Downregulation of *WT1 transcription factor* gene expression is required to promote myocardial fate

ABSTRACT

During cardiac development, cells from the precardiac mesoderm fuse to form the primordial heart tube, which then grows by addition of further progenitors to the venous and arterial poles. In the zebrafish, *wilms tumor 1 transcription factor a* (*wt1a*) and *b* (*wt1b*) are expressed in the pericardial mesoderm at the venous pole of the forming heart tube. The pericardial mesoderm forms a single layered mesothelial sheet that contributes to further the growth of the myocardium, and also forms the proepicardium. Proepicardial cells are subsequently transferred to the myocardial surface and give rise to the epicardium, the outer layer covering the myocardium in the adult heart. *wt1a/b* expression is downregulated during the transition from pericardium to myocardium, but remains high in proepicardial cells. Sustained *wt1* expression impaired cardiomyocyte maturation including sarcomere assembly, ultimately affecting heart morphology and cardiac function. ATAC-seq analysis revealed that wt1 exerts its function through specific repression of regulatory regions of genes associated with myocardial differentiation. Indeed, a subset of *wt1a/b*-expressing cardiomyocytes changed their cell adhesion properties, delaminated from the myocardial epithelium, and upregulated expression of epicardial genes, as confirmed by *in vivo* imaging. Thus, we conclude that active repression of *wt1* gene expression in cardiomyocyte precursor cells is a pre-requisite for myocardial lineage commitment and heart development and that sustained ectopic expression of *wt1* in cardiomyocytes can lead to their transformation into epicardial cells.

KEYWORDS

wt1a, *wt1b,* heart development, cardiomyocyte, epicardium, zebrafish, cell fate

INTRODUCTION

The heart is one of the first organs to acquire its function and it starts beating long before cardiac development is completed. In mammals, its function is essential to promote blood flow

in order to sustain oxygenation and nutrition of the organism. Indeed, heart defects are among the major congenital anomalies responsible for neonatal mortality (1, 2).

The zebrafish is a well-established vertebrate model organism in cardiovascular research given its transparency during early developmental stages and rapid embryonic development (3). Cardiac precursor cells derive from the anterior lateral plate mesoderm (4). At 14 hours postfertilization (hpf), cardiac precursor cells start to express *myosin light chain 7* (*myl7*) (5) and sarcomere assembly begins soon after (6, 7). As the assembly of the sarcomeres continues, the cardiac precursor cells migrate and fuse into a cone that later forms the heart tube, which is contractile at 24 hpf and is comprised of a monolayer of cardiomyocytes lined in the interior with an endocardial layer facing the lumen. Next, the heart tube starts to loop, leading to the formation of the two chambers, the atrium and the ventricle (4). Concomitantly, more progenitors enter the heart tube through the arterial and venous poles (8). Around 55 hpf, the outermost cell layer of the heart, the epicardium, starts to form. Epicardial cells arise from the proepicardium, a cell cluster derived from the dorsal pericardium that lies close to the venous pole of the heart. Cells from this cluster are later released into the pericardial cavity and attach to the myocardial surface, forming the epicardium (9, 10).

Wilms tumor 1 (WT1) is one of the main epicardial and proepicardial marker genes and plays a central role in epicardium morphogenesis (10, 11). Wt1 contains 4 DNA binding zinc-finger domains in the C-terminus and has been shown to act as a transcription factor (12). *Wt1* is expressed in the epicardium during embryonic development and, in the adult heart, is reactivated after cardiac injury (13).

The zebrafish has two *Wt1* orthologues, *wt1a* and *wt1b* (14). These genes are also expressed in the proepicardium and epicardium (9, 15) in partially overlapping expression domains. Using transgenic reporter and enhancer trap lines (16, 17), we previously showed that *wt1a* and *wt1b* are initially expressed in a few proepicardial cells and later in epicardial cells (18). While *wt1a* and *wt1b* mRNA expression was not detected in the myocardium, *wt1b:eGFP* signal was transiently detected in cardiomyocytes of the atrium close to the inflow tract of the heart. Furthermore, some *wt1a* regulatory regions were found to drive eGFP expression in

cardiomyocytes (18). *wt1b* and *wt1a* regulatory elements drive gene expression in the myocardium but endogenous gene mRNA expression is observed only in the proepicardium and epicardium This led us to hypothesize that *wt1* expression needs to be repressed in the myocardium for correct heart development.

To explore whether *Wt1* needs to be downregulated in the myocardium for proper embryonic development, we generated transgenic zebrafish models for tissue specific overexpression of *wt1b* or *wt1a* in cardiomyocytes. We found that sustained *wt1a or wt1b* overexpression in the myocardium induced the delamination and a phenotypic change from cardiomyocytes to epicardial-like cells. Moreover, we observed impaired cardiac morphogenesis, altered sarcomere assembly and delayed myocardial differentiation, which ultimately lead to alterations in cardiac function, atrial hypertrophy and fibrosis. ATAC-seq and bioinformatics analysis revealed that wt1 acts as a break for cardiomyocyte differentiation by repressing chromatin opening at specific genomic loci.

Altogether, our data indicates that *wt1a/b* expression in cardiomyocytes must be downregulated to allow cardiomyocyte specification and the correct development of the heart.

RESULTS

Wt1 is downregulated in cardiac progenitors upon their entry into the heart tube

During heart tube growth, cells from the pericardial mesoderm enter the heart tube at the venous pole (8). These cells can be labelled with the line *epi:eGFP* (9), an enhancer trap line of *wt1a* (Figure 1A). We found that during this process, cardiomyocyte precursors downregulate eGFP expression concomitant with the activation of *myl7:mRFP* (Figure 1B and video 1). We measured the eGFP/mRFP signal intensity ratio in cells of the hearts tube, from the sinus venosus (SV) towards the growing heart tube. We found that the further away the cells were from the SV, the lower the detected GFP signal and the higher the RFP was (Figure 1C, n=3). To further confirm these observations, we perform SMART RNA-seq of cells collected from three distinct regions: pericardium, proepicardium and heart tube at 60 hpf (Figure 1D-F). We detected a gradual decrease in *wt1a* and *wt1b* normalized counts among

these three tissues, with the highest counts in PE cells and lowest in cells from the heart tube The opposite trend was observed for *myl7* expression, being highest in heart tube and lowest in the proepicardium samples.

We next assessed if the observed downregulation of *Wt1* during cardiomyocyte differentiation from a pool of cardiac precursor cells is a conserved mechanism during vertebrate heart development and if it is associated with directed repression of *Wt1* gene expression. For this, we analyzed previously published data on activating and repressing Histone marks at the Wt1 genomic locus during four stages of cardiac differentiation from mouse embryonic stem cells (mESCs) (19) (Figure1, supplement 1). *Wt1* transcript levels were higher in mESCs and gradually reduced through differentiation stages. We observed Histone 3 K27 acetylation (H3K27ac) peaks-that mark active enhancers- in regions up to 5 kb upstream of the *Wt1* transcription start site in mESCs and mesodermal stage cell samples. These peaks overlapped with known enhancer regions driving epicardial gene expression (20). Conversely, Histone H3 K27 trimethylation (H3K27me3) peaks- marking repressed enhancers- were lowest in the earlier undifferentiated stages, but in samples from cardiac precursor stage and differentiated cardiomyocytes H3K27me3 marks decorated heavily the regions covering the 5' region of Wt1 including the epicardial enhancer domains. This analysis reveals that during cardiomyocyte differentiation, *Wt1* expression and epicardial enhancers becomes actively repressed.

In summary, during heart development, *wt1* is downregulated in cardiac precursor upon their entry into the heart tube and this might be a prerequisite for their differentiation into cardiomyocytes (Figure 1G).

Cardiomyocytes that overexpress *wt1* can delaminate from the heart, loose sarcomeric proteins and start expressing epicardial markers

We next aimed to understand if there was a biological relevance for the downregulation of *wt1* in cardiomyocytes. To analyze the impact of *wt1* expression in cardiomyocytes, we generated the line *Tg(b-actin2:loxP-DsRED-loxP-eGFP-T2A-wt1a).* Crossing this line into *Tg(myl7:CreERT2)* (21) allowed the temporally induced overexpression of *wt1a* in

cardiomyocytes. Hereafter, the double transgenic line is called *myl7:CreERT2;eGFP-T2A-wt1a*. We administered 4-hydroxytamoxifen (4-OHT) between 24 hpf and 4 days postfertilization (dpf) to induce recombination of loxP sites and activation of *wt1a* and *eGFP* expression during embryogenesis in cardiomyocytes (Figure 2, supplement 1A). We confirmed *wt1a* and *eGFP* overexpression in the heart by RT-qPCR (Figure 2, supplement 1B-E). Comparison of *eGFP* and *wt1a* expression between *myl7:CreERT2;eGFP-T2A-wt1a* with and without 4-OHT administration revealed a significant increase in *eGFP* and *wt1a* expression in the latter (Figure 2, supplement 1B-E). Moreover, we generated a line to overexpress *wt1b*. We decided to use the Gal4/UAS system in this case, to allow a more homogeneous expression in the myocardium. The line *Tg(eGFP:UAS:wt1b)* allowed overexpression of *wt1b* and *eGFP* under a bidirectional *UAS* promoter. We crossed the line into *Tg(myl7:Gal4)* (22); the double transgenic line will be hereafter called *myl7:Gal4;eGFP:UAS:wt1b* (Figure 2, supplement 1F). *wt1b* and *eGFP* expression in cardiomyocytes of the double transgenic line *myl7:Gal4;eGFP:UAS:wt1b* was four-fold upregulated compared to cells from the single transgenic eGFP:UAS:*wt1b* (Figure 2, supplement 1G-H). As a control, we used the double transgenic line *Tg(eGFP:UAS:RFP);(myl7:Gal4)* (23), hereafter named *myl7:Gal4;eGFP:UAS:RFP*. RT-qPCR analysis also indicated that expression of *wt1b* and *wt1a* could be monitored via GFP imaging (Figure 2, supplement B, D and G).

With these new lines we then analyzed the effect of sustained *wt1a* and *b* overexpression in cardiomyocytes on heart development (Figure 2A). We observed that in *wt1a*-overexpressing hearts at 5 dpf, some eGFP-positive cardiomyocytes were located at an apical position, protruding towards the pericardial cavity. This was not detected in controls (Figure 2B-C'''). Moreover, these delaminating cardiomyocytes showed reduced expression of Myosin Heavy Chain (MHC), suggesting that they lost to some extent a myocardial phenotype (Figure 2C-C'''). We quantified how many of the delaminating cells were GFP^+ or GFP^- and found that only GFP^+ cells were delaminating, indicating that this delamination process is due to a cell-autonomous effect of *wt1a* in cardiomyocytes (Table 1). We detected a similar occurrence in *wt1b* overexpressing hearts, starting at 3 dpf (Figure 2D-E'''). Some GFP-positive cells

delaminated towards the apical myocardial surface. Those cells were not positive for the myocardial marker MHC.

To better understand the origin of these apically positioned eGFP-positive cells we performed *in vivo* imaging in *myl7:Gal4;eGFP:UAS:RFP* and *myl7:Gal4;eGFP:UAS:wt1b* between 2 and 3 dpf (Figure 2F, G and video 2). In *myl7:Gal4:eGFP:UAS:wt1b* hearts, some eGFP-positive cells started to round up and initiated delamination from the myocardium. Cells gradually changed from a flat to a rounded shape and ultimately remained adherent to the outer myocardial layer (Figure 2G and video 2; n=4). This event of cell delamination was not observed in *myl7:Gal4:eGFP:UAS:RFP* control embryos (Figure 2F and video 2; n=2). Apical extrusion of cardiomyocytes can be a consequence of myocardial malformation during which extruded cells are eliminated (24, 25). However, here we found that the extruded cells did remain on the myocardial surface. Between 5 and 6 dpf, these delaminated cells lost their rounded shape and flattened, acquiring an epicardial-like morphology (Figure 2H-I and video 3, n=4).

To confirm that this type of cellular delamination with loss of MHC expression was specific of the overexpression of *wt1* we generated the *Tg(eGFP:UAS:tcf21),* which we then crossed into the *Tg(myl7:Gal4)* (Figure 2, supplement 2 A-D"'). This double transgenic allowed us to overexpress another main epicardial marker, *tcf21* (26) in cardiomyocytes. Contrary to what we observed when overexpressing *wt1a/b* in cardiomyocytes (Figure 2, supplement 2B-B''), in the large majority of these embryos (59/62) we did in not observe apical delamination in the hearts of the *myl7:Gal4;eGFP:UAS:tcf21* fish. In the very few cases where delamination occurred (3/62), the extruded cells still expressed MHC (Figure 2, supplement 2C'').

For a better characterization of a possible switch to an epicardial fate we performed immunofluorescence labeling with the epicardial markers Aldehyde dehydrogenase 2 (Aldh1a2) (27, 28), Caveolin 1 (Cav1) (29) (Figure 3). We detected GFP/Aldh1a2 double positive cells in *wt1b* overexpression hearts (n=2) but not in controls (n=2) (Figure 3B-C'''). Similarly, in *wt1b*-overexpressing hearts (n=4), we identified GFP positive cells that also expressed Caveolin 1 (Figure 3E-E'''), whereas in control hearts (n=6) we could not observe

GFP/Cav1 double positive cells (Figure 3D-D'''). We also detected eGFP$^+$ cells within the epicardium of *wt1a* overexpressing hearts, but not in controls (Figure 3F-G, H-H', I-J', K-K', L-M', N-N', O-P' and Q-Q'). These eGFP$^+$ cells did not express MHC (Figure 3J", K", P" and Q"), and were Aldh1a2-positive (Figure 3J''' and L''') as well as Caveolin 1 positive (Figure 3P''' and Q''') strongly suggesting that *wt1a* overexpressing cardiomyocytes switched their fate to epicardial cells. We tested for the colocalization of *wt1a*-expression in cardiomyocytes with a third epicardial marker, *transglutaminase b tgm2b* (30). We performed *in situ* hybridization against *tgm2b* mRNA followed by immunohistochemistry against eGFP (Figure 3, Supplement 1). In non-recombined *myl7:CreERT2; eGFP-T2A-wt1a* hearts, *tgm2b* expression was only visible in few epicardial cells in ventricle and we could not observe any co-localization with eGFP expressing cells (Figure 3, supplement 1B-C'). However, in embryonically recombined *myl7:CreERT2; eGFP-T2A-wt1a* hearts, we could observe cells co-expressing *tgm2b* and eGFP located within the epicardium (Figure 3, supplement 1D-E').

These results suggest that upon sustained ectopic overexpression of *wt1a/b,* cardiomyocytes can delaminate apically from the myocardial layer and adopt features of epicardial cells that contribute to the formation of the epicardial layer even in the adult heart.

_wt1b_ overexpression disrupts cell-cell contacts and the basement membrane of the cardiomyocytes

We decided to get a better understanding on the cellular mechanisms underlying this process of apical delamination of cardiomyocyte upon *wt1* expression (Figure 4A). Previous reports showed that correct development and morphogenesis of the heart requires cell-cell adhesion and polarization of the cardiomyocytes (31). The proper localization of tight junctions and adherens junctions has conventionally been used to assess the polarization of the cells (32). We first performed immunostainings against ZO-1, a component of the tight junctions (33) (Figure 4B-E'). Whereas the *myl7:Gal4:eGFP:UAS:RFP* control hearts (n=6) showed discrete apical localization of ZO-1 (Figure 4B-C'), in *myl7:Gal4:eGFP:UAS:wt1b* hearts (n=8) ZO-1 levels were reduced, the singal was less defined and we observed lower concentrations of this

protein at the apical junctions between cardiomyocytes (Figure 4D-E'), suggesting defects in the formation and localization of tight junctions. To evaluate the formation of adherens junctions, we crossed the *Tg(myl7:cdh2-tdTomato)*^{bns78}line (34) with *myl7:Gal4:eGFP:UAS:wt1b*. This allowed us to specifically visualize subcellular localization of cdh2-tdTomato in *wt1b*-overexpressing cardiomyocytes and control siblings (Figure 4A and F-M'). At 5 dpf, in control embryos (n=6), tdTomato signal was clearly localized to cell-cell junctions (Figure 4F-G) and detected apically in the cardiomyocytes (Figure 4H-I'). In contrast, in *myl7:cdh2-tdTomato;myl7:Gal4:eGFP:UAS:wt1b* hearts (n=8), we observed a diffused and patchy staining for cdh2-tdTomato, which was not restricted to the apical side of the cardiomyocytes (Figure 4J-J'' and L-M'). More interestingly we observed loss of cdh2-tdTomato signal in the delaminating cells further indicating a loss of polarity in these extruding cells (Figure 4K-K"). To confirm the impairment in the formation of adherens junctions we did an immunostaining against β-catenin, a core component of the adherens junctions (35). Similar to what we had observed for cdh2-td-Tomato, β-catenin staining was located at the apical side of the cardiomyocytes in *myl7:Gal4:eGFP:UAS:RFP* control hearts (n=5) (Figure 4N-O'). However, in *wt1b*- overexpressing hearts (n=5) β-catenin staining was lost (Figure 4P-Q'). Taken together, this data shows that sustained expression of *wt1b* in cardiomyocytes leads to the mislocalization of tight junctions as well as adherens junctions, indicating an impairment of the apical domain in the cardiomyocytes.

To understand the basal domain landscape of the cardiomyocytes we did an immunostaining against Laminin, a component of the basement membrane. Laminins have been associated with myocardial differentiation and with regulating the sarcolemmal properties (36-39). At 5 dpf, in the hearts of control fish (n=5) we observed clear anti-Laminin staining at the basal and lateral domains of the cardiomyocytes (Figure 4 P-Q'), which correlates with previous observations (36, 40). Laminin expression levels were severely reduced in the *wt1b* overexpression hearts (n=5), with no laminin observed in the lateral domains of the cardiomyocytes (Figure 4R-S').

Thus, the improper deposition of the laminins as well as the reduced levels of these proteins point towards an improper basal domain of the cardiomyocytes. Moreover, an incorrect distribution of apical junctions had previously been associated with impaired substratum (41). Perturbed apical and basal domains, indicate that apicobasal polarization of the cardiomyocytes may be disrupted upon *wt1b* overexpression in the cardiomyocytes.

Overexpression of *wt1* in cardiomyocytes hinders cell maturation and disrupts its structural organization

The disruptions in cell junctions and cell extrusion that we observed in the *wt1b* overexpressing cardiomyocytes led us to question the maturation and general architecture of these cells.

Using whole mount immunofluorescence, we observed reduced MHC staining in —*wt1b*-overexpressing hearts at 1 dpf when compared to controls (Figure 5A-C'). The reduction of MHC staining was specific to the heart, as it was not observed in the skeletal muscle of the myotome (Figure 5D). Although at 6 dpf we observed an increase in the MHC expression levels in *wt1b* overexpressing cardiomyocytes, the expression levels never reach the levels observed in the control group (Figure 5E-F'). Quantification of MHC mean fluorescence intensity levels confirmed a decrease upon *wt1b* overexpression at all analyzed stages (Figure 5G). We also analyzed *myl7* mRNA expression levels using whole mount *in situ* hybridization. Consistent with the results obtained using MHC immunostaining, at 3 dpf, *myl7* expression was reduced in *myl7:Gal4;eGFP:UAS:wt1b* (23/25) compared to their single transgenic *eGFP:UAS:wt1b* siblings (Figure 5H-I). We reasoned that the reduced levels in MHC and *myl7* staining could be indicative of an impaired maturation of the cardiomyocytes. To confirm this hypothesis, we performed an immunofluorescence staining against Alcam, a marker for undifferentiated cardiomyocytes (42, 43). At 6 dpf, we observed higher Alcam levels in hearts overexpressing *wt1b* when compared to control hearts (Figure 5J-L).

We next analyzed if sarcomere assembly was impaired in *myl7:Gal4;eGFP:UAS:wt1b* animals. We performed immunofluorescence staining against actinin, a protein known to be expressed in the z line of the sarcomeres (44). Qualitative assessment of actinin expression

revealed that not only the expression levels were lower but also the z lines were thicker and shorter (Figure 5M-P') upon myocardial *wt1b* overexpression. Z line disruption was particularly evident in delaminating cardiomyocytes (Figure 5O). We next sought to analyze sarcomere structure more in detail using serial block face scanning electron microscopy (SBFSEM) (Figure 5Q-R'and videos 4-7). Z-bands were present at the sarcomere boundaries in both groups. While sarcomeres could be easily followed from z-band to z-band (Figure 5Q-Q' and video 5) in the control heart, this was not possible in *wt1b* overexpressing hearts, further supporting our observation of impairment in the sarcomeres (Figure 5R-R'and video 7). A further ultrastructural defect we observed in the *wt1b* overexpression heart was the presence of large intercellular spaces between cardiomyocytes, the epicardium, and endocardium. Moreover, while the control heart revealed a clearly visible basement membrane between the epicardium and the myocardium as well the endocardium and myocardium (dark black line), this structure was not always visible in *wt1b* overexpressing heart (Figure 5A-B'and videos 3 and 5). This observation correlates with the observed impairement in laminin staining (Figure 4P-S').

Altogether, our findings indicate that sustained expression of *wt1b* in cardiomyocytes affects heart development leading to impaired cardiomyocyte maturation and negatively impacts the cardiac ultrastructure, including sarcomere assembly of these cells, as well as the extracellular matrix.

Overexpression of wt1b in cardiomyocytes results in global reduced chromatin accessibility

Having seen that *wt1b* overexpression in cardiomyocytes induced several cardiac malformations and caused a phenotypic change in some cells we decided to explore how the sustained expression of this transcription factor was affecting chromatin accessibility. For that, we performed Assay for Transposase-Accessible Chromatin sequencing (ATAC-seq) in 5 dpf, FAC sorted GFP+ cells from either the *myl7:Gal4;eGFP:UAS:RFP* control or

myl7:Gal4;eGFP:UAS:wt1b larvae (Figure 6A). We identified 1452 differential peaks in *wt1b* overexpressing cardiomyocytes, of which all except for 14 peaks showed repression of chromatin accessibility (Figure 6B). Most of the differential accessible regions were located close to promoter regions (38.87%), in introns (30.37%) or in distal intergenic regions (26.14%) (Figure 6C). We performed Gene Ontology (GO) analysis for the genes lying in close proximity to the regions with altered chromatic accessibility. From the top 25 Biological Pathways that were repressed, five of them account for muscle development (Figure 6D and Supplementary dataset). Within the top 25 Cellular Component pathways we found some to be involved in "actin cytoskeleton", "basolateral plasma membrane", "apical part of the cells", "contractile fiber" or "myofibril" (Figure 6E and Supplementary dataser). Within the top 25 Molecular Function pathways (Figure 6F and Supplementary dataset) five of them are directly implicated in transcription regulation and another four in cytoskeleton formation and cell adhesion, such as "actin binding", "actin-filament binding", "cell adhesion molecule binding" and "beta-catenin binding". All of this repressed pathways define very well the observed phenotype upon *wt1b* overexpression in cardiomyocytes. To identify potential transcription factors that might be binding to the differential regions, we performed a motif analysis, and found WT1 to be one of the top 5 motifs represented (E-value = 7.8e-5). This motif could be identified in 672 (46.25%) of the differentially accessible regions (Figure 6G-G'). To further validate which of the genes identified by ATAC-seq were potential direct targets of WT1, we compared our data with WT1 target genes identified in the CHIP-atlas database (45). We found that 41% of our ATAC-seq genes (426) were common with the CHIP-atlas database for WT1. GO analysis of these common genes identified pathways similar to those observed previously, suggesting a direct regulation of these pathways by Wt1b (Figure 6, supplement 1A-C).

Having seen that overexpression of *wt1b* in cardiomyocytes affected heart development and that these changes correlated well with the observed molecular signature, we looked more closely on how the genetic landscape of some of the ATAC-seq target genes was affected. We had previously seen that apical cell-cell junctions were disrupted in the hearts of the *myl7:Gal4;eGFP:UAS:wt1b* embryos, including expression and localization of *cdh2*, ZO-1, β-

Catenin. In agreement, we observed that regions in *cdh2* and *ctnna* (another core component of adherens junctions) revealed lower accessibility (Figure 6I-I'). We also observed lower accessibility in core apicobasal polarity pathway genes (46) such as *pardb6* and *pard3bb* from the apical polarity pathways and *scrib*, *dlg1* and *dlg1l* from the basolateral pathways (Figure6J-K, Supplementary dataset), supporting a perturbed apicobasal polarity in the overexpression lines.

Moreover, we detected that several genes associated with sarcomere assembly such as e2f3, rbfox2 and rybp (47-49) presented lower chromatin accessibility, which could explain the disrupted sarcomeres observed in the overexpression line (Figure 5M-R'). In conclusion, ATAC-seq data showed that *wt1b* overexpression reduces chromatin accessibility, with Wt1b likely to directly repress gene expression programs controlling muscle development, cell polarity and actin binding.

Overexpression of *wt1* in cardiomyocytes during embryogenesis impairs heart morphogenesis and induces fibrosis in the adult heart

In the *wt1b* overexpression hearts several of the top enriched Biological pathways were associated with muscle development. Moreover, these hearts showed several impairments in cardiomyocyte differentiation and fate. In view of this, we decided to take a closer look at the overall changes in cardiac morphology and growth upon sustained myocardial *wt1b/a* overexpression. We had previously noticed that those animals with strong eGFP expression throughout the myocardium presented impaired cardiac looping (Figure 5F-F') often with a heartstring morphology (observed in n=5 out of 5 embryos by whole mount immunofluorescence). We performed in vivo imaging between 2 and 3 dpf, the time window of cardiac looping (4). We found that, whereas the heart of a *myl7:Gal4;eGFP:UAS:RFP* embryo looped normally, in a *myl7:Gal4;eGFP:UAS:wt1b* larva the heart started to loop, but eventually this process stopped and reverted, resulting in a tubular-like shaped heart (Figure 7, supplement 1A-C and video 8; n=2). We analyzed looping dynamics by quantifying the angle between the ventricle and the atrium (51, 52) (Figure 7, supplement 1D). Whereas in 5 dpf

control hearts the angle between the ventricle and the atrium was, on average, lower than 110o (108o±5), in *wt1b*-overexpressing hearts the angle was larger (142o±15) (Figure 7, supplement 1E).

To validate that these morphological changes were specific to the overexpression of *wt1* in cardiomyocytes we decided to induce *wt1b* expression in other cardiac cell populations (Figure 2, supplement 2). For that, we crossed the *Tg*(*eGFP:UAS:wt1b)* into *Tg*(*fli1a:Gal4)* (50), to overexpress *wt1b* in the endocardium (Figure 2, supplement 2A and C-C"), and into *TgBAC(nfatc1:GAL4ff)*^{mu286} (51), to overexpress *wt1b* in the atrioventricular valves (Figure 2, supplement 2A and G-G") . We could not detect any apical delamination, looping defects or reduced MHC expression in these hearts at 3 dpf and 5 dpf.

Seeing that cardiomyocyte and general heart morphology were affected we decided to evaluate cardiac performance. We did *in vivo* imaging and analyzed different parameters for heart function in *myl7:Gal4;eGFP:UAS:wt1b* and *myl7:Gal4; eGFP:UAS:RFP* larvae. We analyzed cardiac function at 2 dpf, the time point at which we first observed cardiac malformations, as well as at 5 dpf, once looping has concluded (Figure 7, supplement 1F-I; n=14). First, we assessed stroke volume (52, 53), which indicates the volume of blood that the heart is capable of pumping in each contraction. *myl7:Gal4;eGFP:UAS:wt1b* ventricles presented a reduced stroke volume at 2 dpf (0.11±0.04 nl *vs* 0.04±0.03 nl) and this impairment did not recover at 5 dpf (0.39±0.17 nl *vs* 0.22±0.08 nl) (Figure 7, supplement 1G). We next analyzed the heart rate. Although at 2 dpf we could not detect changes in heart rate (114±8 beats per min (bpm) *vs* 119±8 bpm) we observed a significant decrease in the *wt1b* overexpression heart frequency at 5 dpf (166±13 bpm *vs* 141±9 bpm) (Figure 7, supplement 1H). The reduced stroke volume together with the decreased heart rate indicates that the *wt1b* overexpression animals have also an impaired cardiac output. Following on this observation, next we measured the ejection fraction for the ventricle and the atrium (53). We found that in the atrium, at 2 dpf, the ejection fraction did not significantly change between both groups

(43±14 % *vs* 51±8 %). However, at 5 dpf there was a clear reduction in the ejection fraction of the atrium (55±8% *vs* 41±12%). In contrast, the ventricular ejection fraction was initially significantly reduced at 2 dpf in *wt1b* overexpressing embryos (48±13% vs 35±13%), but recovered at 5 dpf (50±8% vs 49±8%) (Figure 7, supplement 1I).

We also noticed that, already at 2 dpf, the atria of wt1b overexpression animals seemed to be much larger than that of the *eGFP:UAS:RFP* line (Figure 7A-C'). This difference in atria size was sustained at 5 dpf (Figure 7D-E'). To confirm that the atria were indeed larger in these animals we first calculated the ratio between the atrium and ventricle volume at 5 dpf. We saw that in the *wt1b* overexpression hearts at 5 dpf the atria were on average 1.5 times larger than the ventricle, whereas in the control group they were only 0.5 times bigger (Figure 7F). To evaluate what could be the cause of this atrial enlargement, we counted the number of atrial and ventricular cardiomyocytes. While the number of MHC-positive cells in the ventricles was only slightly smaller than in the *wt1b* overexpressing hearts (163±47 *vs* 95±24), there was a significant increase in MHC-positive cells in *myl7:Gal4;eGFP:UAS:wt1b* atria (63±8 *vs* 132±11) (Figure 2N,O). This indicated that atrial enlargement in *wt1b* overexpressing hearts might be due to cell hyperplasia. To understand what could be the source of the excess of atrial cells we performed BrDU staining to evaluate proliferation. We calculated the ratio of proliferating cardiomyocytes per total amount of cardiomyocytes for each chamber. Contrary to our expectations, in the atrium of wt1b overexpressing hearts cardiomyocyte proliferation was significantly reduced (Figure 7H), while in the ventricle proliferation was not affected (Figure 7H'). This could indicate that atrial hyperplasia might be due to a continuous inflow of cardiac precursors, rather than over proliferation of the cells in this chamber. Atrial enlargement persisted also in juvenile stages (Figure 7,supplement 2A-G). Due to the severity of the phenotype (Table 2), few *myl7:Gal4;eGFP-UAS-wt1b* animals survived past 6 dpf. Therefore, we used the *myl7:CreERT2:eGFP-T2A-wt1a* line to evaluate the morphology of the adult heart. We analyzed adult hearts from embryonically recombined and 4-OHT untreated *myl7:CreERT2:eGFP-T2A-wt1a* animals (Figure H). Consistent with our result with *wt1b*, we

observed that animals overexpressing *wt1a* in cardiomyocytes starting at an embryonic stage, revealed an enlarged atrium (22/42) (Figure 7 I-J). The increase could be quantified by micro computed tomography scanning (micro-CT) and shown to correspond to a doubling of the normal atrial volume (Figure 7K-O').

We further analyzed *myl7:CreERT2; eGFP-T2A-wt1a* hearts on histological sections (Figure 7, supplement 2H-M). Similarly to what we had seen in the juvenile hearts of the *wt1b* overexpressing fish (Figure 7, supplement 2G-G'), we found a high degree of myocardialization of *wt1a*-overexpressing atria, a feature resembling trabeculation in the ventricle (Figure 7, supplement 2J-J', n=3/4). Furthermore, we detected the deposition of fibrotic tissue around atrial walls (Figure 7, supplement K'-M, n=4/4). Immunolabelling with anti-Col1a1 confirmed these findings. Whereas in the control animals Col1a1 labelling was only detected in the valves (Figure 7, supplement 2N-O"), in hearts of recombined *myl7:CreERT2; eGFP-T2A-wt1a* animals large regions of the atria were also Col1a1-positive. These were in close proximity with eGFP-positive cells, which might indicate that *wt1a*-expressing cardiomyocytes are secreting Col1a1 (Figure 7, supplement 2P-Q"). In sum, induced expression of *wt1a/b* in cardiomyocytes leads to atrial hypertrophy, which in the adult is accompanied by interstitial fibrosis.

Taken together, our data indicates that apart from the induction of cell fate change from cardiomyocytes to epicardial cells, overall, sustained expression of *wt1b* in cardiomyocytes affects heart development leading to impaired cardiomyocyte maturation, increased atrial size due to cardiomyocyte hyperplasia, as well as defective cardiac looping and heart function.

DISCUSSION

During myocardial development, cells from the precardiac mesoderm enter the heart tube. In the zebrafish, the myocardial tube is comprised of an epithelial lining which forms a continuum with the wt1a and wt1b-positive pericardial mesothelium (9, 18). We observed that during heart

tube extension, wt1b-positive mesothelial cells enter the heart tube and differentiate into cardiomyocytes. Concomitantly, wt1b-reporter gene expression is downregulated, suggesting that wt1 downregulation is needed for myocardial maturation. This process seems to be conserved across species as wt1 downregulation we also detected during the differentiation process of mouse embryonic stem cells into cardiomyocytes. Indeed, recently, it has been shown that during early stages of mouse heart development there is also a common progenitor pool that can give rise to both epicardial as well as myocardial cells (54) We found that indeed, sustained expression of *wt1a* or *wt1b* in the embryonic myocardium leads to a phenotypic switch from myocardial to epicardial cells, reduces chromatin accessibility in the heart, impairs cardiomyocyte maturation and causes atrial hypertrophy.

The most striking phenotype we observed upon *wt1* overexpression in cardiomyocytes was that these cells can apically delaminate and convert into epicardial-like cells. During proepicardium formation, *wt1*-positive cells apically extrude from the dorsal pericardial mesothelium giving rise to proepicardial cell clusters that subsequently are transferred to the myocardium (55). Here we find that *wt1*-positive cells in the myocardium undergo a similar process and delaminate apically from the myocardial epithelium. It will be important to further decipher possible parallelisms between these two processes and elucidate the direct role of *wt1* during these cellular rearrangements. Wt1 participates in the mesothelial-to-mesenchymal transition giving rise to epicardial derived cells (EPDCs) (56, 57). Moreover, Wt1 has been suggested to control the retinoic acid (RA) signaling pathway during EPDC formation (57). Indeed, WT1 controls the RA pathway by transcriptionally activating Raldh2 (known as *Aldh1a2* in the zebrafish) (58). The fact that cardiomyocytes overexpressing *wt1* are relocating to the epicardial layer might indicate that the cells undergo EMT-like processes in response to *wt1* overexpression and these might be mediated by RA. However, we did not observe *aldh1a2* expression in the myocardium, prior to delamination suggesting that *aldh1a2* expression might be rather a consequence than a cause of apical delamination of *wt1a* or *wt1b* expressing cells.

Of note, not all eGFP-positive cardiomyocytes undergo delamination and activation of *aldh1a2* expression. It might thus be possible, that not all cardiomyocytes have the capacity to respond to the same extent to *wt1* overexpression. Indeed, in the mouse a small subset of cardiomyocytes has been shown to express *Wt1* and as such, not all cardiomyocytes might be equally sensitive to a change in Wt1 dosage (59, 60).

Previous studies revealed that endocardial *klf2* function is playing a cell non -autonomous role on myocardial cell extrusion (25), where extruded cells are eliminated. Apical delamination of cardiomyocytes correlated with the mislocalization of the adhesion molecules N-cadherin (25). We observed a similar phenomenon of apical extrusion of cardiomyocytes upon *wt1b* overexpression in cardiomyocytes, where we observed a reduction and mislocalization of cdh2-tdTomato in the heart, as well as the repression of chromatin accessibility for this gene. But here, we observed a cell intrinsic effect of *wt1a/b* in cardiomyocytes triggering the event of cell extrusion. Furthermore, here we report that the extruded cells are not eliminated, but remain on the myocardial surface contributing to the epicardial layer.

Wt1 lineage tracing studies using Cre/lox transgenic lines in the mouse, suggested that epicardial derived cells were able to contribute to cardiomyocytes during development and repair (61, 62). Here we report the opposite phenotypic switch, induced by Wt1. In the context of a pathological condition, *Wt1* overexpression in cardiomyocytes had also suggested to trigger a change in cell fate. In arrythmogenic right ventricular cardiomyopathy (ARVC), a disease-causing arrhythmia leading to the accumulation of fat deposits in the heart, a subset of cardiomyocytes has been suggested to start to express *Wt1* and convert into adipocytes. Interestingly, epicardial fat represents an epicardial derivative (63). Together with our results, this indicates that expression of *Wt1* in cardiomyocytes contributes to a phenotypic change, transforming them into epicardial cells or EPDC-like cells. In line with this hypothesis, in the adult heart we observed the deposition of fibrotic tissue in close proximity to *wt1a*-overexpressing cells. The fibrosis might be a consequence of atrial hypertrophy, that is often accompanied by fibrosis (64), or, alternatively, might indicate that *wt1a* overexpressing cells differentiate or adopt features of EPDCs, in this case extracellular matrix producing fibroblasts.

wt1b overexpressing hearts revealed defects such as alterations in muscle cells maturation and sarcomere organization. The fact that sarcomere assembly and stabilization was affected could indicate a general reduced maturity of cardiomyocytes upon overexpression of *wt1b*, which also comes in line with the increased expression of Alcam (43), and reduction in chromatin accessibility of GO pathways related with muscle cell and tissue development and differentiation. Previous work hinted that Wt1 expression prevented the activation of a muscle differentiation program in metanephric-mesenchymal stem cells (65). Also, recent work on the overexpression of *wt1* in an in vitro model of cardiomyocyte differentiation showed reduced cardiomyocyte contractility (66), supporting our observations that wt1 downregulation is a prerequisite to allow myocardial maturation.

A striking phenotypic consequence of *wt1* overexpression is atrial hyperplasia. Enlarged atria might be caused by over proliferation of cardiomyocytes in the atrium, or by increased incorporation of cardiac progenitors from the pericardial mesothelium. Recently it was shown that *lamb1a* mutants have atrial enlargement, most likely due to an excess of second heart field progenitors being added to that region (40).Since we did not observe increased cell proliferation in the atria, the main reason for observing larger atria upon *wt1* overexpression might be that more precursor cells enter the heart during embryogenesis, which might also be linked to the reduced expression of Laminin we observed. This might be secondary to the delay in maturation, which increases the extent of precursors entering the heart.

In conclusion, induced expression of *wt1* in cardiomyocytes during embryogenesis impairs cardiomyocyte maturation and promotes a fate change from cardiomyocytes to epicardial cells. This suggests that during cardiac development, *wt1a/b* expression is turned off in cardiomyocytes once they enter the heart tube to allow their correct differentiation. Dissecting the mechanisms of *wt1a/b* downregulation of expression in cardiomyocyte precursors will

501 further expand our knowledge on the tight temporal control of heart tube expansion and
502 concomitant differentiation.
503
504 **Materials and Methods**

REAGENT/RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-Aldh1a2	GeneTex	Cat# GTX124302
Mouse monoclonal anti-CD166 antigen homologue A (neurolin) (Alcama)	DSHB	Cat # ZN-8
Mouse monoclonal anti α-Actinin (sarcomeric) clone EA-53	Sigma Aldrich	Cat # A7811
Mouse monoclonal anti-Caveolin 1	BD biosciences	Cat# 610406
Chicken polyclonal anti-GFP	Aves Labs	Cat# GFP-1010
Mouse monoclonal anti-myosin, sarcomere (MHC)	DSHB	Cat# MF 20, RRID:AB_2147781
Mouse monoclonal anti-procollagen type I aminoterminal extension peptide (Col1a1)	DSHB	Cat# SP1.D8
Goat anti-Chicken IgY (H+L), Alexa Fluor® 488 conjugate	Thermo Fisher Scientific	Cat # A-11039
Goat anti-Mouse IgG2b, Alexa Fluor® 568 conjugate	Thermo Fisher Scientific	Cat # A-21144
Goat anti-Mouse IgG2b, Alexa Fluor® 647 conjugate	Thermo Fisher Scientific	Cat #A-21242
Goat Anti-Mouse Immunoglobulins/HRP	Dako	Cat # P 0447

Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 568 conjugate	Thermo Fisher Scientific	Cat # A-11036
Goat anti-Mouse IgG1, Alexa Fluor® 568 conjugate	Thermo Fisher Scientific	Cat # A-21124

Primers		
Gene	Forward primer	Reverse Primer
Gfp	CAAGATCCGCCACAACATCG	GACTGGGTGCTCAGGTAGTG
wt1a OE	GAGCCATCCCGGAGGTTATG	GGTACTCTCCGCACATCCTG
tcf21	ATGTCCACCGGGTCCATCAG	TCAGGAAGCTGTAGTCCCGCA

Chemicals, Peptides, and Recombinant Proteins		
4-hydroxytamoxifen	Sigma Aldrich	Cat#H6278
Rhodamine Phalloidin	Thermo Fisher Scientific	Cat# R415
N-Phenylthiourea (PTU)	Sigma Aldrich	Cat# P7629
Proteinase K	Roche	Cat# 03115801001
Heparin sodium salt from porcine intestinal mucosa	Sigma- Aldrich	Cat# H4784
Formamide	Sigma- Aldrich	Cat# 47670-1L-F
Blocking reagent	Sigma-Aldrich	Cat# 11096176001
Ribonucleic acid from torula yeast	Sigma- Aldrich	Cat# R6625-25G
HBSS (10X), no calcium, no magnesium, no phenol red	Thermo Fisher Scientific	Cat# 14185052
Corning™ 0.05% Trypsin/0.53mM EDTA in HBSS w/o Calcium and Magnesium	Thermo Fisher Scientific	Cat# MT25051CI

Collagenase	Sigma	Cat # C8176
BSA	Sigma	Cat# A3059
Kits		
SMARTer® Ultra™ Low Input RNA for Illumina® Sequencing – HV kit	Takara	Cat# 634828
Agilent's High Sensitivity DNA Kit	Agilent	Cat# 5067-4626
Low Input Library Prep Kit	Illumina	Cat# 634947
Illumina Nextera kit	Illumina	Cat# Fc-121-1030
ATAC KITS		
Software and Algorithms		
Fiji	NIH	SCR_002285
GraphPad Prism 7	GraphPad Software	SCR_002798
Imaris 9.5.1	Bitplane	
MATLAB R2017a	MathWorks	
Specialized Material		
U-shaped glass capillaries	Leica microsystems	Cat # 158007061
MatTek imaging dish, 35 mm	MatTek Corporation	Cat # P35G-0-20-C
Wolfram needles		
Microscopes and Imaging machines		
Nikon SMZ800N	Nikon	
Leica TCS SP8 digital light sheet (DLS)	Leica	

Imager M2	Zeiss	
LSM 880 confocal microscope, with Airyscan	Zeiss	
Micro-CT Skyscan 1272	Bruker	
Quanta FEG 250 SEM (serial block face scanning electron microscope)	FEI	
Experimental Models: Organisms/Strains		
Et(-26.5Hsa.WT1-gata2:eGFP)cn1 (epi:eGFP)	(9)	ZDB-ETCONSTRCT-170823-1
Tg(myl7:mRFP)	(67)	ZDB-TGCONSTRCT-080917-1
Tg(fli1a:Gal4); ubs3Tg	(50)	ZDB-ALT-120113-6
Tg(myl7:Gal4)^{cbg2Tg}	(22)	ZDB-TGCONSTRCT-150108-1
Tg(–3.5ubi:loxP-eGFP-loxP-mCherry)^{cz1701}	(68)	ZDB-TGCONSTRCT-110124-1
Tg(eGFP:5xUAS:RFP; gcryst:cerulean)^{cn15}	(23)	ZDB-TGCONSTRCT-190724-4
Tg(bGI-eGFP:5xUAS:wt1b -bGI; cryaa:eCFP)^{brn4}	This manuscript	ZDB-ALT-200327-14
Tg(bactin2:loxP-DsRed2-loxP-eGFP-T2A-wt1a)^{li21}	This manuscript	N/A
Tg(bGI-eGFP:5xUAS:tcf21 -bGI; cryaa:eCFP)	This manuscript	N/A

Zebrafish husbandry

Experiments were conducted with zebrafish embryos and adults aged 3–18 months, raised at maximal 5 fish/l. Fish were maintained under the same environmental conditions: 27.5-28°C, with 14 hours of light and 10 hours of dark, 650-700µs/cm, pH 7.5 and 10% of water exchange

daily. Experiments were conducted after the approval of the "Amt für Landwirtschaft und Natur" from the Canton of Bern, Switzerland, under the licenses BE95/15 and BE 64/18.

Generation of transgenic lines

To generate the transgenic line *eGFP:UAS:wt1b* and the *eGFP:UAS:tcf21* the RFP fragment from the plasmid used to clone *eGFP:5xUAS:RFP* (23) was replaced by either the coding sequence of *wt1b(-KTS)* isoform of the *tcf21* , PCR amplified from 24 hpf and 5 dpf zebrafish embryo cDNA and assembled using Gibson cloning. The final entire construct is flanked with *Tol2* sites to facilitate transgenesis. In this line, tissue specific expression of Gal4 drives the bidirectional transactivation of the UAS leading to the expression of both *eGFP* and *wt1b(-KTS)* coding sequence. The full name of these lines is *Tg(bGI-eGFP:5xUAS:wt1b(-KTS)-bGI; cryaa:eCFP)*^{brn4,} *Tg(bGI-eGFP:5xUAS:tcf21)-bGI; cryaa:eCFP)*^{brn4}.

The construct *bactin2:loxP-DsRed2-loxP-eGFP-T2A-wt1a* was generated by Gateway cloning (MultiSite Gateway Three-Fragment Vector Construction Kit; Invitrogen). As destination vector pDestTol2pA2 was used. The floxed *DsRed2* cassette was derived from vector *pTol2-EF1alpha-DsRed(floxed)-eGFP* (69) and the *wt1a* cDNA was amplified from vector *pCS2P-wt1a* (14). The final construct is flanked with *Tol2* sites to facilitate transgenesis. In the resulting zebrafish line *DsRed* is expressed from the ubiquitous *β-actin* promoter. After Cre-mediated excision of the STOP cassette both *eGFP* as well as *wt1a* are expressed in a tissue-specific manner. The full name of this line is *Tg(bactin2:loxP-DsRed2-loxP-eGFP-T2A-wt1a)*^{li21}*.*

Administration of 4-Hydroxytamoxifen (4-OHT) to embryos and juvenile fish

4-hydroxytamoxifen (4-OHT; Sigma H7904) stock was prepared by dissolving the powder in ethanol, to 10 mM concentration. To aid with the dissolution the stock was heated for 10 minutes (min) at 65°C and then stored at -20ºC, protected from the light. 4-OHT was administered at the indicated times, at a final concentration of 10 µM. For embryos, treatments

were performed continuously. For juvenile fish 4-OHT was administered overnight in E3. Prior to administration, the 10 mM stock was warmed for 10 min at 65 °C (70).

In vivo light sheet fluorescence microscopy and retrospective gating

For *in vivo* imaging of the beating zebrafish heart, 2 dpf old embryos were pipetted with melted 1% low melting agarose in E3 medium (about 45°C), containing 0.003% 1-phenyl-2-thiourea (PTU) (*Sigma-Aldrich*) to avoid pigmentation and Tricaine at 0.08 mg/ml, pH 7 to anaesthetize the fish, into a U-shaped glass capillaries (Leica microsystems). This U-shaped capillary was mounted in a 35 mm MatTek imaging dish. The dish was filled with E3 medium containing 0.003% PTU and Tricaine at 0.08 mg/ml, pH 7.

Imaging was performed with the Leica TCS SP8 digital light sheet (DLS) microscope. We used a 25x detection objective with NA 0.95 water immersion and a 2.5x illumination objective with a light sheet thickness of 9.4 µm and length of 1197 µm. The total field of view is 295 x 295 µm, fitting the size of the embryonic zebrafish heart, allowing space for sample drift. The images were acquired in XYTZL-acquisition (XY: single optical section, T: time series, Z: serial optical sections, L: looped acquisition) mode for later retrospective gating. The parameters as shown in Table 3 were applied.

The images were saved as single *.lif*-file and transferred to a workstation (*HP-Z series, Dual Intel Xeon e5-2667 v4 3.2 GHz, 256 GB, NVIDIA GeForce GTX 1080 Ti*). A quality check of the data was performed, before the data were further processed. The survival of the larva until the end of the acquisition, the sample drift and the degree of bleaching were assessed in the *Processor_6D (*https://github.com/Alernst/6D_DLS_Beating_Heart*)*. The data were only used if the larva survived the acquisition. The single *.lif*-file was converted to XYTC *.tif*-files, using the *Converter_6D (*https://github.com/Alernst/6D_DLS_Beating_Heart*)*. Each XYTC file was named in the following format *"Image_R0000_Z0000"* to be recognized for further processing. Retrospective gating was performed as previously described (71-73). The *MATLAB* (R2017a) tool *BeatSync V2.1* was used for retrospective gating (access to the software can be requested

from the research group of Michael Liebling). The settings for re-synchronization in the BeatSync software were *"Normalized mutual information"*, *"Recursive Z-alignment"* and *"Nearest-neighbor interpolation".* One entire heart cycle was re-synchronized in 3D. After re-synchronization, a 3D time lapse of a virtually still heart was created, using the Fiji (74) tool *Make_timelapse* (https://github.com/Alernst/Make_timelapse) using the *Make_timelapse Fiji* plugin. The time lapse was represented as maximum intensity projection or individual optical slices.

Immunofluorescence

Whole mount immunofluorescence on embryos was done as previously described (23). Shortly, embryos were fixed over-night, at 4 ºC in 4% paraformaldehyde (PFA) (EMS, 15710). Then they were washed with PBS-Tween20 (0.1%) and permeabilized for 30 to 60 min with PBS-TritonX100 (0.5%), depending on the stage and the antibody used. Permeabilization was followed by blocking for 2 hours with histoblock (5% BSA, 5% goat serum, 20mM MgCl2 in PBS). Afterwards, embryos were incubated overnight, at 4 ºC, with the primary antibodies, in 5%BSA. The next day embryos were washed with PBS-Tween20 (0.1%) followed by and overnight incubation in the secondary antibodies, at 4 ºC, in 5% BSA. Finally, embryos were washed with PBS-Tween20 (0.1%) and a nuclear counterstain with DAPI (Merck, 1246530100) 1:1000 was done.

Immunofluorescence on paraffin sections was performed as previously described (75). Briefly, paraffin sections were dewaxed and rehydrated through a series of ethanol incubations, similar to previously described for histological staining. Afterwards, epitope was recovered by boiling the samples in 10mM citrate buffer, pH 6, for 20 min. Next the same procedure was applied as described above for whole mount immunofluorescence.

The following antibodies were used: primary antibodies - anti-eGFP (Aves, eGFP-1010) was at 1:300, anti-Myosin Heavy Chain at 1:50 (DSHB Iowa Hybridoma Bank, MF20), anti-Aldh1a2 at 1:100 (Gene Tex), anti-Alcam at 1:100 (DSHB Iowa Hybridoma Bank, Zn-8), anti-α-actinin at 1:200 (Sigma Aldrich), anti-embryonic Cardiac-Myosin Heavy Chain at 1:20 (DSHB

Iowa Hybridoma Bank, N2.216), anti-Caveolin 1 at 1:100 (BD Biosciences) and anti-Procollagen Type I at 1:20 (DSHB Iowa Hybridoma Bank, SP1.D8). Secondary antibodies were Alexa Fluor 488, 568, 647 (Life Technologies) at 1:250 and biotin anti-rabbit (Jackson Immuno Research, 111-066-003) followed by StreptavidinCy3 or Cy5 conjugate (Molecular Probes, SA1010 and SA1011) at 1:250.

Whole mount in situ hybridization

Whole mount in situ hybridization was performed as described (76), with some minor adaptations. Embryos were selected at 24 hpf and 3 dpf for eGFP expression. After fixation, the embryos were washed with PBS and gradually dehydrated through a methanol series. Embryos were stored in 100% methanol for a minimum of 2 hours, at -20°C. Afterwards, the embryos were rehydrated and permeabilized with proteinase K (10µg/ml in TBST) at 37°C. Incubation times were adjusted according to the stage of the embryos (24 hpf, 10 min and 72 hpf, 20 min). This was followed by a 20 min incubation in 0.1M triethanolamine (pH 8), with 25µl/ml acetic anhydride.

After 4 hours of pre-hybridization, at 68°C, myl7 riboprobe was diluted in pre hybridization solution, at a concentration of 300ng/ml. The embryos were incubated with the riboprobe overnight, at 68°C. The following day, the riboprobe was removed and the embryos were incubated twice for 30 min with post hybridization solution at 68°C. Embryos were then incubated with blocking buffer, freshly prepared, and afterwards with anti-DIG antibody (in blocking solution), at 1:4000, overnight, at 4°C.

The embryos were then washed extensively with Maleic acid buffer (150mM maleic acid pH 7.5, 300 mM NaCl, 0.1% Tween 20). Finally, the embryos were transferred to a 6-well plate and pre-incubated with AP-buffer (0.1M Tris base pH 9.5, 0.1 M NaCl, 1mM MgCl2, 0.1% Tween 20) and then incubated with BM-purple, at room temperature. As soon as color was visible in the heart of either group (overexpression or control), the staining was stopped in both groups by adding TBST and embryos were re-fixed in 4% PFA.

Using a microscope, we could obtain pictures of the hearts of the embryos. For image acquisition, embryos were mounted on 3% methylcellulose for ease of orientation. Embryos were positioned so that the majority of the heart could be observed in a single plane.
Images were acquired with a Nikon SMZ800N stereomicroscope. Illumination conditions and acquisition parameters were maintained for all embryos.

Double in situ hybridization and immunohistochemistry on paraffin sections

In situ hybridization on paraffin sections was done as follows: paraffin sections were dewaxed and rehydrated through a series of ethanol incubations. Sections were then refixed with 4% PFA at room temperature for 20 min. Afterwards they were washed with PBS and the tissue was permeabilized by incubating the slides with 10 µg/ml of Protease K, for 10 min. at 37^{o}C. Afterwards, slides ware washed with PBS and briefly refixed with 4% PFA. The tissue was then incubated for 10 min with triethanolamine 0.1M, pH8 and 0.25% acetic anhydride. After washing the slides with PBS and RNAse free water the slides were incubated for 3 hours with pre-hybridization buffer (50% formamide, 5X SSC pH 5.5, 0.1X Denhardt's, 0.1% Tween20, 0.1% CHAPs and 0.05mg/ml tRNA), at 65^{o}C. Afterwards, pre-hybridization buffer was replaced with hybridization buffer (pre-hybridization buffer with mRNA probe). Slides were left to incubate with hybridization buffer overnight at 65^{o}C. The next day, slides ware washed twice with post-hybridization buffer I (50% formamide, 5X SSC pH 5.5 and 1% SDS) and 2 times with post-hybridization buffer II (50% formamide, 2X SSC pH 5.5 and 0.2% SDS). Each wash was done for 30 min at 65^{o}C. Slides were then washed another 3 times with maleic acid buffer (MABT) and then incubated for 1 hour blocking solution (2% fetal bovine serum, heat inactivated, and 1% blocking reagent, in MABT). Tissue was incubated overnight at 4^{o}C with anti-DIG antibody in blocking solution at 1:2000. Finally, sections were thoroughly washed with MABT and incubated in alkaline phosphatase buffer (AP buffer, NaCl 0.1M, $MgCl_2$ 0.05M, 10% Tri-HCL pH 9.5). Finally, colorimetric assay was performed using BM purple. After the desired staining was achieved, slides were washed with PBS and fixed with 4% PFA, before mounting them with 50% glycerol and imaged using a Zeiss Imager M2, with and Olympus UC50 camera.

After imaging sections were washed and further permeabilized with PBS with 0.5% TritonX-100. Then they were incubated for 2hours with 5% BSA at room temperature and incubated with primary antibody, chicken anti-GFP (1:300 in 5% BSA) overnight at 4ºC. The next day slides were washed in PBS-0.1% Tween20 and incubated for one hour at room temperature with secondary antibody anti-chicken-HRP. Signal was obtained by incubating slides with DAB solution for 30 seconds at room temperature. The reaction was stopped with water. Slides were then mounted in 50% glycerol and imaged.

Embryonic heart function analysis

Heartbeat analysis was performed by assessing the following parameters: degree of rhythmic beating as Root Mean Square of Successive Differences (RMSSD) (77); stroke volume (SV - difference between diastolic an systolic volume); ejection fraction (EF - difference between diastolic an systolic volume relative to the diastolic size); cardiac output (CO - SV multiplied by heart rate); and diastolic volume, and heart rate as described (53).

We recorded 300 frames of the beating heart in the GFP channel in *Tg(myl7:Gal4; eGFP:UAS:wt1b)* and *Tg(myl7:Gal4; eGFP:UAS:RFP)* at 2 dpf and 5 dpf using the fluorescence stereo microscope Nikon SMZ25 (SHR Plan apo 1x objective, 10x zoom, 2880x2048 pixel, 0.44 µm/pixel, 17 frames/s).

For the analysis of heart function, we defined the volume of the heart, which is calculated by measuring the long diameter (D_L) and the short diameter (D_S).

$$Volume\ (nl) = \frac{1}{6} \times \pi \times D_L \times D_S^2$$

The maximal and minimal volume of the ventricle and atrium were measured, to calculate end-diastolic volume (EDV) and end-systolic volume (ESV). The mean EDV and ESV of two heart cycles per fish were averaged to calculate the SV.

$$SV\ (nl/beat) = EDV - ESV$$

The EF was calculated by dividing the SV through the EDV and converted to a percentage.

$$EF\ (\%) = \frac{(EDV-ESV)}{EDV} \times 100 = \frac{SV}{EDV} \times 100$$

We developed the FIJI plugin *Heart beat analysis* to sequentially process all images in a folder and guide the user through each manual step of the analysis. The manual steps are to find the two diastolic and systolic states of the heart, adjust a line to D_L and D_S and to draw one line at the border of the ventricle. The plugin *Heart beat analysis* opens subsets of the data (100 frames and only green channel per fish from the *.nd2* RGB file), applies a Gaussian blur filter (10 px), indicates which manual step to perform, calculates the HR by detecting maxima in a kymograph and subsequently saves all kymograph images as *.tiff*, results as *.csv*, all lines as *.zip* in ROI sets.

To calculate the RMSSD we measured the temporal distance between 12-15 cardiac cycles using instead of a subset of 100 frames all 300 frames from the above described data. The temporal distances between cardiac cycles were measured using the FIJI plugin *RMSSD* (77), two line were drawn crossing one side of the cardiac wall of V and AT. Subsequently, kymographs were generated. The correctness of detected maxima in the kymograph was supervised. All intermediate images and ROIs were saved. The locations of each intensity maximum in the kymograph were exported as *.csv*-file. A Jupyter-notebook (78) was created to calculate the time between two cardiac cycles (R – R; time of cardiac cycle, R; current cycle, i; next cycle, i+1) and variability of these time differences between all frames (total frames, N) as RMSSD.

$$RMSSD\ (ms) = \sqrt{\frac{1}{N-1}\left(\sum_{i-1}^{N-1}((R-R)_{i+1} - (R-R)_i\right)^2}$$

qRT-PCR assay

Hearts from *Tg(eGFP:5xUAS:wt1bOE-KTS;myl7:Gal4)* and *Tg(eGFP:5xUAS:RFP; myl7:Gal4)* were extracted at 40 dpf. Ventricle, atrium and bulbus arteriosus were manually dissected and stored separately in pools of 5. For each sample, between 3 and 7 biological replicates were collected. Total RNA was extracted by using TRI Reagent (Sigma-Aldrich; Cat-No. T9424) according to the manufacturer's recommendations. Afterwards, a total of 200 ng of total RNA was reverse-transcribed into cDNA using High Capacity cDNA Archive Kit

(Invitrogen Life Technologies; Cat-No. 4374966). Quantitative PCR (qPCR) was performed in a 7900HT Fast real-time PCR system (Applied Biosystems). qPCR was done using Power Up SYBR Green Master Mix (Applied Biosystems, A25742).

PCR program was run as follows: initial denaturation step was done for 30 s at 95°C, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. To calculate the relative index of gene expression, we employed the $2^{-\Delta\Delta Ct}$ method, using *e1f2α* expression for normalization.

Histological staining

Hearts were fixed in 2 % paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight at 4°C. Samples were then washed in PBS, dehydrated through graded alcohols (30%, 50%, 70%, 90% and 2 x 100%), and Xylol (2x) and embedded in paraffin wax (3x). All steps were done for 20 min. Histological stainings were performed on 8 μm paraffin sections cut on a microtome (Leica and Reichert-Jung), mounted on Superfrost slides (Fisher Scientific), and dried overnight at 37°C. Sections were then dewaxed in Xylol, rehydrated through graded ethanol (from 100% to 30%) and then washed in distilled water. Connective tissue was stained using Acid Fuchsine Orange G (AFOG) (79).

Serial block face scanning electron microscopy

Zebrafish embryos at 5 dpf were killed with an overdose of tricaine and immediately fixed with 2.5% glutaraldehyde with 0.15M cacodylate buffer and 2mM $CaCl_2$, pH 7.4. Embryos were then processed for serial block face scanning electron microscopy as previously described (80). Briefly we proceed as follows: samples were rinsed 3 times in ice-cold 0.15 M Na-cacodylate for 5 min. They were then incubated in 0.15 M Na-cacodylate solution containing 2% OsO4 and 1.5% potassium ferrocyanide for 45 min, at room temperature, and for 15 min in a water bath, at 50 °C. Samples were rinsed 3 times for 5 min in water. They were then incubated with 0.64 M pyrogallol for 15 min at room temperature, for 5 min in a water bath at 50 °C, and subsequently rinsed with water. The embryos were incubated in 2% OsO4 for 22 min at room temperature and 8 min in a water bath at 50°C. Afterwards they were again rinsed

in water (3 times 5 min) and incubated overnight in a solution of 0.15 M gadolinium acetate (LFG Distribution, Lyon, France) and 0.15 M samarium acetate (LFG Distribution) pH 7.0. The next day the embryos were rinsed 3×5 min with water and incubated in 1% Walton's lead aspartate (81) at 60 °C for 30 min, and rinsed with water (3x 5 min).

After staining, the samples were dehydrated in a graded ethanol series (20%, 50%, 70%, 90%, 100%, 100%) at 4°C, each step lasting 5 min. They were then infiltrated with Durcupan resin mixed with ethanol at ratios of 1:3 (v/v), 1:1, and 3:1, each step lasting 2 h. The embryos were left overnight to infiltrate with Durcupan. The next day, samples were transferred to fresh Durcupan and the resin was polymerized for 3 days at 60 °C. Sample blocks were mounted on aluminum pins (Gatan, Pleasonton, CA, USA) with a conductive epoxy glue (CW2400, Circuitworks, Kennesaw, GA, USA). Care was taken to have osmicated material directly exposed at the block surface in contact with the glue in order to reduce specimen charging under the electron beam. Pyramids with a surface of approximately 500 × 500 μm2 were trimmed with a razor blade.

Three-dimensional (3D) ultrastructural images were produced by serial block face scanning electron microscopy (SBFSEM) on a Quanta FEG 250 SEM (FEI, Eindhoven, The Netherlands) equipped with a 3View2XP in situ ultramicrotome (Gatan). Images were acquired in low vacuum mode (40 mPa), except where indicated otherwise. Acceleration voltage was 5 kV and pixel dwell time was set between 2 μs. Image acquisition was done with a back scattered electron detector optimized for SBFSEM (Gatan). Image stack were aligned, normalized, and denoised by non-linear anisotropic diffusion in IMOD (82). Each field of view consisted of 8192 x 8192 pixels with a dimension of 6 nm/pixel in x-y and zz nm in z direction. Final image montage was done in Fiji.

Micro-computed tomography (microCT)

For each condition, three adult fish were sacrificed in 0.6 mg/ml tricaine (Sigma-Aldrich). Subsequently fish were fixed for 24 hours in 4% paraformaldehyde (PFA), at 4^{o}C. Afterwards, the animals were transversally sectioned bellow the pectoral fins and washed in 1X PBS. They

were then stained in lugol for 24 hours, at room temperature, before being scanned by micro-computed tomography. For this, the six samples were imaged on a Bruker SkyScan 1272 high-resolution microtomography machine (Bruker microCT, Kontich, Belgium). The X-ray source was set to a voltage of 80 kV and a current of 125 µA, the x-ray spectrum was shaped by a 1 mm Al filter prior to incidence onto the sample. For each sample, a set of 948 projections of 2452 x 1640 pixels at every 0.2° over a 180° sample rotation was recorded. Every single projection was exposed for 1593 msec. Three projections were averaged to one to reduce the noise. This resulted in a scan time of two hours per sample and an isometric voxel size of 4 µm in the final data sets.

The projection images were then reconstructed into a 3D stack of images with NRecon (Bruker, Version: 1.7.0.4). The 3D images were analyzed using Imaris software and Fiji. For the analysis, the heart and then the atrium, the ventricle and the bulbus arteriosus were segmented and the volume and surface area were obtained. Volume differences between conditions were analyzed using GraphPad Prism7.

For 3D reconstructions of microCT images we used the Fiji software (74, 83). We first selected the images where the heart was visible and created a z-stack with those images. We then proceed to segment three different areas of the heart: the bulbus arteriosus, the atrium and the ventricle. We created a mask in every 5th z-slice and performed a linear interpolation to generate masks for every z-slice. Subsequently, we applied a macro to set all pixels outside of the masked volume to zero. We repeated this process for each one of the three heart areas. We then attributed a different color to each heart region and merged all three channels. This allowed us to represent the segmented parts of the heart and generate a 3D projection (84). We also used the individually segmented heart regions to calculate the volume of each heart chamber. For this we applied the MorphoLibJ plugin (85).

FAC sorting

myl7:Gal;eGFP:UAS:RFP and *myl7:Gal;eGFP:UAS:wt1b* embryos at 5 dpf were used to obtain GFP+ heart cells. The heart region of these embryos was manually dissected and

placed in Ringer's solution. Afterwards the tissue was briefly centrifuged in a table top centrifuge and the Ringer's solution was replaced by a mix of 20mg/ml collagenase in 0.05% trypsin. The samples were incubated at 32^{o}C for 25 minutes. Every 5 minutes this mixture as gently mixed. The tissue was visually inspected for dissociation. After cell disaggregation the reaction was stopped with Hanks's solution (1xHBS, 10mM Hepes and 0.25%BSA). The homogenized samples were centrifuged at 250g for 10 minutes and re-suspended in Hank's solution. The cells were then passed through a 40 µm filter, centrifuged gain for 10 minutes at 400g and re-suspended in 50 µl of Hank's solution for FAC sorting. Dead cells were marked with 7-aminoactinomycin D (Invitrogen) and discarded. Cells were FAC sorted into Hank's solution, on a Moflo astrios EQ (Beckman Coulter) and analyzed for forward and side scatter, as well as eGFP fluorescence. Between 1200 and 1500 cells were forted for eGFP for each sample.

ATAC-seq

FAC sorted GFP^{+} cells were centrifuged and Han's solution was replaced by lysis buffer (10mM tris-HCL, pH 7.4, 10mM NaCl, 3mM $MgCl_2$, 0.1% IGEPAL CA-630). Cells were immediately centrifuged at 500 g for 10minutes at 4^{o}C. The supernatant was discarded and replaced with the transposition reaction mix (Tn5 in TD buffer) for tagmentation, and incubated at 37^{o}C for 30min. Afterwards, 500mM of EDTA was used for quenching. The solution was incubated for 30 minutes at 50^{o}C. $MgCl_2$ was added to a final concentration of 45mM. Samples were stored at 4^{o}C before proceeding with PCR amplification. For PCR amplification 15 cycles were used due to the reduced amount of material. The amplified library was purified using the Qiagen PCR purification MinElute kit. Quality of the library was evaluated in a bioanalyzer as well as by RT-qPCR. Samples were sequenced using the XP workflow. 100 bp pair-end sequencing was done on all samples.

ATACSeq Data Analysis

All bioinformatics analysis were performed using bash scripts or R statistical software. Quality check of the samples was performed using FASTQC and reports summarized using MultiQC (86, 87). Adapters from the fastq files were trimmed using trimmomatic software (88). Reads were aligned using bowtie2 (89, 90) to GRCz11 danRer11 v102 assembly from Ensembl (91) with flags `--very-sensitive`. Paired end reads were used for downstream analysis. The files were then converted to bam, downsampled to the lowest counts, indexed and the mitochondrial chromosome was removed using samtools (92, 93). Duplicates were removed using Picard tools (94). The samples were processed to select for unique reads using samtools (92, 93). The peaks were identified using Genrich in ATACSeq mode and zebrafish genome size (95). The zebrafish genome size was estimated using faCount script from public utilities from UCSC (96).

To analyze the differential accessible regions, we used DiffBind using background and DeSeq2 normalization and a cutoff threshold of $p<0.05$. To annotate the peaks, we used ChIPSeeker (97). We used the GRCz11 danRer11 v102 assembly from Ensembl and transcription start site region as +/-1kb for annotation. The annotated genes were then converted to mouse orthologous genes using biomaRt and used for pathway analysis using clusterProfiler (98, 99). K-means clustering was performed using SeqMINER software using linear enrichment clustering approach with 10 clusters (100). The bigwig files to visualize the peaks were made using bamCoverage in deepTools2 (101). Interactive Genome Viewer was used to visualize the peaks (102).

To identify the transcription factor binding cite, we used the sequences from the differential accessible regions in Centrimo from MEME-suite. We used CIS-BP 2.0 Danio Rerio Database to identify the potential zebrafish transcription factors (103, 104).

SMARTer-seq

Cells from the dorsal pericardium, proepicardium and heart tube were manually dissected, with wolfram needles, from *epi:eGFP;myl7:mRFP* zebrafish hearts at 60 hpf. A minimum of 10 cells per tissue was collected in ice cold PBS. Cells were centrifuged for 7 minutes at 250 g. The

excess liquid was removed and the cells were stored at -80ºC until further use. RNA was extracted using… kit, according to the manufacturer manual. Afterwards, we used the… kit for cDNA amplification. cDNA quality control was verified using the Agilent 2100 BioAnalyzer and the High Sensitivity DNa Chip from Agilent's High Sensitivity DNA Kit. DNA was sheared to 200-500 bp range using Covaris AFA system. The samples were then processed for sequencing using the Illumina Sequancing Library with the Low Input Library Prep Kit. Sequencing was done in…NGS experiments were performed in the Genomics Unit of the CNIC.

SMARTer-seq analysis

All bioinformatics analysis were performed using bash scripts or R statistical software. Quality check of the samples was performed using FASTQC and reports summarized using MultiQC (86, 87). Adapters from the fastq files were trimmed using fastp software (105). Reads were aligned to GRCz11 danRer11 v102 assembly from Ensembl using STAR (91). The reads were summarized using featureCounts (106). The counts data were imported to Deseq2 and genes who had expression across all samples (rowSums) greater than or equal to 10 were kept ensuring the realiable expression estimates (107). After evaluation of the PCA, one of the samples from the heart tube was determined as an outlier and removed from the downstream analysis. The differential expression analysis was performed using 'ashr' LFC Shrikange (108). A gene was considered as significant if the p adjusted value was <0.05. The plots were plotted using ggplot2 (109).

Imaging and Image processing

Immunofluorescence images were acquired using the Leica TCS SP8 DLS confocal microscopes. For image acquisition of whole mount embryos, larvae were mounted in 1% low melting agarose in a MatTek petri dish. Images were acquired with a 20x water immersion objective. Images were afterwards processed with Fiji software. Figure legends indicate

whether a 3D projection is presented or a maximum intensity projection of a reduced number of stacks is shown. For 3D projections, images were first treated with a mean filter, with a radius of 2.0 pixels. Interpolation was also applied when rendering the 3D projections.

Imaging of AFOG stained sections was done with the Zeiss Imager M2, using a 10x objective. For quantification of mean fluorescence intensity first a mean filter with a radius of 2.0 pixels was applied to smoothen the images. Afterwards we did a maximum intensity projection of all the stacks containing the heart. We then delimited the heart and applied an automatic OTSU threshold. Automatic threshold was evaluated independently for each image, when necessary minor adjustments were applied. Finally mean fluorescence intensity was calculated.

Semi-quantification of signal intensity for whole mount in situ hybridization was done using Fiji software. First the images were inverted, region of interest (ROI) was defined and used for all images. For each image mean signal was measured in six independent areas: three in the background and three in the stained area. Measurements were averaged and then background signal subtracted from the signal measured in the stained area. The fold change was calculated and GraphPad was used for statistical analysis.

Statistical Analysis

Statistical analysis was done with GraphPad Prism 7. When data fitted normality parameters, i.e, passed either the D'Agostino-Pearson or the Shapiro-Wilk normality test, an unpaired t-test was used. If this was not the case, the Mann-Whitney non-parametric test was used to compare differences between conditions. In case a statistically significant difference in the standard deviation between conditions was detected, the Unpaired t test with Welch's correction was applied. In case of multiple comparisons, a One-Way ANOVA was applied, followed by Tukey’s multicomparisons test. For each graph, in each figure, the type of statistical test applied is stated in the figure legend.

The specific test used in each comparison is indicated in the main text or figure legend. Normal distribution was tested to decide if a parametric or non-parametric test needed to be applied.

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AUTHORS CONTRIBUTION

I.M. performed most of the experiments, analyzed data, contributed to interpretation of results and wrote the manuscript

A.E. contributed to in vivo imaging and image processing and quantifications, contributed to writing the manuscript and interpretation of results

P.A. performed sequencing analysis, contributed to writing the manuscript and interpretation of results

T.H. performed qPCR and contributed to other experiments

A.V. performed immunofluorescence and helped with embryo dissociation for FACS

A.S.-M. generated the *eGFP:UAS:wt1b* line

X.L. contributed to histological stainings, sectioning and maintenance of lines

A.O. performed electron Microscopy imaging and image reconstruction

L. A.D. contributed to Smart-Seq

B. Z. supervised electron Microscopy imaging and image reconstruction

D. H. performed micro-CT imaging and imagine reconstructions

R. H. supervised micro-CT imaging and imagine reconstructions

M.O. generated result shown in Fig. 1 S1.

C.T. performed data analysis not included, but with impact to this work

U.N. generated the *Tg(bactin2:loxP-DsRed2-loxP-eGFP-T2A-wt1a)*, line

C.E. supervised the generation of the *Tg(bactin2:loxP-DsRed2-loxP-eGFP-T2A-wt1a)*

N.M. conceived the research question to be addressed, contributed to design experiments and interpretation of results, wrote the manuscript, and secured funding.

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FIGURE LEGENDS

Figure 1. *wt1a* positive cells loose eGFP expression upon entering the heart tube.

(A) Schematic representation of the in vivo imaging of the developing heart tube.

(B) Time-lapse images of the developing heart tube between 52 and 68 hours post fertilization in the double transgenic line *epi:eGFP;myl7:mRFP*. Grey images are single channel zoomed images from the boxes in the merged panels. There is an opposite gradual shift in the expression levels between eGFP and RFP along the time.

(C) Quantification of the ratio of eGFP and mRFP levels in cells of the heart tube according to the distance to the sinus venosus (SV).

(D) Schematic representation of tissue dissection for SMARTer-seq of pericardium, proepicardium and heart tubes of zebrafish embryos.

(E-E') Volcano plots. Magenta dots indicate upregulated genes in the heart tube. Green dots mark genes upregulated in proepicardium (E) or pericardium (E').

(F) Quantification of normalized counts for the epicardial marker genes *wt1a* and *wt1b*, and the myocardial gene *myl7*.

(G) Schematic representation of the downregulation of eGFP and upregulation of mRFP in cardiomyocyte progenitors upon their entry into the heart tube.

Scale bars: 50 µm. dp, dorsal pericardium; ht, heart tube; sv, sinus venosus.

Figure 2. Ventricular cardiomyocytes delaminate from the ventricle and change their fate upon *wt1b* overexpression.

(A) Schematic representation of used transgenic lines and position of the embryos for imaging.

(B-C'") Whole mount immunofluorescence against GFP (green) and MHC (magenta) on *myl7:CreERT2,eGFP-T2A-wt1a* hearts at 5 days post fertilization (dpf), non-recombined (B-B'") and recombined by addition of 4-OHT between 24 hours post fertilization (hpf) and 4 dpf (C-C'"). Shown are maximum intensity projections of 5 optical sections with a distance of 1.5 µm between two consecutive sections. Yellow arrows point GFP positive cardiomyocytes located on the apical myocardial surface revealing reduced MHC staining .

(D-E’’’) Whole mount immunofluorescence against GFP and MHC on a *myl7:Gal4;eGFP:UAS:RFP* (D-D’’’) and a *myl7:Gal4;eGFP:UAS:wt1b* (E-E’’’) embryo, at 3 dpf. DAPI was used for nuclear counterstain. Shown are maximum intensity projections of 20 stacks with a distance of 1 µm between two consecutive optical sections of the heart region. (D-D’’’) and (E-E’’’) are magnifications of the area of the ventricle marked by the dashed bounding boxes in D and E, respectively. Yellow arrowhead points to a GFP-positive cell that is MHC^{+} in D-D’’’ and to a GFP+/MHC- cell in E-E’’’).

(F-G) Time lapse images of the ventricle in a *myl7:Gal4; eGFP:UAS:RFP* (F) or *myl7:Gal4; eGFP:UAS:wt1b* (G) embryo between 2 and 3 dpf. Elapsed time since initial acquisition is stamped in each panel. Arrowhead in G point to a cell extruding from the ventricle.

(H) Time lapse images of the ventricle in a *myl7:Gal4; eGFP:UAS:wt1b* embryo between 5 and 6 dpf. Elapsed time since initial acquisition is stamped in each panel. Notice how a delaminating cell changes morphology along time and flattens down (Yellow arrowhead).

(I) Model of the delamination process of *wt1b* overexpressing cardiomyocytes.

Scale bar, 50 µm. at, atrium; CM, cardiomyocyte; ht, heart tube; IF, immunofluorescence; v, ventricle

Figure 3. Delaminated *wt1* overexpressing in cardiomyocytes start to express epicardial markers.

(A) Schematic representation of the lines used and the time points during which 4-hydroxytamoxifen (4-OHT) was administered to *myl7:CreERT2;β-actin:loxP-DsRed-loxP-eGFP-T2A-wt1a* fish (in short *myl7:CreERT2,eGFP-T2A-wt1a*), as well as embryo orientation for image acquisition.

(B-C’’’) Whole mount immunofluorescence against GFP and Aldh1a2 in a *myl7:Gal4;eGFP:UAS:RFP* (B-B’’’) and *myl7:Gal4;eGFP:UAS:wt1b* (C-C’’’) embryo, at 5 dpf. Shown are maximum intensity projections of 5 images with a distance of 1 µm between two consecutive optical sections. (B’-B’’’) Zoomed view of the boxed area in B. (C’-C’’’) Zoomed

view of the boxed area in C. White arrow, epicardial cells positive for Aldh1a2 and negative for GFP. Yellow arrows, epicardial cells that express both Aldh1a2 and GFP. Green, GFP; magenta, Aldh1a2; blue, DAPI.

(D-E’’’) Whole mount immunofluorescence against GFP and Caveolin 1 (Cav1) in a *myl7:Gal4;eGFP:UAS:RFP* (D-D’’’) and *myl7:Gal4;eGFP:UAS:wt1b* (E-E’’’) embryo, at 6 dpf. Shown are maximum intensity projections of 10 consecutive optical section with a distance of 1.5 µm between them. (D’-D’’’) Zoomed view of the boxed area in D. (E’-E’’’) Zoomed view of the boxed area in E. White arrows, epicardial cells positive for Cav1 and negative for GFP. Yellow arrows, epicardial cells that express both Cav1 and GFP. Green, GFP; magenta, Cav1; blue, DAPI.

(F-K’’’) Immunofluorescence against GFP (green), MHC (white) and Aldh1a2 (magenta) on paraffin sections of (F-H’’’) *myl7:CreERT2;eGFP-T2A-wt1a* and (I- K’’’) *myl7:CreERT2;eGFP-T2A-wt1a* + 4-OHT adult hearts. Shown are sections of the heart (F and I), as well as zoomed views of indicated regions (G-G’’’, H-H’’’, J-J’’’ and K-K’’’). Both, merged and single channels are shown, as indicated in the panel. White arrowheads, cells positive for Aldh1a2 only. Yellow arrowheads point to cells positive for GFP and Aldh1a2 signal that lack MHC staining, and which are located close to the myocardial surface.

(L-Q’’) Immunofluorescence against GFP (green), MHC (white) and Caveolin 1 (Cav1) (magenta) on paraffin sections of (L-N’’’) *myl7:CreERT2;eGFP-T2A-wt1a* and (O-Q’’’) *myl7::CreERT2;eGFP-T2A-wt1a* + 4-OHT adult hearts. Shown are sections of the heart (L and O), as well as zoomed views of indicated regions (M-N’’’ and P-Q’’’). Both, merged and single channels are shown, as indicated in the panel. White arroheads point to cells positive only for Cav1. Yellow arrowheads point to cells positive for GFP and Cav1 signal that lack MHC staining, and which are located close to the myocardial surface.

Scale bars: 500 µm (F, I, L, O) 50 µm (B, C, D, E, G, H, J, K, M, N, P, Q) and 10 µm (B’-B’’’, C’-C’’’, D’-D’’’ and E’-E’’’). at, atrium; Cav1, Caveolin1; MHC, Myosin Heavy Chain; v, ventricle.

Figure 4. Expression of cell junction and polarity markers in *wt1b*-overexpressing hearts.

(A) Schematic representation of the lines used and embryo orientation for imaging.

(B-E') Immunofluorescence against zonnula occludens 1 (ZO-1) and myosin heavy chain (MHC) in 5 days post fertilization (dpf) *myl7:Gal4;eGFP:UAS:RFP* (B-C') and *myl7:Gal4;eGFP:UAS:wt1b* (D-E') embryos. Shown are sagittal single planes of the ventricle. Single channels (B', C', D' and E') show ZO1 expression. (C-C') Zoomed views of the box in B'. White arrows point to ZO1 expression. (E-E') Zoomed views of the box in D'. Yellow arrows point to ZO1 expression.

(F-M') Immunofluorescence against tdTomato (tdT) (using a DsRed antibody) and MHC in 5 dpf *myl7:cdh2-tdTomato* (F-I') and *myl7:cdh2-tdTomato*;*myl7:Gal4;eGFP:UAS:wt1b* (J-M') embryos. (F-G) 3D projections of a heart. (G) Zoomed view of the box region in F'. (H-I') Sagittal single planes of the ventricle. (I-I') Zoomed view of the box in H'. White arrows point do regions with tdT expression. (J-K") 3D projections of a heart. (J") Zoomed view of the box in J'. (K-K") Zoomed views of the box in J. Yellow arrows point to delaminating cells from the ventricle. Notice the absent expression of tdT from the delaminated cells (K"). (L-M') Sagittal single planes of the ventricle. (M-M') Zoomed view of the box in L. Yellow arrows highlight tdT expression.

(N-Q') Immunofluorescence against Beta-catenin (β-cat) and myosin heavy chain (MHC) in 5 dpf *myl7:Gal4;eGFP:UAS:RFP* (N-O') and *myl7:Gal4;eGFP:UAS:wt1b* (P-Q') embryos. Shown are sagittal single planes of the ventricle. Single channels (N', O', P' and Q') show β-cat expression. LUT color shows gradient of β-cat signal intensity. Lower expression is in blue and the higher expression in orange to white. (O-O') Zoomed views of the box in N. (Q-Q') Zoomed views of the box in P. Marked region in P' indicates the ventricle.

(R-U') Immunofluorescence against Laminin (Lam) and MHC in 5 dpf *myl7:Gal4;eGFP:UAS:RFP* (R-S') and *myl7:Gal4;eGFP:UAS:wt1b* (T-U') embryos. Shown are sagittal single planes of the ventricle. Single channels (R', S', T' and U') show Laminin expression. LUT color shows gradient of laminin signal intensity. Lower expression is in blue

and the higher expression in orange to white. (S-S') Zoomed views of the box in R'. White arrows highlight Laminin expression. (U-U') Zoomed views of the box in P. Yellow arrows highlight Laminin expression.

Scale bar: 50 µm (B-B', D-D', F-F', H-H', J-J', L-L', N-N', P-P', R-R' and T-T'); 10 µm (C-C', E-E', G, I-I', J"-K", M-M', O-O', Q-Q', S-S' and U-U').

Lam, Laminin; tdT, tdTomato

Figure 5. Changes in cardiomyocyte maturation and structure upon *wt1b* overexpression.

(A) Schematic representation of the lines used and embryo orientation for stainings and imaging.

(B-F') Immunofluorescence against GFP and myosin heavy chain (MHC) on *myl7:Gal4; eGFP:UAS:RFP* and *myl7:Gal4;eGFP:UAS:wt1b* zebrafish embryos. (B-C') 3D projection of 1 day post fertilization (dpf) embryos. Shown are lateral views of the cardiac tube. Yellow asterisk in C' indicates absent MHC staining in the heart. (D) MHC staining of the myotome region of the *myl7:Gal4; eGFP:UAS:wt1b* embryo at 1 hpf. (E-F') 3D projections of the heart region at 6 dpf. Shown are ventral views, the head is to the top.

(G) Quantification of mean fluorescence intensity in the heart region for *myl7:Gal4; eGFP:UAS:RFP* and *myl7:Gal4;eGFP:UAS:wt1b* zebrafish, at indicated developmental stages. Statistical significance was calculated by unpaired t-test, with Welch's correction (24 hpf) and unpaired t-test for the remaining group comparisons. Means ±SD as well as individual measurements are shown.

(H-I) Whole mount mRNA *in situ* hybridization against *myl7* mRNA in (H) *eGFP:UAS:wt1b* and (I) *myl7:Gal4;eGFP:UAS:wt1b* zebrafish embryos at 3 dpf. Embryos are positioned ventrally, with the head to the top.

(J-K') Immunofluorescence against GFP and Alcam on *myl7:Gal4;eGFP:UAS:RFP* and *myl7:Gal4;eGFP:UAS:wt1b* zebrafish embryos. Shown are 3D projection of the heart region in a 6 dpf old larva (ventral views, the head is to the top).

(L) Quantification of mean fluorescence intensity of anti-Alcam staining as shown in K-L'. Statistical significance was calculated by unpaired t-test, with Welch's correction. Shown are mean ±SD as well as individual measurements.

(M-P') Immunofluorescence against GFP and Actinin. Shown are maximum intensity projections of two consecutive optical sections with a step size of 2 µm of the ventricle of *myl7:Gal4;eGFP:UAS:RFP* (M-N') and *myl7:Gal4;eGFP:UAS:wt1b* (O-P') at 6 dpf. (N,N' and P,P') Maximum intensity projections of boxed regions in (M) and (O), respectively.

(Q-R') Serial block face scanning electron microscope images of zebrafish hearts. Shown are single sections of *myl7:Gal4;eGFP:UAS:RFP* (Q-Q') and *myl7:Gal4;eGFP:UAS:wt1b* (R-R') hearts. Different cell layers are highlighted with colors. (Q' and R'). Zoomed areas highlighting sarcomeres. Green labels the epicardium, magenta marks the endocardial layer and orange highlights the myocardium. Orange arrowheads, z-bands; Cyan arrowhead, basement membrane delimiting epicardium and myocardium.

Scale bars, 50 µm (B-F'H-M and O-O'); 1 µm (Q and R), 500 nm (Q' and R'), 10 µm (N, N and P,P'). at, atrium; BM, basement membrane; CM, cardiomyocyte; dpf, days post fertilization; ECM, extracellular matrix; EnC, endothelial cell; EpC, epicardial cell; Ery, erythrocyte; v, ventricle; ; z, z-line. Green, GFP; magenta, MHC, Alcam; blue, DAPI.

Figure 6. Assay for Transposase-Accessible Chromatin sequencing (ATAC-seq) in *wt1b*-overexpressing cardiomyocytes.

(A) Schematic representation of cell acquisition for ATAC-seq.

(B) Graphical representation of number of differential accessible regions between *myl7:Gal4; eGFP:UAS:RFP* and *myl7:Gal4; eGFP:UAS:wt1b* cardiomyocytes at 5 days post-fertilization (dpf).

(C) Distribution of the genomic regions with differential accessible regions.

(D-F) Gene Ontology (GO) pathways enrichment for differential accessible regions repressed in cardiomyocytes after *wt1b*-overexpression. (D) Shown are selected GO

Biological pathways from the top 25 differential pathways. (E) Shown are selected GO Cellular components from the top 25 differential pathways. (F) Shown are selected GO Molecular functions from the top 25 differential pathways.

(G-G') Wt1 motif analysis. (G') Percentage of the differential accessible regions in which the Wt1 motif is represented.

(H) Venn Diagram comparing the number of differential accessible regions that are common between the ATAC-seq and the CHIP-atlas database for Wt1.

(I-L') Sequencing tracks for genes with differential peaks within their genomic loci. Shown are genes representative of adherens junctions: *cdh2* (I) and *ctnn1* (I'); apical polarity, *pard6b* (J); basal polarity, *scrib* (K); and sarcomere assembly: *rbfox2* (L) and *rybpb* (L'),

Figure 7. Overexpression of *wt1a* or *wt1b* in cardiomyocytes causes morphological changes in the zebrafish heart.

(A) Schematic representation of lines used and embryo orientation for imaging

(B-E') Immunofluorescence against GFP and myosin heavy chain (MHC) on *myl7:Gal4; eGFP:UAS:RFP* and *myl7:Gal4;eGFP:UAS:wt1b* zebrafish embryos. Shown are 3D projections of the heart region of 2 days postfertilization (dpf) embryos (B-C'), 6 dpf larvae (D-E').

(F) Quantification of the ratio of the atrium and ventricle volumes of 5 dpf zebrafish hearts. Statistical significance was calculated by unpaired t-test. Shown are mean ±SD as well as individual measurements.

(G-G') Quantification of the number of atrial (G) and ventricular (G') cardiomyocytes 5 dpf zebrafish hearts. Statistical significance was calculated by unpaired t-test. Shown are mean ±SD as well as individual measurements.

(H-I') Immunofluorescence against BrdU and myosin heavy chain (MHC) on *myl7:Gal4; eGFP:UAS:RFP* (H-H') and *myl7:Gal4;eGFP:UAS:wt1b* (I-I') zebrafish embryos. Shown are 3D projections of the heart region of 5 days postfertilization

(J-J') Quantification of the ratio of the BrDU+ cardiomyocytes per total number of cardiomyocytes in the atrium (H) and ventricle (H') 5 dpf zebrafish hearts. Statistical significance was calculated by unpaired t-test. Shown are mean ±SD as well as individual measurements.

(K) Schematic representation of the time points during which 4-hydroxy-tamoxifen (4-OHT) was administered to *myl7:CreERT2;β-actin:loxP-DsRed-loxP-eGFP-T2A-wt1a* fish (in short *myl7:CreERT2;eGFP-T2A-wt1a)*. Controls are *myl7:CreERT2;eGFP-T2A-wt1a* that were not treated with 4-OHT. Hearts were collected at 12 months postfertilization (mpf).

(L-M) Bright field images of whole mount adult zebrafish hearts untreated (L) and treated with 4-OHT during embryogenesis (M).

(N-O) micro-computed tomography (micro-CT) image of adult heart of untreated (N) and fish treated with 4-OHT (O) during embryogenesis.

(P-Q) 3D volumetric rendering of 3D images acquired with a microCT of adult hearts. (P) untreated, (Q) 4-OHT treated during embryogenesis.

(R-R') Quantification of chambers volumes of adults *myl7:CreERT2;eGFP-T2A-wt1a* that were not treated with *4-OHT* hearts. (R) Shown are the differences in ventricle volume between recombined and non-recombined hearts. Each point represents one heart. (R') Quantification of the differences in atrium volume between recombined and non-recombined hearts. Each point represents one heart. (R") Quantification of the ratio between the volume of the atrium and the ventricle from micro-CT images

acquired from heart of the two experimental groups. Each point represents one heart. Statistical significance was calculated with an unpaired t-test. Shown are means ±SD. Scale bar: 50 μm (B-E' and H-I') and 500 μm (L-Q). at, atrium; v, ventricle; ba, bulbus arteriosus.

FIGURE LEGENDS SUPPORTING FIGURES

Figure 1, supplement 1. Wt1 expression and changes in histone modifications within the regulatory regions of Wt1 during in vitro differentiation of mouse embryonic stem cells towards cardiomyocytes

The graph shows changes in the transcription profile of Wt1 in mouse embryonic stem cells (mESC), mesoderm (M), cardiac precursor (CP) and cardiomyocytes (CM). Also shown are changes in chromatin activation and repression marks, for H3K27ac and H3K27me3 respectively.

Figure2, supplement 1. Validation of the *wt1a* and *wt1b* overexpression lines

(A) Schematic representation of the time points during which 4-Hydroxytamoxifen (4-OHT) was administered to *myl7:CreERT2;β-actin:loxP-DsRed-loxP-eGFP-T2A-wt1a* fish (in short *myl7:CreERT2,eGFP-T2A-wt1a*).

(B-E) qRT-PCR for *GFP* (B,D) and *wt1a* (C,E) on adult heart cDNA from *myl7:CreERT2, eGFP-T2A-wt1a* with and without 4-OHT. qRT-PCR was performed on cDNA obtained from the atrium (B,C) and (D,E) ventricles. Points represent biological replicates, 3 for each group. Statistical significance was calculated using one-way ANOVA. Shown are means ±SD.

(F) Schematic representation of lines used and the time at which RNA was extracted.

(G-H) qRT-PCR for *eGFP* (G) and *wt1b* (H) in *eGFP:UAS:wt1b* and *myl7:Gal4;eGFP:UAS:wt1b* hearts at 40 days post fertilization (dpf). The points represent

biological replicates. Statistical significance was calculated with an unpaired t-test. Shown are also means ±SD.

Figure 2, supplement 2. Overexpression of *tcf21* transcription factor in cardiomyocytes and *wt1b* in non-cardiomyocytes does not affect heart development.

(A) Schematic representation of the lines used and embryo orientation for imaging.

(B-G") Immunofluorescence against GFP and myosin heavy chain (MHC) on *myl7:Gal4;eGFP:UAS:wt1b*, *myl7:Gal4;eGFP:UAS:tcf21, fli1a:Gal4;eGFP:UAS:wt1b* and *nfatc1:Gal4;eGFP:UAS:wt1b* zebrafish embryos, at 3 or 5 days post fertilization (dpf). (B-B") Shown are 3D projections of a *myl7:Gal4;eGFP:UAS:wt1b* heart in a ventral view, at 5 dpf. (B'-B") show single channels for GFP and MHC. The box highlights a zoomed region in the heart where a cluster of delaminating cells can be seen. (B") Notice the absence of MHC in the delaminated cells. (C-D") Shown are 3D projections of a *myl7:Gal4;eGFP:UAS:tcf21* heart in a ventral view, at 5 dpf. (C-C") show single channels for GFP and MHC. The box highlight a zoomed region in the heart with one cell delaminating. Notice in C" that the delaminating cell preserved MHC expression. (E-E") Shown are maximum intensity projections of 5 stacks with a distance of 1.5 µm between two consecutive optical sections of the heart region of a *nfatc1:Gal4;eGFP:UAS:wt1b* heart in a ventral view, at 5 dpf. GFP expression is observed in the valve region. The amount of embryos with delaminating cells is indicates in the panels. Green, GFP; magenta, MHC. Scale bar 50 µm and 10 µm, in the zoom boxes .at, atrium; v, ventricle.

Figure 3, supplement 1. *wt1a* overexpression in cardiomyocytes express epicardial markers in the adult heart

(A) Schematic representation of the time points during which 4-hydroxytamoxifen (4-OHT) was administered to *myl7:CreERT2;β-actin:loxP-DsRed-loxP-eeGFP-T2A-wt1a* fish (in short *myl7:CreERT2,eeGFP-T2A-wt1a*).

(B-E') *in situ* mRNA hybridization against *tgm2b* and immunohistochemistry against eGFP on paraffin sections of *myl7:CreERT2,eeGFP-T2A-wt1a* (B-C') and *myl7:CreERT2,eeGFP-T2A-wt1a* + 4-OHT (D-E') adult hearts. (B,C,D and E) Images of sections after *in situ* mRNA hybridization against the epicardial marker *tgm2b.* (B', C', D' and E'), same section as in B, C, D and E, after eGFP immunohistochemistry. Black arrows in E and E' indicate double positive cells for *tgm2b* and eGFP.

Scale bars: 200 μm (B,B',D and D'), 50 μm (C,C',E and E').

Figure 6, supplement 1. Gene ontology pathways of differential accessible regions for Wt1 motif enriched peaks

Gene Ontology (GO) pathways enrichment for differential accessible regions for the regions that contain Wt1 motif. Shown are the top 25 Biological, cellular components and the Molecular function pathways.

Figure 7, supplement 1. Heart looping and function are is impaired upon overexpression of wt1b in cardiomyocytes.

(A) Schematic representation of the lines used and embryo positioning for image acquisition.

(B-C) Time lapse images of heart looping in (B) *myl7:Gal4;eGFP:UAS:RFP* and (C) *myl7:Gal4; eGFP:UAS:wt1b* embryos between 2 and 3 days post-fertilization (dpf). Elapsed time since initial acquisition is stamped in each panel. Shown are ventral views with the head to the top.

(D) Schematic representation of calculation of heart looping.

(E) Quantification of the looping angle between the ventricle and the atrium at 5 dpf. Statistical significance calculated with unpaired t-test, with Welch's correction. Each point represents one heart. Shown are means ±SD.

(F) Schematic representation of parameters used to determine cardiac function in *myl7:Gal4;eGFP:UAS:RFP* and *myl7:Gal4; eGFP:UAS:wt1b.*

(G) Quantification of ventricular stroke volume at 2 days post fertilization (dpf) and 5 dpf. Statistical significance was calculated with the Mann-Whitney test.

(H) Quantification of the heart rate at 2 dpf and 5 dpf. Statistical significance calculated with an unpaired t-test.

(I) Quantification of ventricular ejection fraction at 2 dpf and 5 dpf. Statistical significance was calculated with an unpaired t-test for the comparison between groups in the atrium and in the ventricle at 2 dpf. Mann-Whitney test was applied to calculate the statistical significance between the groups in the ventricle, at 5 dpf.

In all graphs each point represents one embryo. Shown are also means ±SD.

Scale bars, 50 µm. at, atrium; v, ventricle.

Figure 7, supplement 2. Morphological changes due to *wt1* overexpression in cardiomyocytes are sustained in larval and adult hearts.

(A) Schematic representation of the lines used and the developmental stages at which hearts were analyzed.

(B) 40 day post fertilization (dfp) juvenile *myl7:Gal4:eGFP:UAS:RFP* fish. (C) 40 dpf juvenile *myl7:Gal4:eGFP:UAS:wt1b* fish. Arrow points to pericardial edema.

(D-D') Dissected heart of a *myl7:Gal4:eGFP:UAS:RFP* and (E-E') *myl7:Gal4:eGFP:UAS:wt1b* fish at 40 dpf. Notice the enlarged and dysmorphic atrium.

(F-F') Midline section of a *myl7:Gal4:eGFP:UAS:RFP* and (G-G') *myl7:Gal4:eGFP:UAS:wt1b* fish heart at 40 dpf. Notice the high degree of myocardial tissue within the atrium of the *myl7:Gal4:eGFP:UAS:wt1b* heart.

(H-M) AFOG staining on paraffin sections of *myl7:CreERT2,eGFP-T2A-wt1a* hearts non-recombined (H-J) or recombined (+ 4-OHT during embryogenesis) (K-M). (H) Whole heart section. (K), ventricle. (K), corresponding atrium from the same animal. (I, J, L and M) Zoomed views of boxed areas in H and K.

(N-Q") Immunofluorescence against GFP (green), MHC (white) and Col1a1 (magenta) on adult atria cryosections of non-recombined (N-O") and embryonically recombined (+4-OHT) *myl7:CreERT2,eGFP-T2A-wt1a* fish (P-Q"). (O-O") Enlarged image of the boxed area in N. In *myl7:CreERT2,eGFP-T2A-wt1a* non recombined atria, Col1a1 staining is delimited to the

valves and no GFP signal is detected. (Q-Q") Enlarged image of the boxed area in P. In recombined *myl7:CreERT2,eGFP-T2A-wt1a* atria, Col1a1 staining is visible in myocardial areas close to GFP-positive cells.

Scale bar: 1 mm (B and C); 500 µm (F-H and K); 200 µm (D-E', I, J, L, M-N" and P-P"); 10 µm (O-O" and Q-Q") .

at, atrium; v, ventricle; ba, bulbus arteriosus.

Video 1. *epi*:eGFP-positive cells at the venous pole switch off GFP expression and start expressing *myl7*:mRFP when entering the heart tube.

In vivo time-lapse imaging of a *epi:GFP;myl7:mRFP* heart between 52 hpf and 68 hpf. The yellow arrow highlights a cell that initially is only GFP positive and latter stops expressing GFP and starts to express RFP. The cyan arrows point to cardiomyocytes in the heart tube that are still GFP -positive at the beginning of the video and then loose GFP signal concomitant with increase in mRFP signal intensity. Images were acquired with the Leica TCS SP8 DLS. Shown is a single plane reconstruction of the beating. Scale bar, 50 µm.

Video 2. Apical delamination of a *wt1b*-overexpressing cardiomyocyte in a cardiac ventricle at 2 dpf.

In vivo time-lapse imaging of a *myl7:Gal4;eGFP:UAS:RFP* and a *myl7:Gal4;eGFP:UAS:wt1b* heart between 2 and 3 days post fertilization (dpf) acquired with the Leica TCS SP8 DLS confocal microscope, using the digital light sheet (DLS) mode. Shown is the reconstruction of a single plane of the beating ventricle. Notice the rounded cells extruding from the ventricle in the *myl7:Gal4;eGFP:UAS:wt1b* heart (right panel, arrow). Scale bar, 50 µm.

Video 3. Apical delamination of a *wt1b*-overexpressing cardiomyocyte in a cardiac ventricle at 5 dpf.

In vivo time-lapse imaging of a *myl7:Gal4;eGFP:UAS:wt1b* heart between 5 and 6 days post fertilization (dpf) acquired with the Leica TCS SP8 DLS confocal microscope, using the digital

light sheet (DLS) mode. Shown is the reconstruction of a single plane of the beating ventricle. Notice how the extruded cells flatten down during the time course of the video. Scale bar, 50 µm.

Video 4. Serial block face scanning z-stacks through a control zebrafish heart at 5 dpf.

Serial stacks through a ventricle from a *myl7:Gal4;eGFP:UAS:RFP* control embryo at 5 dpf. Images were obtained by serial block face scanning electron microscopy. Notice the compact organization of the myocardium and the close connection between the myocardium and the endocardium, and how the sarcomeres form a continuous structure between adjacent cardiomyocytes. Of remark is also the dense border between the myocardium and epicardium. EpC, epicardial cell, EnC, endothelial cell, Ery, erythrocyte, CM, cardiomyocyte nuclei. Scale bar 10 µm.

Video 5. Zoomed view of a serial block face scanning z-stacks through a control zebrafish heart at 5 dpf.

Serial stacks through a ventricle from a *myl7:Gal4;eGFP:UAS:RFP* control embryo at 5 dpf. Images were obtained by serial block face scanning electron microscopy. Shown is a magnification of the myocardium in a region where sarcomeres can be observed. Notice the clearly marked z-lines and the longitudinal continuity of the sarcomeres between adjacent cardiomyocytes. Scale bar, 500 nm.

Video 6. Serial block face scanning z-stacks through a *wt1b*-overexpressing heart at 5 dpf.

Serial stacks through a ventricle from a *myl7:Gal4;eGFP:UAS:wt1b* embryo at 5 dpf. Images were obtained by serial block face scanning electron microscopy. Notice the absence of a compact and organized myocardial layer and the enlarged cardiac jelly separating the endocardium and the myocardium. Also visible the extensive areas filled with extracellular

matrix. EpC, epicardial cell, EnC, endothelial cell, Ery, erythrocyte, CM, cardiomyocyte, ECM, extracellular matrix, v, ventricle, at, atrium. Scale bar, 20 µm.

Video 7. Zoomed view of a serial block face scanning z-stacks through a *wt1b*-overexpressing heart at 5 dpf.

Serial stacks through a ventricle from a *myl7:Gal4;eGFP:UAS:wt1b* embryo at 5 dpf. Images were obtained by serial block face scanning electron microscopy. Shown here is a magnification of the myocardium in a region where sarcomeres can be observed. Notice the z-lines. Remarkable is the disorganized arrangement of the sarcomeres between adjacent cardiomyocytes. Scale bar, 500 nm.

Video 8. Heart looping is impaired in *wt1b*-overexpression hearts.

In vivo time-lapse imaging of a *myl7:Gal4;eGFP:UAS:RFP* and *myl7:Gal4;eGFP:UAS:wt1b* heart between 2 and 3 days post fertilization. Images were acquired with the Leica TCS SP8 DLS. Shown is a full 3D reconstruction of the beating heart through the looping process. Scale bar, 50 µm.

Video 9. z-stack through micro-computed tomography (µCT) acquisition of a *myl7:CreERT2;eGFP-T2A-wt1a* heart.

Serial stack of a *myl7:CreERT2;eGFP-T2A-wt1a* heart obtained with a µCT scan of an adult thoraxic cavity, used to evaluate the volume of the chambers of the heart. Marked are the bulbus arteriosus (ba), the ventricle (v) and the atrium (at). Scale bar, 500 µm.

Video 10. z-stack through micro-computed tomography (µCT) acquisition of a *myl7:CreERT2;eGFP-T2A-wt1a* heart recombined during embryogenesis.

Serial stack of a *myl7:CreERT2;eGFP-T2A-wt1a* heart recombined between 24 hours post fertilization (hpf) and 4 days post fertilization (dpf), obtained with a µCT scan of an adult thorax,

1610 used to evaluate the volume of the chambers of the heart. Marked are the bulbus arteriosus
1611 (ba), the ventricle (v) and the atrium (at). Scale bar, 500 µm.
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