Tbx5a lineage tracing shows cardiomyocyte plasticity during zebrafish heart regeneration

Héctor Sánchez-Iranzo, María Galardi-Castilla, Carolina Minguillón, Andrés Sanz-Morejón, Juan Manuel González-Rosa, Anastasia Felker, Alexander Ernst, Gabriela Guzmán-Martínez, Christian Mosimann & Nadia Mercader

During development, mesodermal progenitors from the first heart field (FHF) form a primitive cardiac tube, to which progenitors from the second heart field (SHF) are added. The contribution of FHF and SHF progenitors to the adult zebrafish heart has not been studied to date. Here we find, using genetic tbx5a lineage tracing tools, that the ventricular myocardium in the adult zebrafish is mainly derived from tbx5a+ cells, with a small contribution from tbx5a− SHF progenitors. Notably, ablation of ventricular tbx5a+-derived cardiomyocytes in the embryo is compensated by expansion of SHF-derived cells. In the adult, tbx5a expression is restricted to the trabeculae and excluded from the outer cortical layer. tbx5a-lineage tracing revealed that trabecular cardiomyocytes can switch their fate and differentiate into cortical myocardium during adult heart regeneration. We conclude that a high degree of cardiomyocyte cell fate plasticity contributes to efficient regeneration.
The zebrafish is an established model organism to study heart regeneration, in which pre-existing cardiomyocytes proliferate to replace the lost myocardium. The zebrafish myocardium is formed by an inner trabecular layer, a thin primordial layer, and an outer cortical layer. During embryonic development, cells from the primordial layer give rise to trabeculae. Later, in the juvenile, trabecular cardiomyocytes breach the primordial layer and form the cortical myocardium. Previous clonal analysis suggested that during adult heart regeneration, the cortical myocardium is rebuilt by cardiomyocytes from the same layer, suggesting some degree of commitment to a particular myocardial layer. A higher degree of plasticity had been observed in the embryonic heart, whereby atrial cardiomyocytes were reported to be able to regenerate the cardiac ventricle.

The vertebrate heart is formed from mesodermal precursor cells derived from the first and second heart fields, also called FHF and SHF, respectively. Cells from the FHF form the embryonic heart tube to which cells from the SHF are added to allow further growth. FHF and SHF progenitors have also been described in the zebrafish. Regulatory elements from the draculin (dlr) gene have been used to label a population of lateral plate mesoderm progenitors that give rise to cardiomyocytes of the primitive heart tube, providing a genetic marker to trace FHF-derived myocardium. Latent Tgf beta binding protein 3 (ltbp3) expression marks a distal dlr-negative domain and has been proposed as a SHF marker. Of note, neither dlr nor ltbp3 are established FHF and SHF markers in other vertebrates including mammals.

In the mouse, retrospective clonal analysis revealed that FHF-derived cells predominantly give rise to the left ventricle, whereas the SHF yields the right ventricle and large parts of the atri. Further studies on the contribution of cardiac precursor cells to the mammalian heart come from genetic lineage tracing approaches, which are based on following the fate of cells that expressed a particular gene at a given time point of embryonic development. A key gene used to trace the fate of cardiac precursor cells is Tbx5, encoding a T-box transcription factor, which serves as an early cardiac marker expressed in the developing heart tube. Genetic lineage tracing analysis of Tbx5-expressing cells has revealed a strong contribution to the left ventricular myocardium, recapitulating the fate of FHF progenitors in this cardiac chamber.

Zebrafish have two Tbx5-encoding genes, tbx5a and tbx5b; tbx5a expression levels during heart development are higher than those of tbx5b, and tbx5a mutants recapitulate key phenotypes of mammalian Tbx5 perturbations. We thus decided to generate genetic tools to study the contribution of the FHF to the adult ventricle during homeostasis and regeneration in the zebrafish using regulatory sequences of tbx5a. We describe that in zebrafish the early heart tube is formed by tbx5a-derived cardiomyocytes to which SHF-derived tbx5a cardiomyocytes are added. Even though their contribution to the adult heart is minimal, SHF-derived cells can give rise to a fully functional ventricle if tbx5a-derived cells are genetically ablated. In the adult heart, tbx5a expression is persistent only in the trabecular myocardium, allowing to distinguish this layer from the cortical myocardium. Genetic fate mapping of trabecular tbx5a+ cells shows that these cells contribute to regenerate the outer cortical layer. Together, our findings show a high degree of cardiomyocyte fate plasticity during zebrafish heart regeneration, during both embryonic stages and in the adult.

**Results**

**Contribution of tbx5a+ cardiomyocytes to the adult ventricle.** We used the fluorescent bacterial artificial chromosome (BAC) transgenic reporter line tbx5a:GFP, driving green fluorescent protein (GFP) expression under the control of the tbx5a cis-regulatory elements. The overall GFP expression pattern of tbx5a:GFP recapitulated that of endogenous tbx5a expression in embryos (Supplementary Fig. 1a, b; n = 7/7). We analysed the...
expression of tbx5a:GFP in the early heart tube, before the addition of SHF progenitors. At this stage, the heart tube is formed by a single sheet of cardiomyocytes named the primordial layer. Homogeneous tbx5a:GFP expression was detected in the whole embryonic myocardium at 32 hours postfertilisation (hpf) (Fig. 1a–c; n = 5/5). After this stage, at 56 hpf, (Fig. 1d–f; n = 3/3), a GFP+ region of myocardium started to become visible in the cranial portion of the heart tube, which remained reporter-negative at 72 hpf (Fig. 1g–i; Supplementary Movie 1, n = 5/5), 4 days postfertilisation (dpf) (Fig. 1j–l; n = 9/9), and at 5 dpf (Fig. 1m–o; n = 6/6), concomitant with the reported addition of SHF progenitors to the ventricular myocardium during these stages3,12. The GFP expression pattern was confirmed by gfp messenger RNA in situ hybridisation on heart sections of embryos at 2–5 dpf (Supplementary Fig. 1c–g). Comparison of the expression domains at 56 and 72 hpf between tbx5a:GFP and the previously reported FHF marker drlk:Cherry revealed an overlap of both domains in the proximal part of the ventricle close to the atrioventricular canal (AVC) and in the outer curvature of the ventricle (Supplementary Fig. 2a–h and Supplementary Movies 2 and 3; 56 hpf, n = 5/5; 72 hpf, n = 7/7). Of note, the expression domain of tbx5a:GFP was more extensive toward the distal myocardium. This might indicate that drl expression is downregulated faster in FHF-derived cells than tbx5a, or that part of the proximal SHF-derived myocardium expresses tbx5a. Nonetheless, a clear tbx5a:GFP::drlk:Cherry double-negative domain was present in the inner curvature and distal-most portion of the larval ventricle (Supplementary Fig. 2a–h and Supplementary Movies 2 and 3). This tbx5a:GFP+ region was further expressing transiently injected ltbp3:TagRFP-2A-Cre12, indicating SHF-derived ventricular myocardium (Supplementary Fig. 2i–l and Supplementary Movie 4). Thus, in the embryonic heart, tbx5a expression marks cardiomyocytes in the primitive heart tube and is absent from a population of distally located ventricular cardiomyocytes, which from their expression pattern and temporal appearance match with previous descriptions of the most distal SHF-derived cells12.

We next sought to chart tbx5a expression in the adult zebrafish heart. During zebrafish heart maturation, a third myocardial layer forms enveloping the primordial layer, named cortical layer. Clonal analysis suggested that these myocardial layer derives from trabecular cardiomyocytes13. We still detected high levels of endogenous tbx5a mRNA and tbx5a:Cre expression in the adult zebrafish ventricle (Fig. 2a, b and Supplementary Fig. 3; n = 8/8). tbx5a:GFP expression was restricted to the trabecular myocardium and absent from the cortical layer (Fig. 2c, d), coinciding with the recently reported pattern obtained using an enhancer fragment located 16 kb upstream of the tbx5a transcription start site23. Expression spread through most of the trabecular myocardium, with a gradient from the apex (high expression) to the basal part close to the bulbus arteriosus (BA) (lower expression levels). In addition, we observed a region in the basal ventricle, close to the atrium, in which we detected GFP+ trabecular cardiomyocytes. No GFP expression can be detected in these cells beyond background and we therefore refer to this myocardial territory as basal tbx5a− region (Fig. 2e, f; n = 13/13; Supplementary Fig. 4). tbx5a+ cardiomyocytes in the adult heart could derive from embryonic tbx5a+ cells that switched off their expression or from progenitor cells that never expressed tbx5a. To test these possibilities, we generated transgenics lines for inducible genetic fate mapping of tbx5a+ expressing cells (Fig. 3a). In double-transgenic tbx5a:rnase:tdTomato;p2A-CreERT2;ubbloxP-lacZ-STOP-loxP-GFP26 embryos, mCherry labels tbx5a+ expressing cells and GFP labels the progeny of tbx5a+ cells that expressed CreERT2 recombinase at the time of 4-Hydroxytamoxifen (4-OHT)-induced recombination. This transgenic combination was not leaky and GFP-expressing cells were visible only upon 4-OHT administration (Fig. 3b; n = 5/5). When recombined during embryogenesis, GFP+ cells were found in the atrium and ventricle of the larval heart but never in the distal portion of the ventricular myocardium (Fig. 3c–f; 8/8; Supplementary Fig. 5 and Supplementary Movie 5, n = 9/9). Few epicardial cells were also tbx5a+-derived, consistent with tbx5a:tdTomato expression observed in a subset of embryonic epicardial cells (Supplementary Fig. 6; n = 6/7). When analysed in adult hearts, the cortical layer was mCherry−, in agreement with our observations using the reporter tbx5a: GFP. Nonetheless, most of the cortical layer was GFP+ and thus derived from a tbx5a+ embryonic cell population that switched off tbx5a expression (Fig. 3g–n; n = 5/5; Supplementary Fig. 7, 8).

Akin to the GFP pattern observed in tbx5a:GFP adult hearts, a basal domain of the ventricle close to the BA was also mCherry− (Fig. 3g–i and 3o–r, and Supplementary Fig. 7, 8; n = 5/5). Furthermore, this domain was also GFP+, suggesting that it derives from the tbx5a− distal embryonic ventricle. Thus, we propose that tbx5a+-derived cells in the adult heart predominantly comprise the FHF derivatives of the embryonic heart and the tbx5a− domain in the basal ventricle close to the atrium and BA is SHF-derived. Taken together, our genetic lineage tracing of embryonic myocardial populations into adulthood in zebrafish reveal a fate compartmentalisation of tbx5a− and tbx5a+-derived cardiomyocytes starting in embryogenesis that results in distinguishable ventricle domains in the absence of seption.

SHF progenitors compensate ablated tbx5a+ cardiomyocytes.

Previous results have indicated that atrial cardiomyocytes acquire a ventricular phenotype upon injury in the embryonic heart17. To explore whether SHF progenitors can compensate for a loss of FHF-derived cells, we generated vmhcl:loxP-tagBFP-loxP-mCherry-NTR, a transgenic line to specifically mark and ablate
ventricular cardiomyocytes using the *ventricular myosin heavy chain like* (*vmhcl*) cis-regulatory elements (Fig. 4a; Supplementary Fig. 9; *n* = 10/10). *vmhcl* is expressed exclusively in ventricular and not atrial cardiomyocytes. We crossed *vmhcl:loxP-tagBFP-loxP-mCherry-NTR* with *tbx5a:CreERT2*. This allowed to lineage-trace *tbx5a*+ (mCherry+) and *tbx5a*− ventricular cardiomyocytes (tagBFP+). Furthermore, this genetic strategy allows the ablation of *tbx5a*+ ventricular cardiomyocytes by adding Metronidazole (Mtz) to induce cell death in nitroreductase (NTR)-expressing cells. Administration of 4-OHT from 24 to 48 hpf induced the expression of mCherry-NTR in *tbx5a*-derived cardiomyocytes in the trabecular and primordial layer of the embryonic ventricle, with the exception of the distal-most part at the cranial pole (Fig. 4b, c; *n* = 7/7). In adult hearts, most ventricular cardiomyocytes were mCherry+. Only a small portion of cardiomyocytes close to the BA and AVC was tagBFP + (Fig. 4d, e), coinciding with the results obtained doing lineage tracing using *tbx5a:mCherry-p2A-CreERT2;ubb:loxP-lacZ-STOP-loxP-GFP*. To specifically ablate mCherry+ cardiomyocytes we administered Mtz from 4 to 7 dpf (Supplementary Fig. 10). Surprisingly, 30 days later, a complete ventricle had regenerated from tagBFP+ cardiomyocytes, suggesting that SHF-derived ventricular cardiomyocytes are able to compensate the genetic ablation of the FHF-derived ventricular myocardium (*n* = 6/6; Fig. 4f–h and...
Supplementary Fig. 11). In the experimental setup all atrial cardiomyocytes are tbx5a lineage-derived and thus would become mCherry+ if they contributed to the ventricle (Supplementary Fig. 5 and Supplementary Movie 5, n = 9/9). Thus, our results reveal that ventricular tbx5a− SHF-derived cells compensated for the loss of tbx5a+ ventricular cardiomyocytes. To confirm ablation of tbx5a-derived mCherry-NTR expressing cells, we performed terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining to detect apoptosis (Fig. 5a–g; n = 8 larvae + Mtz group and n = 8 −Mtz group) and detected a significant increase of TUNEL+ mCherry+ trabecular and primordial cardiomyocytes in the Mtz-treated group. In agreement, tagBFP+ ventricular cardiomyocytes incorporated significantly more 5-bromo-2′-deoxyuridine (BrdU) upon Mtz treatment, indicating proliferation of this cell population (Fig. 5h–m).

Ablation of tbx5a-derived ventricular cardiomyocytes led to a transient impairment of cardiac function. At 7 dpf, right after the Mtz treatment, the ventricles revealed an irregular shape and displayed diminished pumping efficiency (Supplementary Fig. 12a–e and Supplementary Movie 6). However, mortality was not significantly increased (Supplementary Fig. 12f). Indeed, in the adults, the regenerated hearts revealed normal ventricular pumping efficiency (Supplementary Fig. 12g). Taken together, our results demonstrate that the loss of FHF-derived cardiomyocytes is compensated by an expansion of SHF-derived cardiomyocytes.

Cardiomyocyte plasticity during adult heart regeneration. We next explored whether this observed cardiomyocyte plasticity remains in the adult. Upon cryoinjury, cardiomyocytes at the site of injury proliferate and rebuild the lost cortical and trabecular

myocardium.1,2. Using our transgenic tools, which genetically discriminate between cortical and trabecular cardiomyocytes, we investigated the plasticity of trabecular cardiomyocyte cell fate during regeneration. First, we analysed the expression of tbx5a: GFP during heart regeneration using cryoinjury (Supplementary Fig. 13a). tbx5a:GFP was highly expressed in uninjured trabeculae at all stages analysed. At 1 days postinjury (dpi), no GFP expression could be found in the cortical layer (Supplementary Fig. 13b–f; n = 4/4). In contrast, at 3 dpi (Supplementary Fig. 13g–k; n = 4/5), 7 dpi (Supplementary Fig. 13l–p; n = 3/3), and 21 dpi (Supplementary Fig. 13q–u; n = 4/4), few tbx5a:GFP+ cardiomyocytes could be observed in the cortical layer close to the injured area. Nonetheless, at 130 dpi, the regenerated cortical myocardium was tbx5a:GFP− (Supplementary Fig. 13v–z; n = 4/4). This transient expression of tbx5a in cortical cardiomyocytes can have two explanations: (a) tbx5a is re-expressed transiently in the cortical layer during regeneration, or (b) trabecular cardiomyocytes are contributing to the new cortical layer.

To evaluate these two possibilities, tbx5a-derived cells were genetically traced using tbx5a:mCherry-p2A-CreERT2; ubb:loxP-GFP double transgenic fish (Fig. 6a). Upon recombination, GFP expression was restricted to the trabeculae in uninjured hearts (Fig. 6b–g; n = 7/7) and we only rarely detected GFP+ cells in the cortical myocardium (two cells in one out of six completely sectioned hearts, Supplementary Fig. 14a–f). By contrast, we observed GFP+/mCherry− cells in the regenerated cortical layer at 21 dpi (Fig. 6h–m; n = 6/7) and 90 dpi (Fig. 6n, o–s; n = 6/6), indicating that these cells derived from tbx5a+ cells had switched off tbx5a expression. In the remote area, GFP+ cells remained restricted to the trabecular layer even upon cryoinjury (Fig. 6t–x; n = 5/6). Based on the percentage of trabecular cardiomyocytes that are GFP+, the recombination efficiency was estimated to be 10 ± 6%. In the newly formed cortical layer, 7 ± 0.0001 by two-tailed unpaired t-test. at, atrium; prim, primordial layer; SHF, second heart field; trab, trabeculae; v, ventricle. Scale bars, 25 μm
4% of cardiomyocytes are GFP* (n = 10). Therefore, trabecular
might contribute to a higher extent to the regenerating cortical
layer than estimated by GFP signal detection.

To confirm that these cells came from tbx5a+ trabeculae and
were not produced by leaky recombination in response to injury,
we repeated the experiment in the absence of 4-OHT adminis-
tration; we found no GFP+ recombinant cells within the
regenerated area (Supplementary Fig. 14g–q; n = 3/3). We further
excluded that the result is caused by 4-OHT remaining in the
body at the moment of injury through treating zebra
fish with 4-OHT and BrdU for 24 h (Fig. 5a) or with 4-OHT and Mtz (red) and terminal
deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) (green). Note that some rounded mCherry+ cells are TUNEL+. Single channels of selected area in c. Quantification of the number of TUNEL+ trabecular and cortical mCherry+ cardiomyocytes per heart from animals of the
+MtZ (n = 8) and −MtZ (n = 8) group, mean ± SD; ***P = 0.0004 by Mann–Whitney non-parametric t-test. Schematic representation of the 5-Bromo-2’
-deoxyuridine (BrdU) treatment to assess proliferation. i. Fish were treated with 4-OHT and BrdU or with 4-OHT, Mtz, and BrdU. Single channels of the boxed area in j, m Quantification of BrdU+/mCherry+ and BrdU+/tagBFP+ cells per heart. Shown are means ± SD (n = 8 for −MtZ hearts and n = 11 for + Mtz hearts, from two separate independent experiments). *P = 0.0240 for tagBFP+ cells and P = 0.0371 for mCherry+ cells by two-tailed t-test. a, atrium; hpf, hours postfertilization; v, ventricle. Scale bars, 25 μm

described to differentiate cortical from trabecular myocardium in the zebrafish. To interrogate whether tbx5a-derived trabecular cardiomyocytes not only relocate into the cortical region and
switch off tbx5a but also adopt the expression of cortical marker
genes, we performed RNA sequencing (RNA-Seq) comparing
+MtZ-positive and -negative cardiomyocytes isolated from adult
trigeminal arteries (Fig. 7a, b). tbx5a was the top gene differentially
expressed between the two populations (Fig. 7c and Supplementary
Data 1), further supporting that the transgenic tbx5a reporter
genes reproduce the endogenous pattern. A number of genes
related to actin cytoskeleton, extracellular matrix, and caveolae
were expressed specifically in tbx5a– cardiomyocytes (Fig. 7c, d): two of these markers, xirp2a36 and lama5, were validated by
immunofluorescence for their protein product (Figs. 8 and 9). In
uninjured hearts, Xirp2a was detected in the cortical myocardium
(Fig. 8a–f, n = 3/3). Strikingly, the tbx5a-derived GFP+ cells
that contributed to the cortical layer and switched off tbx5a:mCherry
also expressed Xirp2a (Fig. 8g–r, n = 6/6). Laminin was detected
in the cortical layer (Fig. 9a–l, n = 3/3) and, upon injury, cortically
located *tbx5a*-derived GFP+ were in a Laminin+ environment; Fig. 9g–r, n = 6/6). This result was further supported using a RNAscope protocol to detect *lama5* mRNA in situ (Supplementary Fig. 15; a–h, n = 4/4; i–p, n = 11/11). A third gene used to determine whether *tbx5a*-derived cardiomyocytes express cortical markers when found in the cortical myocardium was *hey2*, a well-established marker of the compact layer in the embryonic mouse heart11 (Supplementary Fig. 16). *hey2* mRNA in situ detection by RNAscope revealed expression in cardiomyocytes from the cortical myocardium of the adult zebrafish (Supplementary Fig. 16 a–h; n = 3/4). *tbx5a*-derived trabecular cardiomyocytes that were within the cortical region at 90 dpi co-localized with *hey2* staining (Supplementary Fig. 16 i–m; n = 5/6). Of note, *tbx5a*-derived cells within the trabecular myocardium did not reveal similar *hey2* expression levels (Supplementary Fig. 16 n–p; n = 5/6). On the contrary, the expression of the trabecular marker *nppa* was not retained in *tbx5a*-derived cells within the cortical myocardium (Supplementary Fig. 17, n = 7/7), altogether indicating that trabecular *tbx5a*+ cardiomyocytes undergo a true phenotypic switch to become cortical layer cardiomyocytes. Although the cortical myocardium had previously been reported to regenerate from cardiomyocytes within the same layer5, our results reveal that trabecular cardiomyocytes can also contribute to cortical layer repair in response to injury.

**Discussion**

In the developing hearts of birds and mammals, a border between *Tbx5*-positive and *-negative cells determines the region of ventricular septation32. Our results here establish that the embryonic ventricle in zebrafish is concomitantly also split into *tbx5a*+ and *tbx5a*− domains. A similar boundary between *drl*+ and *drl*− cells has been described to separate the embryonic ventricle into domains to control distinct physiological properties11, suggesting that similar to mammals, adult teleost cardiac ventricles maintain FHF and SHF lineage territories (Fig. 10a). Although the zebrafish ventricle is not septated, the presence of a FHF–SHF-like
boundary might represent a state that has been further progressed in evolution to allow morphogenetic events leading to septation.

Our results indicate that cardiomyocyte plasticity allows the ventricle to re-form exclusively from SHF-derived cardiomyocytes (Fig. 10b). Of note, in mammals, Tbx5 expression labels the derivatives of the FHF in the ventricle and also the boundary with the SHF19,20; thus, we cannot exclude that the contribution of the SHF is slightly bigger than described in our study. Nonetheless, our results show that SHF cardiomyocytes can compensate the loss of the whole FHF-derived ventricle.

tbx5a lineage tracing allowed (i) to confirm that the cortical layer forms during development from the trabecular myocardium5 and (ii) to identify that this process involves not only a change in location of cardiomyocytes from the trabecular to the cortical regions but also a change in gene expression (Fig. 10a).

In contrast to development, cardiomyocyte plasticity seems to be reduced in the regenerating adult heart:19 conversion of atrial cardiomyocytes to ventricular cardiomyocytes has not been conclusively reported7 and Gupta et al.5 described that the newly regenerated cortical layer derives from pre-existing cortical layer cardiomyocytes. Indeed, the general rule during regeneration is that every cell contributes to the same cell type it was before injury, as illustrated by the cell types comprising the axolotl limb33. In contrast, our results show that a breach of this behavior is possible, with trabecular cardiomyocytes re-specialising into cortical layer cardiomyocytes during regeneration of the adult zebrafish heart (Fig. 10c). This re-specialisation reuses a...
developmental process in the setting of regeneration to rebuild the injured heart. It is interesting to note that zebrafish hearts are highly trabeculated in comparison with adult mammals. In murine cardiogenesis, the trabecular myocardium is proliferating at a much lower rate than the compact myocardium34. Thus, the switch from a trabecular phenotype to cortical phenotype might additionally allow the cardiomyocytes to re-enter a more active rate of cycling, allowing them to drive the regeneration after cryoinjury in the adult context. We propose that cardiomyocyte plasticity is thus likely a key feature necessary to react efficiently with a regenerative response to injury.

**Methods**

**Animal handling and generation of transgenic lines.** Ethical approval was obtained from the Community of Madrid “Dirección General de Medio Ambiente” in Spain and the “Amt für Landwirtschaft und Natur” from the Canton of Bern, Switzerland. Animals were housed and performed in accordance with Spanish and Swiss bioethical regulations for the use of laboratory animals. Fish were maintained at a water temperature of 28 °C.

The construct to generate Tg(tbXsαGFP) transgenic zebrafish lines was made by recombining the tdTomato cassette (Supplementary Data 2) into the BAC CH73-99A14. The construct for Tg(sbαGFP) was generated by recombining the Tol2Amp cassette15 (Supplementary Table 1, primers 1,2) and mCherry-p2A-CreERT22 (Supplementary Data 3 and Supplementary Table 1, primers 3,4) into the same BAC. The construct to generate Tg(sbαGFP) was made by recombining Tol2Amp-y-crystallin:RFP (Supplementary Data 4 and Supplementary Table 2, primers 2,5) and CreERT22 (Supplementary Data 3 and Supplementary Table 1, primers 3,4) into the same BAC. The construct to generate Tg(sbαGFP) was made by recombining Tol2Amp-y-crystallin:RFP (Supplementary Data 4 and Supplementary Table 2, primers 2,5) and CreERT22 (Supplementary Data 3 and Supplementary Table 1, primers 3,4) into the same BAC. The construct to generate Tg(sbαGFP) was made by recombining Tol2Amp-y-crystallin:RFP (Supplementary Data 4 and Supplementary Table 2, primers 2,5) and CreERT22 (Supplementary Data 3 and Supplementary Table 1, primers 3,4) into the same BAC.

**Imaging, survival, and staining.** Embryos were imaged using a Zeiss AxioImager M1 microscope and ImageJ software. Whole-mount in situ hybridization (ISH) was performed as previously described using a copper enzyme method with digoxigenin-labeled RNA probes. Embryos were photographed at 4 dpf, and embryos with interpretable markers were imaged. For all the experiments involving Cre recombination or cell ablation, homozygous and hemizygous embryos were used interchangeably. BroU was added to E3 water at 5 mg ml⁻¹ with 0.5% dimethyl sulfoxide from 4 to 7 dpf.
In situ mRNA hybridization. In situ mRNA hybridisation on paraffin sections and on whole-mount larvae were performed as described4,5,6 using tbx5a (complementary DNA kindly provided by J.L. Gómez-Skarmeta), and npg47 riboprobes.

Paraffin sections were deparaffinized, postfixed 20 min with PFA 4%, washed with PBS, treated with proteinase K 10 µm ml⁻¹ for 10 min at 37 °C, washed with PBS, postfixed with PFA 4% for 5 min, washed with PBS, treated with HCl 0.07 N for 15 min, washed with PBS, treated with 0.25% acetic anhydride in triethanolamine 0.1 M pH 8 for 10 min, washed with PBS, washed with RNase-free water, and then hybridised with the probe in pre-hybridisation buffer over night at 65 °C. The following day, sections were washed twice with post-hybridisation buffer 1 (50% formamide, 5 × SSC, 1% SDS) for 30 min at 65 °C and twice more with post-hybridisation buffer 2 (50% formamide, 2 × saline-sodium citrate SSC, 1% SDS). Then, sections were washed with maleic acid buffer with 0.1% Tween 20 (MABT) buffer at room temperature and incubated at least 2 h in blocking solution at room temperature. They were next incubated overnight with anti-digalkaline phosphatase (AP) antibody (1 : 2,000) in blocking solution. The third day, they were washed with MABT for several hours, followed by washes in AP buffer. Sections were developed in BM-Purple (Roche) until signal was detected.

For whole-mount in situ hybridisations, embryos were fixed in 4% PFA overnight, dehydrated in methanol series, and stored at −20 °C until its use. On day 1, embryos were rehydrated, washed in PBT, digested with 10 µg ml⁻¹ for 15 min, rinsed in PBT, and fixed in 4% PFA for 10 min. After washing again in PBT, they were pre-hybridised at 67 °C for at least 1 h. The antisense riboprobe was added at 0.3 µg µl⁻¹. After overnight hybridisation, two washes with 50% formamide/2 × SSCT, one wash with 2 × SSCT, and an additional two washes with 0.2 × SSCT were performed, all at 67°C. Then, embryos were transferred to room temperature and washed in MABT. Antibody incubation, washing, and color development conditions were as for in situ on sections. RNAscope (Advanced Cell Diagnostics, Hayward, CA) was performed following the manufacturer’s instructions for formalin-fixed paraffin-embedded samples with standard tissue pretreatment and 2.5 RED detection kit.

Immunofluorescence on sections. Paraffin sections were deparaffinized, rehydrated, and washed in distilled water. Epitopes were retrieved by heating in 10 mM citrate buffer (pH 6.0) for 15 min in a microwave at full power. Gelatine sections were incubated instead 30 min in 0.1% Tween20 in PBS at 37 °C to dissolve the gelatin. Nonspecific binding sites were saturated by incubation for at least 1 h in blocking solution (5% BSA, 5% goat serum, and 20 mM MgCl₂ in PBS). Endogenous biotin was blocked with the avidin–biotin blocking kit (Vector, Burlingame, CA, USA).

Antibodies. Primary antibodies used were anti-Mysin Heavy Chain (MF20, DSHB, 1 : 20 and F59, DSHB, 1 : 20), anti-GFP (AVES, GFP-1010, 1 : 500, 632392, Clontech, Mountain View, CA, USA, 1 : 100), anti-RFP (ab34771, AbCam, 1 : 200), anti-Xirp2a30, a kind gift from C. Otten and S. Seyfried (1 : 500), anti-Laminin (L9393, Sigma, 1 : 200), anti-BrdU (BD Biosciences, B44, 1 : 100), anti-mKate49 to detect tagBFP (1 : 500). Biotin- or Alexa (488, 568, 633)–conjugated secondary antibodies, and streptavidin-Cy3 (Jackson Immuno Research Laboratories) were used at 1 : 300. Nuclear were stained with 4′,6-Diamidino-2-phenylindole (DAPI) and slides were mounted in Fluorsave (Calbiochem).

Imaging and Image analysis. Embryos were imaged with a Zeiss 780 confocal microscope using a × 20 objective with a dipping lens. Z-stacks were taken every 1 µm. Three-dimensional images were reconstructed with ImageJ. A Leica TCS SP-5 or a Nikon A1R confocal microscope was used for imaging of histological sections. The percentage of tagBFP cells was quantified using ImageJ considering the area of tagBFP and comparing it with the area of myosin heavy chain (MHC) staining.
The percentage of tbx5a-derived cells was quantified applying a median filter of radius 1 in ImageJ. The GFP+ and MHC+ areas were measured. The same set of images was used to quantify the percentage of GFP+ cardiomyocytes in trabeculae and in the regenerated compact layer, applying the same threshold for both regions.

In vivo imaging and image processing. Double transgenic Tg(tbx5a:tdTomato)Et (-26.5Hsa.WT1-gata2:EGFP) lines were grown until 60 hpf in E3 culture medium with 0.0033% 1-Phenyl-2thiourea. Embryos were anesthetized with 0.08 mg ml⁻¹ 4-OHT Cryoinjury. Double transgenic Tg(tbx5a:tdTomato); itb5a:mCherry-p2a-CreERT2; ubb:loxP-lacZ-STOP-loxP-GFP lines were grown until 60 hpf in E3 culture medium with 0.0033% 1-Phenyl-2thiourea. Embryos were anesthetized with 0.08 mg ml⁻¹ 4-OHT. Heart dissociation followed by sorter and RNA-Seq library production. Zebrafish hearts were dissected and the atrio-ventricular canal was carefully removed. The probe was lowered until an ultrasound conductive foam holder. Once the animal was anesthetised, the images were obtained with a VEVO 2100 system (VisualSonics) coupled to a 50 MHz ultrasound transducer. The green (GFP) and red (tdTomato) channels were acquired simultaneously with maximum speed in bidirectional mode.

Ultrasound and assessment of cardiac function in larvae. Measurements and analysis were performed as described. A mixture of 60 mM of tricine and 3 mM of isofluorane were added to the tank water in order to anesthetize the animals. A Petri dish was filled with this solution and the fish was placed ventral side up in a foam holder. Once the animal was anesthetised, the images were obtained with a VEVO 2100 system (VisualSonics) coupled to a 50 MHz ultrasound transducer. The probe was lowered until an ultrasound conductive film was formed with the water, so that the skin of the animal was not touched at any time. This way the animal position was rotated till we obtained a long axis view of the heart with a complete visualisation of the ventricular apex. In that moment we recorded cine two-dimensional sequences, with a frame rate of 200 images per second, which were analysed later, in a second step, to minimize anesthesia times.

Imaging of cardiac function in larvae was performed using a Leica AM TIRF MC microscope in epifluorescence mode, using an Andor DU 885K-C50×+VP (1,004 × 1,002) camera and HCX PL APO 20 × 0.7 dry (11,506,166) objective. The maximum (diastolic) and minimum (systolic) ventricular areas were measured in order to determine ventricular function using Image J.

Heart dissociation followed by sorter and RNA-Seq library production. Zebrafish hearts were dissected and the atrio-ventricular canal was carefully removed in order to obtain a pool of exclusively ventricular cells. The ventricles were dissociated according to previous protocols with minor modifications. The enzyme concentration was doubled and time of digestion increased to 1 h and 40 min with gentle agitation while pipetting with a cut 1,000 µl tip every 20–30 min. Then, one volume of PBS + 10% fetal bovine serum (FBS) was added and the mixture was centrifuged for 8 min at 250 g and re-suspended in PBS + 1% FBS. Specifically, the following enzyme concentrations were used: liberase TH (Sigma, 200 mg l⁻¹), Elastase (Serva, 1 : 250), Pronase E (Serva, 1 : 100 dilution of the 26.3 mg ml⁻¹ stock), DNase (Qiagen, 1 : 20,000) and 2,3-butanedione monoxime (Sigma, 10 mM). All reagents were dissolved in Tyrode’s low calcium (in mM): NaCl 140, KCl 5.4, CaCl₂ 0.01, MgCl₂ 1.0, glucose 5.5, and HEPES 5.0; pH was set to 7.4 with NaOH.

Cells were sorted using a SONY Synergy sy3200 sorter and RNA was extracted using the Arcturus Pico Pure RNA isolation kit (Thermofisher) following the manufacturer’s instructions.
Total RNA (0.6 ng) was used to generate barcoded RNA-Seq libraries using the Ovation Single Cell RNA-Seq System (NuGEN) with two rounds of library amplification. The size of the libraries was calculated using the Agilent 2100 Bioanalyzer. Library concentration was determined using the Qubit fluorometer (ThermoFisher Scientific). Libraries were sequenced on a HiSeq2500 (Illumina) platform to generate ≥ 60-base single reads. FastQA files for each sample were obtained using CASAVA v1.8 software (Illumina). Four biological replicates consisting of five pooled hearts were used per sample.

**RNA-Seq analysis.** Sequencing adapter contaminations were removed from reads using cutadat 1.9.1 software and the resulting reads were mapped and quantified on the transcriptome (Ensembl gene-build 10, release 82) using RSEM v1.2.25. Only genes with at least one count per million in at least two samples were considered for statistical analysis. Data were then normalised and differential expression testing using the bioconductor package EdgeR. We considered as differentially expressed those genes with a Benjamini–Hochberg adjusted p-value ≤ 0.05 and log fold change ≥ 1. A heatmap was made using gplot library and heat-map.2 function.

**Data availability.** RNA-Seq data have been deposited in the GEO database under accession code GSE87596. The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files or from the corresponding author upon reasonable request. Raw data corresponding to the Figures are deposited at Mendele: https://doi.org/10.17632/fkm2tvh2sv.1; https://doi.org/10.17632/4fp5f8t7j3.1 and https://doi.org/10.17632/zgdff55m44.1

Received: 10 April 2017 Accepted: 15 December 2017

**Published online: 30 January 2018**

**References**
1. Kitada, K. et al. Primary contribution to zebrafish heart regeneration by gata4 (+) cardiomyocytes. Nature 464, 601–605 (2010).
2. Jopling, C. et al. Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. Nature 464, 606–609 (2010).
3. Gonzalez-Rosa, J. M., Burns, C. E. & Burns, C. G. Zebrafish heart regeneration: 15 years of discoveries. Regeneration (Oxf) 4, 105–123 (2017).
4. Liu, J. et al. A dual role for ErbB2 signaling in cardiac trabeculation. Development 137, 3867–3875 (2010).
5. Gupta, V. & Poss, K. D. Clonally dominant cardiomyocytes direct heart regeneration. Nature 484, 479–484 (2012).
6. Gupta, V. et al. An injury-responsive gata4 program shapes the zebrafish cardiac ventricle. Curr. Biol. 23, 1221–1227 (2013).
7. Zhang, R. et al. In vitro reprogramming contributes to zebrafish heart regeneration. Nature 498, 497–501 (2013).
8. de Pater, E. et al. Distinct phases of cardiomyocyte differentiation regulate growth of the zebrafish heart. Development 136, 1633–1641 (2009).
9. Hami, D., Grimes, A. C., Tsai, H. J. & Kirby, M. L. Zebrafish cardiac development requires a conserved secondary heart field. Development 138, 2389–2399 (2011).
10. Lazic, S. & Scott, I. C. Mef2cb regulates late myocardial cell addition from a second heart field-like population of progenitors in zebrafish. Dev. Biol. 354, 123–133 (2012).
11. Mosimann, C. et al. Chamber identity programs drive early functional partitioning of the heart. Nat. Commun. 6, 8146 (2015).
12. Zhou, Y. et al. Latent TGF-beta binding protein 3 identifies a second heart field in zebrafish. Nature 474, 645–648 (2011).
13. Buckingham, M., Meilhac, S. & Zaffran, S. Building the mammalian heart from mesoderm. Nature 461, 95–99 (2009).
14. Krugl, M. et al. Cells keep a memory of their tissue origin during axolotl limb regeneration. Nature 460, 60–65 (2009).
15. Sedmera, D. & Thompson, R. P. Mycophyte proliferation in the developing heart. Dev. Dyn. 240, 1332–1334 (2011).
16. Suster, M. L., Abe, G., Schouw, A. & Kawakami, K. Transposon-mediated BAC transgenesis in zebrafish. Nat. Protoc. 6, 1998–2021 (2011).
17. Lee, E. C. et al. A highly efficient Eucherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. Genomics 73, 56–65 (2001).
18. Felker, A. & Mosimann, C. Contemporary zebrafish transgenesis with Tol2 and application for Cre/lox recombination experiments. Methods Cell Biol. 135, 219–244 (2015).
19. Kwan, K. M. et al. The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. Dev. Dyn. 236, 3088–3099 (2007).
20. Felker, A. et al. In vivo performance and properties of tamoxifen metabolites for CreERT2 control. PLoS ONE 11, e0152989 (2016).
21. Peralta, M. et al. Heartbeat-driven pericardiac fluid forces contribute to epicardial morphogenesis. Circ. Res. 113, 1762–1773 (2013).
22. Gonzalez-Rosa, J. M. & Mercader, N. Cryoinjury as a myocardial infarction model for the study of cardiac regeneration in the zebrafish. Nat. Protoc. 7, 782–788 (2012).
23. Mahly, J. D., Mohideen, M. A., Burns, C. G., Chen, J. N. & Fishman, M. C. Heart of glass regulates the concentric growth of the heart in zebrafish. Curr. Biol. 13, 2183–2187 (2003).
24. Rohr, S., Otten, C. & Abdelilah-Seyfried, S. Asymmetric involution of the myocardial field drives heart tube formation in zebrafish. Circ. Res. 102, e12–e19 (2008).
25. Mosimann, C. et al. Ubiquitous transgene expression and Cre-based recombination driven by the ubiquitous promoter in zebrafish. Development 138, 169–177 (2012).
26. Gonzalez-Rosa, J. M., Peralta, M. & Mercader, N. Pan-epicardial lineage tracing reveals that epicardium derived cells give rise to myofibroblasts and perivascular cells during zebrafish heart regeneration. Dev. Biol. 370, 173–186 (2012).
27. Mercader, N., Fischer, S. & Neumann, C. J. Pdm1 acts downstream of a sequential RA, Wnt and Fgf signaling cascade during zebrafish forelimb induction. Development 133, 2805–2815 (2006).
28. Gonzalez-Rosa, J. M. et al. Use of echocardiography reveals reestablishment of ventricular pumping efficiency and partial ventricular wall motion recovery upon ventricular cryoinjury in the zebrafish. PLoS ONE 9, e115604 (2014).
29. Jahangiri, L. et al. The AP-1 transcription factor component Fos2 potentiates the rate of myocardial differentiation from the zebrafish second heart field. Development 143, 113–122 (2016).
30. Cai, D., Cohen, K. B., Luo, T., Lichtman, J. W. & Sanes, J. R. Improved tools for Brn2 expression and functional characterization of cardiac pacemaker cells in zebrafish. PLoS ONE 9, e107464 (2014).
31. Martin, C. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMNNet.17, (2011).

ARTICLE | DOI: 10.1038/s41467-017-02650-6 www.nature.com/naturecommunications
Li, B. & Dewey, C. N. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12**, 323 (2011).

Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–140 (2010).

Acknowledgements
We are grateful to the Animal facility, Histology, Microscopy, Cellomics, Bioinformatics, and Genomics Units from CNIC, the Microscopy Imaging Center from the University of Berne, X. Langa, L. Flores, M. Villalba, and R. Costa for experimental assistance; S. Seyfried and C.-B. Chien for reagents and M. Torres, S. Martin-Puig, J. González-Sainz-de-Aja, R.M. Benedito, and A. Jarwinska for discussion. Funding was through FP7/12/03007 and BFU2014–56970–P (Plan Estatal de Investigación Científica y Técnica y de Innovación 2013–2016, Programa Estatal de I+D+i Orientada a los Retos de la Sociedad Retos Investigación: Proyectos I+D+i 2016, del Ministerio de Economía competitividad e Industria), and co-funding by Fondo Europeo de Desarrollo Regional (FEDER) (H.S. and N.M.); Swiss National Science Foundation grant 31003A_159721, ANR-SNF collaborative Project 320030E-164245, and the ERC starting grant 337703–zebra–Heart (N.M.). A. E. is enrolled into PhD specialization Cutting Edge Microscopy offered by the Graduate School for Cellular and Biomedical Sciences (GCB) and the Microscopy Imaging Center (MIC). The CNIC is supported by the Ministry of Economy, Industry and Competitiveness (MEIC) and the Severo Ochoa Center of Excellence (MEIC award SEV-2015–0505). Further support was from the Canton of Zürich, project grant from the Swiss Heart Foundation (A.F. and C. Mosimann); the Swiss National Science Foundation (SNF) professorship (PP00P3_139093) and a Marie Curie Career Integration Grant from the European Commission (CIG PCIG14-GA-2013-631984) (C. Mosimann).

Author contributions
H.S.-I. designed and carried out experiments. M.G.-C., A.S.-M. and A.E. carried out experiments. G.G.-M. carried out ultrasound experiments. A.F. and C. Mosimann generated the drl:mCherry line. C.M. generated the tbx5a:GFP and tbx5a:tdTomato lines. J.M.G.-R. contributed in the generation of transgenic lines. N.M. designed experiments and secured funding. All authors contributed to writing the manuscript.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-017-02650-6.

Competing interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2018