Cardiomyocyte gene programs encoding morphological and functional signatures in cardiac hypertrophy and failure

Seitaro Nomura1,2, Masahiro Satoh2,3, Takanori Fujita2, Tomoaki Higo4, Tomokazu Sumida1, Toshiyuki Ko1, Toshihiro Yamaguchi1, Takashige Tobita5, Atsuhiko T. Naito1, Masamichi Ito1, Kanna Fujita1, Mutsuo Harada1, Haruhiro Toko1, Yoshio Kobayashi3, Kaoru Ito6, Eiki Takimoto1, Hiroshi Akazawa1, Hiroyuki Morita1, Hiroyuki Aburatani2 & Issei Komuro1

Pressure overload induces a transition from cardiac hypertrophy to heart failure, but its underlying mechanisms remain elusive. Here we reconstruct a trajectory of cardiomyocyte remodeling and clarify distinct cardiomyocyte gene programs encoding morphological and functional signatures in cardiac hypertrophy and failure, by integrating single-cardiomyocyte transcriptome with cell morphology, epigenomic state and heart function. During early hypertrophy, cardiomyocytes activate mitochondrial translation/metabolism genes, whose expression is correlated with cell size and linked to ERK1/2 and NRF1/2 transcriptional networks. Persistent overload leads to a bifurcation into adaptive and failing cardiomyocytes, and p53 signaling is specifically activated in late hypertrophy. Cardiomyocyte-specific p53 deletion shows that cardiomyocyte remodeling is initiated by p53-independent mitochondrial activation and morphological hypertrophy, followed by p53-dependent mitochondrial inhibition, morphological elongation, and heart failure gene program activation. Human single-cardiomyocyte analysis validates the conservation of the pathogenic transcriptional signatures. Collectively, cardiomyocyte identity is encoded in transcriptional programs that orchestrate morphological and functional phenotypes.
Organis respond appropriately to external and internal stress to maintain homeostasis, but excessive stress disrupts the adaptive response, leading to organ dysfunction. Hemodynamic stimuli such as pressure and volume overload to the heart initially induce cardiac hypertrophy as an adaptive response to reduce wall stress and prevent cardiac dysfunction\cite{1,2}. However, sustained overload causes cardiac dysfunction leading to heart failure\cite{3}. During this process, cardiomyocytes activate various signaling cascades initially for adaptive morphological hypertrophy, followed by a transition to the failing phenotype characterized by elongation and contractile force reduction\cite{6}. Yet, it remains elusive how individual cardiomyocytes undergo molecular and morphological remodeling in response to stress, contributing to cardiac adaptation and dysfunction.

Because individual cardiomyocytes constitute the basic units of gene regulation, each cardiomyocyte’s phenotype and function are considered to be determined based on its transcriptional programs. Chemical inhibition of transcriptional activation has been shown to suppress cardiac molecular and morphological remodeling after pressure overload\cite{7}. Single-cardiomyocyte gene expression analyses have revealed an increase in cell-to-cell transcriptional variation in the aging mouse heart\cite{8} and partial activation of genes involved in de-differentiation and the cell cycle\cite{9}. These studies have established that cardiomyocyte gene expression underlies cellular phenotypes and determines cardiac function, but it remains elusive what gene programs regulate morphological remodeling and contribute to maintain and disrupt cardiac homeostasis. Furthermore, to reveal the pathogenesis of heart failure and identify novel therapeutic targets, it is important to better understand the molecular and morphological bases of the hypertrophic and failing cardiomyocyte states and to identify regulators of the transition between these states. Uncovering the conserved gene programs involved in cardiomyocyte morphology and cardiac function will enable accurate assessment of the condition of cardiomyocytes and the heart and prediction of treatment response.

Here, through co-expression network analysis\cite{10,11} of mouse and human single-cardiomyocyte transcriptomes, we integrated gene expression profiles with multidimensional data such as single-cell morphology, epigenetic state, and physiological heart function to dissect the molecular and morphological dynamics of cardiomyocytes during cardiac hypertrophy and heart failure. Our study establishes that cardiomyocyte identity is encoded in transcriptional programs that orchestrate morphological and functional phenotypes, and can be controlled by appropriate interventions.

Results

Single-cardiomyocyte transcriptomic profiles in heart failure.

We obtained single-cardiomyocyte transcriptomes from mice exposed to pressure overload (Fig. 1a). In our model, transverse aorta constriction (TAC)\cite{12,13} in 8-week-old C57BL/6 mice induced cardiac hypertrophy at 1–2 weeks after the operation and heart failure at 4–8 weeks (Fig. 1a and Supplementary Fig. 1a, b). Pressure overload increased the cellular width-to-length ratio in early cardiac hypertrophy, which is a morphological feature of cardiomyocytes of the concentric hypertrophic heart\cite{14} (Fig. 1b). After establishing the single-cardiomyocyte transcriptome analysis pipeline (Supplementary Fig. 1c, d), we isolated cardiomyocytes from the left ventricular free wall after sham operation and at 3 days and 1, 2, 4, and 8 weeks after TAC using Langendorff perfusion (Fig. 1a) and prepared 540 single-cardiomyocyte cDNA libraries with the SMART-seq2 protocol\cite{15}. After removing 58 libraries showing incomplete reverse transcription (Supplementary Fig. 1e–g), we sequenced 482 libraries and obtained 396 single-cardiomyocyte transcriptomes (sham, 64 cardiomyocytes; day 3, 58; week 1, 82; week 2, 61; week 4, 73; and week 8, 58) in which >5000 genes were detected (RPKM > 0.1; Supplementary Fig. 1h–n and Supplementary Data 1 and 2). Averaged single-cell expression profiles were tightly correlated with the corresponding bulk expression profiles (Supplementary Fig. 1o, p). Averaged single-cell profiles were also highly correlated between biological replicates (Supplementary Fig. 1q, r), whereas expression profiles substantially differed between individual cells even in the same heart (Supplementary Fig. 1s).

Hierarchical clustering revealed that cardiomyocytes from sham and TAC mice were well clustered (Supplementary Fig. 2a). Principal component analysis showed that cardiomyocytes from different batches at the same time points were located close to each other (Supplementary Fig. 2b). Principal component 1 (PC1) values were correlated with the number of expressed genes and cells with high PC1 values were enriched for cardiomyocytes from mice at 3 days and 1 week after TAC operation (Supplementary Fig. 2c). Differential expression analysis revealed that genes encoding proteins that function in the mitochondria, ribosomes, endoplasmic reticulum, and cytoskeleton were highly expressed at 3 days and 1 week after TAC operation (Supplementary Fig. 3a).

We consider that the increase of the number of detected genes at the early stage after pressure overload might reflect the expression of these stress response genes and/or an enlargement of cell size after pressure overload.

Hierarchical clustering also revealed that some genes thought to be specifically expressed in endothelial cells (e.g., Cav1 and Pecam1) or fibroblasts (e.g., Dcn and Lum) were clustered together with genes essential for transcription; Cav1 and Pecam1 were in G7 and Dcn and Lum were in G8 (Supplementary Fig. 2a). We used single-molecule RNA in situ hybridization (smFISH)\cite{16} to confirm that these genes are expressed in sham and TAC cardiomyocytes (Supplementary Fig. 4). A previous study of single-nucleus RNA-seq of cardiomyocytes also mentioned the presence of cardiomyocytes expressing endothelial marker genes\cite{9}, consistent with our findings. Therefore, we did not eliminate cardiomyocytes expressing these genes from the downstream analysis.

Cell-to-cell transcriptional heterogeneity increased after pressure overload (Fig. 1c). The expression levels of certain genes in the heart failure stage varied considerably (e.g., Nppa, Myh7, and Xirp2; Supplementary Fig. 5a, b). We used smFISH to quantitate single-cell mRNA levels of Myh7 (high variability) and Atp2a2 (low variability), confirming gene expression distributions similar to those obtained from single-cardiomyocyte RNA-seq analysis (Supplementary Fig. 5c–e and Supplementary Data 3).

Network analysis of single-cardiomyocyte transcriptomes.

We applied weighted gene co-expression network analysis (WGCNA)\cite{10} to all single-cardiomyocyte transcriptomes to identify 55 co-expression gene modules (Supplementary Fig. 6a and Supplementary Data 4) and calculated module eigengene expression profiles, which are the first principal components of a given module, across all cardiomyocytes. Unsupervised hierarchical clustering of all modules divided cardiomyocytes into 10 clusters (Supplementary Fig. 6b), but classification accuracy was not high (Supplementary Fig. 6c). To construct a better model, we used the Random Forests machine learning algorithm\cite{17} to identify nine modules significant for cell classification (Supplementary Fig. 6d). These modules were active in many cardiomyocytes, whereas the other modules were active in a few cardiomyocytes (Supplementary Fig. 6b). Through the combination of hierarchical clustering and Random Forests analyses, we classified
cardiomyocytes into seven clusters (Fig. 1d), resulting in highly accurate classification (Supplementary Figs. 6c and 7). We calculated the proportions of clustered cells to reveal cell-type heterogeneity at each time point (Fig. 1e). Gene ontology (GO) analysis showed that each module was enriched for genes involved in specific functions, such as translation, metabolism, heart contraction, and cell junction (Fig. 1f). Therefore, we named each module according to its most characteristic terms (Fig. 1a).

Co-expression gene network analysis revealed a structure highly clustered with these modules (Fig. 1a). Among the nine
modules, M1, M2, and M3 were especially well clustered. We conducted subnetwork analysis to identify the hub genes in each module: Mrpl42, Cfl2, and Timm17a for M1 (mitochondrial ribosome and oxidative phosphorylation), Pdlim5 and Eif5a for M2 (small GTPase signal), and Atp2a2 for M3 (heart contraction) (Supplementary Fig. 8a). Expression of these hub genes was critically correlated with that of the corresponding modules (Supplementary Fig. 8b), indicating that hub gene expression reflects the corresponding module activity.

We used t-distributed stochastic neighbor embedding (t-SNE) dimensionality reduction18 to visualize the relationships among cells (Fig. 1g and Supplementary Fig. 9a). Cardiomyocytes assigned to the same clusters and those isolated at the same time points were located close to each other. Their transcriptomic profiles changed drastically after TAC operation, and returned to the baseline state slowly thereafter, but represented a state distinct from that of sham cardiomyocytes in the heart failure stage. By plotting module expression on the t-SNE map, we found modules expressed in specific cell clusters (Supplementary Fig. 9b). Consistent with this result, differential expression analysis revealed that Atp2a2, an M3 hub gene, was highly expressed in C6 and C7, whereas Mrpl42, an M1 hub gene, was not expressed specifically in C6 or C7 (Supplementary Fig. 3b). Module-to-module correlation analysis showed a negative correlation between M1 and M3 expressions (Supplementary Fig. 10a, b), These results suggest that the balanced expression of mitochondrial translation/metabolism genes and heart contraction genes is an innate feature of cardiomyocytes. We also found M24 specific expression in C7 cells (Fig. 1g and Supplementary Fig. 9b), most of which were cardiomyocytes at the heart failure stage (Fig. 1e), suggesting that M24 is characteristic of failing cardiomyocytes.

Trajectory of cardiomyocyte remodeling. We next used Monocle19,20 to reconstruct the branched trajectory of cardiomyocyte remodeling after pressure overload, which consisted of three cell states and one branch point (Fig. 2a). Cardiomyocytes of the early stage after TAC operation (D3 and W1) mainly belonged to State 1 (Fig. 2b), suggesting that cardiomyocytes enter into State 1 immediately after TAC operation and were bifurcated into distinct cell fates (States 2 and 3) after the branch point. This bifurcation is considered to be related to the increased cell-to-cell heterogeneity (Fig. 1c). Almost all C7 cells were contained in State 3, suggesting that bifurcation into State 3 corresponds to the induction of failing cardiomyocytes.

Pseudo-time analysis revealed the transcriptional dynamics during the trajectory of cardiomyocyte remodeling (Fig. 2c, d). M2 (small GTPase signaling) and M22 (stress fiber formation) were specifically activated during the short period immediately after pressure overload. Module-to-module correlation analysis validated a strong correlation between M2 and M22 (Supplementary Fig. 10a, b). In addition, M1, M11, and M12 were activated, whereas M3 and M5 were repressed after pressure overload (Fig. 2c). The strong correlation between M1 (mitochondrial ribosome and oxidative phosphorylation) and M11 (ribosome and translation) (Supplementary Fig. 10a, b) suggests the synchronized activation of nuclear and mitochondrial translation after pressure overload.

Cardiomyocytes near the branch point were mainly from mice at 2 or more weeks after TAC operation (Fig. 2a), suggesting that the bifurcation occurs in the late stage of hypertrophy. Module expression dynamics along the pseudo-time line (Fig. 2c) showed that the induction of State 2 (cardiomyocytes that escaped from the induction into failing cardiomyocytes) is characteristic for the activation of M5 (tricarboxylic acid cycle and fatty acid degradation). Conversely, the induction of State 3 (failing cardiomyocytes) is characteristic for the activation of M3 (heart contraction) and M24 (actin-binding and contractile fiber) and repression of M1 (mitochondrial ribosome and oxidative phosphorylation). Differential expression analysis with pseudo-time analysis confirmed that M3 genes (e.g., Atp2a2, Myl2, and Myl3) and M24 genes (e.g., Ttn, Myom1, and Alpk3) were activated, whereas M1 genes (e.g., Mrpl42, Cfl2, and Timm17a) were repressed in the induction of State 3 (Fig. 2d). M1 activation and M3 repression, which were observed in State 1, were abolished, suggesting the disruption of adaptive hypertrophy responses at the induction of failing cardiomyocytes. We used smFISH with an mRNA probe for Atp2a2 (M3 hub gene) to validate the temporal dynamics of M3 activity (Supplementary Fig. 10c and Supplementary Data 5). M24 (actin-binding and contractile fiber) was activated specifically in State 3, suggesting the transcriptional signature of failing cardiomyocytes.

Modules and regulators of cardiomyocyte hypertrophy. We next linked the morphological and molecular heterogeneities in cardiomyocyte hypertrophy. To identify the gene modules involved in cardiomyocyte hypertrophy, we again isolated cardiomyocytes from mice at 1 week after TAC (early stage of cardiac hypertrophy), measured their area, obtained their transcriptomes, and integrated them (Fig. 3a and Supplementary Fig. 11a–e). Principal component analysis of their transcriptomes placed cardiomyocytes (Fig. 3b) and revealed that PCI1 was positively correlated with cell area and M1 (mitochondrial ribosome and oxidative phosphorylation) and negatively correlated with M3 (heart contraction) (Fig. 3b–e). Correlation analysis between module expression and cell area showed that M1 and M5 (tricarboxylic acid cycle and fatty acid degradation) were strongly correlated with cardiomyocyte area (Fig. 3f). The expression of M1 hub genes (Mrpl42, Timm17a, and Cfl2; Fig. 3g) was also positively correlated with cardiomyocyte area (Fig. 3h and Supplementary Data 6). GO analysis showed the significant enrichment of mitochondrial genes among the genes correlated with cell area (Fig. 3i and Supplementary Fig. 11f). These results indicate
**Fig. 2** Single-cardiomyocyte trajectory analysis. 

**a** Single-cardiomyocyte trajectory after TAC operation reconstructed by Monocle. Cells are colored by the time when cardiomyocytes were extracted (left), according to the cell clusters (middle), and by the cell states annotated by Monocle (right).  

**b** Bar plot showing the distribution of the cell states (State 1, State 2, and State 3) at each time point (left) and in each cell cluster (right).  

**c** Module expression dynamics along the pseudo-time reconstructed by Monocle. Dot colors indicate state colors, matching the colors in **b**. Single-cell expression levels of each module in sham cardiomyocytes are represented as black dots (left). The fitted curves for the trajectory into State 2 (green) and State 3 (blue), and the number of genes assigned to the corresponding modules are also shown.  

**d** Heatmap showing the expression levels of differentially expressed genes during the trajectory. Corresponding modules are also denoted in parentheses.
**Fig. 3** Hypertrophy-related modules and regulatory factors. 

**a** Experimental scheme to identify hypertrophy-related modules and regulators. 

**b** Principal component analysis (PCA) plot of cardiomyocytes from mice at 1 week after TAC. Arrows denote the correlation coefficients of the respective module with each principal component. 

**c** Correlation coefficient with cell area and proportion of variance for each principal component. 

**d, e** PCA plots colored by the cell area (**d**) and by the expression of each module (**e**). 

**f** Bar plot showing the correlation coefficient between cell area and module expression. Modules are ordered by the correlation levels. 

**g** Hub gene network of M1. The size of the dots represents node centrality. 

**h** Bar plot showing the correlation coefficient between cell area and gene expression. Genes whose expression was detected in at least one of the samples are ordered by correlation levels. 

**i** List of the most enriched GO terms in the top 300 correlated genes with cell area. 

**j** Representative genome browser views of H3K27ac ChIP-seq of cardiomyocytes from mice at 1 week after TAC. The Y-axis indicates reads per million (range, 0–3). 

**k** Transcription factor recognition motifs most significantly enriched in the regulatory elements (REs) for each module. 

**l** Hierarchical clustering of transcription factor recognition motifs. The top 10 significantly enriched motifs for each module are selected. Transcription factors strongly expressed in cardiomyocytes are shown below the heatmap.
that the expression of mitochondrial ribosome and oxidative phosphorylation genes is closely linked to the extent of cell hypertrophy. Mitochondrial biogenesis increases during cardiac hypertrophy\(^{24}\) and cell size correlates with the number of mitochondria\(^{22,23}\). The transcription and translation of nuclear genes involved in mitochondrial oxidative phosphorylation are induced rapidly in response to external stimuli during mitochondrial biogenesis\(^{24}\), suggesting that pressure overload-induced M1 activation might participate in mitochondrial biogenesis to match the increased energy demand during cardiac hypertrophy.

To uncover the regulatory mechanisms regulating cardiomyocyte hypertrophy, we performed chromatin immunoprecipitation followed by sequencing (ChIP-seq) for histone H3 acetylated lysine 27 (H3K27ac) modification\(^{25}\) in cardiomyocytes from mice at 1 week after TAC (Fig. 3a, j). We divided H3K27ac-marked elements into putative regulatory elements for each module and evaluated the enrichment of transcription factor recognition motifs to infer the regulatory factors for each module (Fig. 3k).

Furthermore, the clustering of motif enrichment for the top 10 enriched transcription factors of each module revealed specific combination patterns of transcription factors and their regulating modules (Fig. 3l) and Supplementary Fig. 12). Recognition motifs of ETS domain-containing protein (ELK1) and nuclear respiratory factors 1 and 2 (NRF1/2) were specifically enriched around M1 genes. Extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) signaling induces cardiocyte hypertrophy\(^{26}\) via ELK1 phosphorylation\(^{27}\) and NRF1/2 signaling enhances mitochondrial biogenesis\(^{28,29}\). Collectively, the combination of single-cardiomyocyte RNA-seq and H3K27ac ChIP-seq suggests that the ERK1/2 and NRF1/2 signaling pathways coordinately regulate the mitochondrial ribosome and oxidative phosphorylation module, which is critical for cardiomyocyte hypertrophy.

**Signaling pathway inducing heart failure.** We investigated how cardiomyocytes in the adaptive hypertrophy stage become failing cardiomyocytes. The characteristic module dynamics, such as M1 repression and M3 activation, were observed from the later stage of adaptive hypertrophy (Fig. 2). Heart failure stage-specific cells (C7 cells) began to appear from this stage (Fig. 1e). Thus, we speculated that the activation of some signaling pathway(s) in hypertrophy-stage cardiomyocytes might participate in the transition from hypertrophy to failing cardiomyocytes and the development of heart failure. Therefore, we sought to identify the hypertrophy stage-specific modules activated in even a small population of cardiomyocytes among all modules. If there are hypertrophy stage-specific networks, network analysis using single-cell transcriptomes of all cardiomyocytes except hypertrophy-stage cardiomyocytes cannot identify these network modules. This idea is based on the concept for identifying sample-specific networks\(^{30,31}\). Therefore, we separately performed WGCNA on all cardiomyocytes and on those except hypertrophy-stage cardiomyocytes, and assessed the significance of the overlap between gene modules. We identified 12 hypertrophy stage-specific modules (Fig. 4a and Supplementary Fig. 13a), all of which were active in only a few cardiomyocytes (Supplementary Fig. 6b). Pathway enrichment analysis of these 12 modules revealed that genes involved in the cell cycle and p53 signaling pathways were enriched in M7 (Fig. 4b). smFISH showed that mRNA of Cdkn1a, an M7 network component (Fig. 4c), was specifically highly expressed in certain hypertrophy-stage cardiomyocytes (Fig. 4d, e and Supplementary Data 7). GO analysis revealed that M7 was also enriched for genes involved in the DNA damage response (Fig. 4f). Immunostaining of p21 (encoded by Cdkn1a) and phospho-histone H2AX (DNA damage response marker; \(\gamma H2AX\))\(^{32}\) demonstrated that p21 was specifically detected in hypertrophy-stage cardiomyocytes and that p21-positive cardiomyocytes were also \(\gamma H2AX\)-positive (Fig. 4g and Supplementary Fig. 13b, c). Thus, our unbiased single-cell network analysis uncovered DNA damage-induced p53 signaling activation in hypertrophy-stage cardiomyocytes.

Since the accumulation of DNA damage induced by oxidative stress promotes heart failure progression\(^{33–35}\), we hypothesized that the oxidative stress response might be activated at the hypertrophy stage and be associated with morphological hypertrophy. GO analysis showed that M1 and M5 were specifically enriched for genes involved in the oxidative stress response (Supplementary Fig. 13d). Among these genes, peroxiredoxins, oxidative stress biomarkers\(^{36}\), were critically correlated with cell area during the early stage of hypertrophy (Supplementary Fig. 13e). These results suggest that morphological hypertrophy after pressure overload is involved in both enhanced mitochondrial biogenesis for increased energy demand and excessive oxidative stress leading to the accumulation of DNA damage.

DNA damage accumulation, observed in patients with heart failure\(^{35}\), has been reported as a cause of heart failure progression\(^{34}\). Although p53 has been reported to be detrimental for the heart\(^{12,37}\), its role in cardiomyocyte remodeling during heart failure remains elusive.

**p53-induced molecular and morphological remodeling.** To assess whether p53 activation in cardiomyocytes is critical for the transition to failing cardiomyocytes and development of heart failure, we generated cardiomyocyte-specific p53 knockout (p53CKO) mice (Supplementary Fig. 13f). Baseline features did not differ significantly between p53\(^{flx/flox}\) and p53CKO mice (Fig. 5a, b and Supplementary Data 8). Echocardiography demonstrated that p53\(^{flx/flox}\) and p53CKO mice showed similar cardiac hypertrophy at 2 weeks after pressure overload (Fig. 5a, b and Supplementary Data 8). However, at 4 weeks after pressure overload, cardiac function was impaired in p53\(^{flx/flox}\), but not in p53CKO mice (Fig. 5a, b and Supplementary Data 8).

We assessed the detailed molecular mechanism of p53 in cardiomyocytes by single-cardiomyocyte transcriptome analysis of p53\(^{flx/flox}\) and p53CKO mice at 2 weeks after pressure overload, when the transition from hypertrophic to failing cardiomyocytes occurs (Figs. 1e and 2a). Module expression analysis revealed that p53 downregulated the mitochondrial ribosome and oxidative phosphorylation (M1 and M16) and small GTPase signaling (M2) modules, and upregulated the heart contraction (M3), cell junction (M12) and actin-binding and contractile fiber (M24) modules (Fig. 5c). We used hierarchical clustering and t-SNE to classify and organize these cardiomyocytes into transcriptionally distinct clusters (Fig. 5d, e), and identified differentially expressed modules (Fig. 5f). M1-low, M3-high, and M24-high cardiomyocytes (cluster A; Fig. 5d), which correspond to failing cardiomyocytes (C7 cells; Figs. 1d, e and 2c, d), appeared in p53\(^{flx/flox}\) mice, but rarely in p53CKO mice (Fig. 5d–g). Allocation of cardiomyocytes after pressure overload to predetermined cell clusters using the machine learning Random Forests algorithm\(^{17}\) indicated that cardiomyocyte remodeling progressed to the C5 and C7 stages in p53\(^{flx/flox}\) mice but stopped at the C3 stage in p53CKO mice (Figs. 1d, e and 5h; Fisher’s exact test, \(P < 0.05\)), suggesting that p53 activation is necessary for the induction of failing cardiomyocytes (C7 cells). Increased cell-to-cell transcriptional heterogeneity after pressure overload was diminished by p53 disruption (Fig. 5i). These results indicate that p53 in hypertrophy-stage cardiomyocytes is essential for molecular remodeling into failing cardiomyocytes by disrupting the
Fig. 4 Hypertrophy-stage-specific p53 signaling activation. a Bar plot showing the statistical significance of the overlap between modules detected from co-expression analysis with or without hypertrophy-stage cardiomyocytes. The most strongly overlapping module pairs are selected from Supplementary Fig. 13a. Modules are ordered by significance level. The red line indicates the threshold for hypertrophy stage-specific modules. b KEGG pathway enrichment of 12 hypertrophy stage-specific modules identified in a and Supplementary Fig. 13a. c Co-expression network analysis of M7. d Representative images of Cdkn1a mRNA smFISH in the heart from mice at 2 weeks after pressure overload. Wheat germ agglutinin (WGA) and DAPI are used as markers of the plasma membrane and nucleus, respectively. Scale bar, 20 μm. e Bar plots showing the distribution of cells corresponding to the single-cell fluorescent intensity of Cdkn1a mRNA detected by smFISH in the heart after sham and TAC (weeks 2 and 8) operation. f Heatmap showing the enrichment of GO and KEGG pathway terms in 12 hypertrophy stage-specific modules. g Immunohistochemical staining of gh2A.X and p21 in the heart of mice at 2 weeks after pressure overload. WGA and DAPI are used as markers of the plasma membrane and nucleus, respectively. Arrows indicate the nuclei of gh2A.X and p21 double-positive cardiomyocytes. Scale bar, 20 μm.
adaptive hypertrophy modules and activating the heart failure modules.

M24 is characteristic for failing cardiomyocytes, which are induced by p53 signaling activation. Co-expression network analysis identified *Ttn*, *Xirp2*, and *Kif5b* as central genes of M24 (Fig. 6a). Single-cardiomyocyte RNA-seq validated the heterogeneous downregulation of these genes in p53CKO cardiomyocytes (Fig. 6b). Epigenomic profiling of murine cardiomyocytes in the heart failure stage revealed the enrichment of binding motifs of myocyte enhancer factor-2 (MEF2) and nuclear factor-
p53 mediates cardiac remodeling through Nrf2 activation. Since public Nrf2 ChIP-seq data of macrophages was limited, we hypothesized that activated Nrf2 directly regulates Mef2 gene expression in cardiomyocytes. We conducted ChIP followed by quantitative PCR (ChIP-qPCR) of TAC cardiomyocytes to show the significant enrichment of Nrf2 at the Mef2a promoter (Fig. 6f and Supplementary Data 8), validating our hypothesis that the p53–Nrf2–Mef2a axis is essential for the induction of failing cardiomyocytes.

We investigated whether p53 regulates morphological remodeling into heart failure. Pressure overload initially induced cardiomyocyte hypertrophy (Fig. 1b), whereas sustained exposure caused cardiomyocyte elongation (Fig. 6g), consistent with the morphological characteristics of cardiomyocytes from patients with heart failure45. However, cardiomyocytes from p53CKO mice maintained the morphological characteristics of hypertrophy and did not show elongation even after chronic pressure overload (Fig. 6h). We also measured the cardiomyocyte cross-sectional area of the heart from p53<sup>fl/fl</sup> and p53CKO mice after sham and TAC operation (Supplementary Fig. 15 and Supplementary Data 9), and obtained results consistent with those of the echocardiographic (Fig. 5b) and single-cell cardiomyocyte morphological (Fig. 6h) assessments. We conclude that p53 activation in hypertrophy-stage cardiomyocytes is essential not only for molecular remodeling but also for morphological remodeling into failing cardiomyocytes.

**Distinct gene programs and their pathogenicity in human cardiomyocytes.** We analyzed the gene programs and their pathogenicity in human cardiomyocytes. We isolated cardiomyocytes from normal subjects and patients with dilated cardiomyopathy (DCM) (<i>n</i> = 10) and obtained 411 single-cardiomyocyte transcriptomes. Among the 17 modules detected by WGCNA (Supplementary Data 10), hierarchical clustering identified five essential modules shared by many cardiomyocytes (Supplementary Fig. 16a). Co-expression network analysis revealed the highly clustered structure of module genes (Fig. 7a) and GO analysis identified specific functions of genes in each module (Fig. 7b). t-SNE and hierarchical clustering analyses separated normal and DCM cardiomyocytes and distinguished five cell clusters (Fig. 7c and Supplementary Fig. 16b). Module expression was specific for cell clusters; M1 was specific for C4 and C5, and M2 was specific for C3 (Fig. 7d, e and Supplementary Fig. 16b). RNA in situ hybridization analysis confirmed the heterogeneous expression of MYL2 (M2; Fig. 7e and Supplementary Fig. 16c) in DCM cardiomyocytes (Supplementary Fig. 16d). These results uncover the transcriptional heterogeneity and distinct gene modules in human DCM cardiomyocytes.

**CDKN1A, which is a p53 target gene and belongs to M1 (Fig. 7e and Supplementary Fig. 16c), was highly expressed in a subpopulation of DCM cardiomyocytes (C4 and C5; Fig. 7c, f).** smFISH validated an increase of CDKN1A expression in some cardiomyocytes (Fig. 7g, h). Co-expression network analysis clarified that CDKN1A was strongly correlated with MT2A in the M1 network (Fig. 7i). MT2A was highly expressed in CDKN1A-positive cardiomyocytes (Fig. 7f). Metallothioneins, encoded by MT family genes, are activated by oxidative stress46 and exert cardioprotection47, suggesting that CDKN1A expression in human cardiomyocytes is induced by oxidative stress.

We also revealed that not only cardiomyocytes from normal subjects but also those from responders, who showed an improvement of heart function after mechanical unloading, showed low M1 and M2 expression (Fig. 7j and Supplementary Fig. 16e). Comparison between human and mouse cardiomyocyte modules confirmed that human M1 and M2 significantly overlapped with mouse M1, M2, M3, M5, M11, and M16 (Fig. 7k). These results demonstrate the conservation of the pathogenic gene programs between human and mouse cardiomyocytes.

**Discussion.** Having established a system for the comprehensive analysis of multilayer cardiomyocyte responses to hemodynamic overload in vivo at the single-cell level, we elucidated the trajectory of cardiomyocyte remodeling in response to pathological stimuli, distinguished gene modules for cardiomyocyte hypertrophy and failure, and revealed the coordinated molecular and morphological dynamics of cardiomyocytes leading to heart failure (Fig. 6i).

Upon pressure overload, cardiomyocytes activated the mitochondrial ribosome and oxidative phosphorylation module, the
activity of which is correlated with the extent of morphological hypertrophy and is linked to the ELK1 and NRF1/2 transcriptional networks. This suggests that ERK1/2-induced ELK1 phosphorylation\(^{26,27}\) and NRF1/2 activation\(^{28,29}\) cooperatively regulate mitochondrial biogenesis to generate more ATP, which is required for enhanced protein synthesis to adapt to the enlargement of cell size and for increased contractility to overcome pressure overload. We also revealed the association of morphological hypertrophy and the oxidative stress response, supporting the hypothesis that increased mitochondrial biogenesis is the source of oxidative stress\(^{35}\).

Sustained stimuli induce the accumulation of oxidative DNA damage, leading to p53 signaling activation during hypertrophy. Single-cell analysis of cardiomyocyte-specific knockout mice
Fig. 6 p53 induces molecular and morphological remodeling leading to heart failure. a Co-expression network analysis of M24. b Violin plots showing the expression of Tmn, Xip2r, and Klf5b in cardiomyocytes from p53lox/lox and p53CKO mice at 2 weeks after TAC (43 p53lox/lox and 34 p53CKO cardiomyocytes). c Representative genome browser views of H3K27ac ChiP-seq of cardiomyocytes at 8 weeks after TAC. The Y-axis indicates reads per million (range, 0–4). d The enriched transcription factor recognition motifs at the H3K27ac-positive regions (TAC W8 cardiomyocytes) around the M24 genes. e Western blot analysis of heart tissues using antibodies against Nrf2 and a positive control. Uncropped images of the blots are shown in Supplementary Fig. 14d. f ChiP-qPCR analysis of cardiomyocytes from mice at 8 weeks after TAC operation. Data are represented as mean and standard error of the mean (maximum values. *TAC W4* from three mice each). Horizontal lines indicate the medians. Boxes show the 25th–75th percentiles. Whiskers represent the minimum and maximum values. g Boxplots showing the distribution of morphological parameters of cardiomyocytes from mice after sham and TAC operation (*n* = 1243 [Sham], 1366 [TAC W2], 717 [TAC W4] from three mice each). Horizontal lines indicate the medians. Boxes show the 25th–75th percentiles. Whiskers represent the minimum and maximum values. h Model for the coordinated molecular and morphological dynamics of cardiomyocytes leading to heart failure.

provided strong evidence indicating that p53 in cardiomyocytes increases cell-to-cell transcriptional heterogeneity, induces morphological elongation, and drives pathogenic gene programs by disrupting the adaptive hypertrophy modules and activating the heart failure module, thereby elucidating how DNA damage accumulation leads to heart failure. Heterogeneous p53 activation in vivo may be related to recent live-cell imaging results showing stochastic p53 signaling activation for cell fate determination in vitro. Our single-cell analysis uncovered a small population of hypertrophy-stage cardiomyocytes with transient activation of p53 signaling. Heart failure stage-specific cells dominate in the failing heart, suggesting that p53 signaling might be activated in most cardiomyocytes to induce heart failure. This p53 signaling-induced cellular dysfunction might be associated with findings that p53 activation at the G2–M phase is necessary and sufficient for cellular senescence. The p53-induced heart failure gene program is orchestrated by MEF2 and Nrf2. We showed that p53 is necessary for Nrf2 activation in the heart failure stage, consistent with the previous finding of p21-mediated Nrf2 protection against Keap1-mediated ubiquitination. Barboxolone methyl, an antioxidant Nrf2 activator, increases the risk of heart failure, suggesting that the p53-induced gene program might cause heart failure.

Single-cell analysis of samples from DCM patients revealed distinct gene programs and their pathogenicity in human cardiomyocytes. *CDKN1A* and *MT2A* co-expression suggested the pathogenicity of oxidative stress. The conservation of pathogenic gene programs in mouse and human cardiomyocytes provides the potential for assessing and regulating cardiomyocyte remodeling in heart failure. Further studies are needed to uncover the relationships between module dynamics in mouse cardiomyocytes and the pathogenicity of human cardiomyocytes and to develop novel therapeutic approaches for preventing heart failure. Finally, we note that the integration of single-cell transcriptomes with other multidimensional data deepens our understanding of disease pathogenesis and may be applicable to other diseases and biological phenomena.

Methods

Data reporting. No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during the experiments and outcome assessments.

Mice. All animal experiments were approved by the Ethics Committee for Animal Experiments of the University of Tokyo (RAC150001) and Osaka University (22-056) and adhered strictly to the animal experiment guidelines. C57BL/6 mice were purchased from CLEA JAPAN and α-MHC-CreER mice were from Jackson Laboratory (stock#009074, stock name: STOCK Tg [Myh6-cre]1Jmk/J). Trp53lox/lox mice were obtained from Dr. Anton Berns (The Netherlands Cancer Institute, Amsterdam, Netherlands). All mice were maintained in specific pathogen-free conditions in the animal facilities of the University of Tokyo and Osaka University. Conditional deletion of *Trp53* in cardiomyocytes was achieved by crossing Trp53lox/lox homozygous mice with a MHC-CreER2 hemizygous mice. Trp53lox/lox; α-MHC-CreER2 (Trp53lox/lox-MHC-Cre*) mice were used as p53 cardiomyocyte-specific knockout (p53CKO) mice. Male mice at 8 weeks of age were used for all experiments. p21 knockout mice were obtained from Dr. Philip Leder (Department of Genetics, Harvard Medical School, Boston, MA). All mice were on a C57BL/6 background.

TAC, morphological assessment, and echocardiography. Mice (8 weeks old) underwent TAC to induce heart failure or sham operation [3]. The transverse aorta was constricted with a 7-0 silk suture paralleled with a 27-gauge blunt needle, which was removed after constriction. Sham-operated mice, which were used as controls, had undergone a similar surgical procedure without aortic constriction 2 weeks previously. Transthoracic echocardiography was performed on conscious mice with a Vevo 2100 Imaging System (VisualSonics). M-mode echocardiographic images were obtained from the longitudinal axis to measure left ventricular size and function. The morphological assessment in Figs. 1b and 6g, h was conducted using an Operetta high-content imaging system (Perkin Elmer).

Single-cell RNA-seq analysis of mouse cardiomyocytes. Cardiomyocytes were isolated using Langendorff perfusion from the left ventricular free wall after sham operation and at 3 days and 1, 2, 4, and 8 weeks after TAC. Echocardiography was used to assess whether the heart was appropriately exposed to pressure overload. Non-congested hearts were not appropriately exposed to pressure overload were excluded from single-cardiomyocyte RNA-seq analysis. The results of echocardiographic assessment of the mice analyzed in this study are shown in Supplementary Fig. 1a, b. Enzymatic dissociation using Langendorff perfusion was performed with 37 °C pre-warmed 35 mL enzyme solution (collagenase Type II 1 mg/mL [Worthington], protease type XIV 0.1 mg/mL [Sigma-Aldrich], NaCl 130 mM, KCl 0.5 mM, NaH2PO4 0.33 mM, d-glucose 22 mM, HEPES 25 mM, pH 7.4) at a rate of 3 mL/min. Enzymes were neutralized with fetal bovine serum (FBS) at a final concentration of 0.2%. Cell suspensions were filtered through a 100-μm nylon mesh cell chamber and centrifuged at 100 g for 2 min. The supernatant was discarded. To prevent hypercontraction, the cardiomyocytes were resuspended in medium (NaCl 130 mM, KCl 0.5 mM, MgCl2 0.5 mM, NaH2PO4 0.33 mM, d-glucose 22 mM, HEPES 25 mM, FBS 0.2%, pH 7.4) containing a low concentration of calcium (0.1 mM). Rod-shaped live cardiomyocytes (viability of cardiomyocytes was ≥80% for all time points) were collected immediately after isolation of two mice at each time point with 0.2–0.3 μl pipette (sample volume: 0.5 μl) and incubated in lysis buffer. Single-cell cDNA libraries were generated using the Smart-seq2 protocol and the efficiency of reverse transcription was assessed by examining the cycle threshold (Ct) values of control genes (*Tmnt2, Cox6a2*) from quantitative real-time polymerase chain reaction (qRT-PCR) using a CFX Real-Time PCR Detection System (Bio-Rad) and examining the distribution of the lengths of cDNA fragments using a LabChip GX (Perkin Elmer) and/or TapeStation 2200 (Agilent Technologies). The following primer sets were used for qRT-PCR: Tmnt2 mRNA forward, TCCTGGCAGA GAGGAGGAAG; Tmnt2 mRNA reverse, GGATGCGGAG GCCATTTCTTCAC; Cox6a2 mRNA forward, GCAGGGCGGAG GTTTGATAC. A Ct value of 25 was set as the threshold. The remaining libraries were subjected to paired-end 51-bp RNA sequencing on a HiSeq 2500 in rapid mode. Insert size was 345 ± 40 bp (average ± standard deviation). The remaining libraries were subjected to paired-end 51-bp RNA sequencing on a HiSeq 2500 in rapid mode. Insert size was 345 ± 40 bp (average ± standard deviation). The remaining libraries were subjected to paired-end 51-bp RNA sequencing on a HiSeq 2500 in rapid mode. Insert size was 345 ± 40 bp (average ± standard deviation).
mapped using Tophat57 with the parameters genes were detected with RPKM > 1 (Supplementary Fig. 1l, m). Reads were also the cardiomyocytes that had more than 5000 genes with RPKM > 0.1, over 4000 had more than 4000 genes with RPKM > 1 (Supplementary Fig. 1k). Among all of cardiomyocytes that had more than 5000 genes with RPKM > 0.1 and those that

### Table

| Category | Time | Modules |
|----------|------|---------|
| GO:0006413 | Translational initiation | M1 30.4 0.6 0.0 0.0 0.0 |
| GO:0015629 | Actin cytoskeleton | M2 0.0 0.0 0.0 0.0 0.05 0.6 |
| GO:0005913 | Cell-cell adherens junction | M3 28.7 0.2 0.0 1.6 1.4 |
| GO:0001558 | Regulation of cell growth | M7 0.50 0.04 0.40 0.00 0.0 |
| GO:0043202 | Lysosomal lumen | M10 0.0 0.0 0.0 0.0 0.05 0.6 |
| GO:0031012 | Extracellular matrix | C1 0.0 0.0 36.0 0.0 0.0 |
| GO:0004129 | Cytochrome-c oxidase activity | C2 0.0 10.1 0.0 0.0 0.0 |
| GO:0060048 | Cardiac muscle contraction | C3 0.0 13.4 0.0 0.0 0.0 |
| GO:0032981 | Mitochondrial respiratory chain complex I assembly | C4 0.7 29.9 0.0 0.0 0.0 |
| GO:0051301 | Cell division | C5 6.3 0.0 0.0 0.0 0.0 |
| GO:0060337 | Type I interferon signaling pathway | C6 16.2 0.0 0.0 0.0 0.0 |
| GO:0006457 | Protein folding | C7 12.5 0.0 0.0 0.0 0.0 |
| GO:0000502 | Proteasome complex | C8 17.7 0.0 0.0 0.0 0.0 |
| GO:0000398 | mRNA splicing, via spliceosome | C9 15.8 0.0 0.0 0.0 0.0 |

### Figure

The co-expression network of human cardiomyocytes that had more than 5000 genes with RPKM > 0.1 and those that had more than 4000 genes with RPKM > 1 (Supplementary Fig. 1k). Among all of the cardiomyocytes that had more than 5000 genes with RPKM > 0.1, over 4000 genes were detected with RPKM > 1 (Supplementary Fig. 1l, m). Reads were also mapped using Tophat57 with the parameters “-g 1 -p 8 mm9—no-coverage-search” and the calculated RPKM values and gene modules were compared with those obtained using Bowtie (Supplementary Figs. 1h, i, and n). Two thresholds were used for selecting the cells, and RPKM > 10 was used for selecting the genes. To assess the possibility of batch effects, we added ArrayControl RNA Spikes 1, 4, and 7.
7 (Ambion, cat. No. Am1780) to the lysis buffer at the pre-defined concentrations on two separate plates and reverse-transcribed cardiomycocyte RNA of wild-type mice, confirming a good correlation between RNA spike-in concentrations and their expected RPKM values (normalized by unique reads mapped to the nuclear genome) in both batches (Supplementary Fig. 1c). t-SNE analysis of single-cardio-mycocyte transcripts from normal C57BL/6 mice (RPKM values) in two different batches confirmed that cardiomycocytes could not be classified by batch (Supplementary Fig. 1d). On the basis of these findings, we confirmed that we did not need to control for batch effects and used RPKM normalization for quantitative gene expression analysis in this study.

To assess transcriptional heterogeneity between cells, all genes that were expressed at an RPKM value of ≥10 in at least 20% of the samples were considered. Pearson’s correlation coefficient was calculated using the Cor function in R. Violin plots were generated with the Violplot package in R.

For weighted co-expression network analysis, all genes expressed at an RPKM value of ≥10 in at least one of the samples (n = 11,479) were used to construct a signed weighted co-expression network (WGCNA) R package. The strength of the positive or negative correlation was analyzed with the “pixedSoftThreshold” function and was used to construct a signed network and calculate module eigengene expression using the “blockwiseModules” function. Modules with <30 genes were merged to their closest larger neighboring module. To visualize the weighted co-expression networks, Cytoscape (version 3.0.4) with the “prefuse force-directed layout” was used. Node centrality, defined as the sum of within-cluster connectivity measures, reflected node size. To calculate the module expression data shown in Figs. 3 and 5, the “moduleEigengenes” function was applied to all cardiomycocytes, including the 396 cardiomycocytes that had been used to obtain the co-expression network modules. Assessment of module overlap was performed using Fisher’s exact test with the WGCNA “overlapTable” function.

Cluster 3.0 (ref. 69) and JAVAD Treeview61 were used to perform hierarchical clustering with correlation distance and complete linkage. Minimum cluster size was set to 5% of all samples. To assess the accuracy of the classification, the “randomForest” package in R was used. After obtaining nine modules using the combination of random forest and hierarchical clustering, we performed the subsequent cell clustering analysis based on the PCl values of the nine modules to classify cardiomycocytes. This is because the combination of random forests and hierarchical clustering showed that the accuracy of classification using the PCl values of the nine modules was higher than that using the gene expression profiles of the nine modules (Supplementary Fig. 7a–c). To achieve accurate cell-type classification, in Fig. 1d, we compared the error rates for 4–10 clusters and found that they were maintained at a low level for 4–7 clusters, while a drastic increase of the error rate was observed for 8 clusters (Supplementary Fig. 7b). A comparison of the hierarchical clustering results using the gene expression profiles with those from the using PCl values showed that most of the cells classified into the same cell clusters were well clustered (Supplementary Fig. 7a).

To visualize cell-to-cell variations, the expression of modules identified to be significant for cell classification by the Random Forest algorithm31 were applied to the t-SNE algorithm (Fig. 1d) with the “Rtsne” package in R. Figs. 1g and 5c and Supplementary Fig. 9. Principal component analysis was performed using the “pcompr” function in R.

The Database for Annotation, Visualization, and Integrated Discovery (DAVID)148 was used for GO analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. For Figs. 1f and 4f, the most characteristic GO terms in annotation clusters that ranked in the top 3 in the “Functional Annotation Clustering” function with statistical significance (P < 0.05) were extracted for each module. Enrichment P-values of all extracted GO terms for each module were calculated in DAVID. Monocle252 was used for differential expression analysis (P < 0.05), trajectory analysis, and pseudo-time analysis. For the integrative analysis of single-cell morphology and transcriptomes shown in Fig. 3, images of 48 cardiomycocytes from two mice at 1 week after TAC were obtained using an inverted microscope with a digital camera (CKX31 and XZ-2; Olympus) soon after isolation by Langendorff perfusion. Cardiomycocytes were then incubated immediately in lysis buffer. Subsequent procedures for extracting single-cardio-mycocyte transcriptomes were the same as described above. After removing seven libraries showing incomplete reverse transcription, we sequenced 41 libraries and obtained 36 single-cardio-mycocyte transcriptomes that included over 4500 genes with RPKM > 0.1, which were used for integrative analysis of single-cell morphology and transcriptomes. Cardiomycocyte size was analyzed using NIH Image software (version 1.48)33.

To assess the possibility of morphological changes after isolation in Supplementary Fig. 11, images of single cardiomycocytes were obtained in the medium at 0, 1, 5, 30, and 60 min after isolation using a BX-7000 microscope (Keyence). The data for area, length, width, and length-to-width ratio were analyzed using NIH ImageJ software (version 1.48)33.

Cardiomycocytes from wild type and p53CKO mice (48 wild type and 48 p53CKO cardiomycocytes) were also isolated using Langendorff perfusion at 2 weeks after TAC (Fig. 4f) and two different batches were used and then incubated immediately in lysis buffer. The subsequent procedures for extracting single-cardio-mycocyte transcriptomes were the same as described above. To quantify Trp53 mRNA levels with single-cardio-mycocyte qRT-PCR, the following primer set was used: forward, GACGGGACAG CTTTGAGGTT; reverse, GACGGGACAG CTTTGAGGTT; Gcgr forward, GACGGGACAG CTTTGAGGTT; reverse, GACGGGACAG CTTTGAGGTT. Cells were then cultured for 2 days in a 5% CO2 incubator. After preparation, total RNA was extracted from each culture using TRIzol (Thermo Fisher Scientific) and then reverse-transcribed to cDNA using a PrimeScript Imiwase kit (TaKaRa). Real-time quantitative PCR was performed using SYBR Premix Ex Taq II (Takara). Specific PCR products were detected on agarose gel and analyzed through melting curve analysis. The relative mRNA levels were calculated using the ΔΔCt method. The fold-change in expression was defined as the fold change in the target gene compared with the housekeeping gene GAPDH. All experiments were performed in triplicate.

The Database for Annotation, Visualization, and Integrated Discovery (DAVID)148 was used for GO analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. For Figs. 1f and 4f, the most characteristic GO terms in annotation clusters that ranked in the top three in the “Functional Annotation Clustering” function with statistical significance (P < 0.05) were extracted for each module. Enrichment P-values of all extracted GO terms for each module were calculated in DAVID. Monocle252 was used for differential expression analysis (P < 0.05), trajectory analysis, and pseudo-time analysis. For the integrative analysis of single-cell morphology and transcriptomes shown in Fig. 3, images of 48 cardiomycocytes from two mice at 1 week after TAC were obtained using an inverted microscope with a digital camera (CKX31 and XZ-2; Olympus) soon after isolation by Langendorff perfusion. Cardiomycocytes were then incubated immediately in lysis buffer. Subsequent procedures for extracting single-cardio-mycocyte transcriptomes were the same as those described above. After removing seven libraries showing incomplete reverse transcription, we sequenced 41 libraries and obtained 36 single-cardio-mycocyte transcriptomes that included over 4500 genes with RPKM > 0.1, which were used for integrative analysis of single-cell morphology and transcriptomes. Cardiomycocyte size was analyzed using NIH ImageJ software (version 1.48)33.

To assess the possibility of morphological changes after isolation in Supplementary Fig. 11, images of single cardiomycocytes were obtained in the medium at 0, 1, 5, 30, and 60 min after isolation using a BX-7000 microscope (Keyence). The data for area, length, width, and length-to-width ratio were analyzed using NIH ImageJ software (version 1.48)33.

Cardiomycocytes from wild type and p53CKO mice (48 wild type and 48 p53CKO cardiomycocytes) were also isolated using Langendorff perfusion at 2 weeks after TAC (Fig. 4f) and two different batches were used and then incubated immediately in lysis buffer. The subsequent procedures for extracting single-cardio-mycocyte transcriptomes were the same as described above. To quantify Trp53 mRNA levels with single-cardio-mycocyte qRT-PCR, the following primer set was used: forward, GACGGGACAG CTTTGAGGTT; reverse, GACGGGACAG CTTTGAGGTT; Gcgr forward, GACGGGACAG CTTTGAGGTT; reverse, GACGGGACAG CTTTGAGGTT. Cells were then cultured for 2 days in a 5% CO2 incubator. After preparation, total RNA was extracted from each culture using TRIzol (Thermo Fisher Scientific) and then reverse-transcribed to cDNA using a PrimeScript Imiwase kit (TaKaRa). Real-time quantitative PCR was performed using SYBR Premix Ex Taq II (Takara). Specific PCR products were detected on agarose gel and analyzed through melting curve analysis. The relative mRNA levels were calculated using the ΔΔCt method. The fold-change in expression was defined as the fold change in the target gene compared with the housekeeping gene GAPDH. All experiments were performed in triplicate.
Cardiomyocyte cross-sectional area measurement was performed using a BZ analyzer (Keyence). Immunofluorescence staining, hearts were excised and embedded immediately in Tissue-Tek OCT containing 4% paraformaldehyde for 15 min at 4 °C. The sections were blocked in 5% phosphatase inhibitor cocktails. Lysates were centrifuged at 20,000 × g for 5 min. The sections were transferred onto nitrocellulose membranes. The membranes were blocked with 5% FBS in Tris-buffered saline plus 0.05% Tween and incubated overnight at 4 °C with the following antibodies overnight at 4 °C: mouse monoclonal anti-p21 (H-250, sc-7547; Santa Cruz Biotechnology) and rabbit polyclonal anti-γH2A.X (phospho S139) antibody (1:100; ab28939, Abcam). For γH2A.X staining, a secondary antibody (goat anti-rabbit IgG (H + L) conjugated to Alexa Fluor 488) was used. Alexa Fluor 488-conjugated WGA (1:100) was applied to visualize the plasma membrane. Sections were mounted with ProLong Gold Antifade with DAPI (Thermo Fisher Scientific). Images were obtained as Z stacks using an LSM 510 META confocal microscope (Zeiss) and/or In Cell Analyzer 6000 (GE Healthcare). Quantification of fluorescence intensity was performed using the In Cell Developer Toolbox (GE Healthcare). We defined individual cardiomyocytes using WGA staining, measured fluorescence intensity within each cardiomyocyte, and normalized this intensity by cell area, quantifying the single-cell fluorescence intensity per unit area.

Immunohistochemistry. Fresh sections (5 µm) were fixed in PBS containing 4% paraformaldehyde for 10 min at room temperature and rinsed three times with PBS for 5 min. The sections were blocked with the blocking reagent supplied in a MOM Kit (Vector Laboratories) containing 5% normal goat serum for 1 h at 4 °C and rinsed three times with PBS for 5 min. The sections were incubated with the following antibodies overnight at 4 °C: mouse monoclonal anti-p21 antibody conjugated to Alexa Fluor 647 (1:50; sc-6246 AF647; Santa Cruz Biotechnology) and rabbit polyclonal anti-γH2A.X (phospho S139) antibody (1:100; ab28939, Abcam). For γH2A.X staining, a secondary antibody (goat anti-rabbit IgG (H + L) conjugated to Alexa Fluor 546 (1:200; A11010; Thermo Fisher Scientific)) was used. Fresh sections (5 µm) were stained for WGA and incubated for 5 min at room temperature, with 10% NBF (10% formalin in PBS) for 30 min at 37 °C, washed in distilled water, placed in 0.2 N HCl for 10 min at 37 °C, washed in PBS, treated with 4 µg/mL proteinase K (Wako Pure Chemical Industries) in PBS for 10 min at 37 °C, washed in PBS, and then placed within a Coplin jar containing 1× G-Wash (Genostaff), equal to 1× SSC. Hybridization was performed with probes (250 ng/mL) in G-Hybo-L (Genostaff) for 16 h at 60 °C. After hybridization, the sections were washed in 1× G-Wash for 10 min at 60 °C and in 50% formamide in 1× G-Wash for 10 min at 60 °C. Then, the sections were washed twice in 1× G-Wash for 10 min at 60 °C, twice in 0.1× G-Wash for 10 min at 60 °C, and twice in TBT (0.1% Tween 20 in TBS) at room temperature. After treatment with 1× G-Block (Genostaff) for 15 min at room temperature, the sections were incubated with anti-DIG-AP conjugate (Roche Diagnostics) diluted 1:2000 with G-Block (Genostaff; 1×) at 40 °C in TBT for 1 h at room temperature. The sections were washed twice in TBT and then incubated in 100 µM NaCl, 50 mM MgCl2, 0.1% Tween 20, and 100 µM Tris-HCl (pH 9.5). Coloring reactions were performed with NBT/BCIP solution (Sigma-Aldrich) overnight and then washed in PBS. The sections were counterstained with Kernschtrot stain solution (Muto Pure Chemicals) and mounted with G-Mount (Genostaff).

Statistical analysis. Normality of data distribution were assessed with the Shapiro–Wilkinson test. Statistically significant differences between two groups for continuous variables were determined by a two-tailed unpaired t-test with an F-test to confirm the equality of variance. Statistically significant differences in allocated clusters or genes between groups were assessed with Fisher’s exact test. Significance was considered at P < 0.05 unless otherwise indicated.

Data availability. The data that support the findings of this study are available from the authors on reasonable request. The sequences of the primers used in this study is listed in Supplementary Data 11. Single-cardiomyocyte RNA-seq and cardiomyocyte ChIP-seq data in this study have been deposited in the Gene Expression Omnibus with the accession code GSE95143. Received: 4 November 2017 Accepted: 18 September 2018 Published online: 30 October 2018

References.
1. Komuro, I. & Yazaki, Y. Control of cardiac gene expression by mechanical stress. Am. Rev. Physiol. 55, 55–75 (1993).
2. Mann, D. L. & Felker, G. M. Heart Failure: A Companion to Braunwald's Heart Disease 3rd edn (Elsevier, Amsterdam, 2016).
3. Frey, N. & Olson, E. N. Cardiac hypertrophy: the good, the bad, and the ugly. Annu. Rev. Physiol. 65, 45–79 (2003).
4. Mudd, J. O. & Kass, D. A. Tackling heart failure in the twenty-first century. Nature 451, 919–928 (2008).
5. LaCount, D. J., Dominici, F., Costello, J. F., Segal, D. & Geiger, T. The cell-as-context view of aging. Science 345, 1542–1547 (2014).
6. Haque, Z. K. & Wang, D. Z. How cardiomyocytes sense pathophysiological stresses for cardiac remodeling. Cell Mol. Life Sci. 74, 983–1000 (2017).
7. Anand, P. et al. BET bromodomains mediate transcriptional pause release in heart failure. Cell 154, 569–582 (2013).
8. Bahar, R. et al. Increased cell-to-cell variation in gene expression in ageing mouse heart. Nature 451, 1011–1016 (2008).
9. Langfelder, P. & Horvath, S. WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics 9, 559 (2008).
10. Parikshak, N. N., Gandal, M. J. & Geschwind, D. H. Systems biology and gene regulatory networks. Preprint at https://arxiv.org/abs/1505.06440 (2015).
11. Frey, N. & Olson, E. N. Cardiac hypertrophy: the good, the bad, and the ugly. Annu. Rev. Physiol. 65, 45–79 (2003).
12. Liu, X., Wang, Y., Ji, H., Aihara, K. & Chen, L. Personalized characterization of mitochondrial transcriptome and Nrf2 transcription factor mediates stress-dependent cardiac remodeling in mice. J. Clin. Invest. 118, 124–132 (2008).
13. Tsuchiya, H., Kinugawa, S. & Matsushima, S. Oxidative stress and heart failure. Am. J. Physiol. Heart Circ. Physiol. 301, H2181–H2190 (2011).
14. Perkins, A., Nelson, K. J., Parsonage, D., Poole, L. B. & Karplus, P. A. Peroxiredoxins: guardians against oxidative stress and modulators of peroxide signaling. Trends Biochem. Sci. 45, 435–445 (2010).
15. Mak, T. W., Haack, L., Grothe, D. & Billia, F. p53 regulates the cardiac transcriptome. Proc. Natl. Acad. Sci. USA 114, 2331–2336 (2017).
16. Elson, E. N. & Nordheim, A. Linking actin dynamics and gene transcription to drive cellular motile functions. Nat. Rev. Mol. Cell Biol. 11, 353–365 (2011).
17. Bartsch, S. et al. The MEF2D transcription factor mediates stress-dependent cardiac remodeling. Circulation 118, 124–132 (2008).
18. van Oort, R. J. et al. MEF2A activates a genetic program promoting cardiac dilatation and contractile dysfunction in calcineurin-inhibited heart failure. Circulation 125, 298–306 (2008).
19. Leung, L., Kwong, M., Hou, S., Lee, C. & Chan, J. Y. Deficiency of the Nrf1 and Nrf2 transcription factors results in early cardiomyopathic lesion and severe oxidative stress. J. Biol. Chem. 278, 48021–48029 (2003).
20. Ma, Q. Role of nrf2 in oxidative stress and toxicity. Annu. Rev. Pharmacol. Toxicol. 53, 401–426 (2013).
21. Qin, Q. et al. Nrf2-mediated cardiac maladaptive remodeling and dysfunction in a setting of autophagy insufficiency. Hypertension 67, 107–117 (2016).
22. Eichfeld, D. Z. et al. Tissue damage drives co-localization of Nf-κB, Smad3, and Nrf2 to direct Rev-erb sensitive wound repair in mouse macrophages. eLife 5, e13024 (2016).
23. Francis, G. S. Changing the remodeling process in heart failure: basic mechanisms and laboratory results. Curr. Opin. Cardiol. 13, 156–161 (1998).
24. Ruttay-Nedakey, B. et al. Essential cardiac myocyte transcriptomic signatures to DNA from a biophysical model. Nature 458 (2016).
25. Wang, F. et al. RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J. Mol. Diagn. 14, 22–29 (2012).
26. Bueno, O. F. & Molkentin, J. D. Involvement of extracellular signal-regulated de-differentiation and cell cycle stress-response in vivo. Nat. Commun. 8, 225 (2017).
27. Pettengill, K. A. et al. Cardiac remodeling in preload versus afterload. Circulation 122, 993–1003 (2011).
28. Russell, B., Motlagh, D. & Ashley, W. W. Form follows function: how muscle shape is regulated by work. J. Appl. Physiol. 88, 1127–1132 (2000).
29. Picelli, S. et al. Full-length RNA-seq from single cells using Smart-seq2. Nat. Protoc. 9, 171–181 (2014).
30. Wang, F. et al. RNA-Sequencing reveals a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J. Mol. Diagn. 12, 44–51 (2010).
31. Frey, N. & Olson, E. N. Cardiac hypertrophy: the good, the bad, and the ugly. Annu. Rev. Physiol. 65, 45–79 (2003).
32. Sano, M. et al. p53-induced inhibition of Hif-1 causes cardiac dysfunction during pressure overload. Nature 446, 444–448 (2007).
33. Di Modugno, A. et al. Cardiac hypertrophy in preload versus afterload. Circulation 122, 993–1003 (2011).
34. Van der Maaten, L. & Hinton, G. Visualizing data using t-SNE. J. Mach. Learn. Res. 9, 2579–2605 (2008).
35. Qiu, X. et al. Single-cell mRNA quantification and differential analysis with census. Nat. Methods 14, 309–315 (2017).
36. Qiu, X. et al. Reversed gene expression resolves complex single-cell trajectories. Nat. Methods 14, 979–982 (2017).
37. Dai, D. F. et al. Mitochondrial oxidative stress mediates angiotensin II-induced cardiac hypertrophy and Galphap overexpression-induced heart failure. Circ. Res. 108, 837–846 (2011).
38. Iannetti, E. F., Smetnick, J. A., Beyrah, J., Willems, P. H. & Koopman, W. J. Multiplexed high-content analysis of mitochondrial morphofunction using live-cell microscopy. Nat. Protoc. 11, 1693–1710 (2016).
39. Kitani, T. et al. A chemical screen probing the relationship between mitochondrial content and cell size. PLoS ONE 7, e33755 (2012).
40. Covellion, M. S., Soto, I. C., Shijkovska, G. & Churchman, L. S. Synchronized mitochondrial and cytosolic translation programs. Nature 533, 499–503 (2016).
41. Creyghton, M. P. et al. Histone H3K27ac separates active from poised enhancers and predicts developmental state. Proc. Natl. Acad. Sci. USA 107, 21931–21936 (2010).
42. Bueno, O. F. & Molkentin, J. D. Involvement of extracellular signal-regulated kinases 1/2 in cardiac hypertrophy and cell death. Circ. Res. 91, 776–781 (2002).
43. Babu, G. J., Lalli, M. J., Sussman, M. A., Sadoshima, J. & Periasamy, M. Phosphorylation of erk-1 by MEK/ERK pathway is necessary for c-fos gene activation during cardiac myocyte hypertrophy. J. Mol. Cell Cardiol. 32, 1447–1457 (2000).
44. Rosca, M. G., Tandler, B. & Hoppel, C. L. Mitochondria in cardiac hypertrophy and heart failure. J. Mol. Cell. Cardiol. 55, 31–41 (2013).
45. Wu, Z. et al. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 98, 115–124 (1999).
46. Kuijper, M. L., Tung, M., Yuan, G., Quackenbush, J. & Glass, K. Estimating sample-specific regulatory networks. Preprint at https://arxiv.org/abs/1505.06440 (2015).
47. Liu, X., Wang, Y., Ji, H., Ahara, K. & Chen, L. Personalized characterization of diseases using specific sample networks. Nucleic Acids Res. 44, e164 (2016).
48. Mih, L. J., El-Osta, A. & Karagianis, T. C. gammaH2AX: a sensitive molecular marker of DNA damage and repair. Leukemia 24, 679–686 (2010).
49. Canseco, D. C. et al. Human ventilatory unloading induces cardiomyocyte proliferation. J. Am. Coll. Cardiol. 65, 892–900 (2015).
50. Higo, T. et al. DNA single-strand break-induced DNA damage response causes heart failure. Nature 451, 543–547 (2009).
51. Tsutsui, H., Kinugawa, S. & Matsushima, S. Oxidative stress and heart failure. Am. J. Physiol. Heart Circ. Physiol. 301, H2181–H2190 (2011).
52. Perkins, A., Nelson, K. J., Parsonage, D., Poole, L. B. & Karplus, P. A. Peroxiredoxins: guardians against oxidative stress and modulators of peroxide signaling. Trends Biochem. Sci. 40, 435–445 (2015).
68. Matys, V. et al. TRANSFAC: transcriptional regulation, from patterns to profiles. *Nucleic Acids Res.* **31**, 374–378 (2003).
69. Mathelier, A. et al. JASPAR 2016: a major expansion and update of the open-access database of transcription factor binding profiles. *Nucleic Acids Res.* **44**, D110–D115 (2016).
70. Coppini, R. et al. Isolation and functional characterization of human ventricular cardiomyocytes from fresh surgical samples. *J. Vis. Exp.* **86**, e51116, https://doi.org/10.3791/51116 (2014).

**Acknowledgements**

We thank K. Shiina for next-generation sequencing support and Y. Yokota for experimental support. This work was supported by grants from the Japan Foundation for Applied Enzymology (to S.N.), the SENSIN Medical Research Foundation (to S.N.), the KANAE Foundation for the Promotion of Medical Science (to S.N.), MSD Life Science Foundation (to S.N.), The Tokyo Biomedical Research Foundation (to S.N.), Astellas Foundation for Research on Metabolic Disorders (to S.N.), The NOVARTIS Foundation (Japan) for the Promotion of Science (to S.N.), the Japanese Circulation Society (to S.N.), a Grant-in-Aid for Young Scientists (B) (to S.N.), a Grant-in-Aid for Research Activity Start-up (to M.S.), the Practical Research Project for Rare/Intractable Diseases from the Japanese Agency for Medical Research and Development (to H. Aburatani and I.K.), a Grant-in-Aid for Scientific Research (A) (to I.K.), AMED-PRIME, AMED (JP18gm6210010) (to S.N.), and AMED-CREST, AMED (JP18hm0804010, JP18gm0810013) (to H. Aburatani and I.K.).

**Author Contributions**

S.N., H. Aburatani, and I.K. conceived the project, designed the study, and interpreted the results; S.N. and M.S. collected single cells and generated the single-cell sequencing data; S.N. performed computational analyses; T.F. and H. Aburatani provided support for computational analyses; T.H. generated p53CKO mice, performed TAC, and conducted the functional analysis; T.S. and T.Y. performed TAC and conducted the functional analysis; S.N. and M.S. performed biochemical experiments and analyzed the data; T.K. supported the immunohistochemistry experiments; T.T., A.T.N., M.I., K.F., M.H., H.T., Y.K., K.L., E.T., H. Akazawa, H.M., H. Aburatani, and I.K. provided experimental and analytical support; S.N. and I.K. wrote the manuscript with feedback from all authors.

**Additional information**

**Supplementary Information**
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-06639-7.

**Competing interests:** The authors declare no competing interests.

**Reprints and permission information**
Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

**Publisher’s note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2018