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Supplemental information

Amino acid primed mTOR activity

is essential for heart regeneration

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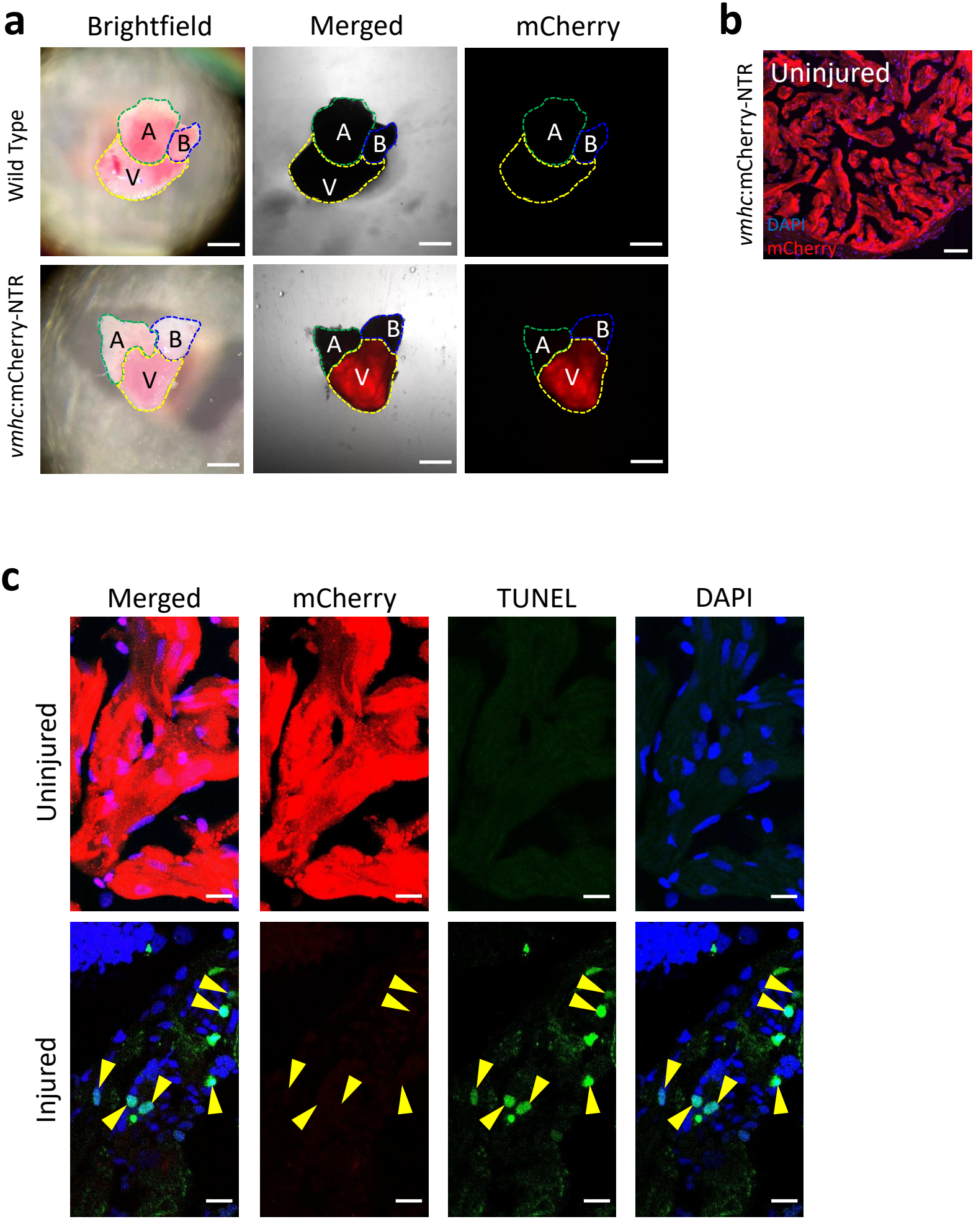
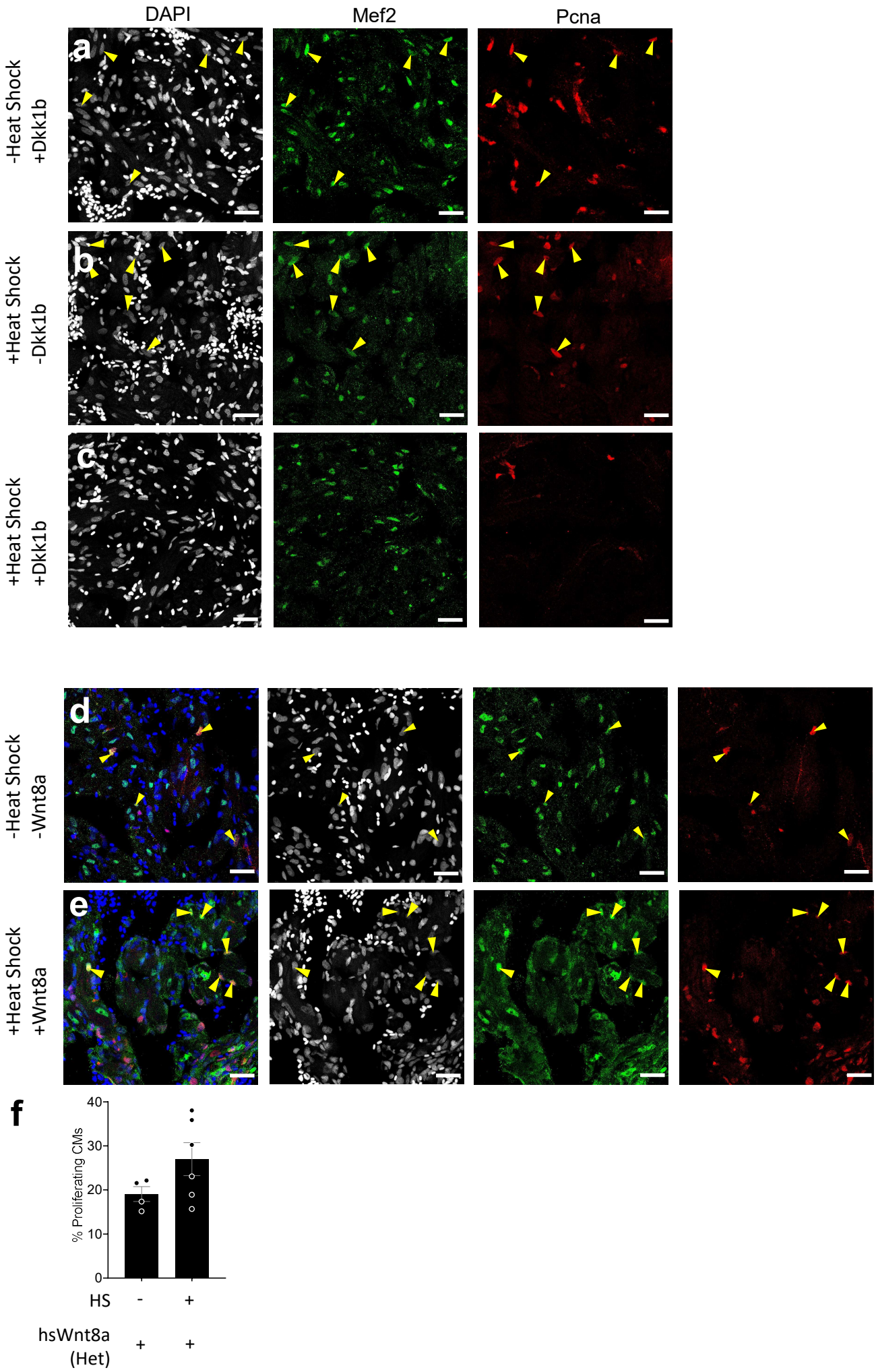
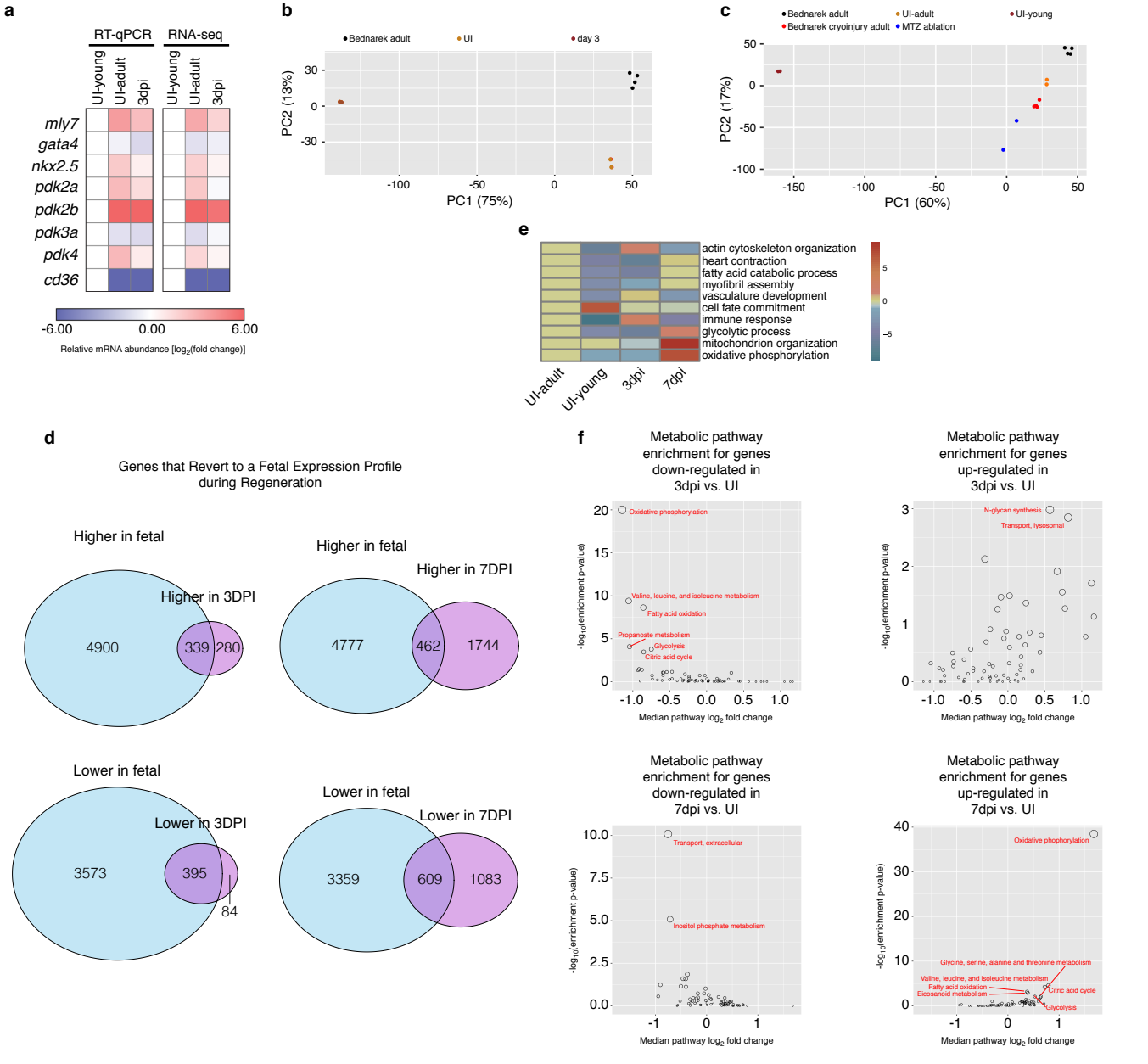

a
Brightfield
Merged
mCherry
Wild Type
vmhc:mCherry-NTR
A
B
V
b
vmhc:mCherry-NTR
Uninjured
DAPI
mCherry
c
Merged
mCherry
TUNEL
DAPI
Uninjured
Injured

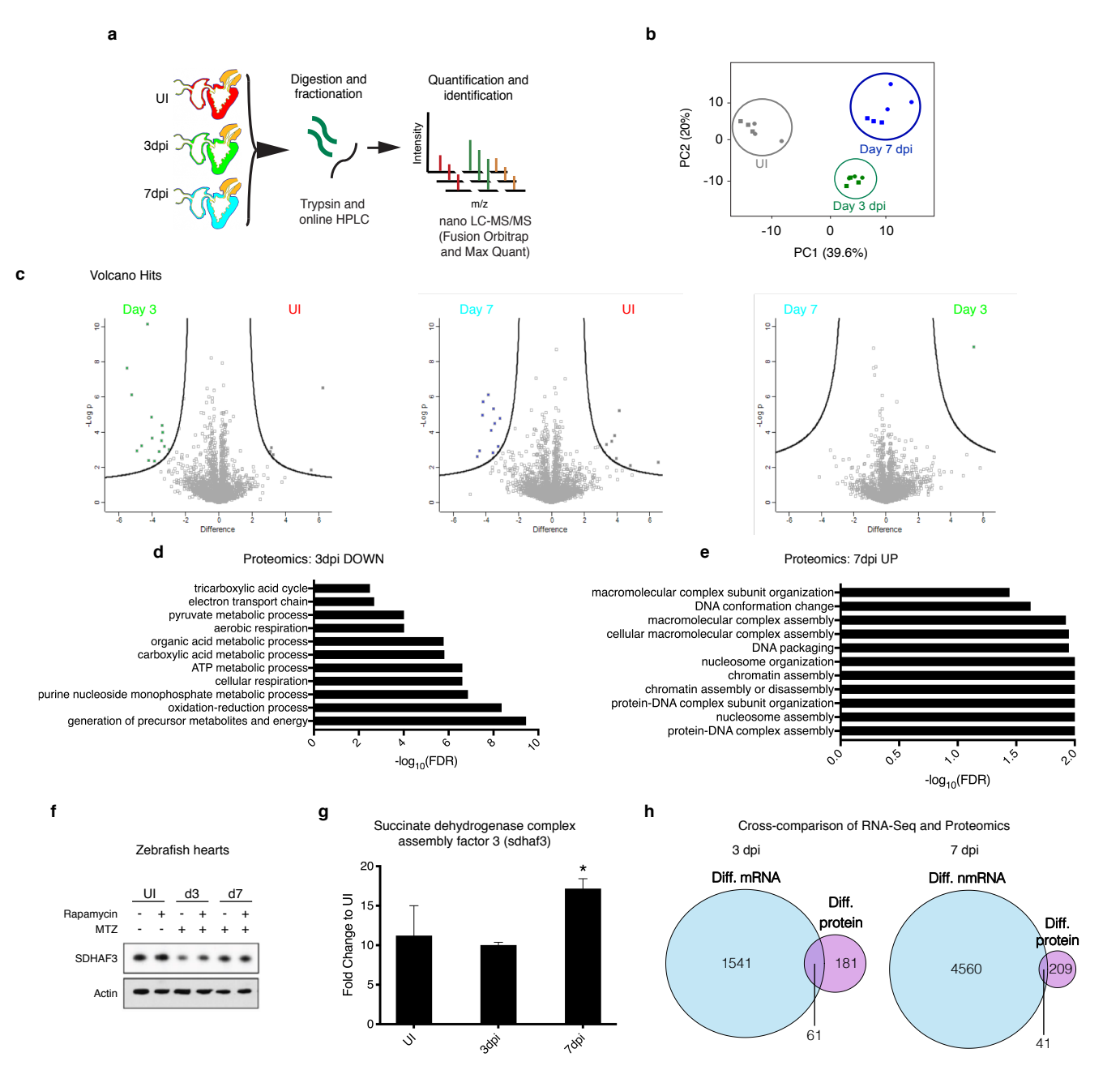
Supplemental Figure 1: Presentation of MTZ to *vmhc*:mCherry-NTR fish results in cell death, related to Figure 1. A) Wild type AB zebrafish heart and *vmhc*:mCherry-NTR zebrafish heart shown in brightfield, merged, and mCherry fluorescence. The mCherry signal shows the tissue specific, ventricle, expression of the mCherry-NTR protein. The chambers of the heart are outlined and labeled: A – atrium, V- ventricle, and B – bulbus arteriosus. The scale bars are: 500 µm. B) Confocal fluorescent microscopy image of a sectioned ventricle of the *vmhc*:mCherry-NTR line. mCherry staining is found throughout the ventricle. Red – mCherry-NTR and Blue – DAPI. Scale bar is 50 µm. C) Confocal microscopy of vmhc:mCherry-NTR zebrafish heart after 48 hours of vehicle (DMSO, uninjured) or MTZ (injured). Injured fish show TUNEL positive cells, yellow arrow heads, while vehicle treated fish do not. During injury there is a loss of ventricular muscle. Red – mCherry-NTR, Green – TUNEL, and Blue – DAPI. Scale bars are 10 µm.


DAPI
Mef2
Pcna
a
-Heat Shock
+Dkk1b
b
+Heat Shock
-Dkk1b
c
+Heat Shock
+Dkk1b
d
-Heat Shock
-Wnt8a
e
+Heat Shock
+Wnt8a
f
% Proliferating CMs
40
30
20
10
0
HS
-
+
hsWnt8a
(Het)
+
+

Supplemental Figure 2: Wnt/β-catenin signaling effects cardiomyocyte proliferation during the early stages of regeneration, related to Figure 1. A-C) Immunohistochemistry images of cardiomyocyte proliferation at 3 days post injury during Wnt/β-catenin inhibition via hsDkk1b overexpression or no Wnt/β-catenin inhibition via no heat shock or no hsDkk1bGFP construct present. D-E) Immunohistochemistry images of cardiomyocyte proliferation at 3 days post injury during Wnt/β-catenin activation via hsWnt8a overexpression or no additional Wnt/β-catenin activation via no heat shock and no hsWnt8aGFP construct present. F) Quantification of cardiomyocyte proliferation in the context of Wnt/β-catenin activation via hsWnt8a. All animals were presented with MTZ. Scale bar: 25 μm. Blue-DAPI, Red-Pcna, and Green-Mef2c. Yellow arrow heads denote proliferating cardiomyocytes, Pcna and Mef2c positive.

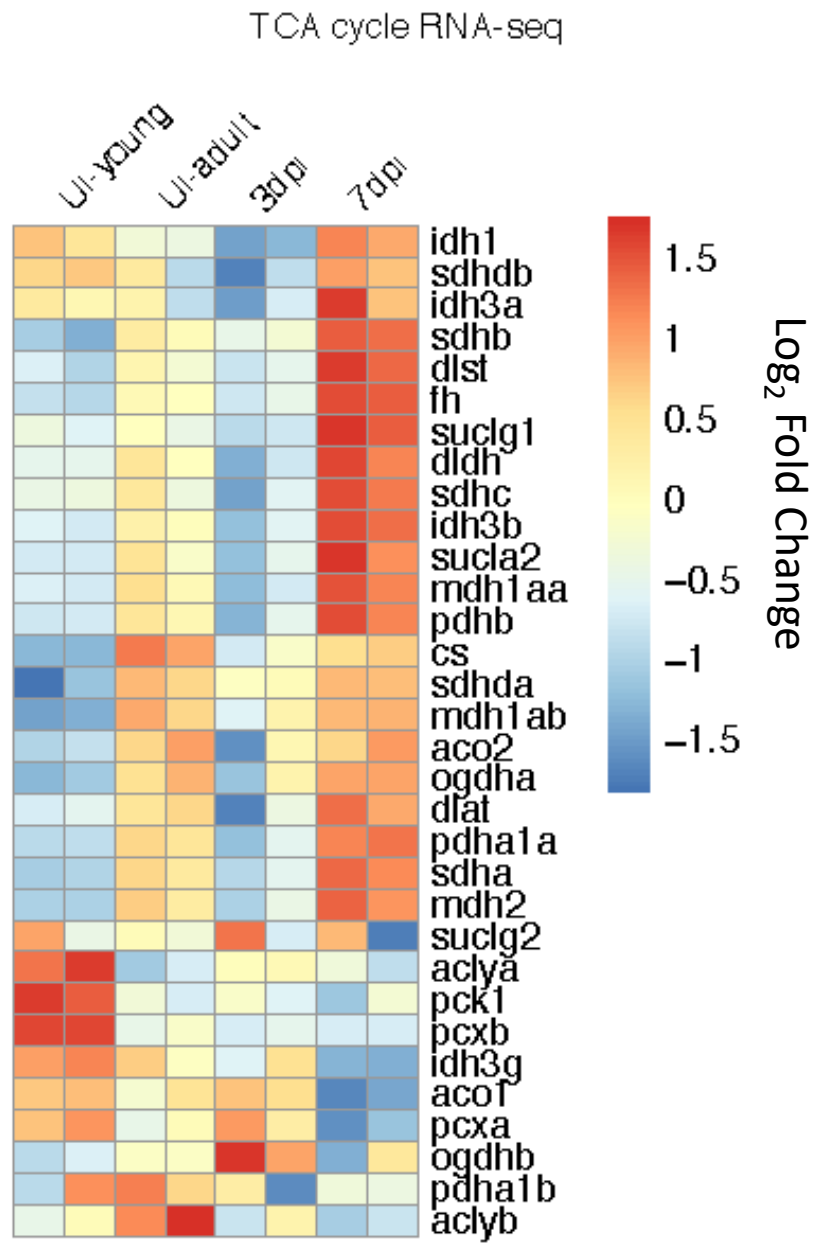

a
RT-qPCR
RNA-seq
UI-young
UI-adult
3dpi
mly7
gata4
nkx2.5
pdk2a
pdk2b
pdk3a
pdk4
cd36
-6.00
0.00
6.00
Relative mRNA abundance [log2(fold change)]
b
Bednarek adult
UI
day 3
PC2 (13%)
PC1 (75%)
c
Bednarek adult
UI-adult
UI-young
Bednarek cryoinjury adult
MTZ ablation
PC2 (17%)
PC1 (60%)
e
actin cytoskeleton organization
heart contraction
fatty acid catabolic process
myofibril assembly
vasculature development
cell fate commitment
immune response
glycolytic process
mitochondrion organization
oxidative phosphorylation
UI-adult
UI-young
3dpi
7dpi
d
Genes that Revert to a Fetal Expression Profile during Regeneration
Higher in fetal
Higher in 3DPI
4900
339
280
Higher in fetal
Higher in 7DPI
4777
462
1744
Lower in fetal
Lower in 3DPI
3573
395
84
Lower in fetal
Lower in 7DPI
3359
609
1083
f
Metabolic pathway enrichment for genes down-regulated in 3dpi vs. UI
Oxidative phosphorylation
Valine, leucine, and isoleucine metabolism
Fatty acid oxidation
Propanoate metabolism
Glycolysis
Citric acid cycle
-log10(enrichment p-value)
Median pathway log2 fold change
Metabolic pathway enrichment for genes up-regulated in 3dpi vs. UI
N-glycan synthesis
Transport, lysosomal
Metabolic pathway enrichment for genes down-regulated in 7dpi vs. UI
Transport, extracellular
Inositol phosphate metabolism
Metabolic pathway enrichment for genes up-regulated in 7dpi vs. UI
Oxidative phophorylation
Glycine, serine, alanine and threonine metabolism
Valine, leucine, and isoleucine metabolism
Fatty acid oxidation
Eicosanoid metabolism
Citric acid cycle
Glycolysis

Supplemental Figure 3: Metabolism is dynamically regulated during the first week of adult zebrafish heart regeneration, related to Figure 2. A) RNA-sequencing data of cardiac transcription factors, structural, and metabolic gene expression during adult zebrafish heart regeneration and RT-qPCR validation of these genes. B) Principle component analysis of isolated young uninjured, 3-day old hearts, and adult zebrafish hearts as compared to Bednarek *et al.* adult zebrafish hearts. C) Principle component analysis of isolated young uninjured 3-day old hearts, uninjured adult hearts, and NTR ablated adult zebrafish hearts as compared to Bednarek *et al.* uninjured and cryo-injured adult zebrafish hearts. D) Venn diagrams for the genes that revert to a fetal like expression level during regeneration. E) Key cardiac pathways that are differentially expressed during adult zebrafish heart regeneration. Log2 fold change shown. F) Metabolic pathways enrichment for up- and down-regulated genes during the first week of adult zebrafish heart regeneration.

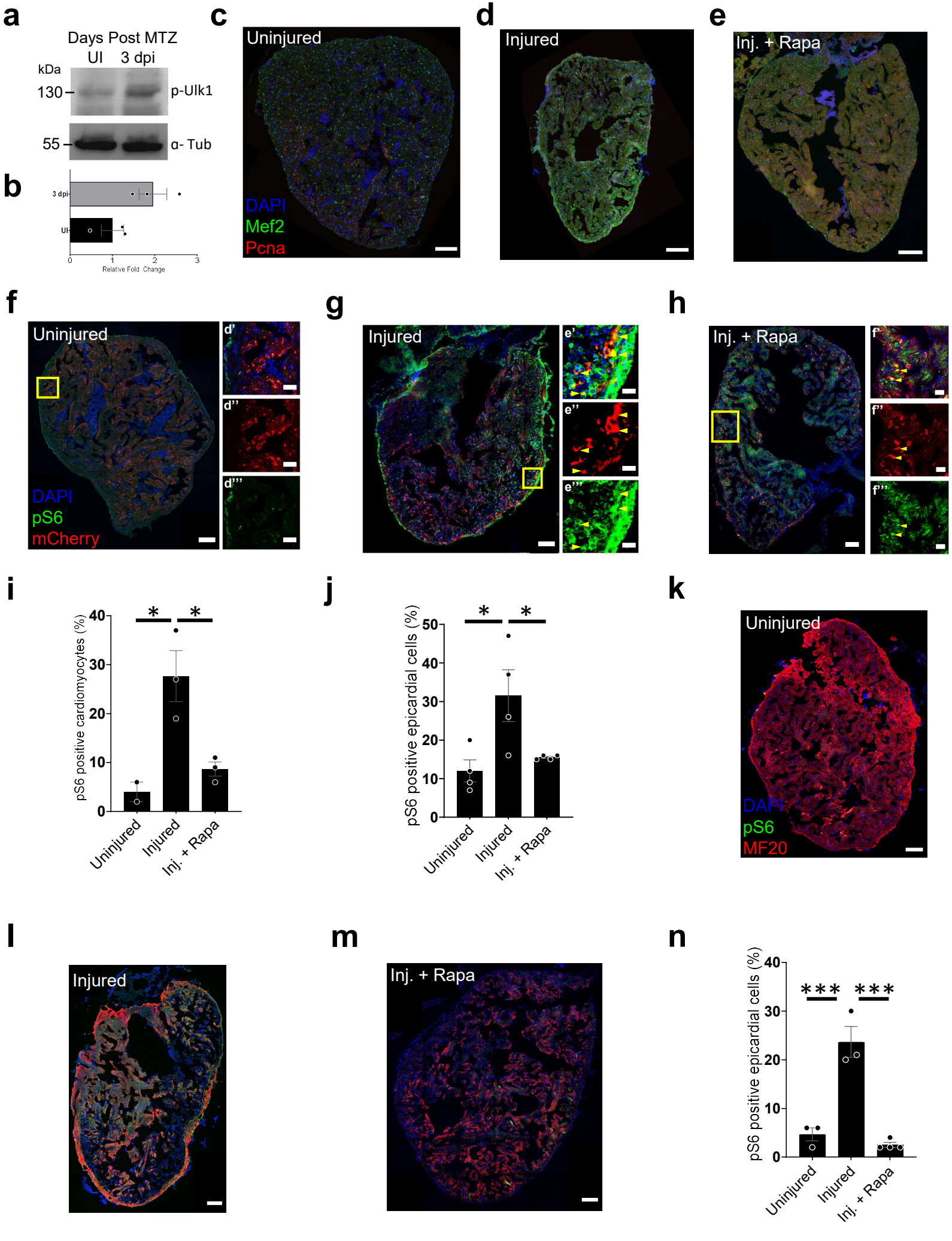

a
UI
3dpi
7dpi
Digestion and fractionation
Trypsin and online HPLC
Quantification and identification
Intensity
m/z
nano LC-MS/MS (Fusion Orbitrap and Max Quant)
b
PC2 (20%)
PC1 (39.6%)
UI
Day 7 dpi
Day 3 dpi
c
Volcano Hits
Day 3
UI
Day 7
UI
Day 7
Day 3
-Log p
Difference
d
Proteomics: 3dpi DOWN
tricarboxylic acid cycle
electron transport chain
pyruvate metabolic process
aerobic respiration
organic acid metabolic process
carboxylic acid metabolic process
ATP metabolic process
cellular respiration
purine nucleoside monophosphate metabolic process
oxidation-reduction process
generation of precursor metabolites and energy
-log10(FDR)
e
Proteomics: 7dpi UP
macromolecular complex subunit organization
DNA conformation change
macromolecular complex assembly
cellular macromolecular complex assembly
DNA packaging
nucleosome organization
chromatin assembly
chromatin assembly or disassembly
protein-DNA complex subunit organization
nucleosome assembly
protein-DNA complex assembly
-log10(FDR)
f
Zebrafish hearts
UI
d3
d7
Rapamycin
MTZ
SDHAF3
Actin
g
Succinate dehydrogenase complex assembly factor 3 (sdhaf3)
Fold Change to UI
UI
3dpi
7dpi
*
h
Cross-comparison of RNA-Seq and Proteomics
3 dpi
7 dpi
Diff. mRNA
Diff. protein
1541
181
61
Diff. nmRNA
Diff. protein
4560
209
41

Supplemental Figure 4: Proteomics analysis of adult zebrafish heart regeneration, related to Figure 2. A) Schematic of adult zebrafish hearts used for proteomics analysis at UI adult, 3, or 7 dpi. B) Principle component analysis of full proteome analysis during the first week of heart regeneration. C) Volcano plots of protein expression comparing adult hearts at UI, 3, or 7 dpi. D) Gene ontology terms that were down-regulated at 3 dpi. E) Gene ontology terms that were up-regulated at 7 dpi. F) Protein validation of succinate dehydrogenase complex assembly factor 3 expression during the first week of adult zebrafish heart regeneration. G) RNA-sequencing expression of succinate dehydrogenase complex assembly factor 3. H) Venn diagrams showing the number of genes and proteins that were differentially regulated and found in both data sets during heart regeneration. Bar graphs error bars represent standard error of the mean.

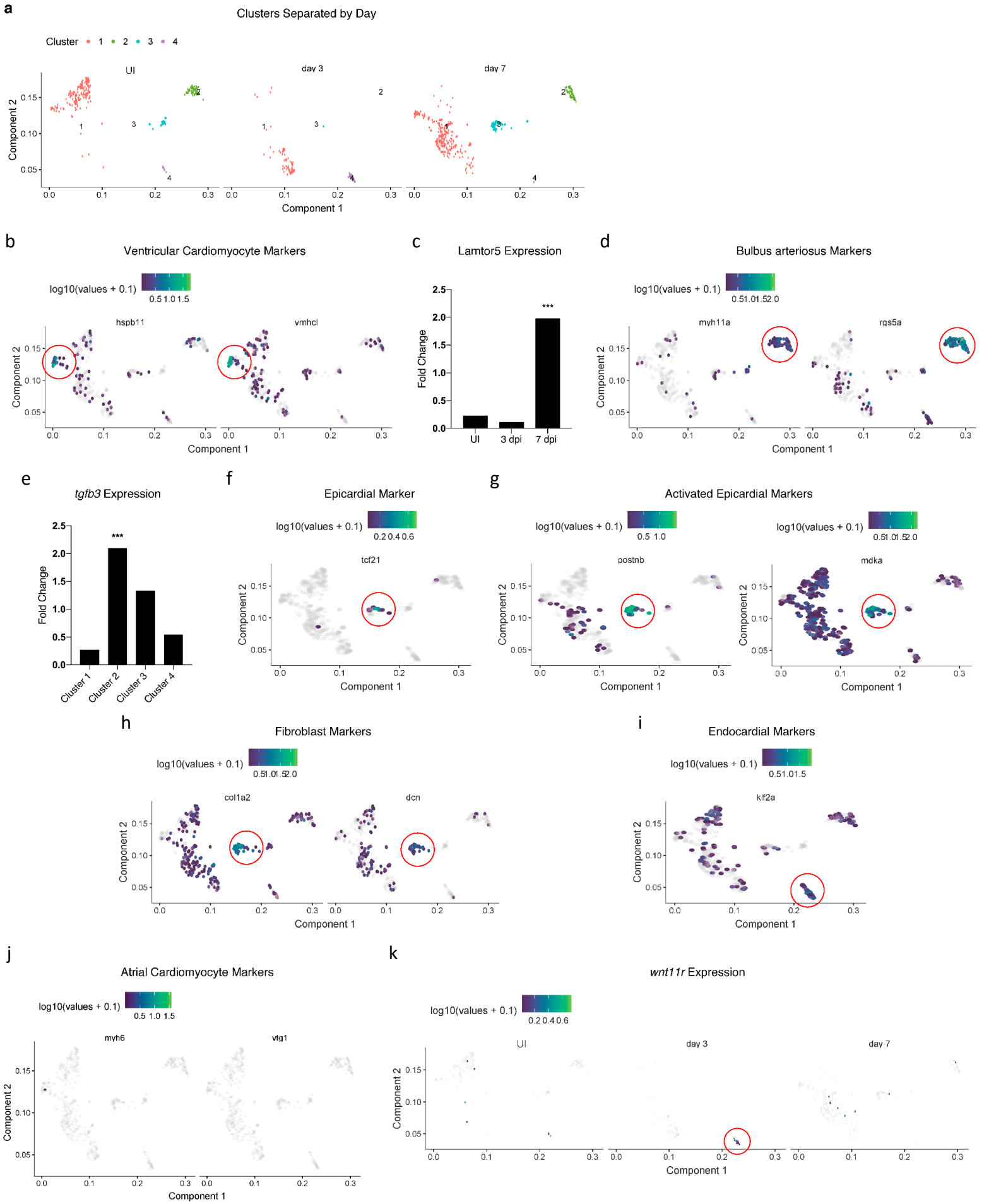
a


TCA cycle RNA-seq
UI-young
UI-adult
3dpi
7dpi
idh1
sdhdb
idh3a
sdhb
dlst
fh
suclg1
dldh
sdhc
idh3b
sucla2
mdh1aa
pdhb
cs
sdhda
mdh1ab
aco2
ogdha
dlat
pdha1a
sdha
mdh2
suclg2
aclya
pck1
pcxb
idh3g
aco1
pcxa
ogdhb
pdha1b
aclyb
1.5
1
0.5
0
−0.5
−1
−1.5
Log2 Fold Change

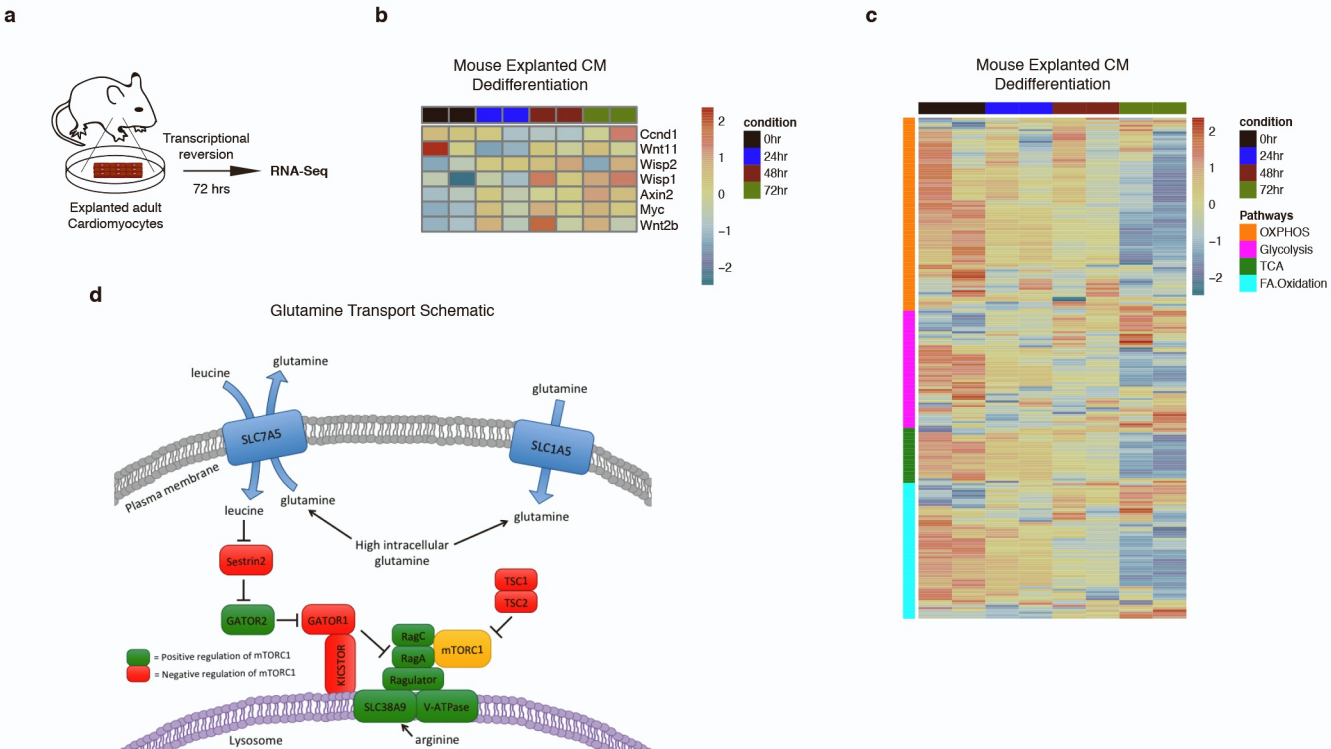
Supplemental Figure 5: Dynamic transcriptional remodeling of metabolic enzymes during the first week of heart regeneration, related to Figure 2. A) Heat map of TCA cycle enzyme gene expression from RNA-sequencing data.


a
Days Post MTZ
UI
3 dpi
kDa
130
p-Ulk1
55
α- Tub
b
3 dpi
UI
0
1
2
3
Relative Fold Change
c
Uninjured
DAPI
Mef2
Pcna
d
Injured
e
Inj. + Rapa
f
Uninjured
d'
d''
d'''
DAPI
pS6
mCherry
g
Injured
e'
e''
e'''
h
Inj. + Rapa
f'
f''
f'''
i
pS6 positive cardiomyocytes (%)
0
10
20
30
40
*
*
Uninjured
Injured
Inj. + Rapa
j
pS6 positive epicardial cells (%)
0
10
20
30
40
50
*
*
Uninjured
Injured
Inj. + Rapa
k
Uninjured
DAPI
pS6
MF20
l
Injured
m
Inj. + Rapa
n
pS6 positive epicardial cells (%)
0
10
20
30
40
***
***
Uninjured
Injured
Inj. + Rapa

Supplemental Figure 6: Cardiomyocytes and epicardial cells have higher TOR activity during zebrafish heart regeneration related to Figure 3. A) Western blot of TOR activity target p-Ulk1 and loading control protein alpha-tubulin. B) Quantification of protein abundance in A shows increased Tor activity at 3 dpi. C-E) Whole heart immunohistochemistry images assessing cardiomyocyte proliferation at 3 dpi in the uninjured, injured, and injured with rapamycin treated conditions. Scale bars: 100 μm. F-H) Immunohistochemistry assessing TOR activity at 3 dpi of heart regeneration. Positive TOR cardiomyocytes are double positive for pS6 and NTR-mCherry (yellow arrows). Positive TOR epicardial cells are positive for pS6 and were determined as the cells along the border of the heart. DAPI – blue, phosphorylation of S6 – Green, and NTR-mCherry – Red. Full heart image scale bars are 100 μm, magnified inset image scale bars are 20 μm. I) Quantification of the percentage of pS6 cardiomyocytes, cardiomyocytes determined by vmhc::mCherry-NTR. One-way ANOVA performed. p=0.023 for uninjured (-Rapa -MTZ) vs injured (-Rapa +MTZ) hearts. p=0.024 for injured (-Rapa +MTZ) vs rapamycin treated injured hearts (+Rapa +MTZ). N=2-3 biological replicates. J) Quantification of the percentage of pS6 epicardial cells. p=0.029 for uninjured (-Rapa -MTZ) vs injured (-Rapa +MTZ) hearts. p=0.050 for injured (-Rapa +MTZ) vs rapamycin treated injured hearts (+Rapa +MTZ). N=4 biological replicates. K-M) Whole heart immunohistochemistry images assessing cardiomyocyte TOR activation at 3 dpi in the uninjured, injured, and injured with rapamycin treated conditions. Scale bars: 100 μm. DAPI – blue, phosphorylation of S6 – Green, and MF20 – Red. N) Quantification of the percentage of pS6 epicardial cells. p<0.001 for uninjured (-Rapa -MTZ) vs injured (-Rapa +MTZ) hearts. p<0.001 for injured (-Rapa +MTZ) vs rapamycin treated injured hearts (+Rapa +MTZ). One-Way ANOVA performed. N=3-4 biological replicates. Bar graphs show individual data points with error bars representing standard error.


a
Clusters Separated by Day
Cluster 1 2 3 4
UI
day 3
day 7
Component 2
Component 1
b
Ventricular Cardiomyocyte Markers
log10(values + 0.1)
hspb11
vmhcl
c
Lamtor5 Expression
Fold Change
***
UI
3 dpi
7 dpi
d
Bulbus arteriosus Markers
myh11a
rgs5a
e
tgfb3 Expression
Cluster 1
Cluster 2
Cluster 3
Cluster 4
f
Epicardial Marker
tcf21
g
Activated Epicardial Markers
postnb
mdka
h
Fibroblast Markers
col1a2
dcn
i
Endocardial Markers
klf2a
j
Atrial Cardiomyocyte Markers
myh6
vtg1
k
wnt11r Expression

Supplemental Figure 7: Single cell RNA-sequencing of the adult regenerating zebrafish heart identifies transient cell populations, related to Figure 4. A) Principle component analysis separated by day. B) Plot showing ventricular cardiomyocyte markers. C) Gene expression of *lamtor5* in cluster 1 cells at different time points. D) Plot showing bulbus arteriosus markers. E) Gene expression of *tgfb3* in each of the four clusters. F) Plot showing epicardial markers. G) Plot showing activated epicardial markers. H) Plot showing fibroblast markers. I) Plot showing endocardial markers. J) Plot showing atrial cardiomyocyte markers. K) Plot showing cells which are expressing the gene for the Wnt ligand *wnt11r*.


a
Transcriptional reversion
72 hrs
RNA-Seq
Explanted adult Cardiomyocytes
b
Mouse Explanted CM Dedifferentiation
Ccnd1
Wnt11
Wisp2
Wisp1
Axin2
Myc
Wnt2b
2
1
0
−1
−2
condition
0hr
24hr
48hr
72hr
c
Mouse Explanted CM Dedifferentiation
2
1
0
−1
−2
condition
0hr
24hr
48hr
72hr
Pathways
OXPHOS
Glycolysis
TCA
FA.Oxidation
d
Glutamine Transport Schematic
leucine
glutamine
SLC7A5
SLC1A5
glutamine
Plasma membrane
leucine
glutamine
glutamine
High intracellular glutamine
Sestrin2
TSC1
TSC2
GATOR2
GATOR1
RagC
RagA
mTORC1
KICSTOR
= Positive regulation of mTORC1
= Negative regulation of mTORC1
Ragulator
SLC38A9
V-ATPase
Lysosome
arginine

Supplemental Figure 8: Adult mouse cardiomyocyte de-differentiation resembles a regenerative transcript profile, related to Figure 5. A) Schematic of adult mouse cardiomyocyte isolation and *in vitro* culture. B) Heat map of cell proliferation, Wnt ligands and Wnt/β-catenin targets during cardiomyocyte de-differentiation. Heatmap shows Log_2 fold change. C) Heat map of the transcriptional change in metabolic pathways during cardiomyocyte de-differentiation. Heatmap shows Log_2 fold change. D) Schematic of glutamine transport and mTORC1 activation in a cell.