains the highly conserved LIM-interaction domain (LID), which mediates high-affinity protein interactions with LIM domains, and can significantly stabilize Isl1 protein levels (Figure 2G,H), but lacks the dimerization domain, necessary to promote long range promoter-enhancer interactions. It has been previously shown that overexpression of this mutant protein results in a competition with wild-type Ldb molecules for binding to LIM domains of LIM-HD proteins, acting in a dominant negative manner (Bach et al., 1999; Becker et al., 2002). Consistently with previous studies, the overexpression of DN-Ldb1 led to defects in eye and brain development

(Figure S3B; (Becker et al., 2002)). Additionally, we observed that the heart contracted irregularly and with a reduced frequency (Figure S3C, S3D) in more than 80% of the embryos expressing DN-Ldb1. Furthermore the atrium of DN-Ldb1-expressing embryos was significantly shorter (Figure S3E). Interestingly, a similar phenotype was observed in *isl1*-mutant zebrafish embryos as a result of a failure of cardiomyocyte differentiation at the venous pole (de Pater et al., 2009). Confocal images of control and DN-Ldb1-expressing embryos stained with anti-Isl1 antibodies at 48 hpf revealed dramatically less Isl1+ cardiomyocytes at the venous pole of the heart. In mutants, Isl1+ cells were found outside of the heart, but did not express the cardiomyocyte marker *myl7*, supporting a key role of Ldb1 dimerization in Isl1+ cardiomyocyte differentiation (Figure 4A). Consistent with this, *bmp4* expression in the sinus venosus was strongly reduced in DN-Ldb1-expressing embryos, although *bmp4* expression at the outflow pole and the atrioventricular canal was unaffected, in striking parallel to *Isl1* mutant embryos (Figure 4B). Additionally, the expression of *mef2cb* was also downregulated (Figure 4C). Since Isl1 plays a key role in cardiac progenitor cells during development, we further addressed whether this phenotype can be attributed to defects at early stages of cardiogenesis by performing *in situ* hybridization and whole mount anti-Isl1 immunostaining of control and DN-Ldb1 overexpressing embryos at the 10 and 15 somites stage. No significant change of *isl1* mRNA expression was detected, suggesting that Isl1+ progenitor cell numbers were not changed in DN-Ldb1 injected embryos (Figure 4D). However, Isl1 staining appeared to be stronger in the injected embryos, consistent with the stabilizing effect of DN-Ldb1 on Isl1 protein levels (Figure 4D, Figure 2). Importantly, we observed strong downregulation of *hand2*, *mef2cb* and *nkx2-5*, which play important roles in the SHF development (Figure 4D), whereas no change was observed in *tbx5a* expression. These findings suggest that not only the stabilization of Isl1 protein levels but also the formation of higher order complexes mediated by the Ldb1 dimerization domain might be important for proper cardiac progenitor cell differentiation during SHF development. To gain further support of this hypothesis, we generated stable Ldb1-deficient ES cell lines

overexpressing GFP alone, Isl1, Ldb1, DN-Ldb1 or in combinations. Consistent with an important role of Ldb1 in regulating Isl1 stability, Isl1 overexpressing *Ldb1*-/- ES cells showed dramatically lower Isl1 protein levels, compared to *Ldb1*-/- ES cells overexpressing Isl1 in combination with Ldb1 or the DN-Ldb1 (Figure S4A), although *Isl1* mRNA levels were similar (Figure S4B). Importantly, Ldb1 overexpression led to a rescue of cardiac differentiation, as measured by the increased percent of beating EBs and mRNA levels of cardiomyocyte marker genes, *Mlc2a*, *Mlc2v* and *Tnnt2* (Figure 4E, 4F). The functional rescue was further potentiated by overexpression of Isl1, confirming a synergistic role of these proteins in cardiomyocyte differentiation. By contrast, overexpression of DN-Ldb1 alone or in combination with Isl1 did not rescue the complete loss of cardiac differentiation of Ldb1-deficient EBs (Figure 4E, 4F). Furthermore, the expression of the endothelial marker genes *CD31* and *Flk1* was increased in *Ldb1*-/- EBs overexpressing Ldb1, whereas smooth muscle genes were decreased by Ldb1 overexpression (Figure 4G), supporting a role of Ldb1 in the differentiation of cardiovascular progenitors into the cardiomyocyte and endothelial cell lineages and suppression of the smooth muscle lineage. FACS analysis for Flk-1 and PdgfR-α showed no significant differences in the Flk-1$^+$PDGFR-α$^+$ cardiovascular progenitor numbers upon Ldb1 and Isl1/Ldb1 overexpression in Ldb1-deficient EBs (Figure 4H), demonstrating that the dramatically increased cardiac differentiation is not due to an increased induction of cardiovascular progenitor cells. However, we observed a pronounced decrease of the Flk-1$^+$/PdgfR-α$^+$ population upon DN-Ldb1 expression (Figure 4H). Interestingly this decrease was rescued by Isl1 overexpression in combination with DN-Ldb1 (Figure 4H), suggesting that the decrease in cardiovascular progenitor cells upon DN-Ldb1 overexpression might be due to interference with the function of LIM only or LIM-HD proteins. Furthermore, we saw rescue of Isl1+ cells and Isl1 expression upon Ldb1 and DN-Ldb1 expression (Figure 4I, Figure S4C), consistent with the role of Ldb1 and DN-Ldb1 in stabilizing Isl1 protein levels, suggesting that the inability of DN-Ldb1 to rescue the cardiac differentiation defects of the Ldb1-deficient cells is not due to lack of Isl1+ cells.

Importantly, the expression of *Mef2c* and *Hand2*, factors crucial for SHF development and differentiation, was significantly upregulated upon Ldb1 overexpression but not upon overexpression of the DN-Ldb1 (Figure 4J). Isl1 has been shown to directly bind to the anterior heart field (AHF) enhancer, which directs the expression of *Mef2c* in the AHF (Dodou et al., 2004, Witzel et al., 2012). Additionally, we found several conserved Isl1 consensus motifs (YTAATGR; TAAKKR (Mazzoni et al., 2013)) between -1,5kb and the Mef2c transcription start site (Figure S5). Chromatin immunoprecipitation (ChIP) analysis of nuclear extracts from day 5 EBs demonstrated specific binding of Isl1 and Ldb1 to the conserved Isl1 binding sites (Figure 4K). Moreover, ChIP experiments in pools of E8-9 embryos showed strong enrichment of Isl1 and Ldb1 at these sites (Figure 4L). Additionally, we found *Hand2* expression to be significantly altered upon loss and gain of function of Ldb1. Hand2 plays a key role in SHF development (Srivastava et al., 1997; Tsuchihashi et al., 2011), and its expression in the heart is specifically driven by a cardiac-specific enhancer located between -4.5 kb and -2.7 kb of the *Hand2* transcription start site (Figure S6; OFTRV enhancer) (McFadden et al., 2000). *In silico* analysis revealed several Isl1 consensus binding sites in the proximal promoter and the OFTRV enhancer of *Hand2* (Figure S6). ChIP analysis of d5 EBs and E8-9 embryos showed strong binding of both Isl1 and Ldb1 at these sites (Figure 4M, 4N). Taken together, our data indicate that *Mef2c* and *Hand2* are direct targets of the Isl1/Ldb1 transcriptional complex.

Ldb1 facilitates long range enhancer-promoter interactions within the Hand2 and Mef2c loci

Using chromosome conformation capture (3C) assays and 3C-based technologies (de Wit and de Laat, 2012; Dekker et al., 2002), it was shown that Ldb1 promotes chromatin looping events that bring distal enhancers into close proximity to promoters, thereby regulating gene expression (Deng et al., 2012; Morcillo et al., 1997; Soler et al., 2010; Song et al., 2007). To assess whether a similar mechanism controls cardiac progenitor cell gene expression gene expression, we analyzed whether binding of the Isl1/Ldb1 complex promotes chromatin loop formation at the

relatively small *Hand2* locus. Using 3C-qPCR analysis with the *Hand2* promoter as a viewpoint we observed a specific close proximity of the promoter with the OFTRV enhancer in d5 EBs from wild-type ES cells, but not in d5 EBs from Ldb1-deficient cells (Figure 5A). A similar interaction pattern was observed in *Ldb1*-/- EBs overexpressing Ldb1 and Isl1/Ldb1 but not in EBs overexpressing DN-Ldb1 or Isl1/DN-Ldb1 (Figure 5A, Figure S7A). No interaction was observed between the promoter and negative regions for Isl1 and Ldb1 binding located upstream of the OFTRV enhancer, between the promoter and OFTRV enhancer and downstream of the promoter. Further, no interaction was detected in wild-type and Ldb1-deficient ES cells, which do not express Isl1 (Figure 5A, Figure S7A).

Next, to characterize the spatial interactions within the Mef2c locus we performed 3C-seq (Stadhouders et al., 2013), using two viewpoints: the *Mef2c* AHF enhancer, which drives *Mef2c* expression specifically in the AHF and the *Mef2c* promoter which drives *Mef2c* expression in different cell types. Multiple promoter-interacting elements were detected in the *Mef2c* locus, of which some showed a lower signal in Ldb1-deficient EBs (Figure 5B). Importantly, we observed interactions of the AHF enhancer with the promoter area and with the 3' end of the *Mef2c* gene, which were decreased in *Ldb1*-deficient EBs (Figure 5B). 3C-qPCR experiments revealed a similar long-range interaction pattern in wild-type versus *Ldb1*-/- EBs during the course of cardiac differentiation, as well as from dissected SHF and hearts of E8-10.5 embryos, confirming the 3C-seq results (Figure 5C). Interestingly, we observed interactions of the AHF enhancer with the *Mef2c* proximal promoter co-occupied by Isl1 and Ldb1 in d4 EBs at the onset of Mef2c expression and in dissected SHF of E8.5 embryos, whereas in d5 EBs and dissected hearts from E9.5-10.5 embryos we observed strong interactions with regions more distal to the transcription start site (Figure 5C-D). Additionally, no interactions of the AHF enhancer with the promoter and with the 3' end of the *Mef2c* gene was observed in the tail of the E8-9 embryos, suggesting that these chromatin loops are specifically formed in cardiac progenitor cells. Importantly, the 3C-seq signals correlated with the binding of Isl1 and Ldb1 at the Mef2c locus

(Figure S7B). Next, we sought to analyze whether the Ldb1 dimerization domain is required for chromatin loop formation by performing 3C-qPCR in d4 and d5 EBs from to *Ldb1*-/- ES cells overexpressing Isl1, Ldb1 and DN-Ldb1 alone or in combinations. Using the AHF enhancer as a viewpoint we observed a specific interaction of the AHF enhancer with the *Mef2c* promoter and the 3' end of *Mef2c* gene in *Ldb1*-/- EBs overexpressing Ldb1 and Isl1/Ldb1, but observed no interaction of these genomic regions in *Ldb1*-/- EBs overexpressing DN-Ldb1 and Isl1/DN-Ldb1 (Figure 5D). To analyze, whether the inability of DN-Ldb1 to promote loop formation was due to its inability to bind to the Isl1/Ldb1 binding sites we performed ChIP analysis of *Ldb1*-/- ES cells overexpressing Isl1 alone or in combinations with HA-Ldb1 or HA-DN-Ldb1 using anti-HA antibody. Importantly, we observed similar enrichment of Ldb1 and DN-Ldb1 at the *Mef2c* promoter and AHF enhancer, showing that the DN-Ldb1/Isl1 complex binds at its target sites, but cannot promote long-range promoter-enhancer interactions, necessary for proper gene regulation (Figure S7C). Finally, luciferase assays revealed significant synergistic effect of Isl1 and Ldb1 on a luciferase construct containing the *Mef2c* promoter upstream and the *Mef2c* AHF enhancer downstream of a luciferase gene, in comparison to a reporter construct harboring the *Mef2c* promoter alone (Figure S7D), suggesting the requirement of Isl1/Ldb1 complexes in promoting long range promoter enhancer interactions to ensure high levels of heart specific *Mef2c* expression.

To understand the functional significance of the dynamic chromatin looping at the Mef2c locus during the course of cardiac progenitor cell differentiation and heart development we analyzed the expression of annotated alternative *Mef2c* transcripts. Interestingly, while the longer reference sequence transcript (Refseq) was highly expressed in d4 EBs and in dissected SHF, the transcript with alternative transcriptional start site 1.5 kb downstream of the AHF enhancer was more abundant later during EB differentiation and in dissected hearts (Figure 5E), implying that the observed dynamic chromatin looping correlates with the expression of alternative transcripts for Mef2c. To better characterize the –14 kb to –5.5 kb genomic region found in close

proximity to the AHF enhancer we screened this region for known enhancer-associated chromatin marks H3K27ac, H3K4me1, p300 and Pol II. We found a marked enrichment of these marks -13 kb upstream of the TSS, which correlated with strong binding of Isl1 and Ldb1 at these sites (Figure 5F, Figure S7B). Sites within the –14 kb to –5.5 kb genomic region which were not bound by Isl1 and Ldb1 did not show significant enrichment of enhancer-associated chromatin marks (Figure 5F).

Ldb1 orchestrate a network for transcriptional regulation and coordination in three-dimensional space during cardiogenesis

Gene Ontology (GO) analysis of sequences found by the 3C-seq in close proximity to the AHF enhancer over-represented in wild-type versus *Ldb1*-/- EBs, showed an enrichment of genes involved in heart development, cardiac muscle development, cell fate commitment and vasculature development within the first ten most enriched GO terms (Figure 6A, p<0.01). By contrast, when similar analysis was performed using the sequences found in proximity to the *Mef2c* promoter, no overrepresentation of GO terms involved in heart development was observed (Figure 6B). Importantly, we found that the AHF enhancer is involved in contacts with multiple genes, which play key roles during cardiac progenitor cell differentiation and heart development (Figure 6C, Table S1). 3C-qPCR analysis of wild-type versus *Ldb1*-/- EBs, as well as of dissected SHF or tails of E8-9 embryos confirmed the specificity and the Ldb1-dependence of these interactions (Figure 6D, 6E). Importantly, in d4 and d6 *Ldb1*-deficient EBs we observed significant downregulation of selected genes, which show significantly higher association with the AHF enhancer in wild-type versus *Ldb1*-/- EBs (Figure 7A). By contrast, genes which showed similar association with the AHF enhancer in wild-type and *Ldb1*-/- EBs (*Rai2*, *Xrcc4*), were not altered. Furthermore, overexpression of Isl1 and Ldb1, but not DN-Ldb1 strongly promoted the expression of the genes that were highly associated with the AHF enhancer in wild type EBs (Figure 7B). Moreover, we observed significant downregulation of these genes in hearts and dissected SHF regions of *Isl1+/-Ldb1+/-* E9.25 embryos compared to wild-type

embryos (Figure 7C, 7D). Taken together, these data provide strong support for a model according to which the Isl1/Ldb1-containing transcription complexes orchestrate a network for transcriptional regulation and coordination in three-dimensional space to regulate cardiac progenitor cell differentiation and heart development (Figure 7E).

DISCUSSION

The differentiation of cardiac progenitor cells into distinct lineages involves a coordinated series of large-scale transcriptional changes, but how these events are coordinated at the molecular level has remained poorly understood. Our study shows that a complex between the central SHF transcription factor Isl1 and the multi-adaptor protein Ldb1 plays a crucial role in directing chromatin organization and coordinating a cardiac lineage specific gene expression program.

We found that Ldb1-deficient ES cells show a markedly decreased expression of SHF marker genes and subsequently impaired differentiation into the cardiomyocyte and endothelial lineages, while differentiation into the smooth muscle lineage was increased. Ldb1 gain-of-function experiments confirmed the requirement of Ldb1 in the differentiation of cardiovascular progenitors in cardiomyocytes and endothelial cells and in suppressing the smooth muscle lineage. FACS analysis for Flk-1 and PdgfR-α and qPCR for *Mesp1/2* revealed no significant differences in cardiovascular progenitor numbers upon Ldb1-deficiency, suggesting that Ldb1 loss affects the differentiation of cardiovascular progenitor cells, but not cardiogenic cell-lineage commitment. Interestingly however, overexpression of Ldb1 in wild-type EBs significantly increased the number of Flk-1+PDGFR-α+ cardiovascular progenitors, suggesting that modulation of Ldb1 levels might affect cardiac lineage commitment. Consistent with this, of DN-Ldb1, which competes with full-length Ldbs for binding to LIM domain proteins led to a significant decrease in Flk-1$^+$PdgfR-α$^+$ cardiovascular progenitor cells, implying functional redundancy between Ldb1 and Ldb2 in cardiac lineage commitment. Mechanistically, we show that the importance of Ldb1 for cardiac differentiation and SHF development is two-fold (Figure 7E): *(i)*

Ldb1 binds to Isl1 and protects it from proteasomal degradation, as a consequence of which Ldb1-deficiency leads to an almost complete loss of Isl1+ cardiovascular progenitor cells. We found that Isl1 is ubiquitinylated at its LIM2 domain and via the binding of Ldb1, mainly to LIM1, Ldb1 protects Isl1 from degradation. This is consistent with previous studies, showing that LIM-HD proteins levels are regulated by the proteasome and that binding of Ldb1 to LIM domain proteins protects them from degradation (Gungor et al., 2007). *(ii)* The Isl1/Ldb1 complex orchestrates a network for transcriptional regulation and coordination in three-dimensional space driving cardiac differentiation and heart development. A truncated Ldb1 protein (DN-Ldb1), which contains the LIM interaction domain and thereby can protect Isl1 protein from degradation, but lacks the dimerization domain and thus cannot promote long-range enhancer-promoter interactions (Krivega et al., 2014) did not collaborate with Isl1 to regulate the expression of their common targets and failed to rescue the cardiac differentiation defects of Ldb1-deficient cells (Figure 4E-F). Importantly, overexpression of DN-Ldb1 in zebrafish embryos leads to defects in the differentiation of Isl1+ cardiac progenitors at the venous pole in striking parallel with *Isl1* mutant fishes (de Pater et al., 2009), presumably by competing with full-length Ldb1 for binding to Isl1 (Bach et al., 1999). Importantly, upon overexpression of DN-Ldb1 we observed significant downregulation of *hand2* and *mef2cb*, transcription factors which play important roles in cardiomyocyte differentiation and heart development (Lin et al., 1997; Srivastava et al., 1997; Tsuchihashi et al., 2011). Similarly, we observed decreased expression of *Hand2* and *Mef2c* in *Ldb1*-deficient EBs, which showed complete blockade of cardiomyocyte differentiation, and in *Isl1/Ldb1* haplodeficient embryos, which developed various cardiac anomalies. Moreover, our results suggest that the Isl1/Ldb1 complex directly regulates *Mef2c* and *Hand2* expression by binding to their heart specific enhancers and stimulating promoter-enhancer interactions. Similarly, Ldb1 is required for the looping of the *β-globin* locus control region (LCR) to the *β-globin* promoter (Deng et al., 2012; Song et al., 2007; Tolhuis et al., 2002), and it was shown that a fusion protein of LMO2 and the Ldb1 dimerization domain is sufficient to restore looping of

the β-globin promoter to the LCR and transcription in Ldb1 knockdown cells (Krivega et al., 2014).

Mef2c plays a key role in regulating anterior heart field development and cardiomyocyte differentiation. Similarly to *Isl1*, *Mef2c*-deficient hearts show outflow tract abnormalities and fail to form the right ventricle (Cai et al., 2003; Lin et al., 1997). 3C-seq and 3C-qPCR using the AHF enhancer of Mef2c, which confers responsiveness to Isl1 and GATA factors (Dodou et al., 2004), as a viewpoint identified close proximity between the promoter region, the AHF enhancer and the 3' end of the gene. Gene looping has been shown to bring promoter and terminator regions together in close proximity in the early stages of transcriptional activation, facilitating RNA pol II re-initiation and high-level expression of the long genes *FMP27* and *SEN1* in *Saccharomyces cerevisiae* (O'Sullivan et al., 2004). Similarly, the observed loop between the promoter and the 3'end of the *Mef2c* gene could generate a functional re-initiation complex for subsequent rounds of *Mef2c* transcription. Further, we observed dynamic chromatin looping at the Mef2c locus during heart development and in the course of cardiac progenitor cell differentiation, in which the AHF enhancer contacts the proximal promoter during the onset of Mef2c gene activation in the anterior heart field, whereas later this contact is lost, correlating with the expression of an alternative transcript with TSS 1.5 kb downstream of the AHF enhancer. A similar developmental switch in chromatin looping was observed during erythroid differentiation at the *β-globin* locus, from favoring the expression of embryonic globin genes in erythroid progenitors to favoring the expression of the adult globin genes in definitive erythrocytes (Palstra et al., 2003).

Remarkably, our 3C-seq data identified surprisingly specific, Ldb1-mediated interactions of the AHF enhancer with multiple genes that play key roles in cardiovascular development, as well as cell fate commitment, suggesting a role of Ldb1 in regulating a cardiac-specific interaction network around the *Mef2c* AHF enhancer. These interactions are cell type specific, as they were observed in cardiac cells but not in other cells types, and are lost upon Ldb1-deficiency. Recent studies analyzing enhancer contacts during *Drosophila* development, revealed that a large

number of enhancer interactions are unchanged between different tissues and developmental stages and only few of them show significant changes (Ghavi-Helm et al., 2014). Notably, Ldb1 appears to mediate cardiac cell-type specific chromatin loops and transcriptional programs, via its diverse DNA binding partners (Figure 5-7, (Soler et al., 2010; Song et al., 2007; Tolhuis et al., 2002)). Importantly, Ldb1 deficiency led to dramatically decreased expression of the genes associated with the AHF enhancer and overexpression of Isl1 and Ldb1 strongly promoted their expression. The dosage-sensitive interdependence between Isl1 and Ldb1 in the expression of these key factors in cardiogenesis, further supports a key role of the Isl1/Ldb1 complex in coordinating chromatin looping and heart-specific gene expression. Notably, we observed significant enrichment for binding of the core cardiac transcription factors Gata-s, Nkx2-5, Mef2c and Tbx-s (Figure S7E) within the genomic regions found in close proximity to the *Mef2c* AHF enhancer. Interestingly, co-occupancy of these factors identifies transcriptional enhancers active in the heart (He et al., 2011) and their overexpression leads to a direct reprogramming of fibroblasts into functional cardiomyocytes, further supporting a role of cell type-specific transcription factor-regulatory networks in the control of cell type-specific genome organization and gene expression (de Wit et al., 2013; Denholtz et al., 2013).

In conclusion, our study highlights a central role for Ldb1 in regulating cardiac progenitor cell differentiation and SHF development and provides exciting novel insights into the molecular machinery that orchestrates chromatin organization and coordinated gene expression during cardiogenesis.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies

For detailed plasmid information, see the Supplemental Experimental Procedures. The following primary antibodies were used: rabbit anti-HA (Y-11; Santa Cruz Biotechnology), mouse anti-Flag (M2, Sigma), goat anti-Ldb1 (N-18, Santa Cruz Biotechnology), mouse anti-Ldb1 (C-9 Santa

Cruz Biotechnology), mouse anti-Islet1 39.4D5 (Developmental Studies Hybridoma Bank); goat anti-Lamin B (C-20; Santa Cruz Biotechnology), mouse anti-tubulin (T5168 Sigma), anti-GFP (ab6556 Abcam), APC-conjugated anti-Flk1 (e-Bioscience 17-5821-81) and PE-conjugated anti-PDGFRα (e-Bioscience 12-1401-81).

Immunoprecipitation and ChIP

Co-IPs and ChIP was performed as described in (Witzel et al., 2012). Detailed protocols are described in Supplemental Experimental Procedures.

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

Embryos were dissected, and 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 StepOnePlusTM real-time PCR detection system.

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

3C-seq and 3C-qPCR was performed as described in (Stadhouders et al., 2013). In brief, 1×10^7 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.

Zebrafish strains

Embryos and adult zebrafish were raised under standard laboratory conditions at 28 ºC. The following mutant and transgenic lines were used: *Tg(myl7:EGFP-HsHRAS)*[s883] (D'Amico et al., 2007) and *isl1sa0029* (Sanger Institute, Zebrafish Mutation Resource).

Mouse lines

The Ldb1 tm1a(EUCOMM)Wtsi line was generated by microinjection of Ldb1 tm1a(EUCOMM)Wtsi ES cells, obtained from the European Conditional Mouse Mutagenesis Program (EUCOMM), into blastocysts.

mRNA injection

mRNA synthesis was performed using mMESSAGE mMACHINE® SP6 Transcription Kit (Ambion) following manufacturer's instructions. mRNA was injected in 1- cell stage of fertilized zebrafish eggs (volume 2 nl, mRNA concentration 200 ng/µl for Flag-HA-DN-Ldb1).

***In situ* hybridization, whole mount immunostaining and confocal microscopy**

In situ hybridization and whole-mount staining was performed as described (Witzel et al.*,* 2012). Confocal images were acquired by a Zeiss LSM 710 system and the Z-stacks were projected by Zeiss LSM 710 software.

Data deposition

All sequencing data have been deposited in GEO (accession number SRP055800).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures and two tables.

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FIGURE LEGENDS

Figure 1. Ablation of Ldb1 results in defects in cardiac progenitor cell differentiation and SHF development. *(A)* Schematic diagram of the experimental setup. *(B)* Percentage of beating EBs in wild-type and *Ldb1-/-* ES cells differentiated in EBs. *(C)* Relative mRNA expression of cardiomyocyte (*Mlc2a*, *Mlc2v*, *Tnnt2*), endothelial (*Flk1*, *CD31*) and smooth muscle (*SM-actin*, *SM-22α*) genes in d9 EBs differentiated from wild-type and *Ldb1-/-* ES cells. *(D)* Relative mRNA expression of mesodermal markers (*Eomes*, *Bry*, *Mesp1* and *Mesp2*) in EBs differentiated from control and *Ldb1-/-* ES cells at different days. *(E)* Relative mRNA expression of cardiac progenitor marker genes in d4 EBs differentiated from *Ldb1+/+* and *Ldb1-/-* ES cells. *(F)* Gross appearance of control (*Isl1$^{cre/+}$/Ldb1$^{+/flox}$*) and *Isl1$^{cre/+}$/Ldb1$^{flox/flox}$* embryos at E10.5, showing developmental arrest of the *Ldb1*-deficient embryos. Scale bars, 500 μm. *(G)* Higher magnification of E9.5 embryos viewed from the right (left panels) and the front (right panels), showing a short outflow tract and a small right ventricle. Scale bars, 200 μm. Abbreviations: OFT, outflow tract; RV, right ventricle; LV, left ventricle. *(H)* Relative mRNA expression analysis of cardiomyocyte genes in dissected outflow tract and right ventricle of E9.25 wild-type and *Isl1$^{cre/+}$/Ldb1$^{flox/flox}$* embryos. Data are mean ± SEMs, n=3 for each genotype. See also Figure S1.

Figure 2. Ldb1 binds to Isl1 and protects it from proteasomal degradation. *(A)* FACS analysis of Flk-1 and PdgfR-α expression in d3.75 EBs differentiated from control and *Ldb1-/-* ES cells *(B)* Relative mRNA expression of *Isl1* in EBs differentiated from control and *Ldb1-/-* ES cells at different days. *(C)* Isl1 immunostaining on vibratome sections from d5 EBs differentiated from control and *Ldb1-/-* ES cells. Scale bars, 100 μm. *(D)* Western blot analysis of total protein extracts of day 4, 5 and 6 EBs differentiated from control and *Ldb1-/-* ES cells. Lamin B1 served as loading control. *(E)* HA-tagged ubiquitin and Isl1 were transiently expressed in HEK293T cells. Cells were either treated with DMSO or with MG-132 6 h before harvesting. Equivalent amounts of total cellular protein were immunoprecipitated with an anti-Isl1 antibody. The co-

immunoprecipitated proteins were detected by an immunoblot analysis with an anti-HA antibody. *(F)* Schematic representation of wild-type Isl1 and Isl1 deletion constructs, lacking either LIM1 or LIM2 or harboring only the homeodomain (top). Western blot analysis of whole cell lysates of HEK293T cells transfected with Isl1, Isl1ΔLIM1, Isl1ΔLIM2 and Isl1Homeo expression plasmids, treated with DMSO or MG-132. *(G)* Schematic representation of wild-type Ldb1 and Ldb1 deletion constructs. Ldb1 harbors three domains that have been shown to be important for its function: dimerization domain (DD), the Ldb1/Chip conserved domain (LLCD) and the LIM-interaction domain (LID) in the C-terminal part of the protein (top panel). HEK293T cells were transfected with constant amounts of Isl1 (10 µg) and GFP (1 µg, used as a control of transfection efficiency) expression plasmids and increasing amounts of Ldb1, Ldb1ΔLID and DN-Ldb1 (5 and 9 µg). Immunoblot analysis of equal amounts of total protein extracts was performed using either anti-Isl1 or anti-GFP antibodies. *(H)* Western blot analysis of whole cell lysates from HEK293T cells transfected with Isl1, Isl1ΔLIM1, Isl1ΔLIM2 and Isl1HOMEO alone or together with DN-Ldb1 and GFP (internal control) using anti-Isl1, anti-FLAG and anti-GFP antibodies. *(I)* FLAG-HA-Ldb1 and Isl1 or Isl1 deletion constructs were transiently expressed in HEK293T cells and immunoprecipitation with an anti-Isl1 antibody was followed by immunoblot analysis with an anti-FLAG antibody.

Figure 3. Ldb1 and Isl1 interact to regulate heart development. *(A)* Co-immunoprecipitation of nuclear extracts from d5 EBs using anti-Ldb1 antibody and detected with anti-Isl1 antibody. *(B)* Percentage of beating d7 EBs derived from ES cells overexpressing either GFP alone (control) or together with Isl1, Ldb1 or in combination. *(C-E)* Relative mRNA expression of cardiomyocyte (*Mlc2a, Mlc2v*) *(C)*, endothelial (*Flk1, VE-Cad*) *(D)* and smooth muscle (*SM-actin, SM-Mhc*) marker genes *(E)* in d7 EBs differentiated from ES cells overexpressing either GFP alone (control) or together with Isl1, Ldb1 or Ldb1+Isl1. *(F, G)* Relative mRNA expression of cardiac progenitor marker genes in d4 EBs *(F)* and *Flk1* and *PdgfR-α* in d3.75 EBs *(G)*

differentiated from ES cells overexpressing either GFP alone or together with Isl1, Ldb1 or Ldb1+Isl1. For qPCR analysis of endogenous *Isl1* levels, primers located in the 5'UTR were utilized. Error bars represent SEMs derived from three biological replicates. *(H-J)* H&E staining of representative paraffin sections of E16.5 hearts of wild-type and *Isl1+/-Ldb1+/-* embryos (*H*, top panels), higher magnification of right and left ventricles showing thinner compact myocardium of the right ventricle in Isl1+/-Ldb1+/- embryos (*H*, bottom panels), DORV *(I)* or OA in E18.5 hearts *(J)* with VSD *(I, J)*. Abbreviations: Ao, Aorta; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle; DORV, double outlet right ventricle; OA, overriding aorta; VSD, ventricular septal defect. *(K)* Morphometric analysis of right ventricle compact myocardial thickness (n=4). *(L)* Relative mRNA expression analysis of cardiac progenitor and cardiomyocyte genes in dissected hearts and SHF of E9.25 wild-type, *Isl1+/-*, *Ldb1+/-* (controls) and double heterozygous *Isl1+/-Ldb1+/-* embryos. Data are mean ± SEMs, n=4 for each genotype. *p≤0.05, **p≤0.01, ***p≤0.005. See also Figure S2.

Figure 4. The dimerization domain of Ldb1 is required for cardiomyocyte differentiation.
(A) Confocal images of control and DN-Ldb1 overexpressing *Tg(myl7:EGFP-HsHRAS)^{s883}* zebrafish embryos stained with anti-GFP and anti-Isl1 (red) antibodies at 48 hours post fertilization (hpf), showing shortened atrium and significant reduction of Isl1+ cardiomyocytes at the venous pole. Arrows indicate Isl1+ cells outside of the heart, which do not express the cardiomyocyte marker *myl7*. Scale bars, 50 μm. *(B, C)* In situ hybridization of control, DN-Ldb1 overexpressing and Isl1 mutant embryos at 48 hpf with *bmp4 (B)* and *mef2cb (C)* probes. The arrows indicates *bmp4* expression at the sinus venosus. *(D)* Confocal images of control and DN-Ldb1 overexpressing embryos stained with anti-Isl1 antibody (leftmost panels) and *in situ* hybridization of control and DN-Ldb1 overexpressing embryos at 10-15s stages with *isl1*, *nkx2.5*, *hand2*, *tbx5a* and *mef2cb* probes. *(E)* Percentage of beating d7 EBs derived from *Ldb1-/-* ES cells overexpressing either GFP alone (control) or together with Isl1, Ldb1, DN-Ldb1 or in

different combinations. *(F, G)* Relative mRNA expression analysis of cardiomyocyte *(F)*, smooth muscle and endothelial marker genes *(G)* in d7 EBs. *(H)* FACS analysis of Flk-1 and PdgfR-α expression in d3.75 EBs. *(I)* Western blot analysis of total protein extracts of d5 EBs. *(J)* Relative mRNA expression of *Mef2c* and *Hand2* in d4 EBs. Error bars represent SEMs derived from three biological replicates. *(K, L)* ChIP of nuclear extracts from d5 EBs *(K)* and pools of dissected SHF from E8-E9 embryos (n=3) *(L)* using anti-Isl1 and anti-Ldb1 antibodies or IgG as control. PCRs were performed using primers flanking conserved Isl1 binding sites in the *Mef2c* promoter and the AHF enhancer. Fold enrichment values for EBs were calculated relative to IgG control and for embryos relative to a genomic region which does not contain conserved Isl1 binding sites. *(M, N)* ChIP of nuclear extracts from d5 EBs *(M)* and pools of dissected SHF from E8-E9 embryos *(N)* using anti-Isl1 and anti-Ldb1 antibodies or IgG as a control. PCRs were performed using primers flanking conserved Isl1 binding sites in the *Hand2* promoter and the OFTRV enhancer (McFadden et al., 2000). See also Figures S3-S7.

Figure 5. Ldb1 facilitates long range enhancer-promoter interactions within the *Hand2* and *Mef2c* loci. *(A)* Schematic representation of the *Hand2* genomic locus and the position of the DpnII restriction sites, used in the 3C assay (top). 3C-qPCR relative crosslinking frequency observed in WT, *Ldb1*-/- ES cells and d5 EBs derived from *Ldb1*-/- ES cells overexpressing either GFP alone or together with Isl1, Ldb1, DN-Ldb1 or in different combinations. The *Hand2* promoter was used as viewpoint to the *Hand2* OFTRV enhancer or to negative control regions downstream of the *Hand2* promoter, between the *Hand2* promoter and the OFTRV enhancer and upstream of the OFTRV enhancer. Values were normalized to the *β-actin* locus and the highest value for the OFTRV enhancer in d5 WT EBs was set as one. Data are mean ± SEMs, n=3. *(B)* Schematic representation of the *Mef2c* genomic locus and the position of the restriction sites of *HindIII*, used in the 3C assay (top). 3C-Seq analysis of *Mef2c* AHF- (middle panel) and *Mef2c* promoter-associated regions (bottom panel) in d5 wild-type and *Ldb1*-/- EBs. The

viewpoint is indicated with an eye symbol. *(C)* 3C-qPCR relative crosslinking frequency observed in EBs at different days (top panels) and in microdissected SHF region, brain or tail from pools of embryos at different developmental stages (bottom panel). Data are mean ± SEMs, n=3. *(D)* 3C-qPCR relative crosslinking frequency observed in d4 (top) and d5 (bottom) EBs derived from WT and *Ldb1-/-* ES cells or *Ldb1-/-* ES cells overexpressing either GFP alone or together with Isl1, Ldb1, DN-Ldb1 in different combinations. Data are mean ± SEMs, n=3. For the 3C-qPCR in *C* and *D* the HindIII fragment containing the *Mef2c*-AHF was used as viewpoint (red bar, eye symbol). *(E)* Schematic representation of alternative Mef2c transcripts (top) and absolute quantification of these transcripts using primers indicated in the scheme (bottom). *(F)* ChIP of d4 EBs derived from WT and *Ldb1-/-* ES cells using antibodies against H3, H3k4me1, H3K27Ac, p300, RNA-PolIIS5p and IgG as a control. (*p≤0.05, **p≤0.01, ***p≤0.005). See also Figure S7.

Figure 6. Ldb1 promotes chromatin looping events between the AHF enhancer and genes which play key roles in cardiovascular development. *(A, B)* Gene ontology analysis of genes interacting with the *Mef2c* AHF *(A)* or the *Mef2c* promoter *(B),* overrepresented in wild-type versus *Ldb1-/-* cells. *(C)* Schematic representation of 3C-seq results showing specific interactions of the *Mef2c* AHF (located at chromosome 13 (Chr13)) with cardiac specific genes on Chr13 or on other chromosomes. The HindIII restriction sites are shown as black bars. Y axes - reads per million. *(D, E)* 3C-qPCR validation of the interactions observed using the 3C-seq approach in WT or *Ldb1-/-* d5 EBs *(D)* or in microdissected SHF regions and tails of E8-9 embryos *(E)* using the *Mef2c*-AHF as viewpoint. Data are mean ± SEMs, n=3. (*p≤0.05, **p≤0.01, ***p≤0.005). See also Figure S7.

Figure 7. The Isl1/Ldb1 complex orchestrates a network for transcriptional regulation and coordination in three-dimensional space during cardiogenesis. *(A)* Relative mRNA

expression analysis in d4 and d6 WT and *Ldb1-/-* EBs of selected genes identified in the 3C-seq analysis to specifically interact with the *Mef2c*-AHF in WT but not in *Ldb1*-/- EBs. *(B)* Relative mRNA expression analysis of selected genes in d5 EBs overexpressing either GFP alone (control) or together with Isl1, Ldb1, DN-Ldb1 or in different combinations. *(C-D)* Relative mRNA expression analysis of selected genes in microdissected SHF *(C)* or heart *(D)* of WT or *Isl1+/- Ldb1+/-* E9.25 embryos. Data are shown as mean ± SEMs, n=4. (*p≤0.05, **p≤0.01, ***p≤0.005). *(E)* Model of the role of Ldb1 in heart development. Ldb1 binds to Isl1 and protects it from proteasomal degradation. The stabilized Isl1/Ldb1 complex orchestrates a network for transcriptional regulation and coordination in three-dimensional space during cardiac progenitor cell differentiation and heart development.

A Control — *myl7:mGFP*, α-Isl1; DN-Ldb1 — *myl7:mGFP*, α-Isl1; Control 48 hpf; DN-Ldb1 48 hpf

B *bmp4* Control 48 hpf; *bmp4* DN-Ldb1 48 hpf; *bmp4* Isl1-/- 48 hpf

C *mef2cb* Control 48 hpf; *mef2cb* DN-Ldb1 48 hpf

D Control / DN-Ldb1: 10s α-Isl1; 10s *isl1*; 10s *nkx2-5*; 10s *hand2*; 10s *tbx5a*; 15s *mef2cb*

E % beating EBs — GFP, Isl1, Ldb1, DN-Ldb1, Ldb1/Isl1, DN-Ldb1/Isl1

F Relative mRNA expression — *Tnnt2*, *Mlc2a*, *Mlc2v*

G Relative mRNA expression — *CD31*, *Flk1*, *sm-actin*, *sm-22a*

H Flk-1 vs PdgfR-α: GFP, Isl1, Ldb1, DN-Ldb1, Ldb1/Isl1, DN-Ldb1/Isl1

I Isl1, HA, Tub — GFP, Isl1, Ldb1, DN-Ldb1, Ldb1/Isl1, DN-Ldb1/Isl1

J Relative mRNA expression — *Hand2*, *Mef2c*

K Fold enrichment — IgG, α-Isl1, α-Ldb1: -1,5 Kb, -1 Kb, -200 bp, AHF, Neg Ctrl

L Fold enrichment — α-Isl1, α-Ldb1: -1,5 Kb, -200 bp, AHF, Neg Ctrl

M Fold enrichment — IgG, α-Isl1, α-Ldb1: OFTRV, Prom, Neg Ctrl

N Fold enrichment — α-Isl1, α-Ldb1: OFTRV, Prom, Neg Ctrl

A Ratio of overrepresentation WT/KO Viewpoint AHF

Cardiac muscle tissue development
Striated muscle tissue development
Regulation of neuron differentiation
Muscle tissue development
Regulation of cell development
Cell fate commitment
Heart development
Blood vessel morphogenesis
Vasculature development
Blood vessel development

B Ratio of overrepresentation WT/KO Viewpoint *Mef2c* Promoter

Cell adhesion
Biological adhesion
Intracellular signaling cascade

p Value cut off 0,01

C

Chr13 Ryr2 — AHF WT — AHF KO
Chr9 Smad3
Chr13 FoxC1 Gmds
Chr1 Nop58 Bmpr2
Chr13 Fgf10
Chr2 Bmp2
Chr13 Isl1
Chr2 Acvr2a
Chr10 Hs3st5 HDAC2
Chr7 Kcnq1 Kcnq1ot1

D ■ *Ldb1* +/+ □ *Ldb1* -/-

Relative Crosslinking Frequency

Bmp2, Bmpr2, Acvr2a, Smad3, Fgf10, FoxC1, Myocd, Isl1, Ryr2, Kcnq1, Kcnj2, Rai2, Xrrc4

E ■ E8-9 SHF □ E8-9 Tail

Relative Crosslinking Frequency

Ryr, Acvr2a, Fgf10, FoxC1, Myocd, Isl1, Rai2, Xrcc4

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. 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.
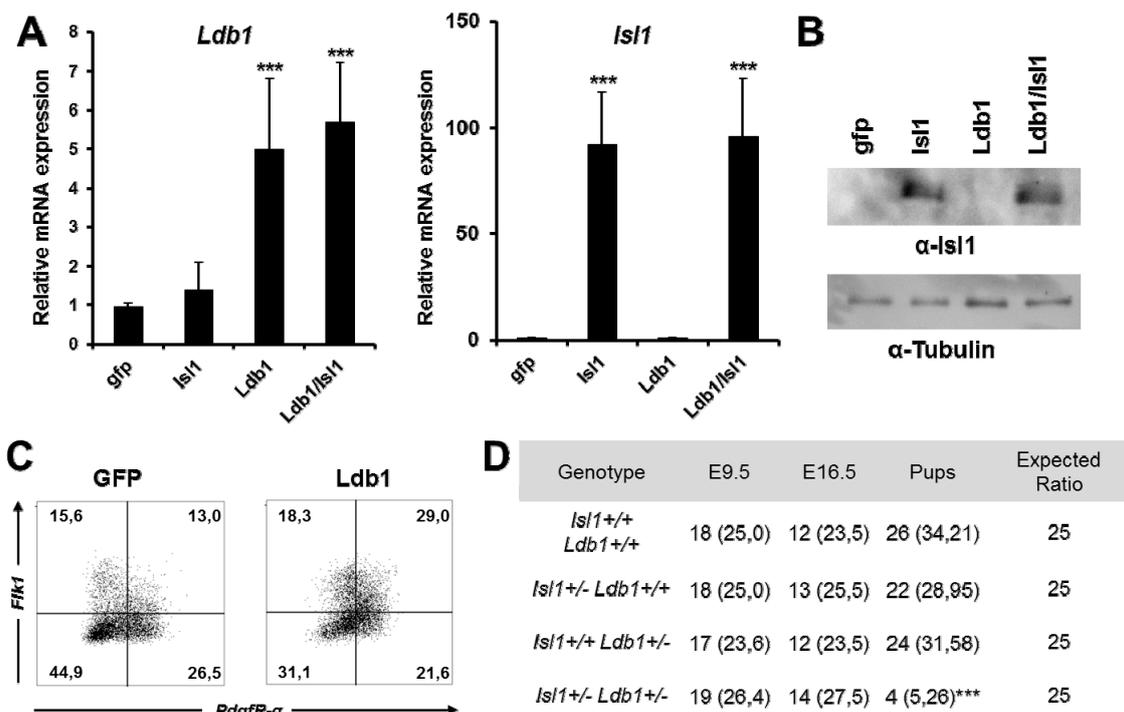
Figure S2, related to Figure 3. Ldb1 and Isl1 interact to regulate heart development. *(A)* Relative mRNA expression of *Ldb1* and *Isl1* in ES cells overexpressing either GFP alone (control) or together with Isl1, Ldb1 or a combination of Isl1/Ldb1. *(B)* Western blot analysis of total protein extracts 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.
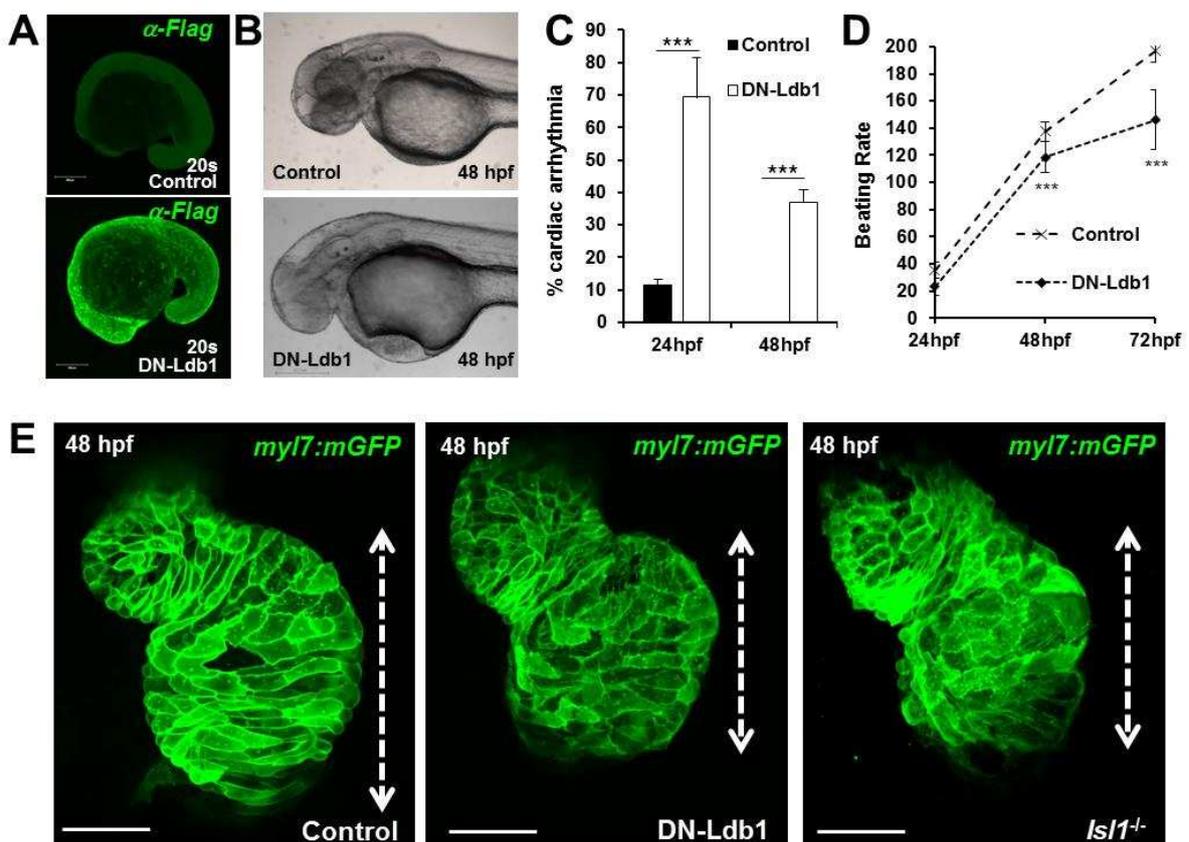
Figure S3, related to Figure 4. Cardiac morphogenesis defects in zebrafish embryos overexpressing DN-Ldb1. *(A)* Confocal images of control and *FLAG-HA-DN-Ldb1* mRNA injected zebrafish embryos, stained with anti-FLAG antibody at 20 somites. *(B)* Control or *DN-*

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.
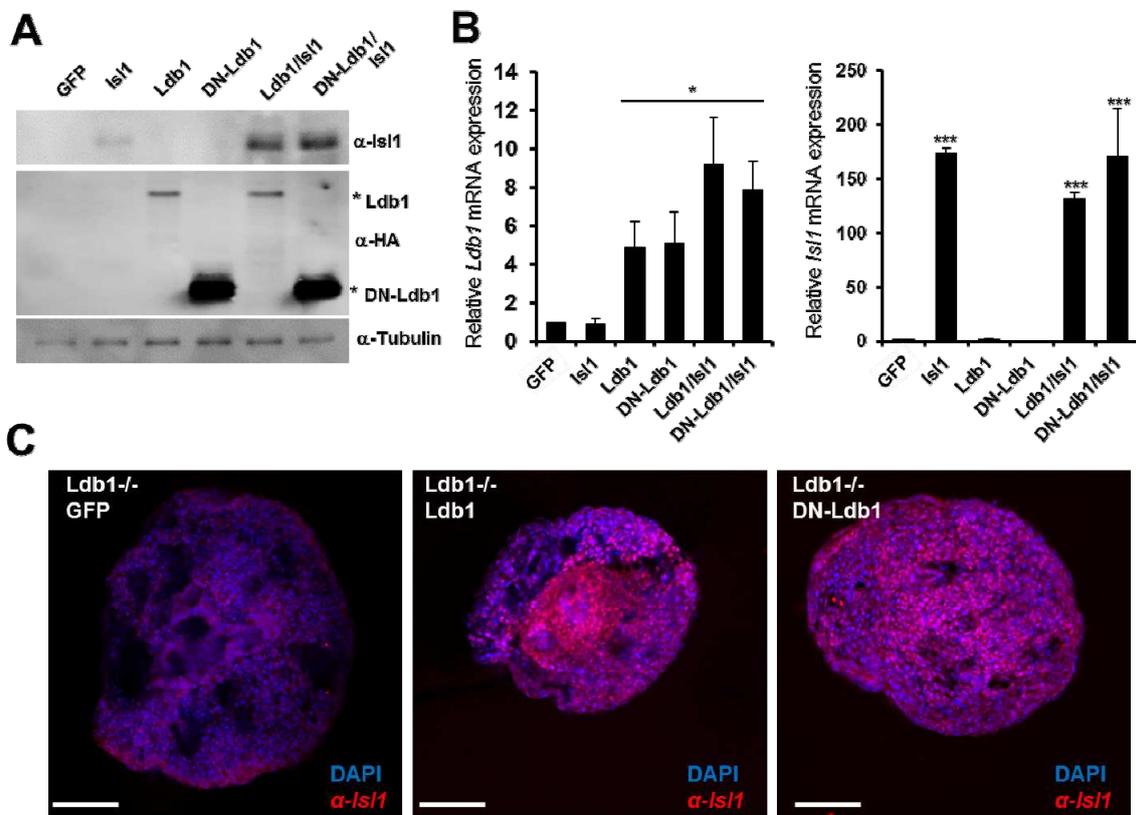
Figure S4, related to Figure 4. Overexpression of Ldb1 and DN-Ldb1 restores Isl1+ cells. **(A)** Western blot analysis of total protein extracts of *Ldb1-/-* ES cells overexpressing either GFP alone (control) or together with Isl1, Ldb1, DN-Ldb1 or in different combinations. **(B)** Relative mRNA expression 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.

Mef2c Promoter

Mouse mm9 chr13 83641449-83643233
Human hg19 chr5 88199875-88201671

```
mouse    TATGAAAGCAATTTCATTTTTTAGGAATGATTTT---GGATAGACTTCCGATTGGATATTTTCCATTGGAACTAACAGTGTAGAGGCTTG
         |||||||||||||||||||||||||||||||||   ||||||||||||||||||||||||||||||||||||||||||   ||| ||||||||
human    TATGAAAGCAATTTCATTTTTTAGGAATGATTTTCATGGATAGACTTCCGATTGGATATTTTCCATTGGAACTAGCAGCATAGGGGGTCG

                                  ⌐→
mouse    GGGTGGGGAGAG---------AGCAGTTCTGTGTTCTTTTGCCAGCACTGACAAAGGTCTGGTTGTCAATGATACCTTTACAGCTAAATT
         ||  ||||  |          |||| ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
human    GGAGGGGGGAGGTCGGAGGGAAGCAATTCTGTGTTCTTTTGCCAGCACTGACAAAGGTCTGGTTGTCAGTGATACCTTTACAGCTAAATT
          +1                          3C-Seg_Mef2cProm
mouse    TACTCCAGAGTGACATGAACAGGTGCACCCTGGCCTGCCAGACACTTGTGCAGGGGATCACGCATCTCACCGCTTGACGATCAAGGGGG
         ||||||||||||||  |||||||||||||  |||||||||||||||||||||||||  ||||||||||||||||  |||||||||||||||
human    TACTCCAGAGTGACAGAAACAGGTGCACCTCGGCCTGCCAGACACTTGTGCAGAGAGATCACGCATCTCACGGCTTGACGATCAAGGGGG
           HindIII
mouse    CAAAGCTTCGGTGTTCATAGAAAAGGAGAGGAGGCGAGCGCAGCCCAAACTGGGGGGTTT
         |||||| ||||| ||||||||||||||||||||||||||| | |||||||||||||||||
human    CAAAGCCTCGGTCTTCATAGAAAAGGAGAGGAGGCAAACGCAGCCCAAACTGGGGGGTTT
```

Mef2c -13Kb

Mouse mm9 chr13 83629799-83630029
Human hg19 chr5 88214651-88214411

```
                                          +13Kb_F
                                 ⌐────────────────────────→
mouse    TATTTGAGAGGGTGGTATCAATTAACTATATTATCTATAAAACCATACTTGCAATTACTACCACTTCACAATTTATCATTAACACATTTG
         |  ||||||||||||||||||||||||||||||||||||||||   ||||||||||||||||||||||||||||||||||||  ||||||
human    TGCTTGAGAGGGTGGTATCAATTAACTATATTATCTATAAAGCCATACTTGCAATTACTACCACTTCACAATTTATCATTAATGCATTTG

mouse    GCAGATTTTTTTTG--CAAAGTAACATTTATTTAAAGGTTAATCAAAATTCTTAGAATAGCATTTATTATAATGTGATTTTTGATAATTG
         ||||||||||||    |||||||||||||||||||||||||||||||||||||||||||  |||||||||||||||||||||| |||||||
human    GCAGATTTTTTTTCTCCAAAGTAACATTTATTTAAAGGTTAATCAAAATTCTTAGAACAGCATTTATTATAATGTGATTTTTGATAATTG
                                     ←────────────────
mouse    ACTAAATCAGTGGCTCTTAAAAACACCCTTGTCTCAGTCCTGCTCAGTATACACCACACT
         |||||||||||||||||||||| |||||||||||||||||| ||||||||||||||||||  +13Kb_R
human    ACTAAATCAGTGGCTCTTAAGAACACCCTTGTCTCAGTCCCACTCAGTATACACCACAGT
```

Mef2c -12Kb

Mouse mm9 chr13 83631203-83631643
Human hg19 chr5 88213106-88212651

```
mouse    AGGCATAACCATCTTGCTTTAATGAGCATTACTAACTACACTCGACTTTATCCCAG----TGGAAGAGCTAAAGACAAGGA--CAGTCTT
         ||| |  |  ||| |   ||||||| |  ||||| | ||||   |  ||  ||  |    |  |||  |   ||   ||  |   |||||
human    AGGTACATACATGTGCATTTAATGAACGTTACTGACTACACTTTACACCATGCAAGGCACTAAAAGGGAGGAGGACAGCCAACAAGTCTC
                                                                          -12Kb_F
                                                                 ⌐──────────────────────────→
mouse    GGCCCTTGGCAAGGATGTAAGAA-------TATCAAGTGCATGTATGTCAAACAAACACTCAGGACAATCA-TGAAAGCACTCAATCCACT
         |  ||   |||| |||   |  |||     ||||   ||| ||||| |||||  ||||| |  ||||  |  || |     |   |  ||||
human    AGTTCTCAACAAGGAAGCTAAAAAGAAATTAACAAGTAACTGCATGCCAAGCACAAAGTCATGAATATCACTGAAAAATTACAAACTGAT
         →
mouse    TCCCATTGGAACCGCAAAGGGAAAAGTCTTCAAGGTCATGG---------AGAAAGTAGGTGTTATT-AGGACACCTCAAGA------TCTC
         ||||  ||| |  ||  ||  ||   |||  |  |||||          | |||||| ||  ||  | | |||||||||||     ||||
human    TCCCTTTGAAGCCTCAGGAAAGAAAGCAGGTGAAATCATGGAAGTCTTAAGGAAGTAGGTGCCATTAAAAATAGTTCAAGAGAATTTCCC

mouse    TGCAGATAGCATGTACTAGTGCCTTTACTAACA-----------------------CCA-------CCACAGTAAGTTTCTTGCTCTCTG
         |  ||| |||  ||| | |||||| | |||                          |||        || ||||||| ||  ||||||
human    T---GATTACATAAATTAGTGAATTCACTAGTGATGAAGGCATTGAAAGTAAAGGTCCAGAGGTTTTCAGAGTAAGTTACTCACTTTCTT
                                                                        -12Kb_R
                                                           ←────────────────
mouse    GGGGACAATTAGTCCATTTGT---TTTCATCAAATTACCTTTGGGGATAC---AAAATGATAATTGGTGATTTAATAGCCACCCAGTAGA
         |||  | | ||  || ||| |   ||| | || | ||  ||| |||  ||   |||||||| |||||| ||||||||| || |
human    AAAAACAG-CAATCTGTTTGTTCATCTCATCAAGTTA-GTTTGGTGCATAAAAAAAATGATAATTGCTGAATTAATGGCCATTCAGTATG

mouse    AGGAACAAGGAAAATTGTTCCTTTCATGGT
         | ||||| ||| |  | |||||| ||| |||
human    AAGAACACTGAAGAAATTTCCTTTCAAGGT
```

Mef2c +2,7Kb

```
Mouse mm9  chr13 83645570-83645865
Human hg19 chr5  88901607-88901315
```

```
                                                          +2,7 F
mouse TAAAAGATTACTTGAAATATATCTTCACAACATAAAGATAA-TTATTTCAGGGGTGGGAATTTAATCATTCTCCTGATTCAGAGAGCCACT
      | |||| |||| ||| || ||||||| | | || |||||  |||||| | | || |||  || ||||| |||||| || ||||
human TTAAACATTATTTGCAAGTTATCTTCCTATCTTACAGATAGCTTATTTACAGTGGAGAAA---AATTGTTTTCCTGTTTCAGAAAGACACT

mouse CAAAAGATTGTTTTGTTC-ATATTCTGTATTCATAAGAAAGCTGTTGATTTTTCTATTAATTAACAATTATTAATGA-ACTGTGGTTACTA
      || |   | ||| || |  || || |  || ||  ||  || ||||| |    |||||| || ||||| |||||| | | || || |
human TGGAAAAACATGTTATTTAACCTTATATATTAATAGGGAAAATCTTGATTCTCCCAGTAGTTAACTTCAATTAATTATATTTTAAATATCA

mouse GACATCTAGTCAATCTAAA-GCAACAGAGTTTCCATGTAAAATAGTTTATTTGTAAGTATTTAAAGAAGCAAAAGCTATTTTGGCAGCTTT
      |   ||| |||||   |||| || | ||||| |||| | ||||||||| |||||||||||||| ||||| |||||||| ||||||||||
human G---CCTATTTAATTTCAGTGCAACAAAATTTCCAGCTAAAATGGTTTACTTGTAAGTATTTAAAGCAACAAAAA-TATTTTGGCAGCTTT

              +2,7 R
mouse ATATGTACCTCCTCATTGACCAGACAC
      |||||| | ||||||||||||||| |||
human ATATGTATCTCCTCATTGACCAGCCAC
```

Mef2c +150Kb

```
Mouse mm9  chr13 83795420-83795807
Human hg19 chr5  88024842-88024459
```

```
                                        +150Kb F
mouse TATTTACTCAAATCGAATTCAATA-----------TTTCAAAGAAACTGAGCTACTGTCTCTTTAATGTAGTAGAACACTCACTTTCTTG
      |||||| ||| || ||  |||||           |||||| || | || || | ||| |  || | |  ||| || |   |CACTTT ||
human TATTTATGCAACCTGAGTTCAAAATAGAAACACACTTTCAAGAAAATTGAG---CTGTTTCTTTGATGTAATAAAAAGCTCACTTTCTCA

mouse AAGATTTTTAATGTAAAACCTATTTCTAGATCATAAAATATATAGAT-CCTTTACACTTTTTAAACAAAGCTAGTA----TAATGTGCTAA
      | |||||            |||||||| ||||| ||||||| |||    |||||||| || | |||   || | ||||TAATG ||
human GGATTGTTTAA---------CATTTCTACATCATCATCAAAATATGTACATACCTTTATACT---------AGATTGTAGTTCTAATGAACTAG

                 +150Kb R
mouse CATTTCAGGATCTATTACTGTGGATCTAGTGTGACATCCTTA-CTCAGATGGATAATATTTTTTTTTTCTTTTTAAATTCAAATTATCCG
      ||| | |  |||| |     |||| || ||| ||  ||| || | ||  ||| ||  |   | ||| |||||||||| ||||| TTATC G
human AATTGCGGACTATGCTACTTCAGAGCATTCGTGGAATACTTATTTTTTGGTTTATAGTA------TATTCCTTTTAAATTAGAATTATCGC

mouse TGGTGTTTTATTTTCTTCTTTTCCTAAATCAAAA-----------AACTTTGATTC--AAAGTATGTTTTAGAGGTTGAAACTGTGTGAT
      || || ||||| ||| |||||  | |||| |||||           ||| | ||    |||  || |||||| |  | ||||| ||||||
human TGATGCTTTATCTCCCCTCCCCTCAAAATTAAAAATATCTATAATAACGATAATCCTAATAGAATATTTTGGAAATTGAAAATGT-TTAT

mouse TTTGCTTACCATTTGACAG--TCTAATTGTCTCTCTCTCTCTCTCTCCTTGCCGCTCCT
      | | ||||||||||| |    |||||| |         | | |  ||||| | |
human TCTATTTACCATTTGATAATTTCTAAAT---------TTAATATTTTCCCTTGTTGTTTA
```

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, for NKE in light blue and for GATA4 in light red. Primers used for ChIP or 3C analysis are shown with grey boxes. HindIII restriction sites are shown in green.

Hand2 OFTRV

Mouse mm9 chr8 59796493-59797943
Human hg19 chr4 174453397-174454860

```
mouse   TATTTCCCCTTAAAAAGAGACTGAAG-----TTTAAAAAAATATTTAGTTTAAAACAGAGC------TTAAA----AGTCATATCTCACA
        ||||| |||| |||  |||   ||||      ||| ||| |||||||     || ||   ||       || ||   | |  ||||||||||
human   TATTTTCCCT-AAAACCTAACTAAAGATTTTTTTAAAA------TTAGTTTAAACCAGATTGATTTTTTCAAGTATAATAATATCTCACA

mouse   TAGGGCAAAAAATGTTTGCAACGGGTTCCCAGACAAATAA[CATG]-GACACAAAGTAAATGTTTGAAACTAGCCTTGCCCCTTCAGCTGAG
        ||  |||||  |||||||| ||| |    | ||||||      ||||  |||| |||||| |||| |||| ||||| |||| |   || || ||||
human   -AGACCAAAA--TGTTTGCATGGCCATGTTAGACAAGAGACATGAGGAGCAAAATAAATGCTTGAACCAGATTCTGTCTATTTGGCTGAA
                                                NlaIII

mouse   C----TTGTGATGTACAGAGCTGTAGATACACCATCAGCCTCAAAGAGGATTATGGACACAGAATCTCCAACACTCCCCTAAACCTGGGC
             ||| |||| || | || |||||||||||||| ||  ||| | |        |||||||| | |||||| ||||| ||||||||||
human   GGCAGTTGACATGTGCAGTACAGTAGGTACACCATCAGCTACAACAAAGGT-------ACAGAATCCCAAACACTCACCCAAACCTGGGC

mouse   TGCCTTCCTGTGATG--ATGTGCAGACCC----AGGGCCA-------CTCGGCCCTTAGCCTCCCGGGGGAG------AGATAATCCTCT
        ||||| | ||||| |    |  |   |      |   || |        ||| |||| |||| |  |||| ||       | || |  | |
human   TGCCTCCTTGTGAAGCAGTTTCTGGACACGAGTAGGCCGGTTTTGGCTCAGCCCTCAGCCTCTGAGGAGAGGCAGAGAAGCACTCCTAG

mouse   ATGGTATTCCTCCCTAGGCACCCAGAATGGCCCACAACCAGCGTCATCACA[GATC]TATGGTGCTT-GAGAATT--CTAAATGTGTATCTT
         |   |||||||||| ||||||||||   || |||| | |||      |||   ||||| |||     ||| ||| | | | | |  |||||
human   CTAAGATTCCTCCACAGGCACCCAGAGGGAGCCCCCAGCAAGC-------CAGTTCCCTGGGCCTTTGAGTACTTACAACATGTGCATC--
                                                           DpnII

mouse   AAAGAGCAGCTCACCAGAGGGCCTGCTTGTACAGCCCAAGAGTGAGGGAGC-AGGCTGACATCTAAGTTAGAAACTATACA------ACC
            |||||||          || | |||||||  ||||| |||| ||||   | |||||||||   || ||| | ||  |||   |
human   ----AGCAGCTA-----------CACTAGTGCAGCCCAGGAGTGGGGTCCCCATGCTGACATCGATGTCAGGGACTGTGCCTTTCTCCCT

mouse   ACATTCTTCCACCTTCTTCCT[GATC]ACCAAATGCCAACTTCCCTGTCAC-ATCACC-CTGTTAGTTCCCTCATAATCACCCCCTGGGGTG
            |||| ||| |||| |||||  ||||| ||  | ||| ||    |   |||||||| |  | |  |||| | |  || |    |
human   TTCTTCTCCCACTTTCCCTCTCATCACCAA-CACCACCCTCTCCTTCATTGCCACCACTGCCAACACCTCCATCACTACCACCAGGAT--
                            DpnII

mouse   GGACAGACTTTCTAAACTAGGGGACTAATTTAAAATAGCATCTTAGATTCAGAGTTTGCCAAAGCAGCAAGTTTACCTGGCAGTCCGAAAT
         |   |||     ||||| |   || || || |||| |||||| | |||| | ||||| |||| |||| |||||| |||| | || |  |||
human   GCCCAGGACACCTAAATTGAGGGCAAACTTGAGATGGCATCCTTGCTTCACAACTTCCCAAAGGAGCAGGCTAACCCAGTATCCTGGAAA

mouse   TACGGAAGTAAAATGTGTCAAAAGATGCTAAAAAGAAAAAA--AATGAACAATTGCAGTAAGTATA----------ACTCAGAGCCAGCC
        |  |  |||| |  ||  || | ||| |   ||  ||||      ||| |   | ||  |   ||          |   | ||||| || ||
human   ACCTGGAACAAAACAGGCCAAAGGACACTGCAAGAGAAAAGTGGATGCGTTTGTGCATTTAGGGTAGAGCTCAACCAGTCAGGGCCAGCC
                                                                                    ChIP_Hand2OFTRV_F

mouse   ----------------AACTACT--[CATG]GTAGATACCACCAAAATTTACCCACTGGTCCCCTCTC-TCCAGCCACCTACAGAACGCTATC
                        | |  |||     ||||  | || |||||  |   |  ||| |||  ||| ||||  ||   | | |    |||| |   ||
human   TGGCCTCCCTGAGTCAGTTACTACAGTTGGGGACACCACTAGGGTTCATCCATTTGTCCCCTCTCATCAAGCCAGCT------------TT
                  NlaIII

mouse   CTACCACTCTAGTCTCTGGGTTTGAGTTCCTAA[GATC][CATG]AAGCTTTAGACCCCTGGATTGTAGTAATTAACTGATAATGGTGCTGTCA
        | |  |||  | ||||||| |||| ||||| |   |||  |||   || |||  |||| || | |  |  || | || ||||||||| | | |
human   CTAAGGCAGTGACCTCTGGATTTGGGTTTCTGAGCCACATCAAGTCTTAGGCCCTTGGACCACAGCAGTTAACTGATAATGACACTGTGA
                 ChIP_Hand2OFTRV_R        DpnII NlaIII

mouse   GTGAGGAGTGATGGTCAGATTAGCATTTTTTACAG--CCCCTTTTCTGTCC[CATG]TTGTCCATTTCTTTTATCACTTGTGAGGATGCTAT
        |||||  | |||| | ||||||||||| ||||| |   | ||||| |||| | ||||  |||||||||| |||| ||||||   | |||||||
human   GTGAGAACTGATGTTCAGATTAGCATCTTTTATGATTTCTTTTTTCTGTACCATGTTGTCCATTTCCTTTACCACTTGC---AATGCTAT
                                              3C_Hand2OFTRV_DpnII  NlaIII

mouse   CTGGAGCTACGAATCCATACCCCTACTTTTTGTTGTTGTTGGTGGTGGTGGTGGTGGTGGTGGTTTCAATTTTAAAAGCAGGAAGCGGGT
        |||||| || || | |  ||| ||| ||||| |||| |                     ||| | |  ||||||||||| | |
human   CTGGAGCCACGAGGTAATTCATTCATTTTTGTTTGTT--------------------TGTTTGTTTCAA--TTAAAAGCATGA--------

mouse   TGGGGGGGAGGCTAAAAGTAGCACCTTCT[G-ATC]CCTAAATCCTGAGGCTG--------------CAAGTGAACAGATAGCTTCTCTATCC
        | |  ||||||| ||| |||| |  |||          || ||| ||||| ||              || || || |||  ||| |||| |||
human   ----GAAAAGCTAAATGTGGCAAGTTCTGAAGCTTTCAAAAGTGAGGCCACTCACCGCTGGTCTCTGGTGGACAG--AGCATCTCAATCC
                                         DpnII

mouse   CCAGCCCTATCTTTCTGTGGATTCTTCTTCTATCTCCCAACGCCCTGAAAGTCTATT---TCCTGAGGCAGCCAAAAAACACCTTTCCTA
        | | |  | ||||||||| || ||||||||  | |   |||| |||  | ||| | ||     ||| | ||||| |||||  || |   |
human   CTAACTCAGCCCTCCTCTGGACTCTTCTTCCTCCCTTCAATTCCCAAAACTCCCTTTGGGTCTTAAAGCAGCCAACCAACTAATTTCCCT

mouse   AGTTTTTTATCTGTGATTGG--ACAGGACATAATCATCTTACCCAGTCTACCTGTTCCCGAGGCCTAAA--TGTTTTTATAAACAAGTTT
         |||||||| | |  ||| |   ||| ||| ||||||| ||| || |||||||||| ||| |||||||||  | ||||||||||||| | ||
human   GAATTTTTATATGTTCCCAGCCCCAGTGCA------ATCTTACTTTGTCTACCTGCTCCCAAGGCCTAAATGTGTTTTTATAAACAGACTT

mouse   TTTTTTAATGTATCAAATGTCAGAGGCTTTAACTACCAATATTTACTGATTCAGGGT
        ||   ||| |||||||||||||||||   ||||||||||| |||||| ||| | |||
human   TTCAAAAACGTATCAAATGTCAGAGAATTGAACTACCAGTATTTACACATTCGGGGT
```

Hand2 Promoter

Mouse mm9 chr8 59799379-59799840
Human hg19 chr4 173530169-173530641

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. Primers used for ChIP or 3C analysis are shown as arrows above in grey boxes. NlaIII and DpnII restriction sites are shown in green and orange respectively. Abbreviations: TSS, transcription start site.

A

Hand2

OFTRV

NlaIII

Relative Crosslinking Frequency

Ldb1+/+ D0	KO + GFP
Ldb1-/- D0	KO + Isl1
Ldb1+/+ D5	KO + Ldb1
Ldb1-/- D5	KO + DN-Ldb1
	KO + Isl1/Ldb1
	KO + Isl1/DN-Ldb1

1 Kb

B

Chr13
HindIII

83,630,000   83,635,000   83,640,000   83,645,000

-14 Kb -13 Kb -12 Kb -9,6 Kb -7,5 Kb -6,8 Kb -1,5 Kb -200bp +2,7 Kb

Fold Enrichment over IgG

IgG
α-Isl1
α-Ldb1

Day 4

Day 5

-14 Kb -13 Kb -12 Kb -9,6 Kb -7,5 Kb -6,5 Kb -1,5 Kb -200 bp +2,7 Kb AHF +150 Kb 3'UTR Neg Ctrl

C

Fold enrichment over GFP control line

α-HA Promoter
α-Isl1 Promoter
α-HA AHF
α-Isl1 AHF

GFP   Isl1   Ldb1   DN-Ldb1   Ldb1/Isl1   DN-Ldb1/Isl1

D

Prom   Luc2
pGL4-Mef2cP-Luc2

Prom   Luc2   AHF
pGL4-Mef2cP-Luc2-AHF

Fold activation

Isl1	-	+	-	-	+	+
Ldb1	-	-	-	+	-	+
DN-Ldb1	-	-	+	-	+	-

E

Overrepresented Motifs

GATA

Mef2

Nkx2-5

Tbx

RTGATAAAA

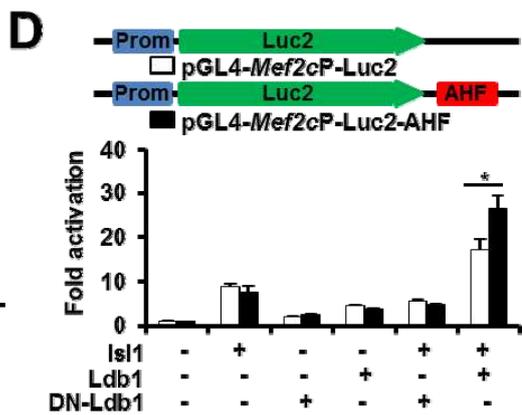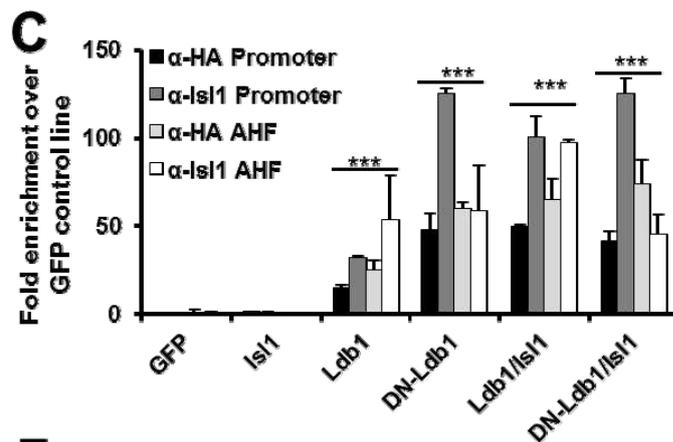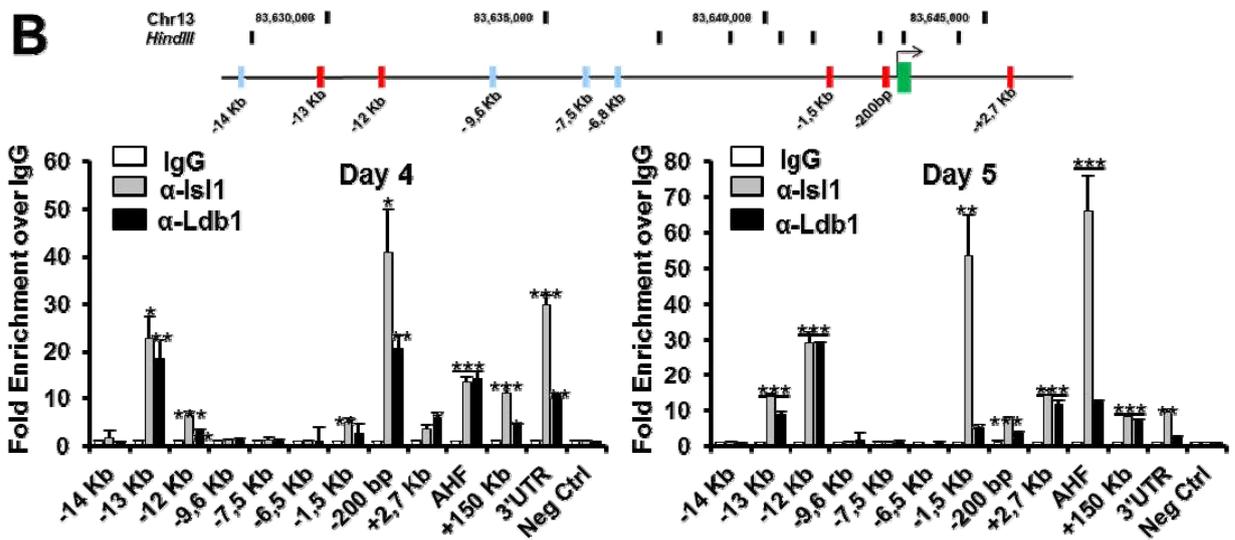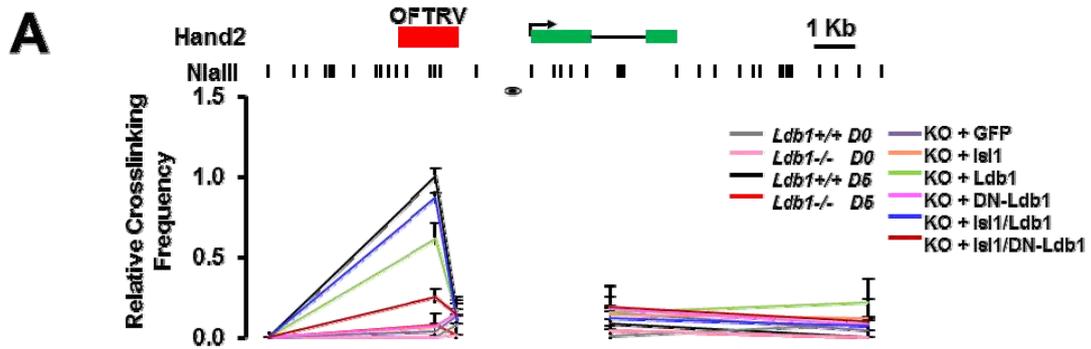AAAATAAAW

CTTGARTTC

CAGGTGACA

9

Figure S7, related to Figure 5 and Figure 6. Ldb1 promotes chromatin looping events between the AHF enhancer and genes which play key roles in cardiovascular development. *(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 in nuclear extracts from d4 EBs using anti-HA and anti-Isl1antibodies. 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. *(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. See also Figure S3, S4 and S5. (*p≤0.05, **p≤0.01, ***p≤0.005). *(F)* Motif enriched in the genomic regions found in close proximity to the Mef2c AHF in d5 EBs.

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

Table S2. 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

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.

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' agatcttctcccccaccccaagcctct 3'

Mef2cAHF_F   5' ggatcccattaaaatagtactctgca 3'

Mef2cAHF_R   5' gtcgacgggccattaactttcgaatc 3'

Cell Culture and Transfection

HEK293T, and COS7 cells were grown in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen), 2mM L-Glutamine, 100U/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 1,000 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 2.10^6 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 Mirthras LB 940 (Berthold Technologies) according to the Luciferase Assay System Manual (Promega). β-galactosidase assays were performed using CPRG as substrate (Sigma).

RNA Isolation

RNA was isolated using the TRIzol Reagent (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). The cycle numbers were normalized to GAPDH (ES/EBs and Embryos). Primer pairs are described in Table S2.

Immunofluorescence of Embryoid Bodies (EBs)

EBs were collected, washed with PBS, embedded in 17% gelatin and fixed O/N 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 MgSO4, 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, 1×10^6 cells were washed with 1 ml PBS and blocked in 100 μl FACS buffer (10% FCS 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% PBS. For chromatin immunoprecipitation 0.5 to 1×10^7 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 Table S2.

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 O/N 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:
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