The careg element reveals a common regulation of regeneration in the zebrafish myocardium and fin

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The existence of common mechanisms regulating organ regeneration is an intriguing concept. Here we report on a regulatory element that is transiently activated during heart and fin regeneration in zebrafish. This element contains a ctgfa upstream sequence, called careg, which is induced by TGFβ/Activin-β signalling in the peri-injury zone of the myocardium and the fin mesenchyme. In addition, this reporter demarcates a primordial cardiac layer and intraray osteoblasts. Using genetic fate mapping, we show the regenerative competence of careg-expressing cells. The analysis of the heart reveals that the primordial cardiac layer is incompletely restored after cryoinjury, whereas trabecular and cortical cardiomyocytes contribute to myocardial regrowth. In regenerating fins, the activated mesenchyme of the stump gives rise to the blastema. Our findings provide evidence of a common regenerative programme in cardiomyocytes and mesenchyme that opens the possibility to further explore conserved mechanisms of the cellular plasticity in diverse vertebrate organs.
A dult zebrafish, axolotls and newts are valuable models in regenerative biology. These animals can survive substantial heart and limb injuries, and efficiently replace the lost parts with new functional tissues\textsuperscript{12-18}. During reconstruction of the missing part, remnant injury-associated cells reactivate morphogenetic programs, proliferate and recreate a replicate of the original structure. Clonal analysis and genetic lineage tracing in salamanders and zebrafish have provided evidence that specialized cell types, such as muscle cells, cardiomyocytes and osteoblasts, undergo reversion to a less mature state, referred to as dedifferentiation, within the same lineage hierarchy\textsuperscript{11-18}. Less-complex cells, such as epithelial, mesothelial and fibroblast-like cells, can more rapidly enter the regenerative programme, and additionally, may exhibit the plasticity to undergo transition to other developmentally related tissues or even more distant lineages through transdifferentiation\textsuperscript{19}. The key regeneration-participating cells of the heart and the appendage, namely cardiomyocytes (CMs) and mesenchymal cells (MES), substantially differ in their architectural complexity, size and metabolism. An intriguing question is whether such diverse cell types activate common genetic regulatory mechanisms during the regenerative response.

Transgenic approaches in the zebrafish heart and fin showed that the regenerated tissues derive from local functional cells that can undergo limited dedifferentiation\textsuperscript{12-15,17,18,20}. In the resected ventricle, analysis of the gata4 transgenic reporter revealed that cortical CMs in the vicinity of the wound reactivate developmental gene expression and contribute to the new myocardium\textsuperscript{13,21}. In the amputated fin, blastema formation requires the activation of MES in the stump. The distal part of the regenerative outgrowth acts as an apical signalling centre, whereas the proximal blastema comprises the proliferation and re-differentiation zone\textsuperscript{22-26}. Chromatin-remodelling factors are required for both organ regeneration\textsuperscript{27,28}. The lineage tracing and genetic clonal analyses have provided no evidence for a contribution of remote stem cells to the heart and fin regenerate\textsuperscript{12,13,20,29}.

Despite the anatomical differences between the heart and fin, a concept of common regulatory mechanisms of regeneration has been recently proposed. This idea is based on the identification of a short DNA sequence upstream to the zebrafish metabolic gene, leptin b, which is induced in the endocardium and wound tissue of the heart, as well as in the blastema of the fin\textsuperscript{30}. No activation of this enhancer element, however, has been observed in CMs. Thus, it still remains unknown whether common regulatory elements exist for injury-responsive cardiac myocytes and fin fibroblasts.

The ventricle of the adult zebrafish heart is a complex structure, containing an outer cortical (compact) layer and inner trabecular (spongy) fascicles\textsuperscript{31}. Both types of myocardium are separated by flattened cardiac cells, called junctional (transitional) CMs\textsuperscript{32,33}. On the basis of multicolour clonal analysis, this cardiac layer has been named ‘primordial’ layer, which is established in the early embryonic heart and maintained throughout ontogenesis\textsuperscript{34}. While the gata4-reporter studies have provided evidence for a regenerative contribution of the cortical myocardium\textsuperscript{13}, the restorative potential of trabecular and primordial CMs remains unclear.

In this study, we identified a 3.18 kb DNA fragment containing an upstream genomic sequence of the ctgfa gene, called the careg element, which is activated in the regenerating ventricular CMs and fin MES in a TGFβ/Activin-β signalling-dependent manner. Our findings yield insight into the mechanisms of heart and fin restoration and provide evidence for the existence of regeneration regulatory programs that are conserved between architecturally diverse cells, such as cardiomyocytes and mesenchymal cells.

Results

A reporter of peri-injured cardiomyocytes and fin mesenchyme.

To uncover the mechanisms underlying the injury-dependent reactivation of morphogenetic programs in distinct organs, we searched for an informative DNA regulatory element that is induced in regenerating myocardium and fin. In both organs, injury-activated tissues upregulate synthesis of specific developmental proteins. In the heart, a portion of the trabecular myocardium within 100 μm from the injury border reactivates the expression of the embryo-specific cardiac myosin heavy chain isofrom, embCMHC\textsuperscript{35}. In the fin, MES reorganization occurs within at least 300 μm from the amputation plane, which is associated with the upregulation of Tenascin C, a de-adhesive extracellular matrix protein\textsuperscript{36}. Using a candidate approach, we identified a transgenic reporter that overlaps with the transient expression of embCMHC and Tenascin C in the regenerating hearts and fins, respectively. The reporter was driven by a 3.18 kb upstream regulatory sequence of connective tissue growth factor a (ctgfa)\textsuperscript{37}. This transgenic line was originally established to investigate notochord development, and was also used to highlight the connective tissue of regenerating fins\textsuperscript{37,38}, but it had not yet been investigated in the heart. Thus, the activation of ctgfa:EGFP in the embCMHC-positive region was an unexpected finding.

To determine the spatiotemporal pattern of the reporter activity during organ regeneration, we analysed the dynamics of ctgfa:EGFP after ventricular cryoinjury and fin amputation. In uninjured hearts, this reporter was detected in a fine sheet of cells along the ventricular circumference (Supplementary Fig. 1a,b). At 1 d.p.c. (days post-cryoinjury), the injured myocardium was recognized by the absence of ctgfa:EGFP expression in the outer heart layer, as opposed to the intact part of the ventricular wall (Fig. 1a,b). No ctgfa:EGFP expression was observed in the trabecular myocardium (Fig. 1c). At 4, 7 and 14 d.p.c., fluorescence persisted in the subcortical layer, and additionally, it emerged in ~60% of the cardiac muscle within a distance of 100 μm from the injury border (Fig. 1d–f; Supplementary Fig. 1c–f). Approximately 20% of these cells also expressed embCMHC at 7 and 14 d.p.c. (Fig. 1f; Supplementary Fig. 1i). Towards the end of the regenerative process at 30 d.p.c., the expression of the reporter became reduced, and it was nearly undetectable within the new myocardium at 60 d.p.c. (Fig. 1g; Supplementary Fig. 1gh). We concluded that ctgfa:EGFP represents a reliable marker of adult CMs that transiently reactivate the regenerative programme in the peri-injured myocardium.

Consistent with previous studies of zebrafish fin regeneration\textsuperscript{38}, live-imaging of caudal fins revealed that ctgfa:EGFP was strongly upregulated in the regeneration zone at 1 and 3 d.p.a. (days post amputation), and declined when the restorative process was completed at 20 d.p.a. (Fig. 1j–o; Supplementary Fig. 2a–l). At 1 and 3 d.p.a., the regeneration zone included not only the outgrowing blastema, but also the peri-injury stump tissue within a distance of three ray segments below the amputation plane. Immunofluorescence analysis of longitudinal fin sections at 3 d.p.a. showed that the expression of ctgfa:EGFP was induced in the Tenascin-C-positive MES of the stump and the blastema (Fig. 1q). No expression was observed in dedifferentiated osteoblasts above the injury plane (Fig. 1s). Little fluorescence was detected in the distal blastema (Supplementary Fig. 2mn). Taken together, ctgfa:EGFP is transiently upregulated during dedifferentiation in both CMs and MES at the injury border of the heart and the fin, respectively.

Although ctgfa:EGFP was strongly induced in the regeneration zone, we observed its homeostatic expression in the intact heart.
Figure 1 | A transgenic reporter of the peri-injured myocardium and the fin stump.  
(a–i) Transversal ventricle sections of transgenic fish 
ctgfa<sup>cre</sup>:EGFP immunostained for embCMHC (red) at different days post-cryoinjury (d.p.ci.). The cardiac muscle is detected by F-actin staining (Phalloidin, blue). 
(b,e,h) The injury-remote part of the ventricular wall displays a subcortical layer of 
ctgfa<sup>cre</sup>:EGFP cells that is located between the thin cortical myocardium (CoM) and the inner trabecular myocardium (TrM). This layer does not express embCMHC and remains unaltered during regeneration. 
(c,f,i) The injury-abutting zone of the ventricular wall shows transient expression of 
ctgfa<sup>cre</sup>:EGFP and embCMHC within a distance of 100 μm from the wound border during regeneration. 

N<sub>z</sub> = 4. 

(j–o) Live-imaging of fins at different days post amputation (d.p.a.). Bright-field (BF) was combined with fluorescence. 
(k,m,o) Higher magnifications of the region at the amputation plane (yellow dashed line) show transient expression of 
ctgfa<sup>cre</sup>:EGFP in the stump and the regenerate. 

N = 4. 

(p–s) Immunofluorescence staining of longitudinal fin sections. 
At 0 d.p.a., 
ctgfa<sup>cre</sup>:EGFP<sup>+</sup> cells are associated with Tenascin C fibres along the border between the bones (B) and mesenchymal cells (MES), but not the epidermis (EP). Some of these cells express an osteoblast marker visualized with Zns5 antibody (red). At 3 d.p.a., Tenascin C demarcates the zone of tissue remodelling in the stump and the outgrowth. 

ctgfa<sup>cre</sup>:EGFP is upregulated in the activated MES at the peri-injury zone, but not in Zns5-labelled osteoblasts of the outgrowth. 

N<sub>z</sub> = 4. Post-infarcted ventricle is encircled with a dotted line. Fin amputation plane is shown with a dashed line. The same rules apply to all subsequent figures.
and fin, namely in the subcortical layer along the ventricular circumference and in cells along the interface between the bones and MES, respectively (Fig. 1b,e,h,p). In intact fins, \textit{ctgfa:EGFP} expressing cells colocalized with Zns5-labelled osteoblasts, predominantly at the interface between acellular bone matrix and MES in the intrarays (Fig. 1r). These cells were associated with extracellular de-adhesive Tenascin C, suggesting local tissue remodelling between MES and the bones (Fig. 1p,r). Thus, \textit{ctgfa:EGFP} is not solely a marker of injury-abutting cells, but also of certain junctional cells located at tissue borders during homeostasis.

A distinct expression of \textit{ctgfa:EGFP} and endogenous \textit{ctgfa}.

To determine whether the \textit{ctgfa:EGFP} reporter matches the endogenous expression pattern of the \textit{ctgfa} gene, we performed \textit{in situ} hybridization on sections of hearts at 14 d.p.c.i. and fins at 3 d.p.a. Unexpectedly, we found that anti-sense probes against \textit{ctgfa} and \textit{enhanced green fluorescent protein (egfp)} labelled different cells (Fig. 2a–h). \textit{ctgfa} mRNA was present solely in the wound tissue of the heart, and in cells associated with the bones of fins (Fig. 2a,b,e,f). By contrast, \textit{egfp} was detected in CMs along the ventricular circumference and in the vicinity of the injury of the heart, and in the blastema of the fin regenerate (Fig. 2c,d,g,h). Thus, in both organs, the \textit{egfp} mRNA distribution reproduced the pattern observed by fluorescence emission, while the endogenous \textit{ctgfa} gene had a distinct expression pattern. We concluded that the \textit{ctgfa:EGFP} transgene contains a sequence that is regulated in a unique manner in the peri-injury zone of regenerating organs. Accordingly, we renamed this reporter, ‘\textit{careg}’ (ctgfa reporter in regeneration), to distinguish it from the endogenous locus that includes additional genomic sequences regulating \textit{ctgfa} mRNA expression. Below, we used \textit{careg} to refer to this reporter.

To verify that the specificity of the \textit{careg:EGFP} expression pattern is related to the 3.18 kb \textit{ctgfa} upstream sequence and not to the genomic integration site of the construct, we created another transgenic line. We used a fluorescent protein with a rapid turnover, namely destabilized monomeric Kusabira-Orange 2 (dmKO2), which contains a C-terminal PEST domain targeted for rapid degradation via ubiquitinylation\cite{39}. Double transgenic fish, \textit{careg:EGFP}dmKO2 displayed an overlapping pattern in regenerating CMs and MES (Fig. 2i–m). The dynamics of \textit{careg:dmKO2} during heart and fin regeneration was similar to that of \textit{careg:EGFP} (Supplementary Figs 3,4). On the basis of the same expression pattern of both constructs, we concluded that \textit{careg} indeed contains a regulatory element that is specifically activated during regeneration.

To determine the features of the \textit{careg} sequence, we performed pairwise alignment of the \textit{ctgfa} genomic region between zebrafish and 9 other fish species from distinct phylogenetic orders, as well as with \textit{Xenopus}, chicken, mouse and human (Fig. 3a,b). This analysis identified two conserved regions within the \textit{careg} sequence, namely, a 150 bp fragment immediately before the transcription start site and a 400 bp region at \(~1\) kb upstream from this position (Fig. 3b). The latter is conserved in five other fish species from different orders, namely, a 150 bp fragment immediately before the downstream signal transducer of this pathway\cite{44}. Ventricle sections at 7 d.p.c.i. revealed that \textit{careg:EGFP} was induced within 100 \mu m in the peri-injury zone of the myocardium, which also upregulated embCMHC expression (Fig. 4a,b). Thus, both methods, cryoinjury and resection, induce a local activation of \textit{careg:EGFP} in CMs along the damaged border.

As opposed to the fin amputation model, the non-surgical exposure to the cold blade along the cryoinjury plane results in a progressive and spontaneous tissue detachment that is apparent between 12 and 48 h after procedure\cite{41}. Analysis of fins shortly after cryoinjury did not reveal any expression of \textit{careg:EGFP} (Fig. 4c,d). During shedding of the damaged distal part, at 1 and 2 d.p.c.i., \textit{careg:EGFP} was weakly induced in the stump, suggesting that the clearance of dead tissues was associated with a concomitant activation of the regenerative programme in the remaining cells (Fig. 4e,h). Remarkably, the expression of \textit{careg:EGFP} became further upregulated at 4 d.p.c.i., when the regenerative process was resumed after reparation of the distorted margin (Fig. 4i,j). As in amputated fins, \textit{in situ} hybridization on longitudinal fin sections showed that the expression of the \textit{careg} reporter was not associated with the induction of the endogenous \textit{ctgfa} gene within the activated MES (Supplementary Fig. 5a–d). At the advanced regenerative phase, at 14 d.p.c.i., \textit{careg:EGFP} was downregulated, consistent with the amputation model (Fig. 4k,l). To further test the regenerative response of the \textit{careg} element, we performed a regeneration assay of the larval tail, in which the posterior tip of the notochord was truncated. Consistent with previous \textit{ctgfa} studies\cite{37}, \textit{careg:dmKO2} displayed a basal expression level in the larval notochord (Supplementary Fig. 5e). The amputation site, however, transiently upregulated \textit{careg:dmKO2} levels during regeneration (Supplementary Fig. 5f–i). Taken together, the \textit{careg} reporter is activated in regeneration-responsive cells in several injury models, suggesting its general suitability for studying organ regeneration in zebrafish.

The \textit{careg} reporter in various injury models.

To test whether the \textit{careg} reporter is activated in other injury models, we switched between the procedures used for each organ, namely, we performed amputations of the ventricular apex and cryoinjury of the caudal fin\cite{40,43}. Examination of longitudinal heart sections at 7 d.p.a. revealed that \textit{careg:EGFP} was induced within 100 \mu m in the peri-injury zone of the myocardium, which also upregulated embCMHC expression (Fig. 4a,b). Thus, both methods, cryoinjury and resection, induce a local activation of \textit{careg:EGFP} in CMs along the damaged border.

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The activation of \textit{careg} is dependent on TGFβ and Activin-β.

Both heart and fin regeneration requires TGFβ/Activin-β signalling\cite{36,42}. In the fibrotic tissue of the injured ventricle, this pathway also induces a transient deposition of certain matrix components, such as a remodelling protein Tenascin C, fibril-forming Collagen I and fibril-associated Collagen XII (refs 42,43). To investigate whether TGFβ/Activin-β signalling is active in the \textit{careg} expression zone of regeneration-activated CMs and MES, we analysed phosphorylation of Smad3, which is a canonical downstream signal transducer of this pathway\cite{44}. Ventricle sections at 7 d.p.c.i. revealed that 57.3% ± 7.1 (N = 6) of \textit{careg:EGFP}-positive cells contained pSmad3-labelled nuclei (Fig. 5a–c). Fin sections at 3 d.p.a. showed 70.3% ± 1.8 (N = 7) of \textit{careg:EGFP} pSmad3 double-positive cells in the MES compartment at 200 \mu m below the amputation plane (Fig. 5o,p). This suggests that the peri-injury zone of both organs is substantially activated by TGFβ/Activin-β signalling.

To test the requirement of this pathway for \textit{careg} expression, we used the pharmacological inhibitor of the TGFβ/Activin-β type I receptor, SB431542 (refs 36,42). In uninjured heart, the prolonged treatment for 7 days with this drug did not affect the homeostatic expression of \textit{careg:EGFP} in the outer layer of the ventricle (Supplementary Fig. 6a–c). Consistently, at 7 and 14 d.p.c.i., the inhibition of the TGFβ/Activin-β pathway did not display any effect on reporter expression along the intact myocardial wall of \textit{careg:EGFP} and \textit{careg:dmKO2} hearts. By
In contrast, the peri-injury myocardium showed a three- to fourfold reduction of careg-labelled CMs (Fig. 5d–h, Supplementary Fig. 7a–j). This phenotype was also associated with attenuated embCMHC immunolabelling. We concluded that the regenerative induction of the careg element and the endogenous embCMHC expression in the peri-injured myocardium is regulated by the TGFβ/Activin-β signalling pathway.

Restorative cardiogenesis requires CM proliferation. To determine the effect of SB431542 treatment on CM proliferation specifically in the careg-positive regenerating myocardium, we assessed BrdU incorporation within the region of 100 μm from the injury border. This analysis revealed a fourfold decrease in the number of Mef2c/BrdU double-positive cells after inhibitor treatment (Supplementary Fig. 7k–o). Thus, the inhibition of TGFβ/Activin-β signalling affects not only dedifferentiation, but also proliferation of CMs in the peri-injury zone.

Next, we analysed the regulation of careg:EGFP in regenerating fins. In uninjured fins, the prolonged treatment for 7 days with SB431542 did not affect the homeostatic expression of careg:EGFP in osteoblasts (Supplementary Fig. 6d,e). At 1 and 3 d.p.a., however, the inhibition of TGFβ/Activin-β strongly prevented the regenerative outgrowth formation36, and the

Figure 2 | careg:EGFP does not reproduce endogenous ctgfa expression in peri-injury tissues. (a–h) In situ hybridization using ctgfa and enhanced green fluorescent protein (egfp) probes on sections of hearts at 14 d.p.c.i. and fins at 3 d.p.a. of ctgfa:EGFP transgenic fish shows distinct expression patterns. (a,b) ctgfa mRNA is not detected in the myocardium, but in a subset of non-myocytes in the post-infarcted tissue. (c,d) egfp mRNA is expressed in the outer wall of the myocardium and at the peri-injury zone (yellow arrowheads). (e,f) ctgfa mRNA is present in a few bone-associated cells of the fin, while (g,h) egfp mRNA is detected in the blastema. N = 6. (i–m) careg:EGFP and careg:dmKO2 double transgenic fish display an overlapping expression pattern in the regenerating heart at 7 d.p.c.i. and the fin at 1 d.p.a. N = 4.
activation of careg:EGFP in the stump (Fig. 5i–l; Supplementary Fig. 8a–d). To determine whether careg:EGFP expression was suppressed due to a general regenerative failure, we tested the fgf20a mutant fish, called dob, which fail to form a blastema. Despite an absence of the regenerative outgrowth, dob mutant fins normally induced careg:EGFP in the peri-injured stump at 1 and 3 d.p.a. (Fig. 5m,n; Supplementary Fig. 8e–f). We concluded that the regulation of the careg reporter in the fin is dependent on TGFβ/Activin-b signalling, irrespectively of the ability to form a blastema.

Although Fgf20a and TGFβ/Activin-β signalling have been described as the early inducers of blastema formation, the interaction between both pathways remains unknown. Using the careg element and pSmad3 immunofluorescence, we addressed the question whether the function of TGFβ/Activin-β signalling is dependent on Fgf20a. Our analysis of longitudinal fin sections revealed that the pSmad3 activity was blocked after SB431542 treatment, but remained unchanged in dob mutant fins (Fig. 5o–t). This finding indicates that TGFβ/Activin-β acts independently of the Fgf20a activity during fin regeneration, providing a
new insight into the molecular network guiding blastema formation.

Taken together, the analysis of hearts and fins revealed that the same molecular pathway, TGFβ/Activin-β, stimulates the upregulation of careg in distinct regeneration-participating tissues, such as CM and MES, but not in the fibrotic tissue.

careg in primordial CMs during heart development. Heart regeneration has been proposed to involve a reactivation of cardiac embryonic programs\(^3\). To determine whether the induction of the careg reporter in the peri-injured CMs demarcates less differentiated CMs, we performed analysis of the embryonic heart. We found that at 1 and 3 days post-fertilization (d.p.f.), careg:EGFP and careg:dmKO2 fully colocalized with embCMHC in embryonic CMs (Fig. 6a–f; Supplementary Fig. 9a,b). This finding suggests that the careg regulatory element represents a common molecular marker of activated CMs during regeneration and immature CMs during development.

To investigate the reporter expression in subsequent developmental stages, we crossed careg:EGFP with the cmhc2:DsRed-nuc line, which labels cardiac nuclei. In larvae at 12 d.p.f., we found that careg expression persisted in CMs of the heart wall, and a few careg-expressing cells were scattered in the ventricular chamber (Fig. 6g–i; Supplementary Fig. 9c). These inner CMs probably detached from the heart surface in the process of trabeculation, which involves delamination of CMs from the outer wall into the heart chamber\(^{46}\). Consistently, after completion of the trabeculation at 30 d.p.f., careg:EGFP and careg:dmKO2 were restricted to the outer myocardial wall (Fig. 6j–o). This result indicates that the reporter expression labels the primordial cardiac wall throughout development.

To genetically test the hypothesis that careg-positive CMs in the larval heart contribute to the delamination process of trabecular CMs, we performed lineage tracing of these cells. We generated a transgenic line with a tamoxifen-dependent Cre recombinase driven by the careg regulatory sequence, named Tg(careg:CreERT2). We crossed this strain with the ubiquitin-loxP-EGFP-STOP-loxP-mCherry lineage-tracing line, referred to as ubi:Switch, which marks cells with red fluorescence after Cre-lox mediated recombination (Fig. 7a). To label embryonic careg-positive cells, we pulsed embryos at 75% epiboly stage with 4-hydroxytamoxifen (4-OHT) for two days and analysed the hearts at 21 d.p.f. (Fig. 7b). We found that nearly 60% of CMs (58.2 ± 2.5; N = 9) within both the trabecular and outer myocardium were randomly demarcated by mCherry expression, whereas no mCherry was observed in embryos treated with the vehicle (Fig. 7c). This fate-mapping result reveals that careg-positive embryonic CMs contribute to the trabecular myocardium during development.

careg in the primordial/junctional layer of adult hearts. Next, we examined both fluorescent transgenic reporters, careg:dmKO2 and careg:EGFP, in the outer wall of the adult heart. Heart sections revealed a strong co-expression of the fluorescent proteins in a single subcortical cell layer between the compact and trabecular myocardium (Fig. 8a–c). This cell layer co-expressed cmhc2:EGFP and Tropomyosin, confirming the cardiac identity. Considering developmental expression of careg and the recent multicolour clonal analysis\(^{34}\), we concluded that the careg reporter is a marker of the ‘primordial’ CMs in the zebrafish ventricle.

To determine the role of the primordial layer in the adult heart, we analysed features of the careg-expressing CMs. First, Tropomyosin revealed a reduced pattern of sarcomeres in
**Figure 5 | The regeneration biosensor careg is dependent on TGFβ/Activin-β signalling.**

(a–c) Immunofluorescence staining of careg:EGFP ventricle at 7 d.p.c. with antibodies against GFP (green), pSmad3 (red) and Tropomyosin (blue) revealed the presence of TGFβ/Activin-β-activated cells in the careg:EGFP-expressing tissue. N = 6. (d–g) careg:EGFP heart sections at 7 d.p.c. treated with 0.1% DMSO or 20 μM SB431542, an inhibitor of TGFβ type I receptors, and immunostained with antibodies against GFP (green) and embCMHC (red). The intact myocardium was detected with F-actin staining (blue). In the magnified images, the upper dotted line demarcates a 100 μm-thick margin of the remaining myocardium from the injury border (lower dotted line). (h) Percentage of careg:EGFP⁺ and embCMHC⁺ area within a distance of 100 μm from the post-infarcted tissue in hearts at 7 d.p.c. treated with DMSO or SB431542. The inhibitor treatment resulted in a significant reduction of careg:EGFP and embCMHC expression in the peri-injured zone, compared to control hearts treated with DMSO. N = 8. ***P<0.001; unpaired t-test. Error bars correspond to s.e. of the mean (s.e.m.). (i–n) Live-imaging of careg:EGFP fins at 3 d.p.a. in a wild-type (WT) background treated with DMSO or SB431542, and in the fgf20a (dob) mutant background. careg:EGFP expression is suppressed in the stump by TGFβ inhibition, but it remains normal in fgf20a mutant fins. (o–p) Longitudinal sections of the fins shown in the left panels display a reduction of pSmad3-positive nuclei (red) and careg:EGFP in the stump of SB431542-treated fins, whereas both markers are unaltered in fgf20a mutants. BV, blood vessel. N = 4.
less differentiated contractile structures. Second, careg-positive CMs displayed a slightly bent shape with their ends facing the ventricular chamber, suggesting the formation of attachments with trabecular CMs (Fig. 8b). To determine whether the careg-positive cell layer indeed interconnects with the trabecular myocardium, we performed antibody staining against N-cadherin (blue). We found an enriched punctate pattern of N-cadherin immunostaining in careg-positive cells, facing the trabecular but not the cortical myocardium (Fig. 8d), suggesting an uneven amount of adhesion on both surfaces of these cells. Thus, careg-expressing CMs contain structural features of junctional/transitional CMs, forming the circumference of the trabecular myocardium.

Electron microscopy analysis has identified the presence of a fibroblast network in the junctional region. To determine the spatial distribution of the primordial layer in this zone, we generated double transgenic fish careg:dmKO2;wt1a-6.8kb:GFP, the latter of which demarcates the subcortical non-myocytes. We found that most wt1a-6.8kbGFP-positive cells were arranged between the cortical myocardium and careg-labelled CMs (Fig. 8e). Thus, a fibroblast layer seems to ‘glue’ together the

careg-expressing cells, especially in comparison to that of cortical CMs, which were typically rod-shaped with a very distinct striation (Fig. 8b). This suggests that careg-positive CMs possess

Figure 6 | careg is expressed in embryonic CMs and the outer wall of developing ventricle. (a–f) Longitudinal sections of caregEGFP;cmic2:DsRed2-Nuc double transgenic hearts at different time points during development. Cardiac nuclei are marked by DsRed expression. The endogenous fluorescence was quenched with HCl treatment before immunostaining. (a–f) At 1 and 3 d.p.f. (days post-fertilization), caregEGFP and endogenous embCMHC are co-expressed in embryonic CMs. (g–i) At 12 d.p.f., a few caregEGFP+ CMs delaminate from the outer heart surface and invade into the ventricle (V) chamber, as seen by the residual EGFP. (j–l) At 30 d.p.f., caregEGFP is restricted to the outer layer of the ventricular wall, and is downregulated in the trabecular myocardium. caregEGFP is also expressed in non-myocytes of the bulbus arteriosus (BA) and a few cells of the atrium (A). (m–o) Longitudinal sections of careg:dmKO2;cmic2:EGFP transgenic heart at 30 d.p.f. immunostained for GFP (green) and dmKO2 (red). The careg:dmKO2 transgenic line has a cardiac developmental expression pattern similar to that of caregEGFP (Supplementary Fig. 10). N=5.

Figure 7 | Embryonic careg-positive CMs contribute to the trabecular myocardium. (a) Schematic representation of the transgenic strains used for lineage tracing. (b) Experimental design. (c,d) Longitudinal sections of hearts at 21 d.p.f. immunostained against mCherry (red), GFP (green) and Tropomyosin (blue). (c) Control embryos treated with the vehicle do not display mCherry fluorescence. (d) mCherry+ CMs are present in the trabecular and outer myocardium at 21 d.p.f. in embryos treated with 4-OHT, suggesting that the trabecular myocardium derives from embryonic careg+ CMs. N=9.
**Figure 8 | Monitoring the primordial CM layer between the compact and trabecular myocardium.** (a) Longitudinal sections of *careg:dmKO2;cmlc2:EGFP* transgenic adult hearts labelled with antibodies against KO2 (red) and Tropomyosin (blue, white). V, ventricle; A, atrium; BA, bulbus arteriosus. (b) *careg:dmKO2* expression is maintained during adulthood in a single layer of thin subcortical CMs (arrowheads) in the junctional zone (JZ) between the compact/cortical (CoM) and the trabecular myocardium (TrM). N = 4. (c) A higher magnification of the adult ventricle wall of double transgenic fish *careg:dmKO2; careg:EGFP* displays an overlapping expression of both markers in subcortical myocytes (arrowheads). N = 4. (d) A magnified adult ventricle wall of *careg:dmKO2* transgenic fish displays an abundant expression of N-cadherin (green, white) on the surface of the junctional CMs on the side facing the trabecular myocardium. N = 4. (e) A fragment of the adult ventricle wall of *careg:dmKO2;wt1a-6.8kb:GFP* transgenic fish displays the separation of compact myocardium and subcortical *careg:dmKO2* + layer by wt1a-6.8kb:GFP-labelled fibroblasts of the junctional zone. N = 4. (f–i) At 90 d.p.ci., transversal heart sections of *careg:EGFP* hearts display a gap in the primordial layer within the regenerated myocardium. The extent of the gap is indicated with arrowheads. N = 4.
cortical and primordial CMs. This suggests that the primordial layer is partially separated from the cortical layer by connective tissue.

Incomplete regeneration of the primordial cardiac layer. After injury, heart regeneration is completed within 30 to 60 days. Closer examination of careg;EGFP or careg;dmKO2 transgenic fish revealed that the primordial/junctional CM layer was interrupted in the new myocardium, indicating its incomplete restoration, even at 90 d.p.c. (Fig. 8f–i; Supplementary Fig. 3i,j). Thus, the regenerative competence of this CM layer is unexpectedly lower than that of other CMs.

To directly test the regenerative contribution of primordial/junctional careg-positive CMs, we performed lineage tracing of these cells. To this aim, we crossed careg;CreERT2 with the cmlc2:loxP-AmCyan-STOP-loxP-ZsYellow lineage-tracing line, referred to as cmlc2:Switch, which labels cardiac cells in yellow after Cre-lox mediated recombination (Fig. 9a). In the absence of 4-OHT, no ZsYellow was detected in the ventricle sections of careg;CreERT2;cmlc2:Switch double transgenic fish (Supplementary Fig. 10a–c). To label the primordial CM layer, we pulsed adult transgenic fish careg;CreERT2;cmlc2:Switch with 4-OHT for 2 days, and we collected the hearts after 7 days (Supplementary Fig. 10d,e). We found that ~30% (29.6 ± 2.5; N = 6) of the entire compact myocardium was demarcated by ZsYellow expression.

To determine whether primordial cells provide a source for the peri-injured trabecular myocardium, we performed cryoinjury 8 days after labelling of CMs, and analysed the hearts at 10 d.p.c. (Fig. 9a,b). Although the primordial layer of the remaining myocardium displayed ZsYellow expression, no fluorescent labelling was detected in the peri-injury zone of the trabecular myocardium (Fig. 9c,d). These fate-mapping results indicate that careg-positive primordial CMs, which have been labelled in the intact heart, do not markedly contribute to the dedifferentiation zone in the regenerating heart.

To examine the contribution of the primordial/junctional CMs in the regenerated ventricles, we used the same experimental design (Fig. 9a,b), and analysed the hearts at 30 d.p.c. We found the main portion of the new myocardium was not markedly labelled by zsYellow expression, suggesting a relatively low regenerative contribution of primordial/junctional CMs. Moreover, the outer wall of the new myocardium contained gaps of zsYellow-labelled cells (Fig. 9c,f). This lineage-tracing analysis indicates that the primordial/junctional cardiac layer is incompletely restored after regeneration, which is consistent with careg;EGFP or careg;dmKO2 reporter results.

Regenerative contribution of peri-injury CMs and MES. To examine whether careg is induced de novo in the dedifferentiated trabecular CMs and to trace the destiny of careg-expressing cells during regeneration, we performed cell labelling after cryoinjury and monitored the distribution of the ZsYellow-positive CMs during new myocardium formation. To this aim, we performed cryoinjury of careg;CreERT2;cmlc2:Switch double transgenic fish, and gave animals a 4-OHT pulse between 5 and 7 d.p.c. (Fig. 9g). Analysis of ventricle sections at 15 d.p.c. revealed 55.1% ± 2.6 (%N = 5) of ZsYellow-positive CMs in the peri-injury zone (Fig. 9h,i). At 30 d.p.c., the injured tissue was replaced with a new myocardium that was abundantly labelled by ZsYellow-positive CMs within the trabecular and cortical regions (Fig. 9j,k). We concluded that the injury-abutting CMs from the trabecular and cortical myocardium were activated and provided a cellular source for the regenerated cardiac muscle. Thus, the lineage-tracing analysis revealed that non-primordial/junctional CMs have a high regenerative capacity and contribute to new myocardium formation.

To examine whether careg is induced de novo in the activated stump mesenchyme of the fin, we performed cell labelling before and after amputation using careg;CreERT2;ubi:Switch transgenic fish. First, we gave animals a 1-day pulse of 4-OHT at 10 days before amputation, and monitored mCherry expression during regeneration (Supplementary Fig. 10h). Live-imaging and immunofluorescence analysis of fin sections did not reveal labelling, except for a few scattered cells (Supplementary Fig. 10g-p). However, when the animals were exposed to 4-OHT during the first day following amputation, the regenerative outgrowth was demarcated by mCherry, as assessed by live-imaging of the fins at different time points, and immunofluorescence analysis of longitudinal sections at 4 d.p.a (Fig. 9i–u). We concluded that careg is induced in the pre-existing MES of the stump, which gives rise to the new MES of the regenerate. Taken together, the regeneration-competent functional cells of the zebrafish heart and fin activate the careg element, indicating a common regulatory mechanism of organ restoration that operates in distinct cell types, such as cardiac myocytes and fin fibroblasts.
**Figure 9 | careg-expressing cells contribute to the regenerating myocardium and fin mesenchyme.** (a,b) Schematic representation of the transgenic strains and the experimental design for lineage tracing of careg-expressing primordial CMs during ventricle regeneration. (c–f) Representative sections of hearts treated with 4-OHT before cryoinjury to label the primordial layer, stained with antibody against Tropomyosin (blue). (c,d) At 10 d.p.c.i., no zsYellow is detected in the trabecular myocardium at the injury site, indicating that primordial CMs do not contribute to the regeneration zone along the post-cryolesioned tissue. (e,f) At 30 d.p.c.i., the zsYellow-positive primordial layer regenerates incompletely. (g) Experimental design for lineage tracing of careg-expressing dedifferentiated CMs during ventricle regeneration. (h–k) Representative sections of hearts treated with 4-OHT after cryoinjury hearts immunostained against Tropomyosin (blue). (j,k) At 30 d.p.c.i., zsYellow-positive CMs are detected in the trabecular (TrM) and cortical myocardium (CoM) of the regenerated muscle. N=8. (l) Transgenic strains and the design of lineage tracing experiments during fin regeneration. (m) Longitudinal section of lineage-labelled fins at 3 d.p.a., stained for osteoblasts using the Zns5 antibody (green) and for nuclei with DAPI (blue). mCherry is detected in the entire mesenchyme of the regenerative outgrowth and below the amputation plane. N=6. (n–u) Live-imaging of the same lineage-labelled fin at different time points of regeneration. The regenerative outgrowth and the stump tissue are labelled with mCherry. N=6.
tissues. Further dissection of the adjacent genomic sequences beyond the careg element is required to identify potential negative regulatory elements. Indeed, a recent study on spinal cord regeneration established a ctgfa:EGFP transgenic fish line with a 5.5 kb sequence upstream of the ctgfa translational initiation codon. It will be interesting to compare the expression of the careg and ctgfa reporters in the context of heart and fin regeneration.

Remarkably, the careg reporter is also constitutively expressed in the tissue-junctional layers, such as the primordial cardiac layer spanning the cortical and trabecular compartments, and the interface cells between bone matrix and MES in the fin. In the adult uninjured heart, our findings provide evidence that the primordial CM layer acts as a junctional/transitional layer, based on the architecture of the single-cell sheet, a flattened U-shaped cell morphology, an enrichment of N-cadherins facing the trabecular fascicles and the presence of overlying connective tissue. These interconnecting CMs are probably exposed to certain stimuli that trigger the activation of the same regulatory elements as during regeneration, although in a TGF/Activin-β independent manner. These stimuli remain unknown but they might be of biomechanical nature. Indeed, the junctional CM layer is thought to anchor the trabeculae with the luminal cavities in order to prevent compaction of the muscle. In the locomotory appendage, osteoblasts connecting bones with MES may also cope with particular inter-tissue tension during swimming. Thus, the careg reporter is expressed in certain integrative cellular layers, which might be exposed to biophysical challenge during their normal function. Molecular mechanisms of such induction require further investigation.

The zebrafish ventricle comprises heterogeneous types of CMs. Cortical CMs display a high regenerative potential, as previously shown using the gata4-reporter. Our findings yielded several discoveries related to the regenerative competence of primordial and trabecular CMs. Lineage-tracing analysis in the embryo revealed that the primordial CM layer gives rise to the growing myocardium during development. In the adult heart, however, the primordial layer was not completely restored after cryoinjury, indicating its low ability to regenerate after traumatic injury. This limitation might be caused by the specialization of the primordial CM layer as the junctional layer of the heart wall.

By contrast, both the inner trabecular CMs, which build the main part of the ventricular chamber, and the cortical CMs, which surround the ventricular wall, all of them efficiently contribute to the formation of the new myocardium. It will be interesting to analyse gata4/careg double transgenic fish, to more accurately specify the relative contribution of cortical and trabecular CMs in the resection and cryoinjury models.

Our study of careg during fin regeneration allowed lineage tracing of the injury-activated MES. We show that MES of the regenerate arise from pre-existing local cells. This finding supports the concept that the newly formed tissue originates from the local stump and not from recruited remote cell populations. The regenerative inadequacy in mammals is thought to result from the evolutionary loss of the intrinsic cellular plasticity that enables the activation of morphogenetic programs after injury. The identification of a common regulatory element in zebrafish heart and fin might provide a valuable resource for transgenic modulation of the regenerative response in other models.

**Methods**

**Zebrafish lines and animal use.** Wild-type AB (Oregon) and transgenic female and male adult zebrafish aged 6 to 12 months were used in this study. Genetically modified lines were: dob5, Tg(ctgfa:EGFP)38, which we renamed Tg(careg:EGFP), Tg(cmlc2:DsRed2-Nuc)37, Tg(cmlc2:EGFP)52, Tg(cmlc2:loxP-AmCyan-loxP-ZsYellow), referred to as cmlc2Switch32, ubi:loxP-EGFP-loxP-mCherry (ubi:Switch)50, Tg(wt1a-6.8kb-GFP)55.

To generate careg lines, 3.18 kb upstream regulatory sequence of ctgfa was PCR-amplified from the plasmid pEGFP-1-ctgfa, which was kindly provided by Antonio Jacinto (Universidade Nova de Lisboa), with primers (F) 5’-CATGAGGAATGTTCCACTG-3’ and (B) 5’-GAGAAGCAACAGTCACTC-3’ by Antonio Jacinto (Universidade Nova de Lisboa), with primers (F) 5’-CATGAGGAATGTTCCACTG-3’ and (B) 5’-GAGAAGCAACAGTCACTC-3’. To establish caregdmKO255, the PCR fragment was cloned into pDestToZa2 upstream of dmKO2 (ref. 39), kindly provided by Brian Link (Medical College of Wisconsin). To create careg:CreERT2Mercury, the same PCR fragment of the ctgfa upstream regulatory sequence was cloned into pSE-MCS (#475, zcre.org). The pDest_cya3:Venus_careg:CreERT2 construct was generated using multisite Gateway assembly of pSE-ctgfa, pMECreERT2 (#396, zcre.org), p3E_SV40polyA (#302, zcre.org) and pDestTo2xcreaVenus (#480, zcre.org). Each plasmid was co-injected with the pCS2FA-transposase mRNA into one-cell-stage wild-type embryos.

All assays were performed using different animals that were randomly assigned to experimental groups. The exact sample size (N) was described for each experiment in the figure legends, and was chosen to ensure the reproducibility of the results. During invasive procedures and imaging, fish were anesthetized with buffered solution of 0.6 mM tricaine (MS-222 ethyl-m-amino-benzoate, Sigma-

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**Figure 10 | CMs and stump MES activate a common regulatory element during regeneration.** (a,b) A schematic representation of ventricle sections and whole fins at different time points during regeneration. The peri-injury zone of the ventricle and the stump of the fin activate a transient expression of the careg reporter in a TGF/Activin-β-dependent manner. In the heart, the careg reporter also demarcates the primordial layer, which fails to be completely restored in the new myocardium.
Aldrich) in system water. Immediately after the procedures, zebrafish were transferred into a tank with system water, and were continuously monitored until fish restarted swimming. Fish normally resumed breathing within 30 s after transfer into water. In cases where the breathing was not observed after this time, we stimulated the animals by squirting water into their gills with a plastic pipette until they started spontaneous breathing. During regeneration, fish were maintained at 26.5 °C. Animal procedures were approved by the cantonal veterinary office of Fribourg, Switzerland.

**Ventricular cryoinjury and resection.** Myocardial cryoinjuries were performed according to the video protocol56. Briefly, anaesthetized fish were placed ventral side up in a damp sponge under the stereomicroscope. To access the heart, a small incision (approximately 1–2 mm) was made through the chest with iridectomy scissors (Roboz Surgical Instrument Co.), a procedure described as thoracotomy57. The beating heart was well visible, and no extensive bleeding occurred during the thoracotomy. Then, the ventricular wall was directly frozen by applying for 23–25 s a stainless steel cryoProbe (custom-made) pre-cooled in liquid nitrogen. The tip of the cryoProbe was 6 mm long with a diameter of 0.8 mm, the handle of the cryoProbe was 4 cm long with a diameter of 8 mm and is covered with a plastic surface. To stop the freezing of the heart, system water at room temperature was dropped on the tip of the cryoProbe, and fish were immediately returned into a tank with water. It was not necessary to suture the incision of the chest, as the thoracic wound healed spontaneously within a week.37,56

**Fin cryoinjury and amputation.** The fin cryoinjury was performed with a steel cryoProbe blade, (22 cm, 260 g, Product 14021660077, Leica). The cryoProbe blade was immersed for 90 s in liquid nitrogen, removed and held for 5 s in the air to avoid the dispersion of liquid nitrogen droplets. The pre-cooled blade was placed for 15 s on the fin surface perpendicularly to the proximo-distal axis of the appendage at an equidistant position between the fin base and the central clef31.1 Fin sections were performed using a razor blade. Adult caudal fins were amputated proximally to the first bone bifurcation point. Larval tails were cut at the posterior end of the notochord. Wound healing was spontaneous without significant bleeding. Live images of regenerating fins were taken with a Leica AF M205 FA stereomicroscope.

**Drug treatments.** The TGFB type I receptor inhibitor SB33482 (Tocris) was dissolved in DMSO at a stock concentration of 20 mM and used at a final concentration of 20 μM. Control animals were kept in water with 0.1% DMSO. For lineage tracing, fish were incubated in 5 μM 4-hydroxytamoxifen (4-OHT, Tocris), which was made from a 10 mM stock solution dissolved in DMSO, for 24 or 48 h at the time points indicated. For BrdU incorporation, fish were incubated for 4 h in water containing 50 μg/ml BrdU (Sigma-Aldrich). Zebrafish were treated with drugs at a density of 3 fish per 100 ml of water, and then returned to circulating system water.

**Immunofluorescence analysis.** Embryos and fins were fixed in 4% paraformaldehyde (PFA), adult heart in 2% PFA, except the samples for N-cadherin immunostaining, which were fixed in Dent’s fixative (80% methanol, 20% dimethyl sulfoxide). The fixation was performed overnight at 4 °C. followed by washing in PBS (3 × 10 min each). Embryos and heart specimens were embedded in 30% sucrose at 4 °C. Fin specimens were fixed for 15 min with 4% paraformaldehyde (4-OHT, Tocris), then washed twice in PBS, and then returned to circulating system water.

**In situ hybridization.** The following forward (F) and reverse (R) primers (5′ to 3′) were used to synthesize the template for the probes: ctf6 (NM_001015041) F: 5′-TGGTGCTCAAGGTGCACTG-3′ and R: 5′-CATGTCGCCAACCATCTCTC-3′, cggf (pEFP-F1); F: 5′-ACGTTAAGCCGCAACATGCCT-3′ and R: 5′-CTGTTGCTGCTAGTAGTGG-3′, nsxB (NM_131260) F: 5′-GAGAATTGGGACATGGTCAGG-3′ and R: 5′-CCGCTTCCCTCAAGATAAA-3′. To synthesize the anti-sense probes a sequence of T3 RNA polymerase promoter (5′-ATTAAAGCTCACTAAAGGGAGA-3′) was added to the 5′ end of the reverse primers. The digoxigenin-RAA labelling mix (Roche) was used to generate the probe, which after purification was dissolved in hybridization solution and stored at −20 °C. Ventricular apoptosis was performed according to the established protocol56. Briefly, after thoracotomy, the apex of the ventricle was removed using curved iridectomy scissors. To collect the heart for fixation, fish were killed in tricaine solution. A large (~4 mm) incision was made above the heart through the branchial cartilage and the heart was pulled from the body cavity as shown in the video protocol56.

**Image and statistical analysis.** Fluorescent images of sections were taken using a Leica SP5 confocal microscope and a Zeiss microscope, respectively, and were superimposed using Adobe Photoshop.
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Author contributions
C.P. planned, performed experiments and analysed the data. A.J. conceived and supervised the study, designed experiments and wrote the manuscript with input from C.P.

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