Methods

Constructs and zebrafish lines

All constructs were generated using the Tol2 Kit as described [19]. For the tg-cmlc2a-Cre-Ert2 construct the 5´ entry clone *cmlc2a* contained a 1kb PCR fragment of the *cmcl2a* promoter [20], the middle entry clone contained Ert2-Cre-Ert2 amplified from pCAG-ERT2CreERT2 [21] and the 3´ entry clone contained IRES mCherry.

The tg-cmlc2a-LnL-GFP reporter construct consisted of the *cmlc2a* 5´entry clone, a middle entry clone containing a floxed stop cassette amplified from pCALNL-GFP [21] and the 3´entry clone p3E-EGFPpA [19]. Multisite recombination reactions into pDestTol2Pa2 were performed as described [19].

The NTR-GFP fusion construct was generated using the *cmlc2a* 5´entry clone, a middle entry clone pME-EGFP ´no stop´ and a 3´entry clone containing *nitroreductase* amplified from pECFP-*Nitro* [14]. Multisite recombination reactions into pDestTol2Pa2 were performed as described [19].

The Ert-GFP construct was generated using a 5´ entry clone p5E-*bactin2* [19], a middle entry clone containing Ert2 fused to GFP amplified from pErt2-GFP (pErt2-GFP was generated by cloning Ert2 from pCAG-ERT2CreERT2 [21] into the EcoR1 and BamH1 sites of pEGFP-N1 (clontech)) and a 3 ´ entry clone containing Ert2 amplified from pCAG-ERT2CreERT2 [21]. Multisite recombination reactions into pDestTol2Pa2 were performed as described [19].

To generate stable transgenic lines the tg-cmlc2a-Cre-Ert2 and tg-cmlc2a-LnL-GFP plasmids were injected into 1 cell stage embryos with transposase RNA[19].

Cre/tamoxifen in zebrafish

Transgenic zebrafish were generated in which 4-OHT inducible Cre is under the control of the cardiomyocyte-specific *cmlc2a* promoter [20]. Concomitantly, the reporter construct used the same *cmlc2a* promoter followed by a floxed stop cassette and eGFP. Removal of the stop cassette by Cre allows the *cmlc2a* promoter within the transgene to drive the expression of GFP. Subsequently, any cell that is derived from these GFP^{pos} cardiomyocytes will also be genetically labelled and as such also express GFP. However, cells in which the *cmlc2a* promoter was silent at the time of 4-OHT treatment (e.g. progenitor cells), would not have expressed any Cre and thus, the reporter in these cells will not be recombined and will remain silent. Any cell derived from these unlabelled cells will also remain unlabelled. To test the efficacy of this system, embryos from a heterozygous incross were treated with a range of concentrations of 4-hydroxy-tamoxifen (4-OHT) (Sigma-H7904). We found that 2μM of 4-OHT is sufficient to produce an efficient Cre mediated recombination of the floxed stop cassette and subsequent uniform expression of GFP in cardiomyocytes (supp fig.1.a,b). Prior to treatment with 4-OHT, embryos show no discernable expression of GFP (supp fig.1.a) indicating that this system does not leak. 24hrs after treatment with 4-OHT, cardiomyocytes in the embryonic heart express GFP (supp fig.1.b) with no discernable ectopic expression, demonstrating the specificity of the tg-cmlc2a-Cre-Ert2/cmlc2a-LnL-GFP line. Consequently, treated embryos were grown to adulthood (3months/sexually mature) at which stage cardiomyocytes within the heart are still uniformly GFP^{pos} (supp fig.1.c). Similar findings using this system have also been recently reported [22,23]. Furthermore, examination of cells isolated from GFP^{pos} transgenic hearts shows that all GFP^{pos} cells are MF20 ^{pos} (n=999) (supp fig.2.a-d). The specificity of Cre-Ert2 expression in the tg-cmlc2a-Cre-Ert2 line was directly tested by crossing these fish with animals from 2 different ubiquitous Cre reporter lines

((Tg(eab2:[EGFP-T-mCherry])[23] and EF1α loxP-DsRed2-loxP EGFP[22], kind gifts of Wenbiao Chen and Michael Brand, respectively). Zebrafish embryos from either intercross displayed strong reporter activation in the heart, and no ectopic expression was detected after detailed analyses using UV and confocal microscopy (supp fig.3 and 5). Identical results were also obtained by crossing a 2nd tg-cmlc2a-Cre-Ert2 line with the EF1α loxP-DsRed2-loxP EGFP line (supp fig.6 a-j). Analyses of regenerating hearts from this 2nd tg-cmlc2a-Cre-Ert2 line indicate that all the regenerated cardiac tissue is GFP^{pos} (supp fig.6.k). These findings reduce the chance of mislabelling to negligible levels. To further ensure Cre-Ert2 expression was restricted to cardiomyocytes, embryos from the tg-cmlc2a-Cre-Ert2 / Tg(eab2:[EGFP-T-mCherry]) analysis detailed above were raised until 2months old. We subsequently isolated hearts from these animals and performed immunohistochemistry using anti-RFP(to label mCherry expressing cells) and anti-α sarcomeric actin (to label cardiomyocytes). We were unable to detect any cells labelled with anti-RFP alone (n=1564) indicating that Cre expression is restricted to cardiomyocytes (supp.fig.4. a-c). Furthermore, we isolated cells from tg-cmlc2a-Cre-Ert2 / Tg(eab2:[EGFP-T-mCherry]) transgenic hearts and found that all $mCherry^{pos}$ cells were also positive for α sarcomeric actin (n=843) (supp fig.4.d-f). This confirms that Cre expression is restricted to cardiomyocytes.
Next we sought to determine whether this system was also effective in adult fish. Untreated GFP^{neg} embryos were raised to adults and then outcrossed to wildtype zebrafish. Embryos from this outcross were treated with 4-OHT to induce GFP expression in the heart allowing us to identify the positive transgenic adults (supp fig.1.d and inset). Before treatment with 4-OHT transgenic adult fish show no GFP expression in the heart (supp fig.1.d), again indicating no leakage. Previous findings have proposed that under certain conditions reporter systems such as those utilising the heat shock promoter can become inadvertently activated. However, we found no expression of GFP in cardiomyocytes following heart amputation (7day recovery) in adult transgenics, indicating that even after a stressful procedure, the reporter remains silent (supp fig.1.e and inset). To induce the expression of GFP in cardiomyocytes, adult transgenics were treated every 2 days with 1μM 4-OHT for one week followed by a one week wash period after which GFP is readily visible in cardiomyocytes (supp fig.1.f). To rule out the possibility that labelling cardiomyocytes at embryonic stages also resulted in progenitor cells being labelled, GFP^{neg} transgenic fish were treated with 4-OHT as adults and subsequently amputated. At 30dpa we assessed regeneration and found again that all cardiomyocytes within the regenerate were GFP^{pos} (supp fig.8.a,b).
To establish that Cre is removed from the nucleus following 4-OHT withdrawal, we fused the same estrogen receptors to GFP (GFP-ER) allowing us to visualise, in-*vivo*, the dynamics of 4-OHT treatment. Under the control of the *βactin* promoter and in the presence of 4-OHT, GFP-ER is concentrated in the nucleus of a variety of cells in embryonic zebrafish (supp fig.7.a). 3 days after 4-OHT withdrawal, GFP-ER returns to its pre-treatment cytoplasmic status (supp fig.5.b) similar to time windows established in the mouse (between 2-4 days until removal from nucleus) [6,24].

Amputation
Amputations were performed as described previously[5].

Inhibitor treatments
Cyclapolin 9 (Sigma C6493) was dissolved in DMSO and added to 400ml system water to a final concentration of 3μM. Water and inhibitor or DMSO alone were changed daily throughout the experimental procedures.

BrdU labelling

BrdU treatment was performed essentially as described [5]. Metamorph® software (Molecular Devices) was used to count the total number of BrdU labelled cardiomyocytes in each section (17 regenerating sections from 7 different animals and 9 control sections from 3 animals).

Cardiomyocyte islolation

Fish were sacrificed in tricaine and injected intraperitoneally with 20µl of a 1000U/ml of heparin solution in PBS to avoid blood clotting upon heart extraction. Hearts were collected and put to PBS with penicillin and streptomycin and 10U/ml heparin. The outflow tracts were then removed and ventricles and atriums were opened to get rid of the blood. They were then washed three times in perfusion buffer [PBS, 10mM HEPES, 30mM taurine, 5,5mM glucose and 10mM 2,3-butanedione monoxime (BDM)] and placed into digestion buffer [perfusion buffer plus 12,5µM calcium chloride and 0,2 Wünsch units/ml Liberase Blendzyme 3 (Roche)] to digest for 40 minutes at 27ºC in a thermomixer at 800rpm. Next, an equal volume of stop buffer 1 (perfusion buffer plus 12,5µM calcium chloride and 10%FBS) was added and cells were mechanically separated. Undigested material was left to sediment and cells suspended in the supernatant were pelleted by centrifugation at 250g for 5 minutes. The cells were then resuspended in stop buffer 2 (perfusion buffer plus 12,5µM and 5%FBS) and calcium reintroduction was performed by gradually raising the concentration to 62, 112, 212, 500 and 1000µM respectively. Cells were then pelleted again and resuspended in plating medium (MEM, 5%FBS, 10mM BDM, 2mM Glutamax, 100U/ml penicillin and 100µg/ml streptomycin). Undigested material was washed once in perfusion buffer and digested and subsequently treated again following the same steps as previously described. Both cell preparation batches were combined, plated onto matrigel-coated chamber slides (Nunc, ref.177445), and allowed to attach overnight. Immunofluorescence was performed the following day.

Immunohistochemistry

Immunohistochemistry was performed on 10 µm cyro-sections as previously described [4]. The antibodies used in this manuscript are anti-MF20(DSHB MF20), anti-GFP(AVES GFP-1020), anti-BrdU(Accuratechemical OBT0030), anti-phopsho histone H3(Upstate 06-570), TUNEL(Roche 11684817910) anti-PCNA (Sigma P8825), anti-α sarcomeric actin (Sigma A2172), anti-RFP (Abcam ab34771).

In situ Hybridisation

In situ hybridisation was performed as described previously [5]. The *plk1* probe was generated by cloning zebrafish *plk1* from RZPD clone- IRBOp991A0269D into the EcoR1 and Xho1 sites of pBSK- .

Semi-thin section analysis

For each section, 6 images were taken in the positions indicated in supp fig.10.d inset. Structurally altered cardiomyocytes were identified manually using Metamorph® software (Molecular Devices). Each time point in supp fig.10.c represents the average of all 6 images +/- SEM. For 3 and 7dpa, 4 sections were analysed from 2 different hearts. For control, 1, 14 and 30 dpa, 4 sections were analysed from 1 heart for each time point. For positional analysis (supp fig.10.d) the average was taken from positions 1+2 and 5+6 from all the 3 and 7dpa sections.

Confocal microscopy

Confocal microscopy was performed using a Leica SP5. For co-localisation analysis the formula used to calculate the axial resolution is as follows.

$Dz= \sqrt{((l*n/NA^2) + (AU*n*\sqrt{2}*1,22*l/NA^2)^2)}$

Emission (l)= 500 nm

Refractive index 1.518

NA= 1,4

Airy Units: 1

This results in a section thickness of z= 0,773µm

Transmission electron microscopy

Transmission electron microscopy was performed as described previously[25].

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