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Redifferentiated cardiomyocytes retain residual dedifferentiation signatures and are protected against ischemic injury

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Fiji script for Sirius Red Scar Analysis

Movie S1.

Functional Improvement Follows caERBB2 shut-off. Short axis echocardiography recording of the 4WPMI timepoint (left video) and 7WPMI timepoint (right video) of the same mouse, demonstrating the functional improvement seen from dedifferentiation to redifferentiation.

Movie S2.

Dedifferentiatoin-Redifferentiation cycle confers cardioprotection 1 month after ERBB2 shut off. The first video sequence contains two panels of representative long axis echocardiography recordings of the same WT mouse at Baseline (before injury) and 4 weeks after permanent LAD ligation. The second video sequence is the same format, but for a tOE-DR (1 month) mouse. These videos correspond to the data shown in Fig. 3, B and C.

Movie S3.

Dedifferentiatoin-Redifferentiation cycle confers cardioprotection 5 months after ERBB2 shut off. The first video sequence contains two panels of representative long axis echocardiography recordings of the same WT mouse at Baseline (before injury) and 4 weeks after permanent LAD ligation. The second video sequence is the same format, but for a tOE-DR (5 month) mouse. These videos correspond to the data shown in Fig. 3, O and P.

Movie S4.

Hippo inhibition of ERBB2 overexpressing cardiomyocytes increases proliferation. Timelapse microscopy of tdTomato labelled CMs. Left video shows CMs treated with DMSO and right video shows CMs from the same mouse treated with TRULI (corresponding to the 2nd and 4th panel of Fig. 5B respectively). Mitosis counter reflects the number of mitotic events that occurred in the field of view as the video progresses. White arrows appear shortly before a mitotic event. Most, but not all mitotic events resulted in cytokinesis. Only cytokinetic events were counted during the quantification shown in Fig. 5B. Time counter in top left corner of each video represents time elapsed (hh:mm).

Movie S5.

LATS1/2 cKO in cardiomyocytes following transient ERBB2 overexpression prevents redifferentiation. From left to right, video panels show short axis echocardiography recordings of mice at the 7WPI (weeks post induction) timepoint (see Fig. 5D) for WT, tOE and tOE-LATS1/2 cKO mice to highlight the failure to redifferentiate in the absence of LATS1/2. Each panel is accompanied (below) by a representative immunofluorescent stain of the heart for cardiac troponin T (green) and DAPI (blue) (also shown in Fig. 5G), to reflect the gross loss of sarcomeric integrity that accompanies tOE LATS1/2 cKO hearts. R-diff = redifferentiation.

Extended Data Fig. 1 | Hundreds of genes and proteins show differential expression after redifferentiation. a-h, Left Ventricular Posterior Diastolic Wall thickness (LVPW;d) (**a**,**f**), Left Ventricular Anterior Diastolic Wall thickness (LVAW;d) (**b**,**e**), Ejection Fraction (**c**,**g**), and long-axis Fractional Shortening (**d**,**h**) of WT MI, tOE MI, pOE MI, WT Sham and tOE Sham mice, measured by echocardiography. Gray shaded area represents time period of caERBB2 activation (dedifferentiation). Blue shaded area represents the redifferentiation phase. Overall, n = 3 - 8 per group. For (**a**), WT MI vs pOE MI p = 0.0002, tOE MI vs pOE MI p = 0.0005, for (**b**), WT MI vs pOE MI p = 0.0018, tOE MI vs pOE MI p = 0.0461, for (**c**), WT MI vs pOE MI p = <0.0001, tOE MI vs pOE MI p = 0.0161, WT MI vs tOE MI p = 0.0001, for (**d**), WT MI vs pOE MI p = <0.0001, tOE MI vs pOE MI p = 0.0003, WT MI vs tOE MI p = 0.0005. i, RT-qPCR analysis of *Erbb2* from sham WT and tOE adult heart lysates for each time point. All values are normalised to their in-time point average WT value (black dashed line). All groups had minimum n = 3. For WT vs tOE, Dediff p = 0.004606, Intermediate p = 0.016849. **j**, Western blot quantification of tOE/WT FC of ERBB2 and pERBB2 (Tyr-1248) protein. All values are normalised to their in-time point average WT value (black dashed line). n = 4 - 8 per group. For WT vs tOE for ERBB2, Dediff p = 0.00189, Intermediate p = 0.00551, for pERBB2, Dediff p = 0.00016. **k**, Representative western blot images of data from (**j**). **l**,**m**, Heatmaps of differentially expressed genes (**l**) and proteins (**m**) from MI-injured samples, compiled as described in Fig. 1**g**,**h**. **n**-**q**, Principal component analysis and dendrograms of sham RNAseq (**n**,**o**), and sham proteomics groups (**p**,**q**). In all panels numerical data are presented as mean ± SEM; statistical significance was calculated using one-way ANOVA with Sidak's multiple comparison test at the 7WPMI time point in (**a**-**h**), two-tailed unpaired Student's t-test between tOE and WT of each time point in (**i**,**j**). *$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Uncropped blots for (**k**) are provided in supplementary source data.

Extended Data Fig. 2 | Dedifferentiated phenotypes are largely reversed in functionally redifferentiated hearts. a,**b**, tOE/WT at all timepoints (**a**) and pOE/WT at Rediff (**b**) RNA expression fold change of 'return to normal' genes, involved in metabolism, proliferation and EMT-like features, determined by RT-qPCR. All values are normalised to their in-time point average WT value (black dashed line). *n* = 3 - 4 mice per group. **c**, Western blot quantification for data in Fig. 2b in order to validate the 'return to normal' behaviour of proteins involved in proliferation, EMT-like features and metabolism. *n* = 4 – 7 mice per group **d**, Representative immunofluorescence images of isotype controls (WT) for the tOE hearts at Int. and Rediff timepoints shown in Fig. 2c, stained for Ki67, Nestin and Tomm20. Full quantification is provided in Fig. 2c. Scale bars = 50μm for Ki67 and Nestin, 100μm for Tomm20. **e'-f''**, Metabolic analysis of cultured P7 WT (*n* = 3) and OE (*n* = 5) CMs using an XFe96 Seahorse analyser. Upper left panel shows the OCR (oxygen consumption rate) during the Cell Mito Stress Test and lower left panel shows the ECAR (extracellular acidification rate (glycolysis proxy)) during the Glycolysis Stress Test. Maximal respiration/OCR and Glycolysis are represented in the upper right and lower right panels respectively. For (**e''**) WT vs OE p = 0.0320. **g**, H&E-stained histological sections of WT Dediff and tOE Dediff, Int. and Rediff hearts. Images were acquired in the remote zones of MI injured hearts as a proxy for sham injury. Scale bars = 50μm. *n* = 3 for each group. **h**,**i**, Scatter plot of immune-related (**h**) and angiogenesis-related (**i**) GO term z-scores against enrichment significance ($\log_{10}$ p-value) for tOE/WT across all timepoints, based on Ingenuity Canonical Pathway Analysis of RNAseq data using a threshold fold change (FC) ≥ 1.5; adjusted p ≤ 0.05. Arrows on each line indicate the direction of the GO term from Dediff to Int. to Rediff. Z-scores below -2 are predictive of pathway inactivation and above +2 are predictive of pathway activation. Values above the horizontal dashed black line represent statistically significant enrichment. **j**,**k**, Heatmaps of differentially expressed genes corresponding to the IPA analysis in (**i**), (**j**) and an independently curated list of angiogenesis genes (**k**), compiled as described in Fig. 1g. **l**, Western blot quantification for data in Fig. 2g in order to validate the proteins involved in metabolism, cytoskeletal signalling and heart function that remain differentially expressed at Rediff. In all panels numerical data are presented as mean ± SEM; statistical significance was calculated using two-tailed unpaired Student's t-test in (**a-c**,**e''**,**f''**,**l**) between the in-time point WT and tOE values. **p* ≤ 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

Extended Data Fig. 3 | Expanded RNAseq evidence of effects for EMT-like GO terms that 'return to normal'. Heatmaps based on log_2 transformed normalised counts from Sham RNAseq data of differentially expressed genes that appear within at least one of the listed EMT-like category GO terms (indicated by a tick). Rows represent genes. Columns represent each biological sample. Colour bars represent z-score for each timepoint.

Extended Data Fig. 4 | Expanded RNAseq evidence of effects for mitochondrial metabolism GO terms that 'return to normal'. Heatmaps based on log_2 transformed normalised counts from Sham RNAseq data of differentially expressed genes that appear within at least one of the listed mitochondrial metabolism GO terms (indicated by a tick). Rows represent genes. Columns represent each biological sample. Colour bars represent z-score for all timepoints.

Extended Data Fig. 5 | Expanded proteomics evidence of effects for mitochondrial metabolism GO terms that partially 'return to normal'. Heat maps based on log10 transformed intensity values from Sham proteomics data of differentially expressed proteins that appear within at least one of the listed mitochondrial metabolism GO terms (indicated by a tick). Rows represent proteins. Columns represent each biological sample. Colour bars represent z-score for each row across all timepoints.

Extended Data Fig. 6 | Expanded proteomics evidence of effects for EMT-like category GO terms that partially 'return to normal'. Heat maps based on log10 transformed intensity values from Sham proteomics data of differentially expressed proteins that appear within at least one of the listed EMT-like category GO terms (indicated by a tick). Rows represent proteins. Columns represent each biological sample. Colour bars represent z-score for each row across all timepoints.

Extended Data Fig. 7 | Expanded proteomics evidence of effects for heart related category GO terms that partially 'return to normal'. Heat maps based on log10 transformed intensity values from Sham proteomics data of differentially expressed proteins that appear within at least one of the listed heart related category GO terms (indicated by a tick). Rows represent proteins. Columns represent each biological sample. Colour bars represent z-score for each row across all timepoints.

Extended Data Fig. 8 | Dedifferentiation-Redifferentiation cycle confers robust protection against ischaemic injury. a-d, Relative stroke volume (**a**), Left Ventricular Anterior Diastolic Wall thickness (LVAW;d) (**b**), Left Ventricular Posterior Diastolic Wall thickness (LVPW;d) (**c**) and Left ventricular diastolic volume (LV Volume;d) (**d**) of WT and tOE-DR, measured by echocardiography. $n = 14$ for each group. For WT vs tOE-DR in (**a**), 2DPMI p = 0.000369, 2WPMI p = 0.002282, 4WPMI p = 0.00133. For WT vs tOE-DR in (**b**), 4WPMI p = 0.010216. **e**, Scar classification quantification for WT and tOE-DR mice. $n = 14$ for each group. For WT vs tOE-DR, No Scar p = 0.0442, Transmural scar p = 0.0051. **f**, Average perimeter and area per aSMA+ vessel. $n = 5$ for each group. **g**,**h**, Average perimeter (**g**) and area (**h**) per blood and lymphatic vessel, as measured by CD31 and LYVE1 immunofluorescence. $n = 3$ for each group. **i-l**, rSV (**i**), LVAW;d (**j**), LVPW;d (**k**) and LV Volume;d (**l**) of WT and tOE-DR 5 months after ERBB2 shut-off, measured by echocardiography. $n = 21$ for WT, $n = 16$ for tOE-DR. For WT vs tOE-DR in (**i**), 2WPMI p = 0.013785, 4WPMI p = 0.001556, (**j**), 2DPMI p = 0.021647. **m**, Scar classification quantification for WT and tOE-DR mice, 5 months after ERBB2 shut-off. $n = 19$ for WT, $n = 15$ for tOE-DR. Data are presented as mean ± SEM; statistical significance was calculated using a one-tailed Mann-Whitney test in (**e**) and (**m**) for 'No scar', a two-tailed Mann-Whitney test in (**m**) for transmural scar, and a two-tailed unpaired Student's t-test in (**e**) for 'non-transmural' and 'transmural' scar and (**m**) for 'non-transmural' scar counts and (**f** to **h**). $*p \leq 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$

Extended Data Fig. 9 | ERBB2 signalling promotes a multi-faceted negative feedback response. a, RNAseq FPKM values for negative feedback regulators from ventricular lysate of various ages. Data re-analysed from O'meara et al. 2015[40]. **b**, RNAseq normalised counts for negative feedback regulators from bulk-RNAseq of purified CMs from sham injured P1 and P56 mice. Data re-analysed from Quaife-Ryan et al. 2017[41]. **c**,**d**, *In situ* hybridisation for Hippo pathway genes *Lats1, Lats2* and *Sav1* (**c**) and ERK negative feedback regulators *Dusp6* & *Spry4* (**d**) mRNA in 7DPI (days post injury) adult zebrafish hearts. RZ = Remote Zone. BZ = Border Zone. Zones are delineated by the black dotted lines. Middle and bottom panels show higher magnification images of the corresponding dashed black boxes in the top panel. Black arrows highlight the presence of detected mRNA. Scale bar in top panels represent 100µm, scale bars in middle and bottom panels represent 10µm. $n = 3$ for each group. **e**, Quantification of combined FISH and immunofluorescence staining for PCNA and either *Dusp6* (top panel) or *Spry4* (bottom panel) positive CM nuclei in RZ and BZ of 7DPI adult zebrafish hearts ($n = 4$). For *Dusp6* Border zone vs Remote zone, PCNA+ CMs p = 0.0033, Dusp6+ CMs p = 0.0371, PCNA+/Dusp6+ CMs p = 0.0421. For *Spry4* Border zone vs Remote zone, PCNA+ CMs p = 0.0241, Dusp6+ CMs p = 0.0240, PCNA+/Dusp6+ CMs p = 0.0369. **f**, Representative images of remote and border zones for *Dusp6* (upper) and *Spry4* (lower) stained sections in (**e**). White arrows indicate double-positive CM nuclei. Scale bars represent 20µm. **g**, To-scale Venn diagram for *Dusp6* (upper) and *Spry4* (lower) positive nuclei overlapping with PCNA positive nuclei between the RZ and BZ. In all graph panels numerical data are presented as mean ± SEM; statistical significance was calculated using a two-tailed unpaired Student's t-test in (**b**) between the P1 and P56 values, and in (**e**) between the corresponding RZ and BZ values. $*p \leq 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Extended Data Fig. 10 | LATS1/2 negative feedback signalling is required for redifferentiation. a, Representative immunofluorescence images of Ki67 in LATSi or DMSO treated WT and OE P7 cardiac cultures, with full quantification. All groups had minimum $n = 3$. Scale bars = 50μm. **b**,**c**, Representative western blot of whole-heart lysates for general Yap (gYAP) and pYAP S112 (a target residue of LATS1/2) from WT, tOE, WT LATS1/2 cKO and tOE LATS1/2 cKO mice (**b**), with quantification, normalised to the average WT value (**c**). WT $n = 3$, tOE $n = 3$, WT cKO $n = 4$, OE cKO $n = 3$. In all panels numerical data are presented as mean ± SEM; statistical significance was calculated using a paired two-way ANOVA followed by Sidak's test in (**a**) and a one-way ANOVA followed by Tukey's test in (**c**). *$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Uncropped blots for (**b**) are provided in supplementary source data.