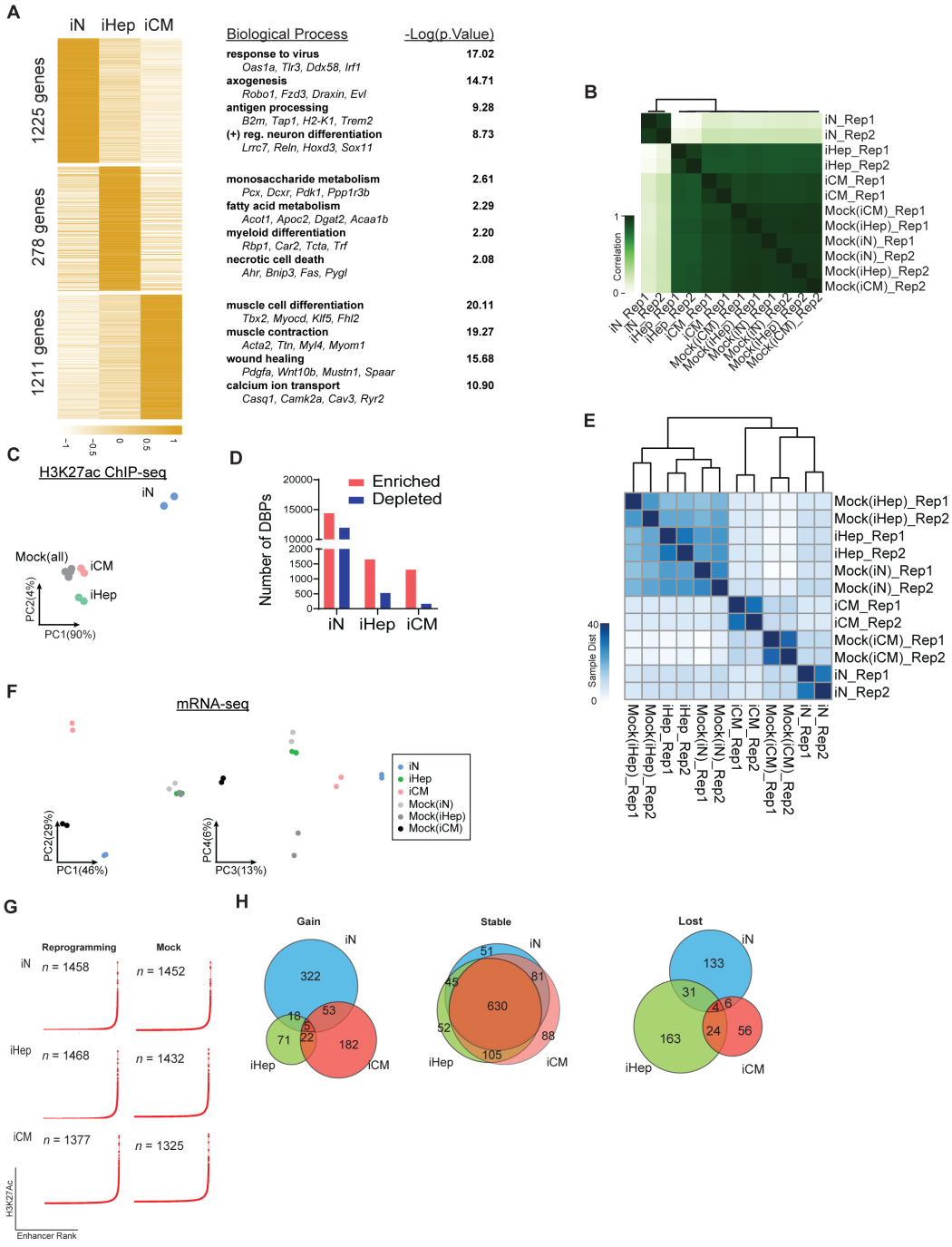


Supplementary Figure 1

Supplementary Figure 1. Characterization of global regulatome dynamics during iN, iHep and iCM reprogramming，Related to Figure 1

A) (Left) Heatmaps showing the expression of upregulated genes specific to iN (top), iHep (middle), and iCM (bottom) reprogramming, respectively. (Right) Enriched biological processes from gene ontology analysis of each group of genes, with representative genes also listed.

B) Heatmap of Pearson correlation analysis of the H3K27Ac ChIP-seq libraries of iN, iHep, and iCM reprogramming and their corresponding mock samples.

C) PCA analysis of H3K27Ac ChIP-seq libraries of iN, iHep, and iCM reprogramming and their corresponding mock.

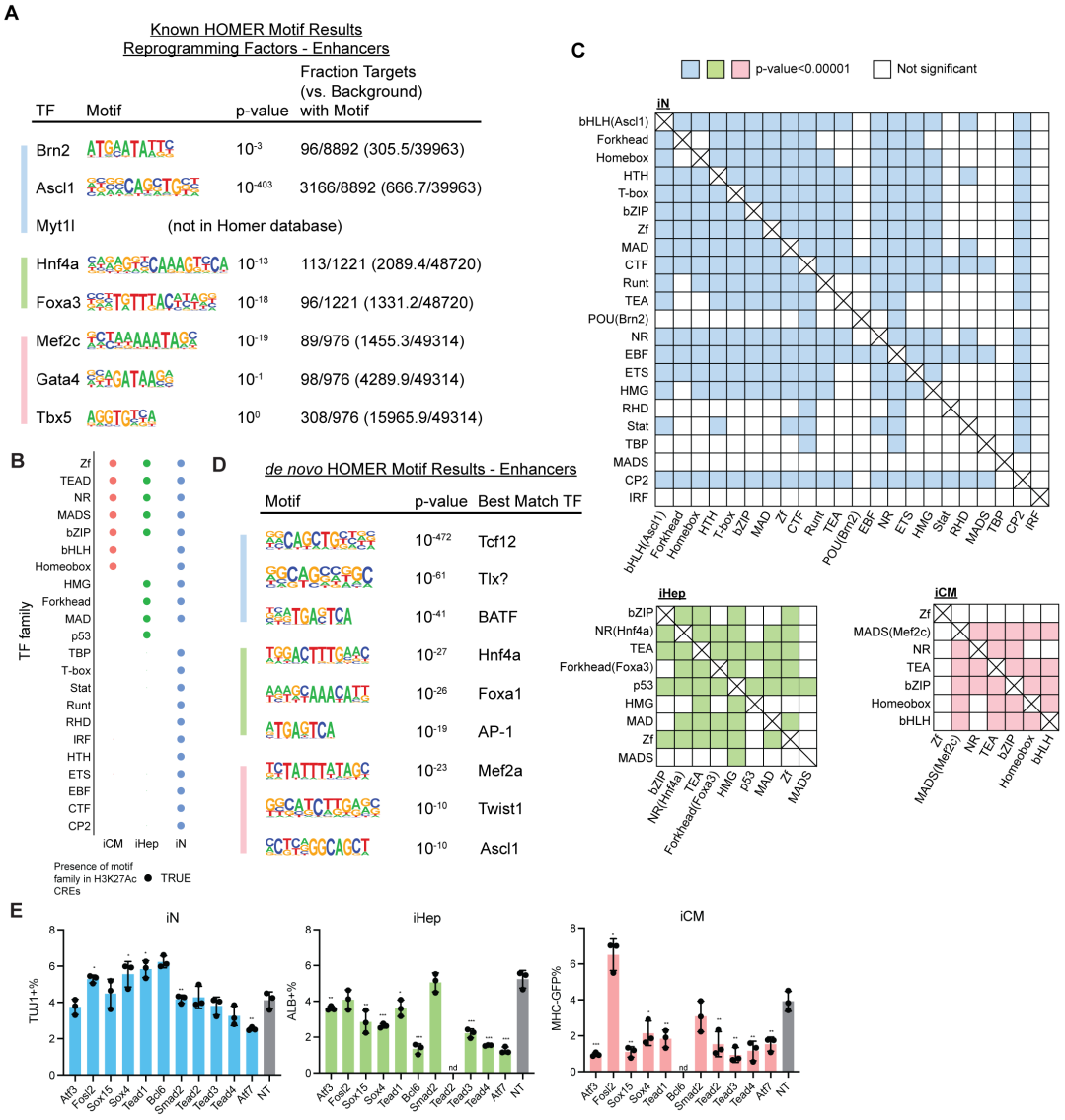
D) The number of enriched and depleted H3K27Ac differentially bound peaks (DBPs) for iN, iHep, and iCM reprogramming samples compared to mock controls.

E) Heatmap showing the sample distance between the RNA-seq libraries of iN, iHep, and iCM reprogramming and their corresponding mock samples.

F) PCA analysis of the RNA-seq libraries of iN, iHep, and iCM reprogramming and their corresponding mock samples.

G) Plots showing the distribution of H3K27ac ChIP-seq signal at enhancers in iN, iHep, and iCM reprogramming and their corresponding mock controls. Enhancer regions are plotted in increasing order based on their input-normalized H3K27ac ChIP-seq signal. Super-enhancers are defined as the population of enhancers above the inflection point of the curve. The number of super-enhancers identified in each sample is noted in the top-left of each plot.

H) Venn diagrams showing the overlap of gained, stable, and lost super-enhancers between iN (blue), iHep (green), and iCM (red) reprogramming.



Supplementary Figure 2

Supplementary Figure 2. Integrated analysis reveals common non-cocktail TFs shared by different direct reprogramming, Related to Figure 2

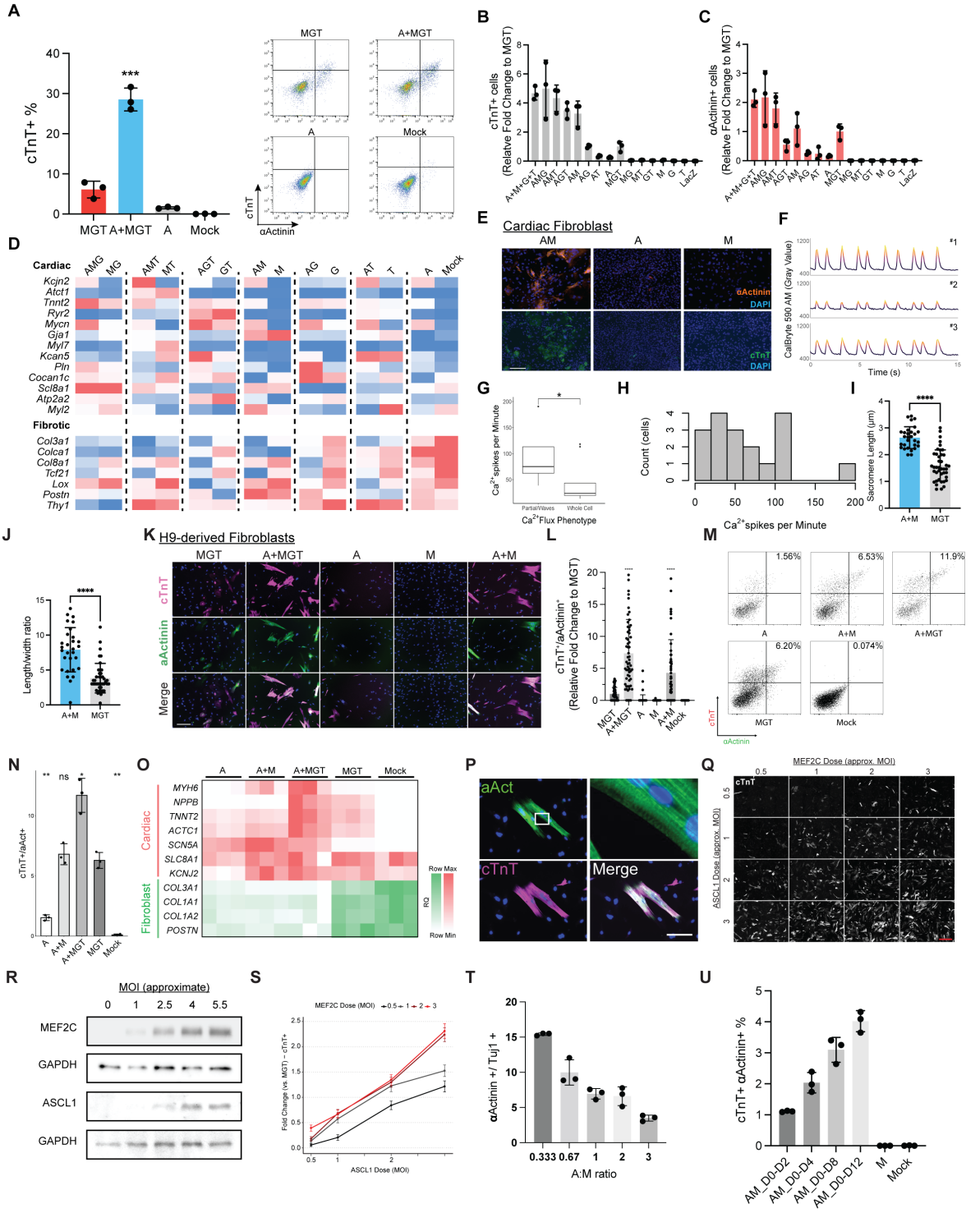
A) Results of HOMER known motif analysis for the reprogramming TFs used in each type of reprogramming. Color code: blue = iN, green = iHep, red = iCM.

B) Dot plot showing which TF families have motif enrichment in the gained cis-regulatory elements (CREs, i.e., enhancers and promoters with increased H3K27Ac signal) for each type of reprogramming.

C) Matrix showing results of co-occurrence analysis amongst TF motifs enriched in gained CREs during iN, iHep, and iCM reprogramming. Results have been summarized by TF family.

D) Results of HOMER *de novo* motif analysis of the enhancers gained during iN, iHep, and iCM reprogramming. The top 3 motifs (by p-value) for each type of reprogramming are shown.

E) Bar charts showing the effect of knockdown of non-cocktail TFs on reprogramming efficiency in iN, iHep, and iCM. n=3. Each dot represents a replicate. Error bars represent the standard deviation. n.d. = no data, due to substantial cell death over the course of the experiment.



Supplementary Figure 3

Supplementary Figure 3. Discovery of the cross-lineage potential of Ascl1, Related to Figure 3

A) Bar chart showing the flow cytometry of cTnT+ cells on Day 12 post the infection of neonatal mouse cardiac fibroblasts with MGT ± Ascl1. n=3. Represented flow cytometry results were shown on the right. Error bars represent standard deviation. The student's t-test was performed between A+MGT and MGT. ***, p-value < 0.001.

B) Bar chart showing the flow cytometry of cTnT+ cells on Day 12 post-infection of MEF with all combinations of Ascl1, Mef2c, Gata4, and Tbx5. Replication of samples (n=3). Error bars represent standard deviation.

C) Bar chart showing the flow cytometry of αActinin+ cells on Day 12 post-infection of MEF with all combinations of Ascl1, Mef2c, Gata4, and Tbx5. n=3. Error bars represent standard deviation.

D) Heatmap showing the relative expression by qRT-PCR of cardiac and fibroblast genes on Day 12 post-infection of MEF with different combinations of Mef2c, Gata4, Tbx5, with and without Ascl1.

E) Fluorescent images showing $cTnT^+$ and $\alpha Actinin^+$ cells on Day 12 post-infection of neonatal mouse cardiac fibroblasts Ascl1 (A), Mef2c (M), or A+M. cTnT staining is shown in green. αActinin+ staining is shown in orange. DNA is shown in blue. Scale bar: 275 μm

F) Plots of CalBryte 590 fluorescence intensity at each of the three labeled points labeled in Figure 3Q showing the flux of intracellular calcium during iCM contraction. Scale bar: 300 μm.

G) The box plot showing the frequency of calcium spikes in beating loci with partial calcium burst and whole-cell calcium burst. The student's t-test was performed. *, p < 0.05. Plots

of CalBryte 590 fluorescence intensity at each of the three labeled points labeled in Figure 3Q showing the flux of intracellular calcium during iCM contraction. Scale bar: 300 μm.

H) Bar chart showing the counts of beating loci with different frequencies of the calcium spikes.

I) Bar chart showing the percentage of reprogrammed cells with organized sarcomere on Day 12 post the infection of neonatal mouse cardiac fibroblasts with A+M or MGT. n=8. Error bars represent standard deviation. The student's t-test was performed between A+M and MGT. ns, $p > 0.05$.

J) Bar chart showing the length of sarcomere on Day 12 post the infection of neonatal mouse cardiac fibroblasts with A+M or MGT. The number of cells was quantified (n=29 for A+M, n=41 for MGT). Error bars represent standard deviation. The student's t-test was performed between A+M and MGT. ****, p-value < 0.0001.

K) Fluorescence images of cTnT+ and αActinin+ cells on Day 14 post-infection of H9-derived fibroblasts (H9F) with different combinations of MGT, Ascl1, or Mef2c, plus miR-133. Mock infection with lacZ was used as a negative control. cTnT staining is shown in magenta. αActinin+ staining is shown in green. DNA is shown in blue. Scale bar: 275 μm.

L) Bar chart showing automated quantification of (S).30 images were taken for each treatment. Error bars represent standard deviation. The student's t-test was performed between each TF combination and MGT. *, $p < 0.05$; **, $p < 0.01$.

M) Dot plots of flow cytometry results showing $cTnT^+/\alpha Actinin^+$ cells on Day 14 post-infection of H9F with miR-133 plus different combinations of MGT, Ascl1 (A), and Mef2c (M). The percentage of $cTnT^+/\alpha Actinin^+$ cells is labeled on the upper-left corner of each plot.

N) Bar chart showing quantification of (C). Each dot represents a biological replicate. Error bars represent the standard deviation. The student's *t*-test was performed as the statistical test. *, $p < 0.05$. **, $p < 0.01$.

O) Heatmap showing relative expression of cardiac and fibroblast genes by qRT-PCR on Day 14 post-infection of H9F with different combinations of MGT, Ascl1, and Mef2c, plus miR-133. Mock infection with lacZ was used as a negative control. cTnT. n=3. The color intensity in each square represents gene expression in a single replicate.

P) Fluorescence images showing iCM on Day 14 post-infection reprogrammed from H9F with A+M plus miR133. cTnT staining is shown in magenta. αActinin+ staining is shown in green. DNA is shown in blue. Scale bar: 150 μm. (Inset) Close up view of banding pattern in αActinin staining.

Q) Fluorescence images of cTnT+ cells on Day 14 post-infection of H9F infected with varying doses of Ascl1 and Mef2c, plus a constant amount of miR-133. cTnT staining is shown in white. 16 images were taken for each treatment. Images from each sample have been tiled from 16 separate images to better represent overall reprogramming efficiency. Scale bar (red): 600 μm.

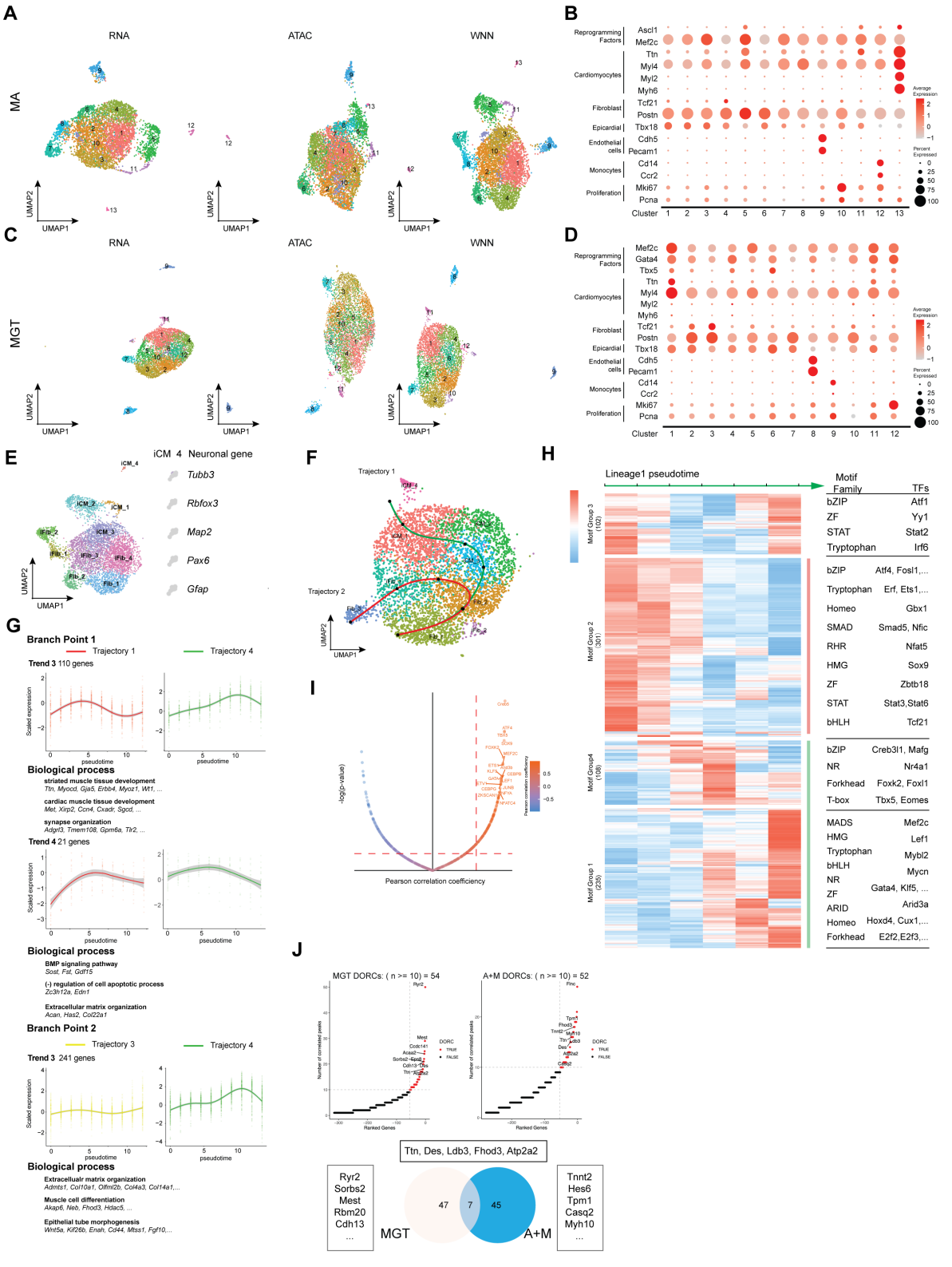
R) Western blot image showing the expression of MEF2C and ASCL1 when the fibroblast is being infected at different multiplicity of infection (MOI). GAPDH is used as the loading control.

S) Line plot showing automated quantification of (W). Each line represents a different MEF2C dose. Error bars represent standard deviation.

T) Bar charts showing the effect of different Ascl1-to-Mef2c ratio on the reprogramming outcome. The iCM to iN ratio is accessed by the ratio of αActinin + to Tuj1 + cells.

Biological replication of samples, n=3. Each dot represents a replicate. Error bars represent the standard deviation.

U) Bar chart showing the flow cytometry of αActinin+cTnT+ cells on Day 12 post-infection of mouse cardiac fibroblast with doxycycline treatment for the first two (AM_D0-D2), four (AM_D0-D4), eight (AM_D0-D8), or twelve days (AM_D0-D12). n=3. Error bars represent standard deviation.



Supplementary Figure 4

Supplementary Figure 4. Successful A+M reprogramming terminates in a more mature iCM fate, Related to Figure 4

A) UMAP visualization of A+M single-cell multi-omics library. UMAP based on the scRNA-seq portion of the multi-omics library is shown on the left. UMAP based on the scATAC-seq portion of the multi-omics library is shown on the left. UMAP of the integrated multi-omics library using weighted nearest neighbor (WNN) analysis is shown on the right.

B) Dot plot of the expression of cell-type-specific markers, reprogramming factors, and proliferation-related genes for each cell cluster in A+M reprogramming. Representative genes of each cell type were selected based on previous publications. Individual dots are sized to reflect the proportion of cells of each cluster expressing the gene and colored to reflect the average expression of each gene across all cells, as indicated in the key.

C) UMAP visualization of MGT single-cell multi-omics library. UMAP based on the scRNA-seq portion of the multi-omics library is shown on the left. UMAP based on the scATAC-seq portion of the multi-omics library is shown on the left. UMAP of the integrated multi-omics library using the WNN analysis is shown on the right.

D) Dot plot of the expression of cell-type-specific markers, reprogramming factors, and proliferation-related genes for each cell cluster in MGT reprogramming. Representative genes of each cell type were selected based on previous publications. Individual dots are sized to reflect the proportion of cells of each cluster expressing the gene and colored to reflect the average expression of each gene across all cells, as indicated in the key.

E) Zoomed in view of the neuronal gene expression in iCM_4 cluster in A+M reprogramming.

F) Pseudotime trajectories of cells originating from fibroblast (Fib_1) in MGT reprogramming. Two trajectories were identified using Slingshot and are labeled with

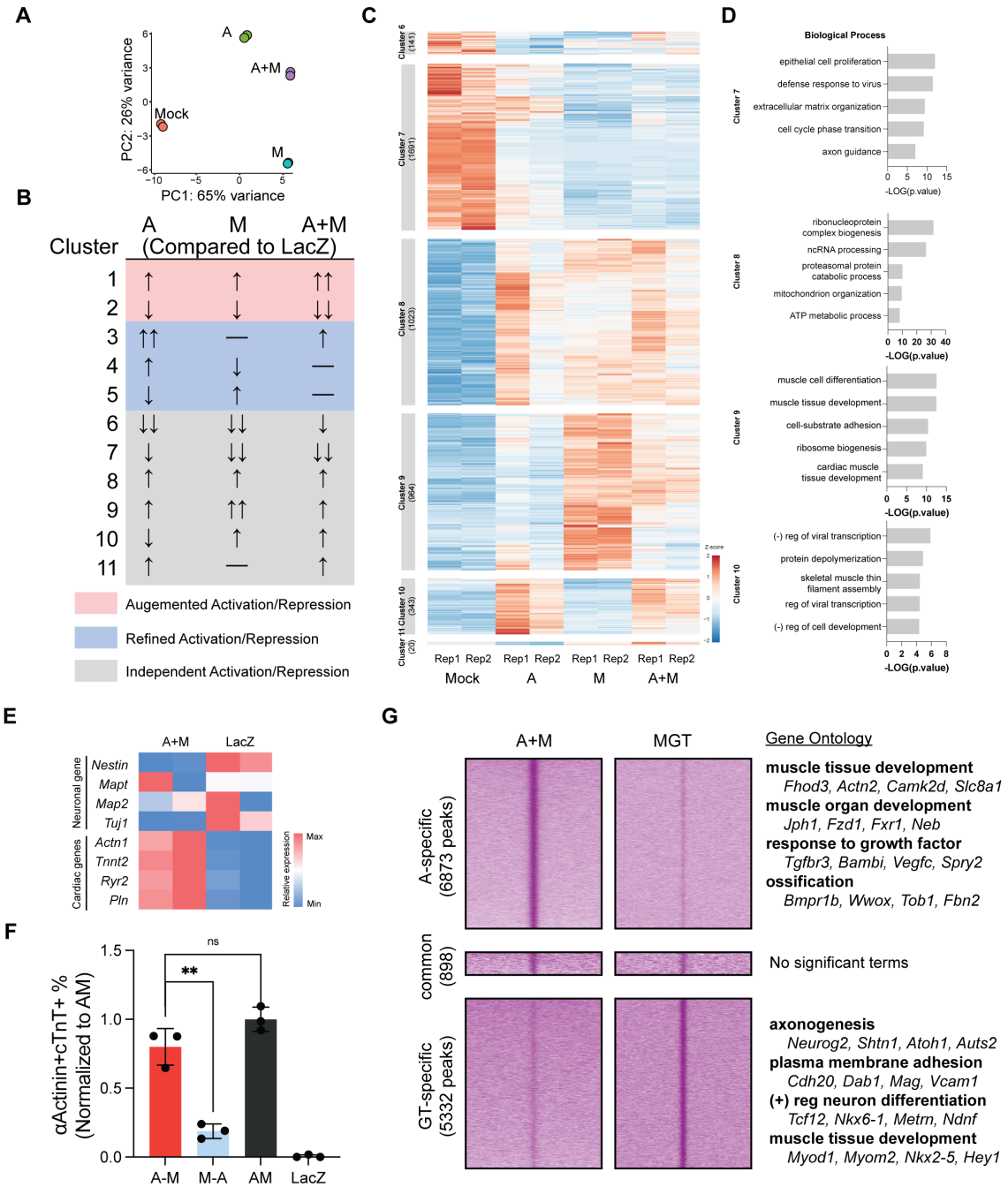
different colors. Trajectory 1 represents the successful reprogramming trajectory of MGT-mediated cardiac reprogramming.

G) Line plot of expression of the differentially expressed genes (DEGs) before and after the branch points along the pseudotime trajectories. The DEGs were clustered into different trends using unsupervised hierarchical clustering. Y-axis is the scaled gene expression normalized of each gene's expression along pseudotime. The expression of genes within each cell is shown as dots. The average expression of genes across all cells along each reprogramming trajectory is plotted as a smoothed solid line. Biological processes enriched for each cluster of genes are shown below the corresponding line plot.

H) Heatmap showing the motif enrichment along the MGT-mediated cardiac reprogramming trajectory 1. The trajectory was divided into six bins and then the motif enrichment score was calculated for all the vertebrate motifs in the JASPAR database. The motifs were clustered using unsupervised hierarchical clustering. Group 2 was classified as motif lost during MGT reprogramming (indicated by the red line). Groups 1 and 4 were classified as motifs gained during MGT reprogramming (indicated by the green line). Group 3 contains motifs showing transient loss during MGT reprogramming. In each motif group, enriched motif families and their representative TFs are shown to the right of the heatmap.

I) Scatter plot of TF motifs. X-axis is Pearson correlation between the motif's enrichment score with its expression along trajectory 1 of MGT-mediated cardiac reprogramming. Y-axis is the log transformation of the motif enrichment p-value calculated using chromVar. Only motifs with a Pearson correlation co-efficient larger than 0.6 and -log(p-value) larger than 0.1 (indicated with red dashed lines) were considered active motifs.

J) Line plot of DORCs identified in MGT (left) and A+M (right) reprogramming. DORCs are labeled as red dot. Venn diagram showing overlap of DORCs between MGT and A+M reprogramming.



Supplementary Figure 5

Supplementary Figure 5. Ascl1's bi-lineage potential is restricted by Mef2c, Related to Figure 5

A) PCA analysis of RNA-seq libraries of MEFs infected with Ascl1 (green), Mef2c (blue), A+M (purple), or Mock (red) infection.

B) After unsupervised hierarchical clustering of the TPM data, the differentially expressed genes (DEGs) in A, M, and A+M infected cells were categorized into three categories: Augmented, refined, and independent activation /repression. Augmented activation/repression are genes that showed increased expression/decreased expression when A and M are co-expressed compared to A or M expression alone. Refined activation/repression are genes that showed less change in expression when A and M are co-expressed compared to A or M expression alone. Independent activation/repression are genes that did not fit into the augmented or refined patterns. Arrows in the plot indicate the degree of change in gene expression in each sample compared to mock.

C) Heatmap of the DEGs in A, M, and A+M infected MEFs compared to mock. DEGs were clustered by unsupervised hierarchical clustering. Clusters 6,7,8,9,10, and 11 are classified as genes showing independent activation/repression when A and M are co-expressed. (See the main figure for clusters that exhibited augments or refined expression patterns.) Heatmap columns represent biological duplicates of each sample type.

D) Bar plots showing the results of gene ontology (GO) analysis of the clusters in (C). Cluster 11 is not shown as its genes were not significantly enriched for any GO terms.

E) Heatmap showing relative expression of cardiac and neuronal genes by qRT-PCR on Day 58 post-infection of A+M reprogramming. Mock infection with lacZ was used as a negative control.

F) Bar chart showing the flow cytometry of αActinin+cTnT+ cells on Day 12 post-infection of mouse cardiac fibroblast with Ascl1 on Day -2 and Mef2c on Day -1(A-M), Mef2c on Day -2 and Ascl1 on Day -1(M-A), Ascl1 and Mef2c on Day -2(AM), and LacZ(LacZ). n=3. Error bars represent standard deviation. **, $p < 0.01$. ns, non-significant.

G) Heatmap of the Mef2c ChIP-seq peaks specific to A+M reprogramming, common between AM and MGT reprogramming, or specific to MGT reprogramming. Biological processes significantly enriched among the genes annotated to these regions are shown on the right, along with representative genes.