Complementary functions of the mechanosensitive factors *egr1*, *klf2b* and *klf2a* instruct the endocardial program

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Abstract

Mechanical forces are key modulators of valvulogenic developmental programs. However, the mechanosensitive gene network underlying this process remains unclear. It is well established that contractile and flow forces activate endocardial expression of the transcription factor *klf2a* during valve morphogenesis. We report two novel transcription factors with a function in heart valve formation in zebrafish: *egr1* and *klf2b*. Genome-wide analysis of gene expression reveals that the endocardial transcriptional programs modulated by *klf2a*, *klf2b*, and *egr1* mainly contain non-redundant targets. Several of these targets have been implicated in endothelial-to-mesenchymal transition (EMT). Many of the deregulated genes exhibit changes in chromatin accessibility pointing to potential direct effects of these factors. Finally, *in vivo* phenotypic analyses show that VEGF receptor 1 (*flt1*) is a target of *egr1* and *klf2b* during early valvulogenesis. These findings suggest that *klf2a*, *klf2b*, and *egr1* cooperate for the activation of EMT program in response to mechanosensitive inputs. We propose that the combinatorial action of these factors mediates flow mechanotransduction to control the endocardial program, especially for valve development.

Introduction

Atrioventricular septal defects (AVSDs) resulting from abnormal fetal and embryonic valve development are the leading cause of Congenital Heart Disease (CHD) [1]. CHD is the most frequently occurring congenital disorder at approximately 1% of live births, with similar prevalence throughout the world (CDC statistics) [2, 3]. Although the diagnosis and treatment of CHDs have significantly improved over recent decades, the complexity of this disorder still represents a challenging medical issue. A complete understanding of the molecular mechanisms governing heart septation and valve development is therefore of particular importance.

In vertebrates, the heart is one of the first organs to be formed. It is well established that the mechanical forces induced by the development of the heart trigger cell signaling events. Although the mechanisms controlling valve leaflet growth and shape are well known, how those mechanical forces interact with signaling pathways to regulate valve formation remains unclear. Zebrafish is emerging as an invaluable model system to study the influence of mechanical forces in cardiac valve formation [4-8]. In zebrafish, the atrioventricular (AV) valves emanate from the endocardial cells (EdCs) [9]. EdCs first cluster at the AV boundary (or atrioventricular canal, AVC) and form an endothelial ring that lines the AV canal between 24 and 48 hpf [4]. This initial clustering is followed by the appearance of cellular extensions towards the extracellular matrix, or cardiac jelly, which is the elastic acellular layer separating myocardium and endocardium [10, 11]. Subsequent tissue remodeling occurs to form multilayered endocardial cushions consisting of ventricular and AVC-derived EdCs within the cardiac jelly, which in turn overlays the atrial-derived EdCs exposed to the lumen [12, 13].

Heart valve development begins with the formation of cardiac cushions at the sites of future valves [14, 15]. In mouse, cushion formation is initiated with endocardial-mesenchymal transformation (EMT), a process in which endocardial cells delaminate and transform into mesenchymal cells to invade the matrix that separates the endocardial and myocardial cell layers [16]. In zebrafish the hypothesis that heart valve development relies on EMT processes remains to be elucidated. Recent studies describing how migratory cell behaviors are involved in heart valve morphogenesis suggest that EdCs may undergo partial EMT and migrate into the cardiac jelly as a coordinated sheet of cells [12, 13]. Given the clinical potential of stem cell-based valve repair, there is a growing interest in understanding the genetic programs and developmental pathways regulating valve formation [15]. Endocardial cells respond to local signals generated by the myocardium, such as components of the BMP and TGF- β signaling pathways, by delaminating and migrating while undergoing EMT. This complex process also

involves inputs from the Notch and Wnt signaling pathways, in addition to Erbb and VEGF signaling operating within the EdCs [17]. Endothelial cell remodeling is usually controlled by VEGF signaling [18, 19] *Flt1* is one of the four VEGF receptors in zebrafish. Both zebrafish forms of the receptor (membrane mFlt-1 and soluble sFlt-1) were shown to be essential to establish and maintain angiogenesis within a VEGF-Notch Feedback Loop [20]. In heart, *flt1* expression is first detected in the endocardium at 26 hpf, and seems involved in heart development acting upstream of the phospholipase C gamma 1 (PLCgamma1) to control cardiac ventricular contractility [21]. To date, however, VEGF signaling role during cardiac valve formation is unclear and endocardial growth is thought to occur independently of VEGF signaling[22]. Despite the well-established link between EdCs and valve development, how mechanical forces control EMT to modulate valve morphogenesis *in vivo* remains unclear.

Valve morphogenesis relies on the coordinated interplay between transcription factors and mechanical forces generated by the blood flow [7, 23]. Blood flow is critical for cell shape change and cell migration occurring at the onset of valvulogenesis in the cardiac jelly [24]. EdCs can sense a variety of biomechanical stimuli that reflect different patterns of blood flow, such as wall and fluid shear stress and transmural pressure and transduce them into internal signals [25]. These signals trigger the activation of flow-responsive genes in specific regions of the valve, including the zinc-finger transcriptional regulator *klf2* [24, 26]. Interestingly, KLF transcription factors have been associated with cerebral cavernous malformations (CCM), which are lined by endothelial cells undergoing EMT [27, 28]. However, *klf2* expression and function are not restricted to the AVC [24, 25]. Indeed, *klf2* also plays a role in ventricular trabeculation [29] and in controlling the shape of endothelial cells located in the ventricle [22], suggesting that other flow-inducible transcription factors are required in the AV canal to modulate specific AVC cell responses to flow. A possible candidate could be the only paralog of *klf2a* in zebrafish, *klf2b*, of unknown function in valve morphogenesis. The stress-activated transcription factor *egr1* is expressed in the developing zebrafish AVC, and it is therefore likely to be involved in valvulogenesis [30]. Moreover, *egr1* has been associated with pathologic cardiac hypertrophy and atherosclerosis in human adults [31], and Egr1 protein has been detected in calcified but not normal aortic valve cusps [32]. The potential roles of *egr1* and *klf2b* in cardiac valve formation therefore need to be investigated.

In this study, we uncover a novel function for *egr1* and *klf2b* as mechanosensitive factors during AV valve morphogenesis and identify their downstream transcriptional targets in the endocardium. When compared to *klf2a*, *egr1* and *klf2b* have a wide range of unique targets involved in different aspects of endocardial signaling, in particular in the regulation of EMT,

including WNT, BMP, Notch, and TGF- β pathways. Importantly, we show that chromatin accessibility is differentially affected in *egr1* and *klf2a* mutants. Our results on gene expression and chromatin accessibility suggest that these transcription factors regulate different networks involved in endocardial development. Finally, we identified *flt1* as a target of *egr1/klf2b* but not *klf2a* and found it is important for early valve morphogenesis. These data uncover the gene networks and signaling pathways that control endocardial response and heart valve morphogenesis downstream of mechanical stimuli.

Results

***Egr1* is a flow-responsive gene**

We analyzed *egr1* expression at the AVC in wild-type (WT) zebrafish embryos during valvulogenesis between 24 and 48 hpf (hours post fertilization). Whole-mount In Situ Hybridization (WISH) revealed that *egr1* is expressed in the central nervous system and heart from 30 hpf onwards (S1 Fig). At 48 hpf, *egr1* expression becomes restricted to a small subset of cells at the AV constriction initiation and an additional region in the Out-Flow Tract (OFT) similarly to *klf2a* [24] and *klf2b* (S1 Fig), where the second set of zebrafish valves develop. *egr1* expression was maintained at a high level at 72 hpf in the cardiac area (S1 Fig).

To assess whether *egr1* responds to mechanical forces associated with blood flow, we examined *egr1* expression in mutants with abnormal blood flow. Lack of *troponin T2A* in *silent heart* (*sih*^{-/-} or *tnn2a*^{-/-}) mutants causes the loss of cardiac contractility and blood flow [33], whereas the "bloodless" *Vlad Tepes* mutant (*vlt*^{m651} or *gata1*^{-/-}) [34] has few to no blood cells at the onset of circulation due to a severe reduction in blood cell progenitors [35]. *egr1* expression was absent in *silent heart* mutants (S2 Fig) and was decreased in *Vlad Tepes* mutants (S2 Fig). Together these results reveal that *egr1* is specifically expressed in valve-forming areas in the same developmental time-window as *klf2a* [24]. Moreover, this *egr1* expression pattern is dependent on cardiac contractility and blood flow.

***Egr1* and *klf2b* are required for valve formation**

Considering their expression patterns in the AVC, we generated *klf2b* mutants by targeting the first exon to create a premature stop codon (*klf2b*^{ig5}) and used a mutant allele of *egr1* (Figure 1A) to investigate their function in valvulogenesis. We examined the cardiac phenotype of *klf2b*^{ig5} and *egr1*^{sa64} mutants. We found that 20% of the *egr1*^{sa64} maternal zygotic mutants exhibited a pericardial edema visible at 72 hpf and 80% survived to adulthood. Homozygous viable adults were also obtained with *klf2b*^{ig5} single mutants (Figure 1A). We analyzed valve morphology with BodiPy dye staining [13] at 96 hpf, when the AV valve leaflets can be seen extending into the lumen of the AVC in WT. 50% of *egr1*^{sa64} and *klf2b*^{ig5} maternal zygotic mutants showed valvular defects similar to those described previously for *klf2a*^{ig4} mutants [13] (Figure 1B and C).

To investigate whether defects in EdCs remodeling cause the AV valve phenotypes, we performed photo-conversion experiments using *egr1* mutant and WT embryos expressing a photo-convertible protein in endothelial cells, including the endocardium (*Tg(fli1a:Gal4FF*,

UAS:Kaede) [36]. In WT embryos, EdCs maintain cell-cell contact and form a multilayered structure during valve morphogenesis. *egr1* mutants mainly show “clump with mixing” phenotypes, indicating that cell migration during valve morphogenesis is abnormal in these mutants. “Mixed” phenotypes occur when cell adhesion is not properly controlled during cell migration, resulting in mixing of cells from different valve regions. In contrast, in “thick” and “clump” phenotypes, the ability of cells to migrate as a coordinated sheet seems unaffected, but an additional cell layer appears inside the valve leaflet (Figure 1D and E). The *egr1* mutant valve morphology phenotypes suggest a potential role of these factors in regulating the cellular remodeling processes driving mature cushion formation.

***egr1*, *klf2a*, and *klf2b* control diverse transcriptional programs in the endocardium**

Since the expression of *egr1* and *klf2* is restricted to the EdCs that overlie the cardiac jelly, the valve morphology phenotypes of *egr1* and *klf2* mutants likely reflect perturbations in signaling from the endocardium. To identify these signaling events, we performed RNA-seq in EdCs isolated from 48 hpf *egr1* and *klf2a* and *b* mutants and control embryos (*fli:gal4FF;UAS:Kaede*) (Fig 2A). We chose this time point because *egr1*, *klf2a*, and *klf2b* are all strongly expressed in the AVC. By using a stringent cut-off of 3-fold, we identified 675 up- and 719 down-regulated genes ($p < 0.05$) in *egr1* mutants (Fig 2B and Supplementary Table 1), with down-regulated genes enriched for the categories “heart development” and “heart morphogenesis” ($P = 3.94E-04$ and $P = 2.2E-03$, respectively) (Fig 2C and Supplementary Table 1). Moreover, we identified 485 up- and 1137 down-regulated transcripts in *klf2a* EdCs (cut-off 3-fold, $p < 0.05$) (Fig 2B, and Supplementary Table 1) with a distinct enrichment for categories such as “embryonic heart tube development” ($P = 1.04E-04$), “heart development” ($P = 2.36E-04$), and “heart morphogenesis”, ($P = 0.033$) (Fig 2C and Supplementary Table 1) in the down-regulated genes. Similar experiments in *klf2b* mutants identified 2369 up- and 652 down-regulated genes more than 3-fold ($P < 0.05$) (Fig 2B). Thus, in contrast to *klf2a*, which mostly activates gene expression, *klf2b* appears to retain a repressive role. Nevertheless, similar to *klf2a* mutants, genes involved in “heart development” ($P = 2E-02$) were found exclusively in the down-regulated list (Fig 2C and Supplementary Table 1). These results show that *egr1* is both an activator and a repressor of gene expression, whereas *klf2a* is mostly an activator and *klf2b* is mostly a repressor. Importantly, genes involved in cardiac development are down-regulated in all these mutants.

To further explore the function of *egr1* and *klf2* in valve morphogenesis, we compared the sets of deregulated genes between the mutants. 47 genes were upregulated (hypergeometric test [HGT] $P=4.628E-18$) and 99 genes were down-regulated (HGT $P=3.114E-31$) in both *egr1* and *klf2a* (Fig 2D). Additionally, 126 genes were up-regulated (HGT $P=1.594E-14$) and 55 genes were down-regulated (HGT $P=1.417E-15$) in both *egr1* and *klf2b* mutants (Fig 2D). From the 1622 deregulated genes in *klf2a* and 3021 genes deregulated in *klf2b*, 197 genes were common to both datasets (HGT $P=6.10E-22$). Specifically, 58 genes were up-regulated (HGT $P=0.026$) and 139 down-regulated (HGT $P=3.663E-64$) (Figure 2D) in both *klf2a* and *klf2b*. As these KLF factors seem to have opposing roles, we asked whether genes up-regulated in *klf2a* mutants were down-regulated in *klf2b* mutants and vice-versa. Only 14 genes were up-regulated in *klf2a* mutants and down-regulated in *klf2b*, whereas 62 genes were down-regulated in *klf2a* mutants and up-regulated in *klf2b* (Supplementary Table 1). This comparative transcriptional analysis suggests that despite their similar cardiac development phenotypes, *egr1*, *klf2a* and *klf2b* regulate different gene networks. To further explore this possibility, we compared the datasets between the three mutants using Ingenuity Pathway comparative Analysis (IPA). We first analyzed the canonical pathways enriched in each mutant when compared to the others. Surprisingly, *egr1* and *klf2a* mutants showed similar trends with negative activation z-score, pointing to pathway inhibition, for instance in “Role of NFAT in cardiac hypertrophy” or “VEGF family ligand-receptor interactions”. However, *klf2b* mutants exhibited positive z-score, pointing to activation, in the same pathways (Fig 2E). These results suggest an overall trend whereby *egr1* and *klf2a* regulate the same pathways as *klf2b* but in opposite ways (Fig 2E). Moreover, several developmental pathways were affected in the mutants (Fig 2E). Additionally, we performed upstream regulator analysis using IPA to discover potential transcription factors that could lie upstream of our deregulated genes lists. Upstream regulators involved in developmental pathways such as Wnt, BMP, and TGF- β were enriched in all datasets, and these results were confirmed by qPCR for many genes (Fig 2F and S3A Fig). Again, this analysis showed similarities between *egr1* or *klf2a* in comparison to *klf2b* mutants (Fig 2F).

Collectively these results show that *egr1*, *klf2a*, and *klf2b* regulate several developmental signaling pathways in cardiac development, and suggest that *klf2a* has an activator role in contrast to *klf2b*.

***egr1*, *klf2a*, and *klf2b* control unique valve-related transcriptional programs and developmental signaling pathways**

To gain a better mechanistic insight into the function of *egr1*, *klf2a*, and *klf2b* in heart and valve development, we analyzed specific target genes with known roles in these processes. Considering that genetic compensation can significantly alter gene expression in ZF mutants[37], we assessed potential crossregulation between *egr1*, *klf2a*, and *klf2b*. Contrary to Rasouli et al. [29] we did not detect significative compensatory expression of klf factors in our *klf2* mutants, suggesting that our alleles do not activate genetic compensation [38]. Rather, we found that *egr1* was significantly downregulated in *klf2b* mutants (S3A and Supplementary Table 1), potentially explaining some of the shared common targets.

We next focused on the wnt signaling pathway which is key for valve development[12, 26]. We found that 18 genes in the Wnt signaling pathway were deregulated in at least one of the mutants (Fig 3B and Supplementary Table 1). Interestingly, there was little overlap of deregulated genes between the mutants, except for *wnt9b*, which was down-regulated in *egr1*, *klf2a*, and *klf2b* mutants (Fig 3A and 3B and Supplementary Table 1). *wnt2ba* and *wnt10a* were both significantly up-regulated in *egr1* mutants, while *wnt16* was up-regulated in *klf2a* mutants. *daam2* and *wnt5a* were the only genes in this pathway up-regulated in *klf2b* mutants (Fig 3B and Supplementary Table 1). A number of other Wnt-related genes were significantly down-regulated in at least one of the three mutants. Thus, although *egr1*, *klf2a*, and *klf2b* converge in activating *wnt9b* expression, they regulate distinct genes of the Wnt pathway.

Our previous research shows that the major endocardial response during AV valve maturation between 48 and 56 hpf relies on the activation of ECM-related genes [13]. Consistent with these findings, we found that *has2*, a key biosynthetic enzyme of the ECM component hyaluronan [39], was significantly down-regulated in *egr1* mutants, however, *fibronectin1b* (*fn1b*) was specifically up-regulated (Fig 3C and Supplementary Table 1). A number of collagen-encoding genes were down-regulated in *klf2a* mutants (S3B Fig and Supplementary Table 1). Strikingly, although *has2* was down-regulated in *klf2b* mutants, more than 20 different collagen-encoding genes were up-regulated (S3B Fig and Supplementary Table 1). These results indicate that *egr1*, *klf2a*, and *klf2b* have distinct ECM targets and likely cooperate to modulate ECM synthesis in the endocardium.

TGF- β signaling is a well-known regulator of valve morphogenesis [40]. As expected, a number of TGF- β signaling pathway members were deregulated in *egr1*, *klf2a*, and *klf2b* mutants (Fig 3D and Supplementary Table 1). Moreover, we also identified targets in Notch signaling, which is an essential regulator of valve development and cardiac maturation (Fig 3E and Supplementary Table 1). Interestingly, although Notch receptors, ligands and effectors

were deregulated in *klf2a* and *klf2b* mutants, these targets were not overlapping. Indeed, *klf2b* seems to modulate Notch ligands and receptors (*dll4* is down-regulated but *notch1a* and *jag1a* are up-regulated), whereas *klf2a* regulates Notch effectors, such as *her 3, 6, 9, 4.3* (all down-regulated) (Fig 3E and Supplementary Table 1). Similarly, BMP signaling components and its SMAD effectors were deregulated in *klf* mutants, but these targets did not fully overlap. For instance, effectors such as *bmp2a, 7b, and 7a* were down-regulated in *klf2a* mutants, whereas *bmp5* and *smad4a* were down-regulated in *klf2b* mutants (Fig 3F and Supplementary Table 1). Only *bmpr1ba* was down-regulated in both *klf* mutants. These results indicate that Klf2a and Klf2b regulate the expression of different genes in the Notch and BMP pathways and therefore have a non-redundant role in cardiac development.

Our Notch pathway target analysis suggests that Klf2a and Klf2b act independently of Egr1. In agreement with these findings, a number of genes in the prostaglandin signaling pathway, which is also involved in modulating valve development in response to mechanical forces [41], was significantly affected in *klf2a* and *klf2b* but not in *egr1* mutants (S3C Fig and Supplementary Table 1). Similarly, FGF signaling was abnormal in *klf2a* and *klf2b* [29] but minimally affected in *egr1* mutants (S3D Fig and Supplementary Table 1).

ErbB signaling plays an important role in trabeculation and EMT. Several genes in this pathway coding for tyrosine kinase receptors (*erbb3b, 4a, 4b, and 3*) were up-regulated in *klf2b* mutants. Consistent with this, ligands of *erbb3* and *erbb4*, *nrg* (encoded by *nrg2a* and *nrg2b*) required for normal heart trabeculation [42, 43] were up-regulated in *klf2b* mutants (S3E Fig and Supplementary Table 1). In contrast, these Erb ligands were down-regulated in *klf2a* mutants (S3E Fig and Supplementary Table 1). As these genes have been implicated in EMT, we next examined all the EMT-related genes identified in mouse. Out of 46 deregulated genes in this list, only a single gene was commonly misregulated between *klf2a* and *klf2b* (*tbx20*) or between *egr1* and *klf2a* (*msx2a*) (S3F Fig).

Together, these results suggest that whilst Klf2a, Klf2b, and Egr1 share a number of common targets, each of these factors exhibits unique regulatory mechanisms to modulate genes involved in EMT.

***egr1* mutants exhibit reduced chromatin accessibility in TGF- β and Wnt pathway-related genes**

Given that *egr1* and *klf2a* mutants show specific alterations in gene expression, we asked whether they have defects in chromatin accessibility by using Assay for Transposase-Accessible Chromatin sequencing (ATAC-seq) in isolated endothelial cells (Fig 4A). We

identified 20383 differential peaks in *egr1* and *klf2a* mutants, when compared to *fli:life-act-eGFP* controls. 20099 of these peaks exhibited lost or reduced accessibility, and only 284 peaks gained chromatin accessibility sites (Fig 4B). The majority of the lost and common peaks were localized around the transcription start site (TSS), in introns, or intergenic regions. However, the few sites that gained accessibility were mostly intergenic (Figure 4C). For instance, *bmp6*, a gene in the BMP signaling pathway, shows decreased accessibility in *egr1* mutants (Fig 4D). IPA analysis of the genes that are assigned to the differentially accessible regions (100kb upstream and 25kb downstream of the TSS) revealed enrichment for “cardiac hypertrophy signaling” ($P=1.6E-14$), “Notch signaling pathway” ($P=1E-05$) and “Wnt/Ca⁺ pathway” ($P=1E-03$) (Fig 4E and Supplementary Table 2). Motif analysis showed that different GATA motifs were highly enriched in the differential peaks (Fig 4F and Supplementary Table 2). To identify potential direct targets of *egr1*, we searched for the core EGR motif (TGCGTGGGYG) in all the differentially accessible regions. We were able to identify 1600 regions containing this motif (cut-off $P=1E-04$). After assigning these regions to the closest gene, we obtained a list of 1007 potential *egr1* direct targets. GO analysis on this list of genes revealed categories associated with heart development, including “cardiovascular system development” ($P=6.3E-03$), “heart morphogenesis” ($P=9.3E-03$), and “Wnt signaling pathway” ($P=1.03E-05$) (Supplementary Table 3).

To gain a better understanding of the differential occupancy of transcription factors, we performed digital footprinting analysis. We identified 12297 control-specific and 1294 *egr1*-specific footprints assigned to 5081 and 394 genes respectively (Supplementary Table 4). Next, we identified 46 *egr1* motif-containing footprints with reduced occupancy in *egr1* mutant EdCs when compared to the control (Fig 4G). Transcription factor motifs for different developmental pathways, such as SMAD, RBPJ and LEF1 factors, showed reduced occupancy in *egr1* mutants (S4A Fig). These data demonstrate that *egr1* mutants exhibit decreased chromatin accessibility in regions containing *egr1* motifs, but also in regions with motifs for pathways involved in EMT, in agreement with our RNA-seq results.

We compared our ATAC-seq and RNA-seq results to determine whether chromatin accessibility was associated with gene deregulation. We found an overlap of 404 genes with genes deregulated more than 3-fold, and an overlap of 406 genes with genes deregulated with a 1.5 fold cut-off ($HGT\ p < 5.503e-07$) (S4B Fig). GO analysis of these genes revealed categories involved in “heart development” ($P=2E-03$) and “heart morphogenesis” ($P=2E-03$) (S4C Fig). Importantly, some of these genes were both deregulated and had differences in chromatin accessibility in *egr1* mutants, for instance, *tgfb2* and *tgfb2a*, and *bcl9l*, which have

been associated with the WNT pathway and EMT (S4D Fig and Supplementary Table 1, 2). Thus, these results suggest that *egr1* plays an important role in modulating the TGF- β and Wnt pathways in EdCs.

***klf2a* mutants have alterations in chromatin accessibility in developmental and heart-associated pathways**

Next, we conducted a similar chromatin accessibility analysis in *klf2a* mutant endothelial cells. We identified 8576 differential peaks with altered accessibility in *klf2a* mutants, with 7813 lost and 763 gained peaks, when compared to the control (Fig 4H and Supplementary Table 2). Notably, genes in various developmental pathways lose accessibility in *klf2a* mutants (Fig 4J). GO analysis showed enrichment in genes of the Wnt ($P=3.4E-08$), Notch ($P=1.7E-06$) and BMP ($P=3.7E-05$) signaling pathways, including *wnt5a*, *wnt3*, and *axin1/2* for the Wnt signaling pathway, *notch1a*, *notch1b*, and *jag1a* for the Notch signaling pathway, and TGF- β receptors and Smad transcription factors for the BMP signaling pathway (Fig 4K and Supplementary Table 2). Analysis of *de novo* and known motifs in differentially accessible ATAC-seq peaks revealed motifs for transcription factors including SOX ($P=1E-116$), KLF14 ($P=1E-29$), and TEAD ($P=1E-27$) (Figure 4L and Supplementary Table 2). The core KLF motif (CACCC) was found in 299 differentially accessible regions within 249 genes, suggesting they could potentially be direct targets of *klf2a*. Consistent with this, GO analysis in these genes showed enrichment for developmental pathways such as “Wnt signaling pathway” ($P=9E-03$) and “Notch signaling pathway” ($P=1E-02$) (Supplementary Table 3).

Next, we performed footprinting analysis to examine the overlap of transcription factor occupancy between control and *klf2a* mutants. We detected 10029 lost and 3793 gained footprints in *klf2a* mutant endothelial cells, which were assigned to 4462 and 1867 genes, respectively (Supplementary Table 4). After mapping sequence motifs corresponding to binding sites for various transcription factors, we analyzed KLF motifs to validate our approach. As expected, footprints containing the KLF motif showed reduced occupancy in *klf2a* mutants (Fig 4M). Similar results were obtained for footprints of the TGF- β , BMP (SMAD3), and Notch (RBPJ) signaling pathways and GATA4 motif in *klf2a* mutants (S4E Fig). Additionally, footprints containing LEF1 motifs, a transcription factor that is primarily involved in the canonical Wnt/ β -catenin signaling pathway, were less accessible in *klf2a* mutants (S4E Fig). These results show that chromatin accessibility is altered in *klf2a* mutants in KLF motifs as well as motifs for transcription factors required for EMT.

As for *egr1* mutants, we compared our ATAC-seq and RNA-seq results *klf2a* mutants to determine whether chromatin accessibility was associated with transcriptional deregulation. We found an overlap of 328 genes (HGT, $P < 4.903\text{e-}11$) when we selected a 3-fold expression cut-off, and 466 genes (HGT, $P < 6.889\text{E-}19$) with a 1.5 fold cut-off (S4F Fig and Supplementary Table 2). Genes involved in “heart development” ($P=9\text{E-}03$), such as *tbx20*, *tbx2a* and *hand2*, were among these genes. Genes in the Wnt signaling pathway was also enriched ($P=1\text{E-}03$), including *lef1* and *fzd9b*, in the BMP signaling pathway ($P=6\text{E-}06$), such as *bmp7b* and two BMP receptors, and in the Notch signaling pathway ($P=5.8\text{E-}05$), namely *her2*, *her6*, and *her9* (S4G and H Fig and Supplementary Table 2). Additionally, EMT genes such as *tgfb1b*, *bmpr1ab*, *epb41l5*, *vasna*, *tbx20*, *snai2*, *nfatc1*, *isl1*, *msx2b*, and *lef1* were significantly deregulated in *klf2a* mutants and exhibited changes in chromatin accessibility (Supplementary Table 1,2). Thus, *klf2a* is a strong candidate as a direct upstream regulator of elements of the BMP, Notch, and Wnt signaling pathways involved in controlling EMT.

***klf2a* and *egr1* regulate different developmental programs**

The above transcriptomic and chromatin accessibility analyses reveal that whilst *egr1* and *klf2a* have different programs, they also share some characteristics at the chromatin level. To further characterize these similarities in chromatin accessibility, we conducted IPA analysis to compare the genes with differentially accessible regions between *egr1* and *klf2a* mutants. Interestingly, one of the top canonical pathways in both mutants is “cardiac hypertrophy signaling”, but we also found developmental pathways, such as Wnt and Notch (mostly enriched in *klf2a* mutants), TGF- β signaling pathway (enriched in both mutants), Hippo and BMP signaling pathways (enriched in *egr1* mutants) (Fig 5A). Additionally, TGF β 1 is enriched as a possible upstream regulator, albeit mostly in *klf2a* mutants (Fig 5B). These findings are consistent with our ATAC-seq data showing that developmental pathways are more enriched in differentially accessible regions in *klf2a* than in *egr1* mutants (Supplementary Table 2).

We then compared the differentially accessible regions of *egr1* and *klf2a* mutant endothelial cells. First, we compared the sites that gain accessibility and identified common regions between the mutants. *egr1* and *klf2a* only shared 18 regions that gained accessibility, suggesting these sites are highly specific to each factor (Fig 5C and 5D and Supplementary Table 5). Second, we compared the regions that lose or have reduced accessibility in the mutants. 3669 sites were lost in both *egr1* and *klf2a* mutants (Fig 5E and Supplementary Table 5). These regions can be located around the TSS or in intronic and intergenic regions, revealing

they have no preference for genomic location (Fig 5F). Genes important for developmental pathways, such as *jag1a* (Notch), *tcf7l2* (Wnt), and *bmpr1ab* (BMP), show loss of accessibility in both mutants (Fig 5G). We assigned these common regions between *erg1* and *klf2a* mutants to 2534 genes and identified their known and *de novo* motifs. Interestingly, EGR1 (P=1E-24) and SMAD4 (P=1E-15) motifs come up in *de novo* motif analysis (Fig 5H) suggesting that some of the regions that lose accessibility could be directly regulated by *egr1* and the BMP signaling pathway.

Subsequently, we identified the regions in *erg1* and *klf2a* mutants that exhibit loss or decreased accessibility and are also transcriptionally deregulated. We detected 99 genes when down-regulation was more than 3-fold (HGT P<3.114e-31), and 102 genes when the cut-off was 1.5 fold (HGT P<7.878e-27). The overlap between these genes and the lost ATAC-seq peaks in both mutants was 9 or 10 genes, for a cut-off of 3 or 1.5, respectively. *evala*, *irx7*, and *foxd2* were among these genes. We then performed similar analyses for the genes up-regulated in both *klf2a* and *egr1* mutants. We detected 47 genes with a 3-fold cut-off (HGT P<4.628e-18) and 57 genes with 1.5-fold cut-off (HGT P<1.578e-14). Only one up-regulated gene overlapped between the RNA-seq and ATAC-seq datasets of *egr1* and *klf2a* mutants, and this gene was not relevant for cardiac development. Assuming that some of the direct targets of *egr1* and *klf2a* lie within the regions with altered accessibility, our results suggest that these factors regulate different developmental programs.

***egr1* and *klf2b* regulate VEGF-R1/*flt1* gene expression**

flt1 is an important regulator of endothelial function and angiogenesis [44-46] and of EMT during valve development in mammals [47, 48]. Expression of the VEGF receptor *flt1* was down-regulated in *egr1* and *klf2b* mutants, but not in *klf2a* mutants. Moreover, a genomic region ~20kB downstream of the *flt1* TSS had reduced accessibility in *egr1* mutants (Supplementary Table 2). WISH revealed that *flt1* is expressed in the heart tube from 30 hpf and becomes restricted to the ventricle and AVC/OFT at 48 hpf (Figure 6A). To investigate a possible function for *flt1* in valvulogenesis, we analyzed *flt1*^{sa1504} mutants, which have a point mutation in an essential splice site of exon 9. 60% of the maternal zygotic mutants had cardiac edema from 48 hpf, which is indicative of abnormal cardiac development (Fig 6B). Indeed, *flt1* mutants show some important valve defects at 96 hpf (Fig 6C and 6D) similar to those of *egr1* and *klf* mutants. Together, these results indicate that *flt1* acts downstream of *egr1* and *klf2b* during valve leaflet maturation.

Discussion

We have identified the gene networks downstream of the transcription factors Klf2a and Egr1, involved in modulating heart valve morphogenesis in response to mechanosensitive stimuli. Genome-wide analyses suggest that Klf2a, Klf2b, and Egr1 cooperate non-redundantly to regulate EMT-associated genes expression during valve morphogenesis. Interestingly, although Egr1 and Klf2a control the expression of different genes, these genes belong to signaling pathways implicated in remodeling of AV EdCs and cardiac jelly, such as Notch, TGF β and Wnt. These findings suggest that mechanical forces activate EMT programs through multiple flow-responsive transcription factors, including Egr1 and Klf2a. Such a mechanism could explain the diversity of cellular responses to blood flow in the developing endocardium.

Several of the misregulated EMT-associated genes identified in *egr1* and *klf2* mutants have previously been involved in modulating EMT processes and in regulating EMT during AVC morphogenesis, including Wnt [12, 26] and VEGF signaling pathways [47, 48]. We show that EMT markers are expressed in ZF EdCs and are differentially regulated by Egr1, Klf2b, and Klf2a. EMT is essential for cardiac valve formation in mouse and has also been associated with vascular diseases, including CCM [49], pulmonary hypertension [50], vascular graft remodelling, and atherosclerosis [51]. It has been shown that shear stress can activate EMT targets in endothelial cells [52, 53] and in cushion cells of the outflow tract [54]. Notably, Snail and Twist are both activated in response to shear stress in cultured EC [52] and Twist1 promotes EdCs proliferation [55]. We show here that the zebrafish homologues *snail2* and *twist1* are misregulated in the EdCs of *klf2a* mutants, suggesting a link between Klf2a function and EMT in EdCs. *klf4* and *klf2* are both up-regulated in CCM endothelial cells displaying abnormal EMT [27, 28], which suggests the expression of these factors needs to be tightly repressed during EMT. Since *klf* expression is repressed by CCM1 in endocardial cells (Donat et al., 2018), we postulate that a similar regulatory mechanism may occur in the endocardium to control EMT during valve morphogenesis. In addition, our findings further suggest that other regulatory processes may be at play to modulate EMT progression in addition to *klf2a*. For instance, we found that Klf2b represses a number of genes associated with EMT, such as elements of the FGF pathway and collagens. Similarly, we found that Egr1 regulate genes of the Wnt and Tgf beta pathways that are also associated with EMT. EMT has been suggested to occur during sprouting angiogenesis in tip cells, as they lose apical-basal polarity, delaminate and migrate [56] and during ZF heart valve morphogenesis [12]. Further work will be needed to determine how the EMT proceed in ZF cardiac valve at the cellular scale, and whether *klf2* and *egr1* act in all endocardial cells or only in specific cell populations undergoing EMT. Answering these

questions would help elucidate the dynamics of gene expression that organise the rapidly changing cellular behaviour driving valve morphogenesis.

Endothelial cell remodeling is key for cardiovascular morphogenesis and is typically controlled by VEGF signaling [18, 19]. *Flt1* is one of the four VEGF receptors in zebrafish. Interestingly, we identified *flt1* as an essential downstream target of *Egr1* and *Klf2b*, but not *Klf2a*. Both zebrafish forms of the receptor (membrane mFlt-1 and soluble sFlt-1) have been shown to be essential for the establishment and maintenance of angiogenesis within a VEGF-Notch feedback loop [20]. In the zebrafish heart, *flt1* modulates endothelial cell proliferation and regulates vessels formation [57, 58] and cardiac ventricular contractility [59]. However, the role of VEGF signaling during cardiac valve formation remains unclear, and endocardial growth is thought to occur independently of VEGF signaling in zebrafish [22]. In mouse, VEGF is a negative regulator of EMT transformation during the formation of endocardial cushions [47]. Consistent with this, we found that *flt1* expression in the AVC between 30 and 48 hpf mimics the expression pattern of *egr1* and *klf2a*. Moreover, *flt1* expression is down-regulated in *egr1* and *klf2b* mutants. Since *flt1* is required for AVC valve formation, we hypothesise that *flt1* acts as a flow-responsive gene and therefore defines a mechanosensitive axis *egr1/klf2b-flt1*. Future experiments should address the potential function of *flt1* in normal and pathological EMT.

In this study, we paid particular attention to heart-associated developmental pathways and genes implicated in EMT regulation. However, our findings highlight the versatility and multi-functionality of *egr1* and *klf2a/b*. Moreover, our data sets provide a powerful resource for the community to explore other potential roles for these transcription factors. For instance, our RNA-seq analysis on *klf2a* and *klf2b* mutant EdCs (and to a lesser extent *egr1* mutant EdCs) identify several differentially expressed genes involved in various metabolic processes, and these results were corroborated by the chromatin accessibility analysis. This result raises the exciting possibility that these factors may control EdCs metabolism and, conversely, that EdCs metabolism may play a regulatory role in valve morphogenesis. Finally, calcium signaling another enriched pathway in *klf2b* mutants. Given that calcium signaling acts downstream of mechanosensitive channels during valve formation [25], it would be interesting to study the role of calcium during valve formation in the context of *klf2b* function. Further mining of these datasets should provide new mechanistic insights into the crosstalk between mechanotransduction and heart development.

Author Contributions

N.F. and J.V. designed the experiments. N.F. performed the experiments, with the help of the following persons for some experiments: R.C. for the photoconversion experiments, S. R. for the qPCR experiments. A.P. performed all the bioinformatics analysis. E.T. provided guidance and help on the genome-wide experiments and analysis, as well as reagents and critical insights. N. F., J.V. and E.T. wrote the paper.

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Figures and legends

Figure 1

Figure 1. Description of the valve defects observed in *egr1* and *klf2b* mutants. **A**, MZ (maternal zygotic) mutant at 72hpf for *egr1^{sa64}* showing pericardial edema compared to a wild-type sibling. **B**, Valvular defects observed for AV valves used to classify the observed phenotype in *egr1* and *klf2b* mutants. **C**, Statistical data of valvular defects observed in *egr1^{sa64}* at 96hpf using Bodipy staining. **D**, Photoconversion of AVC EdCs at 48hpf displaying the phenotypes used to classify the cell layering defects observed. **E**, Morphological observation of the leaflets at 98hpf showed that the organization of cells within the cardiac jelly is affected by the loss of *egr1*.

Figure 2

Figure 2. Analysis of the transcriptomic landscape of *egr1* or *klf2a* and *klf2b* mutant endocardial cells **A**, Experimental set-up for the RNA-seq experiments. **B**, Scatter plots depicting the significantly upregulated and downregulated genes for the three mutants (3-fold cut-off, p-value <0.05). X-axis depicts the log2 mean expression level for each control, Y-axis

depicts the log2 mean expression level for each mutant. Upregulated genes are depicted in red and downregulated in blue. **C**, Bar graph depicting enrichment according to DAVID for downregulated genes in each mutant. X-axis depicts the p-value. **D**, Venn diagrams depicting the overlap between the upregulated genes (upper panel) and the downregulated genes (lower panel) in *egr1* or *klf2a* and *klf2b* mutants (3-fold cut-off, p-value <0.05). **E**, IPA Canonical pathway analysis heatmap for *egr1*, *klf2a* and *klf2b* mutants. **F**, IPA Upstream regulator analysis heatmap for *egr1*, *klf2a* and *klf2b* mutants

Figure 3

Figure 3. Mis-regulated pathways relevant to cardiac valve morphogenesis A, *wnt9b* WISH showing cardiac expression of the *wnt9b* gene in OFT and AVC area in WT 48hpf-embryo. *egr1* and *klf2* mutants did not express *wnt9b* gene anymore. B-F, Heatmaps showing expression fold change of *egr1* and *klf2* mutants versus the respective controls for Wnt pathway

(B), valve related genes (C), TGF- β pathway (D) Notch pathway (E), and BMP pathway (F). * marks the significantly deregulated genes (Fold cut-off=3, p-value<0.05)

Figure 4

Figure 4. ATAC-seq and Footprinting analysis on *egr1* and *klf2a* mutant endocardial cells
A, Schematic of the ATAC-seq experiment. B Heatmap depicting the differentially accessible

regions in *egr1* mutant endocardial cells. ATAC signals are centered around ± 3 kb of the center of the peak (COP) and grouped into three clusters as indicated. The “lost” cluster represents sites that are accessible in the control but not the *egr1* mutant. The “common” cluster represents accessible regions in both control and *egr1* mutant and the “gained” cluster represents regions that gain accessibility in the *egr1* mutant. The heatmap density is represented as RPKM (reads per kilobase per million reads) mean score. **C**, Pie charts depicting the genomic location distribution of the lost (upper pie chart), common (middle pie chart) and the gained (lower pie chart) accessible regions in *egr1* mutant endocardial cells compared to control. TSS=transcription start site. **D**, ATAC-seq gene track depicting a differentially accessible region on *bmp6* gene. Y-axis represents RPKM. **E**, Bar graphs of selected gene ontology enriched categories for the differentially accessible ATAC-seq regions in *egr1* mutants. X-axis represents p-value. **F**, Exemplary motif discovered upon motif analysis of the differentially accessible regions in *egr1* mutants. **G**, Average normalized Tn5 insertion profiles around footprinted EGR motif in merged ATAC peaks in control and *egr1* mutants. Footprints occupancy score (FOS) indicates significance versus the control. Footprints number is indicated on the top. Insertions on the forward and reverse strands are indicated in red and blue, respectively. **H**, Heatmap depicting the differentially accessible regions in *klf2a* mutant endocardial cells. ATAC signals are centered around ± 3 kb of the center of the peak (COP) and grouped into three clusters as indicated. The “lost” cluster represents sites that are accessible in the control but not the *klf2a* mutant. The “common” cluster represents accessible regions in both control and *klf2a* mutant and the “gained” regions gain accessibility in the *klf2a* mutant. The heatmap density is represented as RPKM (reads per kilobase per million reads) mean score. **I**, Pie charts depicting the genomic location distribution of the lost (upper), common (middle) and the gained (lower) accessible regions in *klf2a* mutant endocardial cells compared to control. TSS=transcription start site. **J**, ATAC-seq gene track depicting a differentially accessible region on *lef1* gene. Y-axis represents RPKM. **K**, Bar graphs of selected gene ontology enriched categories for the differentially accessible ATAC-seq regions in *klf2a* mutants. X-axis represents p-value. **L**, Exemplary motifs discovered by motif analysis of the differentially accessible regions in *klf2a* mutants. **M**, Average normalized Tn5 insertion profiles around footprinted KLF motif in merged ATAC peaks at control and *klf2a* mutants. FOS indicates significance versus the control. Footprint numbers are indicated on the top. Insertions on the forward and reverse strands are indicated in red and blue, respectively.

Figure 5

Figure 5. *egr1* and *klf2a* have some common targets. A, Heatmap depicting the enriched IPA canonical pathways of genes assigned to differentially accessible regions in *egr1* and *klf2a* mutants. B, IPA upstream regulator analysis of genes assigned to differentially accessible regions in *egr1* and *klf2a* mutants. C, Heatmap depicting regions that gain chromatin

accessibility in *egr1* and *klf2a* mutants compared to controls. ATAC signals are centered around ± 3 kb of the center of the peak (COP) and grouped into three clusters as indicated. The “gained *egr1*” cluster includes sites that gain accessibility in *egr1* mutants. The “common” cluster represents regions with similar accessibility in both *egr1* and *klf2a* mutant and the “gained *klf2a*” regions that gained accessibility specifically in the *klf2a* mutant. The heatmap density is represented as RPKM. **D**, Pie charts depicting the genomic distribution of the clusters in Figure 5C. **E**, Heatmap depicting chromatin accessibility on the sites of *egr1* and *klf2a* mutants that lose or have diminished accessibility compared to controls. ATAC signals are centered around ± 3 kb of the center of the peak (COP) and grouped into three clusters as indicated. The “lost *egr1*” cluster includes sites that loose accessibility in *egr1* mutants. The “common” cluster represents regions with lost accessibility in both *egr1* and *klf2a* mutant and the “lost *klf2a*” regions are regions that lost accessibility specifically in the *klf2a* mutant. The heatmap density is represented as RPKM. **F**, Pie charts depicting the genomic distribution of the clusters in Figure 5E. **G**, Gene tracks depicting common chromatin accessibility changes in *egr1* and *klf2a* mutants. The scale of the Y-axis represents RPKM. **H**, Exemplary motifs from motif analysis on regions that lose or have diminished accessibility in both *egr1* and *klf2a* mutants.

Figure 6

Figure 6. *flt1* modulates AV valve development. **A**, *flt1* expression in the AVC at 30 and 48 hpf. **B**, *flt1* MZ mutant at 72hpf showing pericardial edema compared to a wild-type sibling. **C**, **D**, Statistical data of valvular defects observed in *flt1* MZ mutants at 96hpf using Bodipy staining (C) and photoconversion of EdCs at 48hpf (D).

Supplementary Figures and legends

S1 Figure

S1 Fig. *egr1* expression at 30, 48 and 72 hpf and *klf2b* expression at 48 hpf

S2 Figure

S2 Fig. *egr1* cardiac expression at 48hpf is restricted to valve forming area in WT embryos. *egr1* expression is abnormal in mutants where blood flow is altered (*tnnt2*, *gata1a*). This indicates that *egr1* expression is flow dependent (upper panel). Statistical analysis of *egr1* WISH in the different mutants. The control presented here is the sum of all respective controls for the mutants.

S3 Fig. A, qPCR verification of deregulated genes from the RNA-seq data. **B-F**, Heatmaps showing expression fold change of *egr1* and *klf2* mutants versus their respective controls for collagen genes (B), prostaglandin signaling pathway (C), FGF signaling pathway (D) Tyrosine kinase signaling pathway (E) and EMT signaling pathway (F). * marks the significantly deregulated genes (Fold cut-off=3 p-value <0.05).

S4 Fig. A, Average normalized Tn5 insertion profiles around footprinted SMAD, RBPJ and LEF1 motifs in merged ATAC peaks at control and *egr1* mutants. FOS indicates significance versus the control. Footprint numbers are indicated on the top. Insertions on the forward and reverse strands are indicated in red and blue, respectively. **B**, Venn diagram showing the

correlation between genes assigned in differentially accessible regions and differentially expressed genes (FC=Fold cut-off 1.5) between control and *egr1* mutant endocardial cells. **C**, Bar graphs of selected gene ontology enriched categories for the genes that are differentially expressed and exhibit also changes in chromatin accessibility in *egr1* mutants. X-axis represents p-value. **D**, Gene track depicting the deregulated *tgfb2* gene that also exhibits changes in chromatin accessibility in control and *egr1* mutants. The scale of the Y-axis represents RPKM. **E**, Average normalized Tn5 insertion profiles around footprinted SMAD, GATA, RBPJ and LEF1 motifs in merged ATAC peaks at control and *klf2a* mutants. FOS indicates significance versus the control. Footprint numbers are indicated on the top. Insertions on the forward and reverse strands are indicated in red and blue, respectively. **F**, Venn diagram showing the correlation between genes that are assigned in significantly differentially accessible regions and differentially expressed genes (FC=Fold cut-off 1.5) between control and *klf2a* mutant endocardial cells. **G**, Bar graphs of selected gene ontology enriched categories for the genes that are differentially expressed and exhibit also changes in chromatin accessibility in *klf2a* mutants. X-axis represents p-value. **H**, Gene track depicting the deregulated *her2* gene that also exhibits changes in chromatin accessibility in control and *klf2a* mutants. The scale of the Y-axis represents RPKM.

Supplementary tables

Supplementary Table 1. RNA-seq data. Excel file containing the results of the transcriptomic landscape of *egr1* or *klf2a* and *klf2b* mutant endocardial cells.

Supplementary Table 2. ATAC-seq. Excel file containing the results of the ATAC-seq performed on *egr1* and *klf2a* mutant endocardial cells.

Supplementary Table 3 Core motif analysis. Excel file containing the results of the core motif analysis performed on *egr1* and *klf2a* mutant endocardial cells.

Supplementary Table 4. Footprinting analysis. Excel file containing the results of the footprinting analysis performed on *egr1* and *klf2a* mutant endocardial cells.

Supplementary Table 5. Comparison ATAC RNA *egr1* and *klf2a* common. Excel file containing the results of the ATAC-seq and RNA combined analysis performed on *egr1* and *klf2a* mutant endocardial cells.

Materials and Methods

Zebrafish accession gene numbers

klf2a: ENSDARG00000042667

klf2b: ENSDARG00000040432

egr1: ENSDARG00000037421

flt1: ENSDARG00000019371

Zebrafish husbandry, fish strains and embryo treatments

The zebrafish (*Danio rerio*) lines used in the experiments were the following: wild-type AB, *egr1^{sa64}* – (ZIRC), *klf2a^{ig4}* [13], *klf2b^{ig5}* (further information below), *flt1^{sa1504}* – (ZIRC), *pkd2^{tc321}* or *cup* mutants from ZIRC, *vlt^{m651}* or *gatala* [60], *tnnt2a^{b109}* or *sih* [33], *tg(fli:gal4FF^{ubs}; UAS:kaede)* [61] and *Tg(kdrl:mCherry)* [62].

Embryos were staged according to hours (hpf) and days postfertilization (dpf). They were incubated at 28.5°C in 0.3% Danieau medium supplemented 5h after birth with 0.003% (wt/vol) 1-phenyl-2-thiourea (PTU) (Sigma Aldrich) to inhibit pigment formation. All zebrafish strains were maintained at the IGBMC fish facility under standard husbandry conditions (14h light/10h dark cycle). The Animal Experimentation Committee of the Institutional Review Board of IGBMC approved all animal experiments performed in this project.

klf2b^{ig5} line was generated using a TALEN pair (left and right arms: 5'GGACATGGCTTTACCT-3' and 5'AACGTTTGCAAACCAG-3') were designed to target exon 1 of the *klf2b* gene and injected into single cell wild-type (AB) first cell. We identified the alleles generated and confirmed that potential targeting events could be transmitted through the germline by out-crossing the F0 fish with AB animals and sequencing genomic DNA from pools of 6 F1 embryos. After screening the first generation, we focused on a 28pb-deletion mutation (5'-GGACATGGCTTTACCTTGCCTTTTGCCT-3') leading to a premature stop codon in *klf2b* transcript. Studies were performed from F4 fish and later generations, and on transgenic lines resulting of out-crossings. A PCR-based genotyping strategy was established using the following primers to identify the wild-type and mutant alleles, the length of the deletion allowing their visualization directly on a 3%-agarose DNA gel: forward 5'-GGAAAGCGCGTATATTTGGA-3', reverse 5'-CAAGTAGGAAATGCAAGTGT-3' and sequencing forward primer 5'-AGAGCGCACTGTGCCTTATA-3'.

In Situ Hybridization (ISH)

ISH assay was performed as in [63] using the following anti-sense probes:

klf2a probe: obtained by PCR amplification of the plasmid IRBOP991B0734D, provided by RPDZ, Berlin, using forward primer: 5'-CAGGCGACTACAGAATGCA-3' and reverse primer: 5'-TAATACGACTCACTATAGGGAGTGAC. Transcription with T7 polymerase.

klf2b probe: obtained by PCR amplification of the plasmid #1343 pSCB-*klf2b*, provided by the Seyfried group), 640pb fragment amplified with reverse primer 5'-CTACGGTCCGGTGATAGGCATG-3' and forward primer 5'-AGCATTTAGGTGACACTATAGTCACAGGTGTCTCTTCATGTGCAG-3'. Transcription by SP6 polymerase.

egr1 probe: from pBSK plasmid, PCR amplification using forward primer: 5'-ATGACCCGTGAGTCAGTAA-3' and reverse primer: 5'-ATTAACCCTCACTAAAGGGACTTGGTGCCCTGAGTTCTGAT-3'. Transcription using T3 polymerase.

flt1 probe: from a partial cDNA fully sequenced ordered at Science Biosource (IRCYp5023H065D, 9038840 IMAGE ID), pCR4-TOPO plasmid, linearized by NCOI (NEB). Transcription using SP6 polymerase.

wnt9b probe: forward primer: 5'-TATTGCCCTCTGCATCCTTC-3' and reverse primer: 5'-TGACATTCAACGTGACAGCA-3'.

To optimize the imaging of the heart after staining, in certain cases the embryos were made more transparent using fructose. A first bath in 45%-fructose solution (D-fructose, Sigma, ref F0127) containing 1/100 1-Thioglycerol (Sigma M6145) was performed for 15 min followed by a second bath in 90%-fructose solution from 30min to 1 hours depending the stage of the embryo. Imaging was performed using a Leica M165 macroscope with a TrueChrome Metrics (Tucson) with a Leica 1.0X objective (10450028).

High throughput dissection of 48hpf-hearts

Hearts were dissected from *klf2a^{ig5}*, *klf2b^{ig5}* and *egr1^{sa64}* lines in background *fli:lifeact-eGFP*; *kdrl:nls-mcherry* and respective control embryos at the desired stage (48hpf) using high-throughput extraction technique, optimized from Lombardo et al. (2015) protocol. Hearts were separated mechanically by pipetting up and down a batch of 200 embryos in culture medium (L-15 Leibovitz, 10% FCS 9150, 1.25mM CaCl₂, 800mg/L glucose, 50microg/mL penicillin, 0.05mg/mL streptomycin), medium prepared fresh for each experiment. Hearts were then separated from debris after passages on two different filters: first purification through a 100um nylon cell stainer (Falcon, 352360), and further collection on a pre-separation filter 30um (Miltenyi Biotec). After centrifugation 10 min at 2.5g, culture medium was replaced by cold FACS medium (PBS with 2% Fetal Calf Serum 9150, 1% Penicilline/streptavidine, 1mM EDTA) and final filtration was performed with tip strainers (40um) (Scienceware, Bel-Art, flowmi tip strainers)

Fluorescence-activated cell sorting (FACS)

Further separation of endocardial cells from the other cardiac cell types was achieved directly after heart extraction by FACS. FACSaria Fusion (BD Biosciences) device was used. Cells were sorted on a FITC-A detector and flow cell passed through 70um-nozzle.

Sorted cells were collected at 4°C in PBS: 9,5uL cold PBS + 0,24uL RNAsin and immediate freezing in dried ice of 1000 cell-samples for mRNA sequencing assay; or 50uL cold PBS for the samples directly treated after sorting with the transposase for ATACseq assay.

mRNA-sequencing

1000 endocardial cells were collected in 9.4 uL PBS-RNase free supplemented with 0,24uL RNAsin and immediately frozen after FACS sorting in dried ice and stored at -80°C. cDNA transcription was performed using Clontech SMART-Seq v4 Ultra Low Input RNA Kit. Paired-end 100 bp reads for control and experimental samples were generated with Illumina HiSeq 4000. RNA-seq raw sequencing data from zebrafish were trimmed with Trimmomatic (version 0.36) [64] and aligned to zebrafish genome version GRCz10/danRer10, with the tophat algorithm (version 2.1.1) [65] and the use of «--b2-very-sensitive» parameter. Samtools (version 0.1.19) [66] were used for data filtering and file format conversion, while the HT-seq count (version 0.5.4p3.) [67] algorithm was applied to assign aligned reads to exons using the following command line «htseq-count -s no -m intersection -nonempty». Differentially expressed genes were identified with the use of the DESeq R package [68], and genes with fold change cut-off 1.5 and P-value<0.05 were considered to be differentially expressed (DEGs). Heatmaps that were constructed to depict DEGs or selected genes were generated with

R/Bioconductor. A summary of the differentially expressed genes can be found in Supplementary Table 1.

ATAC-sequencing

After FACS sorting, cells harvested in PBS were treated according to Buenrostro et al. protocol [69]. Transposition reaction and purification were directly performed the day of cell collection, DNA resuspended in Buffer EB from the MinElute kit was stored at -20°C upon further treatment. Custom Nextera PCR Primer were used during PCR amplification to label the samples. Library quality control was done using Bioanalyser, a purification step was performed using MiniELute Qiagen kit and size separation on Agencourt AMPure XP beads (A63882, Beckman Coulter, Inc) to remove PCR Nextera primers.

Sequencing was performed on one lane on Illumina HiSeq 4000 sequencing, Nextera, HS-2x50 millions bases for control and mutants. Bowtie2 algorithm (version 2.1.0) [70] and «--very-sensitive» parameter were used for aligning ATAC-seq data to the zebrafish genome version GRCz10/danRer10. Samtools (version 0.1.19) (Li *et al.*, 2009) were used for data filtering and file format conversion. Duplicate reads were removed before peak calling. The MACS2 (version 2.1.0) algorithm [71] was used for ATAC-seq peak identification with default p-value=1-E05. All .bam files were converted to bedgraphs with genomeCoverageBed, a subcommand of BEDTools [72] and MACS2 bdgdiff command was used in order to identify differential enrichment in the accessible regions between control and mutants, with default options. Gene annotation (100 kB upstream and 25kB downstream from the TSS) and genomic distribution of accessible regions identified by MACS2 was performed with BEDtools (Quinlan and Hall, 2010) and -closetBed and -intersectBed subcommands were used respectively. Clustering of regions was generated with ComputeMatrix function of DeepTools [73], using reference point --referencePoint center -b 3000 -a 3000 -R <bed files> -S <bigwig files> as parameters. The function plotHeatmap from the same package was used for displaying the average profiles heatmap. A summary of ATAC-seq peaks can be found in Supplementary Table 2.

Gene ontology, pathway and network analysis

Gene ontology and pathway analysis of the differentially expressed genes from RNA-seq and the genes that were assigned in differential peaks from ATAC-seq was performed with DAVID knowledgebase [74, 75] and Ingenuity Pathway Analysis software (IPA, Ingenuity® Systems, www.ingenuity.com) with the default settings. Only pathways and biological processes with p-value ≤0.05 were considered to be significantly enriched.

Motif analysis

Differentially accessible chromatin regions were searched for enriched short-sequence motifs using HOMER software with “findMotifsGenome.pl” command. [76]. To identify potential direct targets of mutants, core motifs were scanned with FIMO (Find Individual Motif Occurrences) [77], taking 1E-04 as a threshold. A summary of mutant direct targets can be found in Supplementary Table 3.

Digital genomic footprinting for ATAC-Sequencing

To attain sufficient sequencing depth to perform digital genomic footprinting (DGF) on ATAC-Seq data produced in this work, aligned .bam files were merged using samtools merge (version 1.3.1) (Li *et al.*, 2009) following sorting via samtools merge and subsequently indexed using samtools index. DGF was performed using dnase_footprints of the Wellington pyDNase package (version 0.2.4) [78] on total merged ATAC peaks using -A as a parameter to enable ATAC mode, resulting in coordinate shift 5' and 3' by +4 and -5 bp, respectively. Motif

overrepresentation and average profile analyses were performed with `dnase_average_profile.py` of the Wellington pyDNase package[78], on WT-only footprints versus all mutant footprints and vice-versa. For footprinting occupancy scores (FOS) between two experiments, a python script from Wellington pyDNase package (`wellington_score_heatmap.py`) was used and they were computed first by log2 transformation and then performed two-tailed, paired t-test in R, as each value correspond to the same chromosomal region, and not assuming any direction in the relationship between both samples. Gene annotation (100 kB upstream and 30kB downstream from the TSS) of the footprints sites was performed with BEDtools and `-closetBed` subcommand was used. A summary of the footprint analysis can be found in Supplementary Table 4.

Venn diagrams

Proportional venn diagrams for overlapping genes were generated with BioVenn (<http://www.biovenn.nl/>). Statistical significance of the overlap between two groups of genes was calculated with Hypergeometric statistical test.

qPCR

Whole hearts were extracted as explained previously. RNA was extracted directly after heart collection inside RNA lysis buffer from Quick RNA micro-prep (Zymo kit, R1050). Around 1mg of each RNA mix was used to synthesized cDNA using SuperScript VILO kit (Invitrogen, ref: 11754-050) in 20uL final volume according to the manufacturer's instructions. Products were amplified in a real-time PCR reaction with Light Cycler 480 Real-Time PCR System (Roche) using a UPL Probes Master mix (Roche) according to the manufacturer's instructions. Sequence of primer pairs were as follows:

<i>gene</i>	Forward primer	Reverse primer
<i>egr1</i>	agtttgatcaccttgctggag	ggtgaaacggcctgtgtaag
<i>flt1</i>	agtcacgtccaccacaatc	gccaaactgcagaactccaac
<i>gata5</i>	ggacgccaggggaactctac	acacggcaggtcatccag
<i>has2</i>	agcatccctgttcaactaacg	gtgaccgctttatcacatct
<i>klf2a</i>	ccgtctatttcacattttcg	tccagttcatccttcacct
<i>klf2b</i>	cgtggacatggctttacctt	ttgtgctcctcaatcttcca
<i>sox17</i>	caaccacagttctgggtcaa	ccccaaaagagagacagtgc
<i>tbx20</i>	gacttatgctggagatgaagagact	tgcagtgaacgctgaacc
<i>Wnt9b</i>	cttattgcctctgcatcct	gaggctcgcgacctgtaa
<i>polr2d-2</i>	aacgcaagtgggagatgtg	agcgtctctgcgttctcaa

Valve imaging

Embryos were incubated with 4mM BODIPY-FL C5-ceramide (Molecular Probes from ThermoFischer, D3521) overnight and then anesthetized with 0.02% Tricaine (Sigma Aldrich, Saint-Louis, USA) solution and were mounted on Petri dishes embedded in 0.7% low melting point (LMP) agarose (Sigma Aldrich). To visualize the valve shape and to observe their motion, confocal imaging was performed on a Leica SP8 confocal microscope. Images were acquired with a low-magnification water immersion objective (Leica HCX IRAPO L, 25X, N.A. 0.95). For four-dimensional imaging, time series were acquired at a random time in the cardiac cycle at 35fps for 3 s. The optical plane was moved 2 mm between the z-sections until the whole AVC was acquired. AVC area was visually defined as in (Steed et al., 2016b). The number of cells within each AVC was analyzed using Imaris software (Bitplane).

AVC photoconversion

Photoconversion was performed at 48 hpf and follow-up imaging experiments were performed at 98 hpf using the protocol described in Chow et al., 2018 (Jove Paper). Briefly, Tg(fli1a:Gal4FF; UAS:Kaede) embryonic hearts were induced to stop beating by treating embryos with 50 mM 2,3-Butanedione monoxime (BDM) (Sigma Aldrich), a myosin inhibitor, for 5-10 minutes. They were then mounted in 0.7% low melting-point agarose supplemented with 50mM BDM to inhibit heart contraction for the duration of the photoconversion procedure. 48hpf- ventricular cells were selected using the Region of Interest tool and exposed to 405 nm light (400 Hz, 25% laser power) using the FRAP module on a SP8 confocal microscope and a Leica HCX IRAPO L, _25, NA0.95 water immersion objective. For each selected region, one pre-bleach frame was acquired, followed by 3–6 bleach pulses with acquisition to convert kaede protein in the illuminated region from its green to red form. A z-stack of the photoconverted heart was then acquired in the standard confocal mode to record the starting point of each experiment. Embryos were then carefully removed from the agarose using a glass suction pipette, washed in fish water, and then placed in fresh fish water containing 0.003 % PTU. Heart contraction soon resumes, and the fish are allowed to develop normally at 28.5 °C under standard conditions until the second timepoint of interest (98hpf).

Data availability-Accession numbers

The data have been deposited to the Short Read Archive SRA under the BioProject Accession code PRJNA529416.

Reviewer

link:<https://dataview.ncbi.nlm.nih.gov/object/PRJNA529416?reviewer=k0s8mhiojujhtb1r90k1q8s0qe>

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