HHS Public Access

Author manuscript

Sci Transl Med. Author manuscript; available in PMC 2021 May 19.

Published in final edited form as:

Sci Transl Med. 2019 October 09; 11(513): . doi:10.1126/scitranslmed.aaw6419.

Control of cytokinesis by β-adrenergic receptors indicates an approach for regulating cardiomyocyte endowment

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H.L., C.-H.Z., S.S., G.M.U., M.S., and B.K. developed the research strategy.

S.S., G.M.U., M.A., and S.W. developed assays.

H.L., C.-H.Z., S.S., G.M.U., M.A., N.A., B.G., L.H., K.R., N.C., C.L., S.W., Y.W., D.Y., and S.C. performed experiments.

S.S., J.S., and S.C. performed transcriptional analysis of single mouse cardiomyocytes, with direct input and supervision by J.H.E.

N.A. performed mouse surgery, stereology, and transcriptional analysis of single human cardiomyocytes.

M.K., J.K., A.B., D.K., J.D., Z.B.-J. performed bioinformatics analysis of single-cell transcriptomes.

K. L., K.F., N.A., and B.K. developed the approach for identification of human study subjects and ascertaining of myocardium.

K.L. and K.F. identified human subjects.

M.S. and M.V. assisted K.L. and K.F. in identification of human study subjects and assisted N.A. in ascertainment of human heart samples.

M.L.S. and B.K. designed the human labeling and MIMS approach; C.G. and M.L.S. performed MIMS analysis.

H.L., C.-H.Z., S.S., G.M.U., M.A., N.A., C.L., and B.K. wrote parts of the manuscript, which B.K. assembled, and all authors edited.

B.K. supervised and coordinated the entire study.

Competing interests

The authors declare that they have no competing interests.

Data availability

The datasets generated or analyzed for this study are available from the corresponding author upon request. The single-cell transcriptional profiling data have been deposited in NCBI's Gene Expression Omnibus (GEO) with the dataset identifiers GSE108359 and GSE56638.

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ABSTRACT/SUMMARY

One million patients with congenital heart disease (CHD) live in the US. They have a lifelong risk of developing heart failure. Current concepts do not sufficiently address mechanisms of heart failure development specifically for these patients. We show that cardiomyocyte cytokinesis failure is increased in tetralogy of Fallot with pulmonary stenosis (ToF/PS), a common form of CHD. Labeling of a ToF/PS baby with isotope-tagged thymidine showed cytokinesis failure after birth. We used single-cell transcriptional profiling to discover that the underlying mechanism is repression of the cytokinesis gene ECT2, and show that this is downstream of β-adrenergic receptors (β-AR). Inactivation of the β-AR genes and administration of the β-blocker propranolol increased cardiomyocyte division in neonatal mice, which increased the endowment and conferred benefit after myocardial infarction in adults. Propranolol enabled the division of ToF/PS cardiomyocytes. These results suggest that β-blockers should be evaluated for increasing cardiomyocyte division in patients with ToF/PS and other types of CHD.

INTRODUCTION

Congenital heart disease (CHD) is the most common birth defect. Improvements in diagnosis and treatment of CHD have increased survival, and 1 million patients live in the US with CHD[1]. Patients with CHD have a high lifetime risk for developing heart failure, and current thinking about this is based on research on heart failure in adult patients[1]. We have considered the possibility that CHD may alter cellular growth of the myocardium, i.e., cardiomyocyte proliferation and differentiation, because these mechanisms are active in infants and children without heart disease[2, 3].

Tetralogy of Fallot with pulmonary stenosis (ToF/PS) is a common form of CHD with relatively uniform structural defects (anterior deviation of the infundibulum, pulmonary stenosis, ventricular septal defect, and right ventricular hypertrophy). Despite extensive progress in understanding the genetic causes of CHD, the majority of ToF/PS remains genetically unexplained[4]. Infants and children with ToF/PS rarely have heart failure, but it is a well-documented cause of morbidity and mortality in adults[5–12]. Current thinking is that the sequelae of cardiac surgery cause an increased risk of heart failure. However, classical studies showed severe cardiomyocyte changes in ToF/PS patients prior to surgery[13, 14], but did not examine cardiomyocyte proliferation and differentiation. This suggested to us that myocardial changes happen before surgery. We have considered this possibility and found changes in cardiomyocyte proliferation and differentiation in ToF/PS.

Recent studies have shown that cardiomyocytes divide in human infants and children in contrast to the extremely low cardiomyocyte division rate in adults[2, 3, 15]. When cardiomyocytes stop proliferating, they undergo incomplete cell cycles, leading to binucleated cardiomyocytes. Although the mechanisms of formation of binucleated cardiomyocytes are unknown, it is thought that they do not divide further[16]. Mice and rats form binucleated cardiomyocytes in the first week after birth[17–19]. Zebrafish have only mononucleated cardiomyocytes, which can divide and regenerate myocardium[20], which has led to the hypothesis that a high percentage of mononucleated cardiomyocytes is the foundation for myocardial regeneration. However, humans have 70% mononucleated cardiomyocytes and yet do not regenerate myocardium. In addition, cardiomyocytes differentiate by endocycling, which increases the DNA content of nuclei without nuclear division, i.e., they become polyploid. Humans show a high degree of endocycling around 10 years after birth[2, 3, 15]. Two recent papers have altered the percentage of binucleated cardiomyocytes in mice and zebrafish, but this resulted in additional large changes of polyploid cardiomyocytes[21, 22]. Although multiple studies have suggested that cardiomyocytes become binucleated by incomplete cytokinesis[23–25], the precise mechanisms and relevance of cardiomyocyte binucleation are still unknown. During cytokinesis, a contractile ring forms at the future division plane[26]. Contraction of this ring is triggered by the cytokinesis protein ECT2, a RhoA guanine-nucleotide exchange factor. RhoA-GTP activates, via Rho-associated protein kinase (ROCK), non-muscle myosin II, which constricts the cleavage furrow.

Different pathways regulate cardiomyocyte proliferation, with the Hippo tumor suppressor pathway taking a central position[27]. The Hippo pathway is regulated by G protein-coupled

receptors (GPCR), and, in the heart, activated by β-adrenergic receptors (β-AR)[28]. β-AR regulate cardiomyocyte contractile function, by adjusting the intracellular second messenger cyclic adenosine monophosphate (cAMP)[29]. In CHD, and specifically in ToF/PS, β-AR signaling is overactivated[30–34]. Adrenergic signaling has also been evaluated in the context of heart regeneration in mice[35, 36] and cell cycle activity in cultured rat cardiomyocytes[37–39]; however, these results were obtained without genetic disruption of signaling pathways. Our results extend these findings by demonstrating a function of β-adrenergic receptors (β-AR) signaling in regulating cardiomyocyte cytokinesis *in vivo*.

Using formation of binucleated cardiomyocytes as read-out for the definitive endpoint of cell division, we discovered extensive changes of cardiomyocyte proliferation in ToF/PS. We identified the mechanisms of formation of binucleated cardiomyocytes, establishing a new connection between β-AR signaling and regulation of cardiomyocyte cytokinesis.

RESULTS

Infants with Tetralogy of Fallot with pulmonary stenosis have increased binucleated cardiomyocytes

We examined samples from the right ventricle of patients with ToF/PS and made the surprising observation that the percentage of binucleated cardiomyocytes was increased to 50–60% (Fig. 1A–C), suggesting extensively increased cytokinesis failure. The temporal pattern of this increase shows that babies with ToF/PS were born with the appropriate percentage of 20% binucleated cardiomyocytes[2, 3], but that the increase happened in the first 6 months after birth (Fig. 1B). All ToF/PS patients > 2 months had cardiomyocytes with > 2 nuclei, a very rare phenotype in humans without heart disease (Fig. 1C), suggesting that multiple serial cytokinesis failures occurred. Bi- and multi-nucleated cardiomyocytes were present in 6 and 13-year-old patients, i.e., after the decline of cardiomyocyte cell cycle activity to the very low levels present in adults. This shows that bi- and multi-nucleated cardiomyocytes generated in the first 6 months after birth live for at least one decade. To directly assess the generation of mono- and binucleated cardiomyocytes, we labeled a 1-month-old ToF/PS baby with ^{15}N-thymidine and examined uptake and retention with multiple-isotope imaging mass spectrometry (MIMS) at 7 months of age (Fig. 1D, E). ^{15}N-thymidine labeling was twice as high in the binucleated cells compared to mononucleated cardiomyocytes (Fig. 1F), indicating extensive cardiomyocyte cytokinesis failure corresponding to a 20–30% reduction of the number of cardiomyocytes (endowment). These findings motivated us to determine the mechanisms controlling cytokinesis in cardiomyocytes.

Cytokinesis failure in cardiomyocytes is associated with low levels of the Rho guanine nucleotide exchange factor Ect2

To determine the cellular mechanisms of cytokinesis failure in cardiomyocytes, we performed live cell imaging with neonatal rat ventricular cardiomyocytes that undergo binucleation (NRVM, Fig. 2A, Video S1). Cleavage furrow ingression was observed in 80% of the cardiomyocytes studied, followed by cleavage furrow regression. We used a transgenic mouse model expressing the fluorescent ubiquitination-based cell cycle indicator

(FUCCI) to highlight cell cycle progression [40], which showed normal cell cycle progression until cleavage furrow regression (Videos S2, S3). This finding demonstrates that failure of abscission generates binucleates from mononucleated cardiomyocytes.

To identify the molecular mechanisms of cleavage furrow regression, we separated cycling from non-cycling cardiomyocytes and took a single cell transcriptional profiling approach to compare the expressed genes (Fig. S1). We isolated embryonic (Embryonic day 14.5, E14.5) and neonatal (Postnatal day 5, P5) cardiomyocytes and identified cycling cardiomyocytes with the mAG-hGem reporter of the FUCCI indicator [40](Fig. S2). We performed deep, genome-wide, single-cell transcriptional analysis with the Eberwine method [41–43], followed by validation of the results (Fig. S3). Because RhoA activation is required for cleavage furrow constriction, we examined the expression of Dbl-homology Rho-Guanine Nucleotide Exchange Factors (GEF) in the single cell transcriptional dataset (Fig. 2B, Table S1). Ect2 mRNA was present in cycling E14.5 cardiomyocytes but not in binucleating P5 cardiomyocytes (Fig. 2B). Other genes controlling cytokinesis, *i.e.*, *Racgap1* (inactivating RhoA), *RhoA*, *Anillin*, *Aurkb,* and *Mklp1,* were present in P5 cycling cardiomyocytes (Fig. S4), indicating that *Ect2* is uniquely regulated. In accordance with the decreased Ect2 levels, active RhoA (RhoA-GTP) was decreased in binucleating cardiomyocytes (Fig. 2C). Taken together, these results show insufficient Ect2 levels in cardiomyocytes lead to less RhoA activation, weakening their cleavage furrows[26].

We tested whether increasing Ect2 expression enables cardiomyocyte abscission by expressing *GFP-Ect2* [44]. Live cell imaging showed the functionality of GFP-ECT2 in cardiomyocytes (Fig. 2D and Video S4) and increased cardiomyocyte abscission (Fig. 2E) without inducing apoptosis (Fig. S6). *GFP-Ect2* did not alter cardiomyocyte BrdU uptake (Fig. 2F) or H3P (Fig. 2G). In conclusion, increasing *Ect2* expression in cardiomyocytes has a specific effect on abscission without changing cell cycle entry or progression.

Lowering Ect2 expression reduces cardiomyocyte endowment and heart function

We next tested the hypothesis that lowering the expression of Ect2 induces cytokinesis failure *in vivo*. To this end, we inactivated the $Ect2^{flox}$ gene in mice with αMHC-*Cre* [45], (Fig. S6A). $\alpha MHC\text{-}Cre;\ Ect2^{flox/flox}$ mice showed a 3.2-fold increase of binucleated cardiomyocytes (23.3%, Fig. 2H), compared to $\alpha MHC\text{-}Cre;\ Ect2^{wt/flox}$ mice (7.4%, P<0.0001), at P1. *Ect2* inactivation did not change the DNA contents of nuclei (Fig. 2I). $\alpha MHC\text{-}Cre^{+};Ect2^{flox/flox}$ pups had 583,000 ± 15,379 cardiomyocytes (n=5 hearts) at P1, a 49% decrease compared to $\alpha MHC\text{-}Cre^{+};Ect2^{wt/flox}$ mice (1,140,833 ± 58,341 cardiomyocytes, n=12 hearts, P<0.0001, Fig. 2J). The mean cardiomyocyte size in $\alpha MHC\text{-}Cre;Ect2^{flox/flox}$ mice was increased by 65% (Fig. 2K). These results show that the lower endowment in $\alpha MHC\text{-}Cre;Ect2^{flox/flox}$ pups triggered cardiomyocyte hypertrophy, and not a compensatory increase in cell cycling. The heart weight was unchanged (Fig. 2L). Echocardiography showed that $\alpha MHC\text{-}Cre^{+};\ Ect2^{flox/flox}$ had a significantly decreased heart function, measured by ejection fraction (EF=49.6%), compared with control (EF=85.9%, Fig. 2M, Video S6 and S7). All the $\alpha MHC\text{-}Cre^{+};\ Ect2^{flox/flox}$ died before P2 (Fig. 2N, S6B). $Ect2^{flox}$ inactivation did not change cardiomyocyte M-phase activity, as measured by quantification of H3P-positive nuclei (Fig. 2O). We observed binucleated $Ect2^{flox/flox}$

cardiomyocytes with both nuclei being in M-phase, indicating that forcing cytokinesis failure does not prevent entry into another cell cycle and advancement to karyokinesis (Fig. 2O). This finding suggests a mechanism for how cardiomyocytes with four and more nuclei are generated, *i.e.*, by serial karyokinesis and failure of abscission. To determine whether Ect2 gene inactivation alters cardiomyocyte cell cycle entry, we inactivated an $Ect2^{flox46}$ gene with αMHC-MerCreMer[47] (tamoxifen P0, 1, 2) *in vivo*, thus circumventing the lethality of inactivating with αMHC-Cre. We isolated cardiomyocytes at P2 and cultured for 3 days in the presence of BrdU. Ect2 inactivation did not alter cell cycle entry (Fig. 2P) or cell viability (Fig. S9). In conclusion, Ect2 inactivation induced cytokinesis failure in cardiomyocytes, which decreased endowment by 50% and led to severely decreased ejection fraction and death.

ß-adrenergic receptors control cardiomyocyte abscission and endowment by regulating the Hippo tumor suppressor pathway and *Ect2*

We next sought to identify the mechanisms responsible for decreasing transcription of the *Ect2* gene. Previous publications suggested that the Hippo tumor suppressor pathway regulates cardiomyocyte proliferation [48–50]. YAP1, the central transcriptional co-regulator controlled by the Hippo pathway, forms a protein complex with TEAD transcription factors [49]. We identified five binding sites for the transcription factors TEAD 1 and 2 in the *Ect2* promoter (Fig. S7). Removing these TEAD-binding sites individually or *en bloc* decreased Ect2 promoter activity in Luciferase assays (Fig. 3A). siRNA knockdown of TEAD1/2 reduced Ect2 mRNA levels and increased the proportion of binucleated cardiomyocytes (Fig. 3B–C). Adenoviral-mediated overexpression of wild type YAP1 (YAP1-WT) and a non-degradable version (YAP1-S127A) in NRVMs increased Ect2 mRNA levels and reduced the proportion of binucleated cardiomyocytes (Fig. 3D–E). These results show that YAP1 and TEAD1/2 regulate the expression of Ect2 and cardiomyocyte abscission.

The Hippo pathway is activated by G protein coupled receptors (GPCR) *via* the stimulatory G protein, Gs [28]. Accordingly, we treated cultured NRVMs with forskolin, a mimic of active Gs [51], which decreased Ect2 mRNA levels (Fig. 3F). We administered forskolin in newborn mice and found a 37% increase in the proportion of binucleated cardiomyocytes after 4 days and a 21% increase after 8 days (Fig. 3G). Because β_1- and β_2-adrenergic receptors (β_1-, β_2-AR) are the major Gs-activating GPCR in cardiomyocytes, we examined β_1-$AR^{-/-}$; β_2-$AR^{-/-}$ (double-knockout, DKO, [52, 53] pups. These mice showed a lower proportion of binucleated cardiomyocytes (Fig. 3H) and a higher endowment at P4 (Fig. 3I). Their cardiomyocyte M-phase activity was not changed (Fig. 3J). β_1-$AR^{-/-}$; β_2-$AR^{-/-}$ DKO hearts showed increased transcription of the Hippo target genes *Cyr61* and *CTGF*, as well as *Ect2* (Fig. 3K). We then administered propranolol, a blocker of β_1- and β_2-AR, in newborn mice. Propranolol decreased the proportion of binucleated cardiomyocytes by 21% after treatment from P1 to P4, and by 17% after treatment from P1 to P8 (Fig. 3L). This was associated with a 22% and 30% increase of cardiomyocyte endowment at P4 and P8, respectively (Fig. 3M), without a change of cardiomyocyte M-phase (Fig. 3N) or heart weight (Fig. 3O). These results show that reducing β-adrenergic receptor signaling enables abscission, thus increasing the endowment.

Increasing cardiomyocyte endowment by administration of propranolol in neonatal mice improved heart function and reduced adverse remodeling due to myocardial infarction in adulthood

Although the propranolol-increased cardiomyocyte endowment did not alter cardiac function (Fig. 4A), a larger endowment should confer a benefit after large-scale cardiomyocyte loss, for example, after myocardial infarction (MI). We tested this by administering propranolol in the first week after birth and then inducing myocardial infarction in adult mice (Fig. 4B). We determined cardiac structure and function with MRI and histology (Fig. 4B). Twelve days after MI, mice with propranolol-induced endowment growth had an ejection fraction of 42%, compared with 18% in control mice (Fig. 4C). The thinned region of the LV myocardium after myocardial infarction was significantly smaller (Fig. 4D), and the relative systolic thickening was higher (Fig. 4E), indicating less adverse remodeling. Importantly, the region of myocardium affected by ischemia, visualized by late Gadolinium enhancement (Fig. 4F), and the scar size, determined by histology (Fig. 4G), were not different. Propranolol-treated hearts had a 30% higher cardiomyocyte endowment after MI (determined by stereology, Fig. 4H), in keeping with the increased endowment before MI. The heart weight was not changed (Fig. 4I), indicating that the higher endowment reduced the maladaptive hypertrophy, which drives adverse remodeling after MI. Taken together, these results demonstrate that rescuing cardiomyocyte cytokinesis failure with propranolol at the end of development reduces adverse ventricular remodeling in adult mice.

β-blockers rescue cytokinesis failure in cardiomyocytes from infants with ToF/PS

We determined if the molecular mechanisms of cardiomyocyte cytokinesis failure we discovered are responsible for the increased proportion of binucleated cardiomyocytes in ToF/PS. To this end, we transcriptionally profiled single cardiomyocytes from infants with ToF/PS. ToF/PS cardiomyocytes showed lower Ect2 mRNA levels compared with dividing human fetal cardiomyocytes (Fig. 5A). The frequency of Ect2-expressing cycling cardiomyocytes in ToF/PS infants (24.4%) was significantly lower than in human fetal cardiomyocytes (75.6%, Fig. 5B). Thus, cardiomyocytes in ToF/PS infants exhibit decreased Ect2 levels, similar to cardiomyocytes in neonatal mice (see Fig. 2B). This prompted us to examine the regulation by β-receptors. We used cultured human fetal cardiomyocytes and added forskolin to maximally increase cardiomyocyte cytokinesis failure (Fig. 5C). We then treated with dobutamine to mimic the *in vivo* microenvironment of increased β-AR stimulation, which increased binucleated cardiomyocytes to 95.2% of the forskolin-induced increase (Fig. 5C). Addition of propranolol blocked the dobutamine-stimulated increase of cardiomyocyte cytokinesis failure completely (Fig. 5C). We examined cardiomyocytes in cytokinesis by immunofluorescence microscopy, which showed that Ect2-positive midbodies were increased with propranolol (Fig. 5D). We then generated organotypic cultures of heart pieces from infants with ToF/PS and added BrdU to label cycling cardiomyocytes (Fig. 5E). Forskolin and dobutamine induced a maximal increase of binucleated cardiomyocytes, and propranolol inhibited the dobutamine-stimulated increase completely (Fig. 5E). In conclusion, β-receptors regulate cytokinesis failure in cardiomyocytes from infants with ToF/PS and propranolol decreases this effect.

DISCUSSION

Our results show that ToF/PS infants develop increased cardiomyocyte terminal differentiation, measured by increased formation of binucleated cardiomyocytes. This happens in the first 6 months after birth, which shows that these changes do not result from surgical or medical interventions. The changes persisted in older ToF/PS patients. The increased binucleation indicates a proportionate failure of cytokinesis, reducing cardiomyocyte proliferation by 25%. By identifying the upstream molecular regulators, we show that β-blockers could turn cytokinesis failure to increased division.

The results in mice demonstrate that promoting the progression of cytokinesis to abscission in the post-natal period increases the endowment, which improves remodeling due to myocardial infarction. This raises the possibility that a decreased or increased cardiomyocyte endowment connects to outcomes in human patients. This could be tested with β-blocker administration in human infants with CHD to increase the endowment, followed by measuring clinical outcomes, such as myocardial function and risk of heart failure development. β-blockers have been used acutely to treat and prevent cyanotic spells in ToF/PS[54]. Although this demonstrates that β-blockers are safe in this population, an effect on myocardial growth mechanisms was not evaluated. Our results also predict that administration of β-blockers should produce the largest effect on cardiomyocyte cytokinesis in the first 6 months after birth, which is in line with our previously demonstrated effectiveness of stimulating cardiomyocyte cell cycling in CHD cardiomyocytes in the same period[55]. The duration of this period should also be assessed in types of CHD other than ToF/PS.

Elucidating the mechanisms generating binucleated cardiomyocytes allows us to compare this process in other cell types. Formation of binucleated cells is also an early event in cancer formation, leading, *via* entrapment of lagging chromosomes in the cleavage furrow, to aneuploidy [56]. In this process, chromosome entrapment lowers Rho A activity in the cleavage furrow, leading to relaxation of the contractile ring and cytokinesis failure. However, we have no evidence for chromatin entrapment cardiomyocyte cytokinesis failure. In cancer cells, cytokinesis failure activates the Hippo tumor suppressor pathway[57]; however, we found that Hippo pathway activation triggers cytokinesis failure in cardiomyocytes. Cytokinesis failure is also a step in platelet formation, also induced by repression of Ect2 [58]. This suggests a generalizable molecular mechanism of Ect2 repression in cytokinesis failure in somatic cells.

Cardiomyocyte cell cycle withdrawal in mice happens in the first 3 weeks[59] and formation of binucleated cardiomyocytes in the first 2 weeks after birth[18, 19]. This is nearly coincident, which suggests that they could be mechanistically connected. However, we show that by increasing or decreasing cardiomyocyte binucleation, cardiomyocyte cell cycle entry does not change, thus demonstrating that formation of binucleated cardiomyocytes and cell cycle withdrawal are distinct molecular processes. This is supported by the increase of multinucleated cardiomyocytes (>2 nuclei) in ToF/PS, which shows that in ToF/PS, binucleated cardiomyocytes re-enter the cell cycle. This is consistent with the literature

demonstrating that pig cardiomyocytes have up to 16 nuclei[60], which shows that binucleated cardiomyocytes can re-enter the cell cycle multiple times.

Our results place the Hippo pathway downstream of β-AR signaling and upstream of *Ect2* gene regulation. This does not involve regulation of cell cycle entry and, as such, is distinct from direct genetic modulation of the central Hippo kinases and scaffolds, or regulation of the dystroglycan/agrin complex[27], which all have a significant effect on cell cycle entry. Because of the large number of different G protein coupled receptors (GPCR), it is possible that other GPCR may have a function in regulating cardiomyocyte cell cycle entry, progression, and division.

Low levels of cardiomyocyte proliferation in mammals continue to be a barrier for heart regeneration [27]. To overcome this, molecular interventions to stimulate cardiomyocyte cell cycle entry in adults have been proposed: increase of individual positive cell cycle regulators (cyclins A2 and D2, [61, 62] and combinations [63]), removal of negative cell cycle regulators (p53, pocket proteins, [64, 65]), and administration of mitogenic growth factors (FGF, NRG1, Oncostatin M, FSTL1, [55, 66–68]). All of these approaches involve oncogenes or tumor suppressors and are associated with the risk of inducing uncontrolled proliferation. The findings presented here indicate that targeting the final stage of the cell cycle, *i.e.*, abscission, is a viable strategy that could synergize with any of these interventions to increase cardiomyocyte generation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Tae Kyung Kim (University of Pennsylvania) for help with initial single cell amplifications. We thank Channing Der (University of North Carolina Chapel Hill) for providing $Ect2^{flox}$ mice and Mark Petroncski (Clare Hall Laboratories, London), Buzz Baum (University College London), and Toru Miki (Nagaoka University of Technology) for providing Ect2 expression constructs. We thank Maria Magaro (Harvard Medical School) for technical assistance with phenotyping of cardiomyocytes. We thank the patients and families for participating in this research and the operating room staff and cardiac surgeons for assistance with identifying study subjects and ascertaining samples. We thank Sean Lal and Cris dos Remedios (University of Sydney) and Charles McThiernan (University of Pittsburgh) for providing human myocardial samples. This project used the UPMC Hillman Cancer Center and Tissue and Research Pathology/Pitt Biospecimen Core shared resource which is supported in part by award P30CA047904. We thank members of the Kuhn laboratory for support, helpful discussions, and critical reading of the manuscript.

Funding

This research was supported by the Richard King Mellon Foundation Institute for Pediatric Research (UPMC Children's Hospital of Pittsburgh), by a Transatlantic Network of Excellence grant by Fondation Leducq (15CVD03), Children's Cardiomyopathy Foundation, and NIH grant R01HL106302 (to B.K.) and Health Research Formula Funds from the Commonwealth of Pennsylvania which had no role in study design or interpretation of data (to J.H.E.). This project was supported in part by UPMC Children's Hospital of Pittsburgh (to H.L.), Genomics Discovery Award (to B.K. and D.K.), and UPP Physicians (to B.K.).

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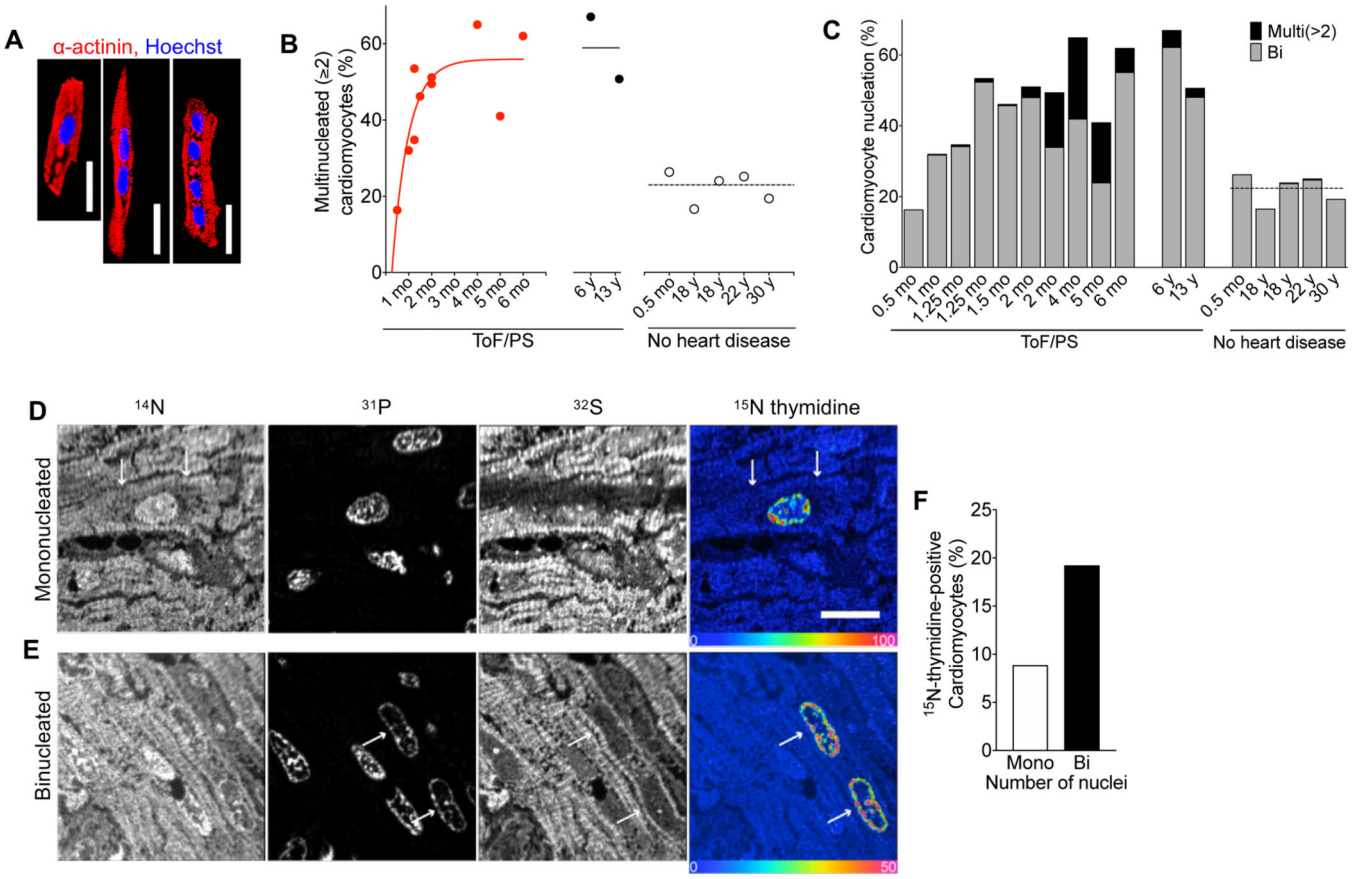

Figure 1. Cardiomyocytes in infants with Tetralogy of Fallot with pulmonary stenosis (ToF/PS) fail to divide.

(A-C) Patients with Tetralogy of Fallot and pulmonary stenosis (ToF/PS) show an increased proportion of multinucleated (≥2) cardiomyocytes (filled symbols and solid lines in **B)**. Each symbol in **(B)** and bar in **(C)** represents one human heart (ToF/PS: n = 12; No heart disease: n = 5). **(D-F)** A 4-week-old infant with ToF/PS was labeled with oral ^{15}N-thymidine. Myocardium was analyzed by multiple-isotope imaging mass spectrometry (MIMS) at 7 months. **(D-E)** ^{31}P reveals nuclei and ^{32}S morphologic detail, including striated sarcomeres. Nuclei are dark in the ^{32}S image. The ^{15}N/^{14}N ratio image reveals ^{15}N-thymidine incorporation. The blue end of the scale is set to natural abundance (no label uptake) and the upper bound of the rainbow scale is set to 50% above natural abundance. The white arrows in **(D)** indicate the boundaries of a labelled mononucleated cardiomyocyte and in **(E)** the nuclei of a binucleated cardiomyocyte. **(F)** The percentage of labeled binucleated cardiomyocytes is higher than mononucleated cardiomyocytes (Mononucleated cardiomyocytes analyzed: n = 282; 15N+ mononucleated cardiomyocytes: n = 25; Binucleated cardiomyocytes analyzed: n = 104 (208 total nuclei); 15N+ binucleated cardiomyocytes: n = 20 (40 total nuclei)), indicating that this patient experienced extensive cytokinesis failure. Scale bar: 20 μm **(A)**, 10 μm **(D)**.

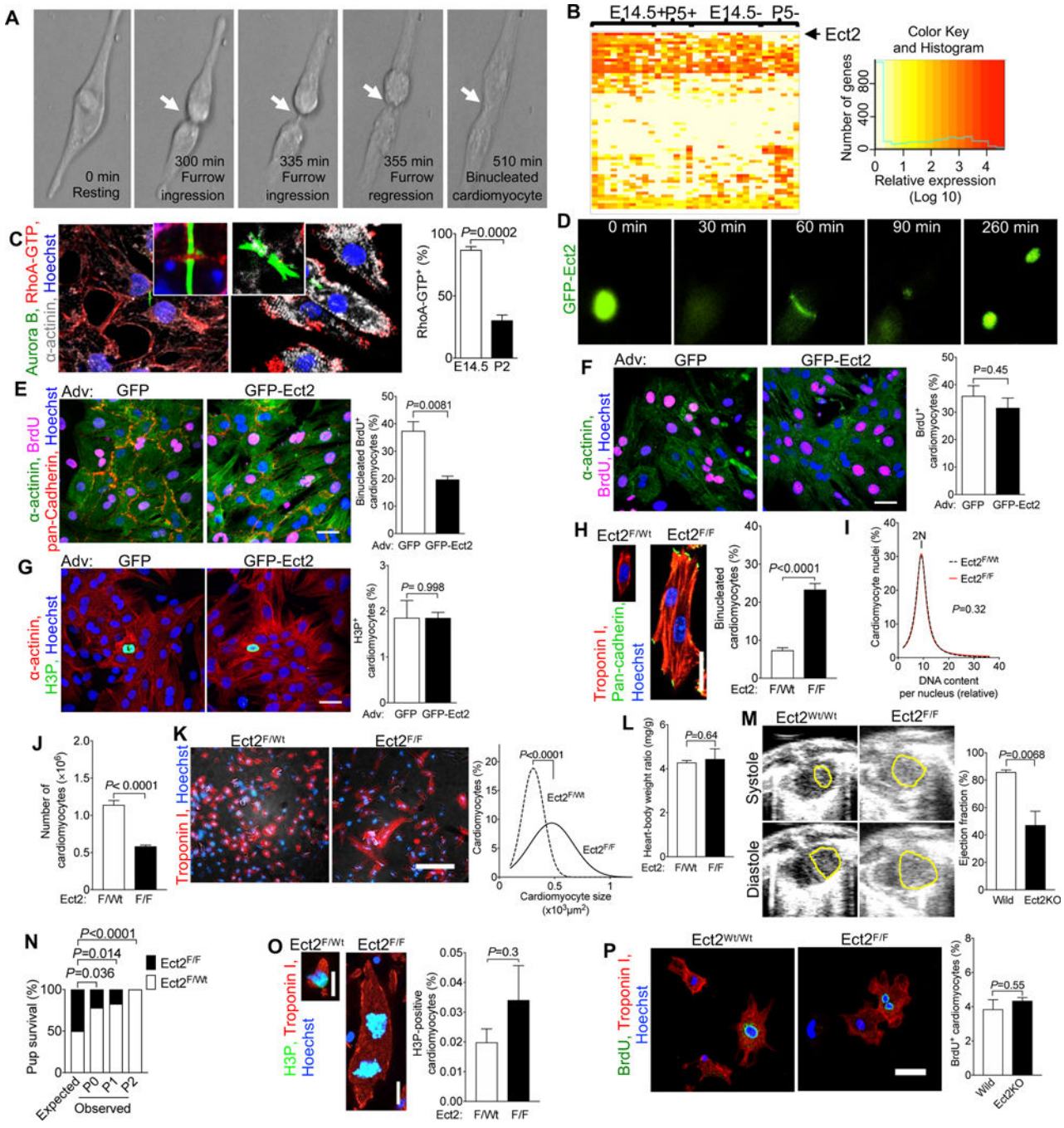

Figure 2. Ect2 levels regulate cardiomyocyte cytokinesis and Ect2 gene inactivation lowers endowment and is lethal in mice.

(**A**) Live cell imaging of neonatal rat cardiomyocytes (NRVM, P2-P3, 52 cardiomyocytes) shows that cleavage furrow regression precedes formation of binucleated cardiomyocytes, corresponding to Video S1. Cleavage furrow ingression is between 300–335 min, regression at 355 min, and formation of a binucleated cardiomyocyte at 510 min. (**B**) Transcriptional profiling of single cycling (+) and not cycling (−) cardiomyocytes at embryonic day 14.5 (E14.5) and 5 days after birth (P5) reveals that of 61 Dbl-homology family RhoGEF, Ect2 is

significantly repressed in cycling P5 cardiomyocytes ($P < 0.05$). (**C**) Binucleating cardiomyocytes exhibit lower RhoA activity (RhoA-GTP) at the cleavage furrow (E15.4: 59 midbodies; P2: n = 56 midbodies). (**D**-**G**) NRVM were transduced with Adv-CMV-GFP-Ect2. Live cell imaging shows appropriate and dynamic localization of GFP-ECT2 in cycling NRVM (**D**, corresponding to Video S4), reduced cytokinesis failure and reduced generation of binucleated cardiomyocytes. (**F, G**) Overexpression of GFP-Ect2 does not alter cardiomyocyte S- (**F,** GFP: n = 407; GFP-Ect2: n = 285) or M-phase (**G,** GFP: n = 432; GFP-Ect2: n = 443). (**H**-**P**) Ect2 gene inactivation in the αMHC-Cre; $Ect2^{F/F}$ mice at P1 (**H-O)** showed increased binucleated cardiomyocytes (**H**, $Ect2^{F/wt}$ n = 6, $Ect2^{F/F}$ n = 6 hearts) without change of DNA content per nucleus (**I**, $Ect2^{F/wt}$ n = 642 cardiomyocytes, $Ect2^{F/F}$ n = 647 cardiomyocytes), and a 50% lower cardiomyocyte endowment (**J**, $Ect2^{F/wt}$ n = 12, $Ect2^{F/F}$ n = 5 hearts). The reduced endowment triggers compensatory cardiomyocyte hypertrophy (**K**, $Ect2^{F/wt}$ n = 1,138 cardiomyocytes from 6 hearts, $Ect2^{F/F}$ n = 1,015 cardiomyocytes from 6 hearts), without change of heart weight (**L**, $Ect2^{F/wt}$ n = 14, $Ect2^{F/F}$ n = 6 hearts). (**M, N**) The lower cardiomyocyte endowment leads to myocardial dysfunction at P0 (**M**, left ventricular endocardium outlined in yellow, $Ect2^{wt/wt}$ n = 4, $Ect2^{F/F}$ n = 3 mice) and lethality before P2 (**N,** Video S5), but does not alter the M-phase (**O**, $Ect2^{F/wt}$ n = 6, $Ect2^{F/F}$ n = 6 hearts) and cell cycle entry (**P**, BrdU uptake, $Ect2^{flox}$ gene inactivation with αMHC-MerCreMer, tamoxifen DOL 0, 1, 2, followed by 3 days culture). Statistical significance was tested with Student's *t*- test if not specified, and Fisher's exact test (**N**). Scale bars 30 μm (**E, P**), 50 μm (**F, G**), 100 μm (**K**).

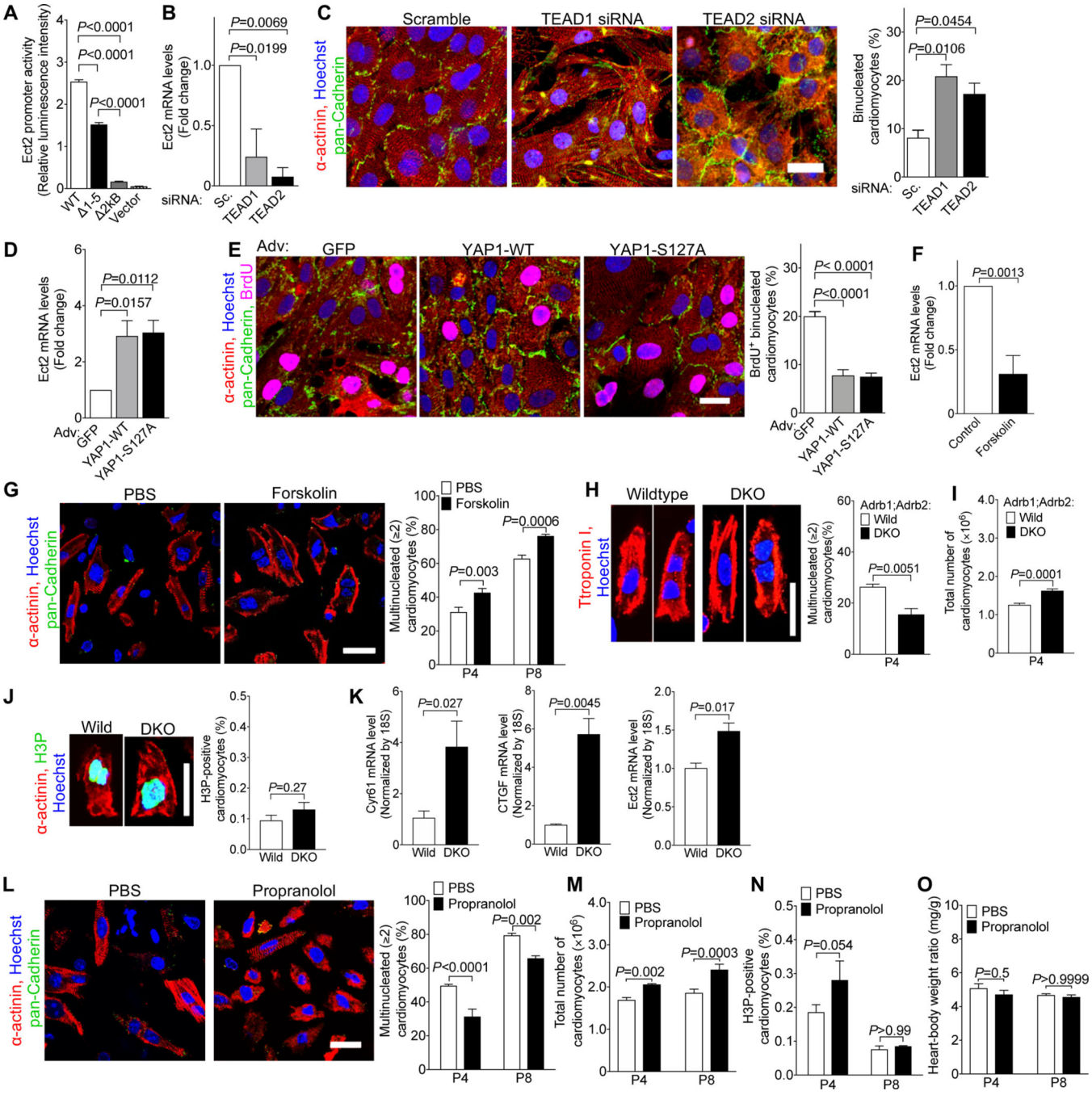

Figure 3. β-adrenergic receptor signaling regulates cardiomyocyte abscission and endowment in mice.

(**A**) Removal of the five TEAD1/2-binding sites (Fig. S7) reduced the activity of the Ect2 promoter in luciferase assays in HEK293 cells. WT: wild type Ect2 promoter; Δ1–5: All five putative TEAD-binding sites were removed; Δ2kB: the continuous 2kB DNA sequence containing all five TEAD-binding sites was removed; Vector: Empty vector that did not contain Ect2 promoter (n = 4 cultures). (**B, C**) Knockdown of TEAD1 and TEAD2 by siRNA reduced Ect2 mRNA (**B**), and increased the proportion of binucleated NRVMs (P2,

C, n = 3 cardiomyocyte isolations). (**D-E**) Adenoviral overexpression of wild type YAP1 (YAP1-WT) and a mutated version containing a S127A mutation (YAP1-S127A) in NRVMs (P2) increased the level of Ect2 mRNA (**D**) and reduced the percentage of binucleated cardiomyocytes (**E**, n = 4 cardiomyocyte isolations). (**F**) Forskolin reduced the mRNA level of Ect2 in cultured NRVMs (n = 5 cardiomyocyte isolations). (**G**) Forskolin administration (1 μg/g, 1 i.p. injection per day) increased the proportion of binucleated cardiomyocytes *in vivo* (n = 6 hearts/group). (**H-K**) Inactivation of β_1- and β_2-adrenergic receptor genes (DKO) decreased formation of multinucleated cardiomyocytes *in vivo* (**H**, n = 4 hearts/group), increased the total number of cardiomyocytes (**I**, n = 7 hearts for wild, n = 5 hearts for DKO), did not change M-phase (**J**, n = 4 hearts/group), and increased the expression of the YAP target genes Cyr61 and CTGF (**K**, n = 3 hearts/group). (**L-O**) Propranolol administration (10 μg/g, 2 i.p. injections per day) reduced the proportion of multinucleated cardiomyocytes (**L,** n = 6 hearts/group), and increased the number of cardiomyocytes (**M,** n = 6 heart/group for P4, n = 4 hearts/group for P8), but did not alter M-phase activity (**N**, n = 4 hearts/group) or heart-body-weight ratio (**O**, P4: n = 7 hearts for PBS, n = 6 hearts for Prop; P8: n = 4 hearts/group). Scale bar: 20 μm (**C, E, G, H, J, L**). Statistical significance was tested with one-way ANOVA with Bonferroni's multiple comparisons (**A-E**), Student's *t*- test (**F, H-K**), and two-way ANOVA with Bonferroni's multiple comparisons test (**G, L-O**).

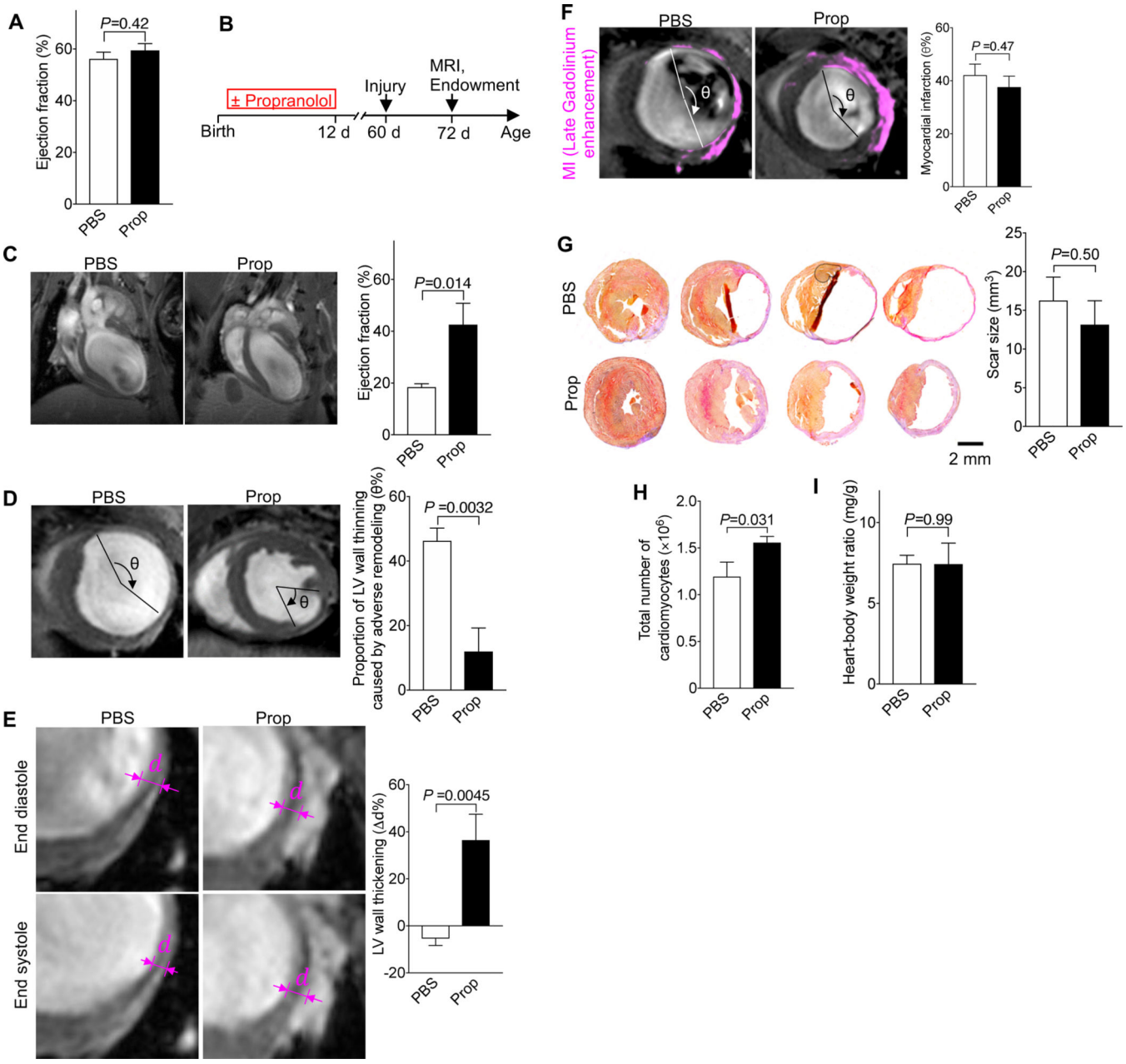

Figure 4. Mice with a 30%-increased cardiomyocyte endowment have better cardiac function and reduced adverse remodeling after myocardial infarction (MI).

Mice received propranolol (Prop, 10 μg/g, 2 i.p. injections per day, P1–12). (**A**) The ejection fraction (EF) at P60 was not changed (n = 11 hearts for PBS, n = 6 hearts for Prop). (**B**) Diagram of experimental design. MI was induced at P60 and MRI and histology were performed 12 days after MI. (**C**) After MI, EF was higher in propranolol-primed mice. (**D-G**) The region of stretched-out myocardial wall is significantly smaller (**D**) and systolic myocardial thickening is greater (**E**), despite same scar size measured in vivo (**F**) and ex vivo (**G**, n = 5 hearts for PBS, n = 4 hearts for Prop). (**H, I**) Propranolol-primed hearts show a higher number of cardiomyocytes, determined by stereology (n = 5 hearts/group), after MI

without change of heart weight (n = 5 hearts for PBS, n = 6 hearts for Prop) (**I**). Statistical significance was tested with *t*-test.

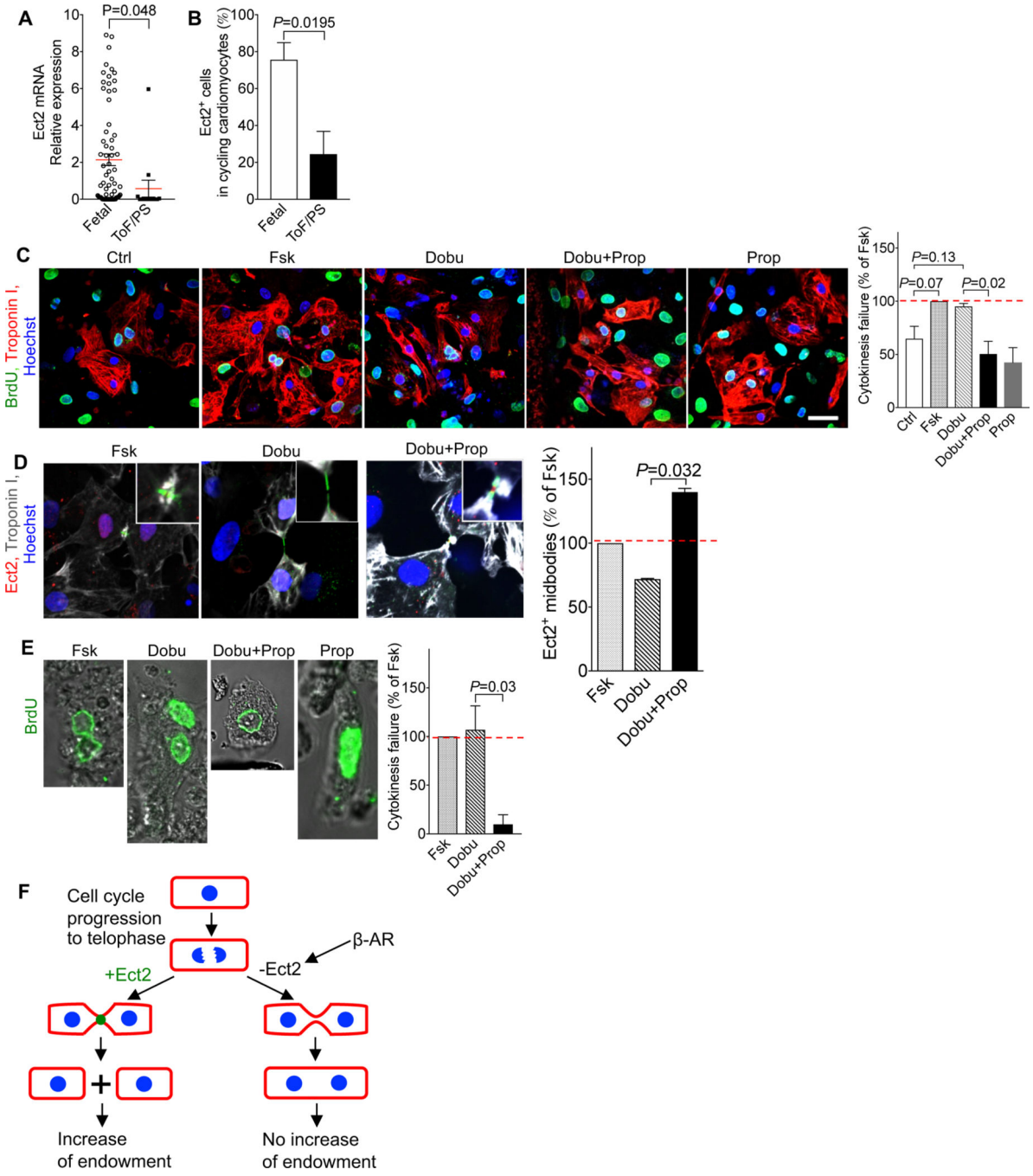

Figure 5. β-adrenergic signaling regulates cytokinesis in cardiomyocytes from ToF/PS patients.

(**A**) Cardiomyocytes from ToF/PS patients exhibit decreased levels of Ect2 mRNA. Each symbol represents one cycling cardiomyocyte. (**B**) The percentage of Ect2-positive cycling cardiomyocytes was reduced in ToF/PS patients, compared with fetal human hearts. (**A-B**, Fetal: n = 71 cardiomyocytes from 4 hearts, ToF/PS: n = 13 cardiomyocytes from 3 hearts). (**C, D**) β-AR signaling regulates cytokinesis failure in cultured human fetal cardiomyocytes (n = isolations from 3 hearts; Ctrl: control; Fsk: Forskolin, Prop: Propranolol; Dobu: Dobutamine: 10 μM), measured by reduced formation of binucleated daughter cells (**C**) and

higher prevalence of Ect2-positive midbodies (**D**). (**E**) Propranolol increases completion of cytokinesis in cultured ToF/PS cardiomyocytes (n = cultures from 3 patients). (**F**) Cellular model connecting cytokinesis failure to endowment changes. (**G**) Molecular model of cytokinesis failure in cardiomyocytes. Scale bar: 40 μm (**C**). Statistical significance was tested with T-test (**A**) and one-way ANOVA with Bonferroni's multiple comparisons test (**C-E**).