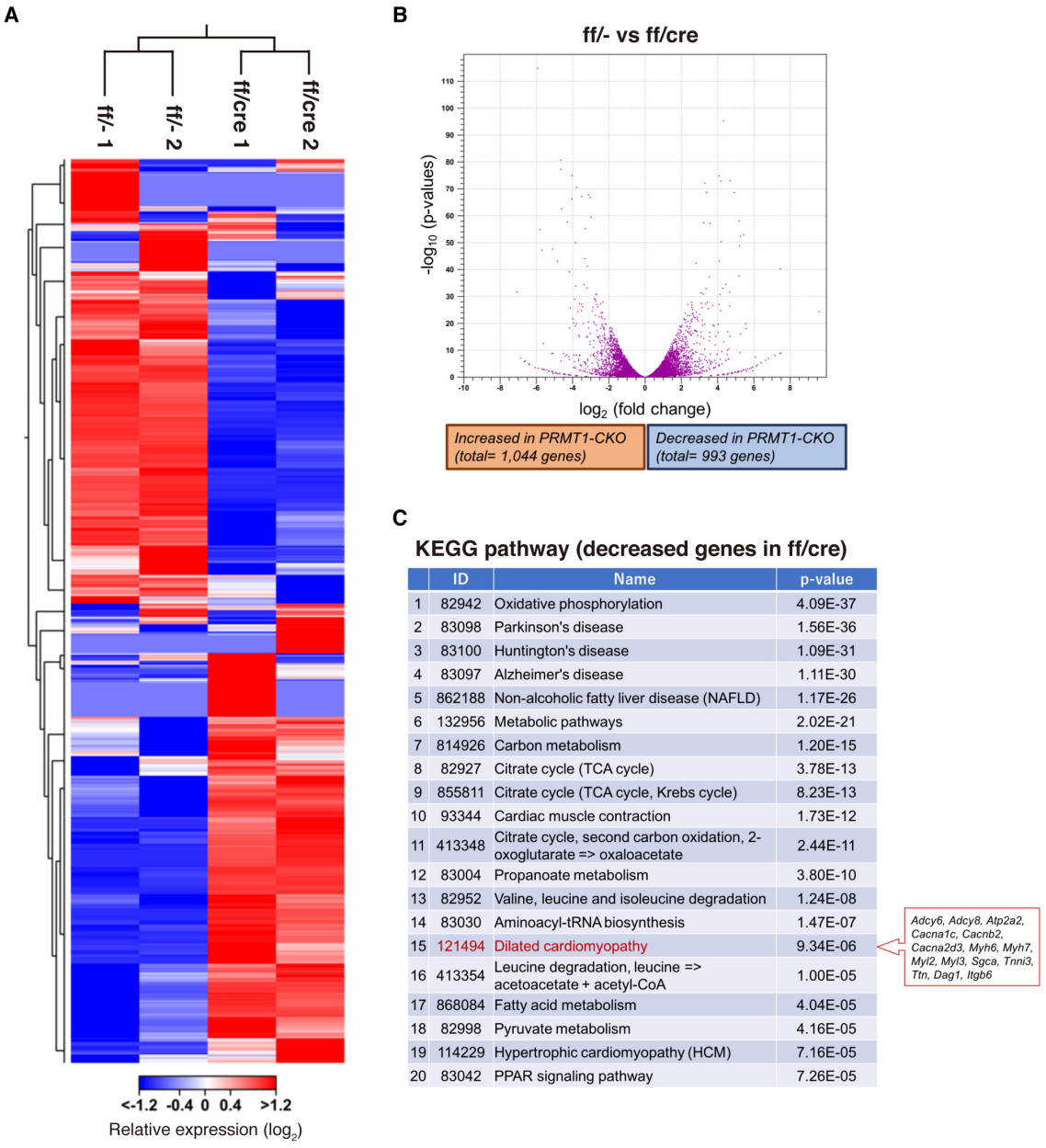
ISCI, Volume *8*

Supplemental Information

PRMT1 Deficiency in Mouse Juvenile Heart Induces Dilated Cardiomyopathy and Reveals Cryptic Alternative Splicing Products

Kazuya Murata, Weizhe Lu, Misuzu Hashimoto, Natsumi Ono, Masafumi Muratani, Kana Nishikata, Jun-Dal Kim, Shizufumi Ebihara, Junji Ishida, and Akiyoshi Fukamizu

Supplemental Figures and Tables



	ID	Name	p-value
1	82942	Oxidative phosphorylation	4.09E-37
2	83098	Parkinson's disease	1.56E-36
3	83100	Huntington's disease	1.09E-31
4	83097	Alzheimer's disease	1.11E-30
5	862188	Non-alcoholic fatty liver disease (NAFLD)	1.17E-26
6	132956	Metabolic pathways	2.02E-21
7	814926	Carbon metabolism	1.20E-15
8	82927	Citrate cycle (TCA cycle)	3.78E-13
9	855811	Citrate cycle (TCA cycle, Krebs cycle)	8.23E-13
10	93344	Cardiac muscle contraction	1.73E-12
11	413348	Citrate cycle, second carbon oxidation, 2-oxoglutarate => oxaloacetate	2.44E-11
12	83004	Propanoate metabolism	3.80E-10
13	82952	Valine, leucine and isoleucine degradation	1.24E-08
14	83030	Aminoacyl-tRNA biosynthesis	1.47E-07
15	121494	Dilated cardiomyopathy	9.34E-06
16	413354	Leucine degradation, leucine => acetoacetate + acetyl-CoA	1.00E-05
17	868084	Fatty acid metabolism	4.04E-05
18	82998	Pyruvate metabolism	4.16E-05
19	114229	Hypertrophic cardiomyopathy (HCM)	7.16E-05
20	83042	PPAR signaling pathway	7.26E-05

Figure S1 (related to Figure 3). Gene expression profile of RNA-Seq data

(A and B) A hierarchical clustering and a volcano plot based on the 2,037 differentially expressed genes between control and PRMT1-cKO group (adjusted FDR *P* value < 0.05). (C) Functional enrichment analysis of 993 downregulated genes in PRMT1-cKO mice. The top 20 enriched pathways are shown in the table. A list of matched genes with DCM-related gene sets in the box insert. See also Table S1.

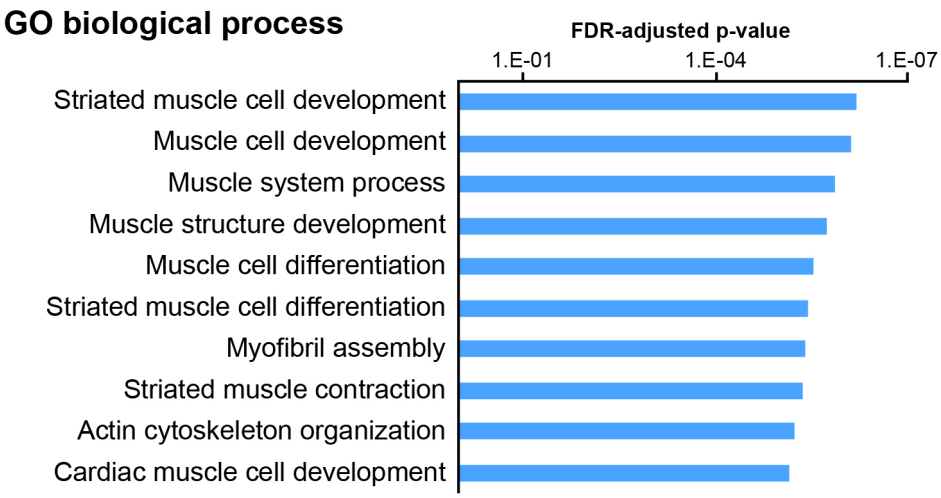


Figure S2 (related to Figure 4). Gene ontology (GO) analysis of alternatively expressed isoforms between control and PRMT1-cKO mice

GO analysis of 74 genes was performed using the PANTHER web tool (Mi et al., 2013). The top 10 enriched GO terms are shown in the graph.

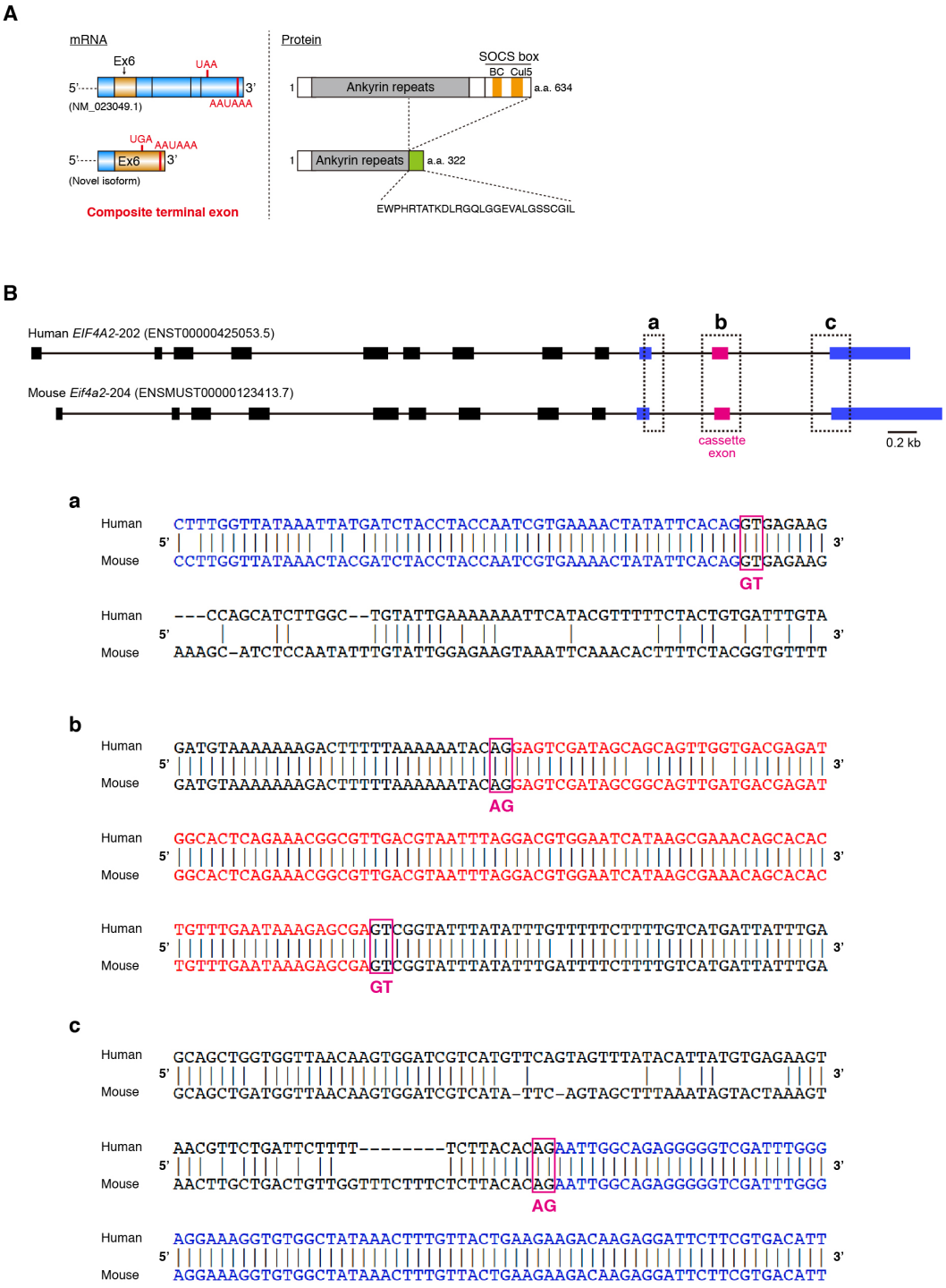


Figure S3 (related to Figure 4). Schematic diagrams of *Asb2* mRNA/protein and *Eif4a2* gene

(A) 3' side of *Asb2* mRNA and domain structure of ASB2 protein. The extended exon 6 contains a stop codon and a putative poly(A) signal. (B) Structures of human and mouse *Eif4a2* transcripts. (a-c) DNA sequences surrounding splice donor and acceptor sites.

Alternatively spliced genes	Animal models	Reference	
Tmed2, Snap23	CELF1-overexpressing mice	*Nat. Commun.* 2014	doi: 10.1038/ncomms4603.
Mef2a	SRSF10-KO mice	*Cell Rep.* 2015	doi: 10.1016/j.celrep.2015.10.038.
	RBFOX2-KO mice	*Cell Rep.* 2015	doi: 10.1016/j.celrep.2015.02.013.
Ktn1	CELF1-KO mice	*Sci. Rep.* 2016	doi: 10.1038/srep35550.
	rbfox1-KD zebrafish	*J. Cell Sci.* 2015	doi: 10.1242/jcs.166850.
Lmo7	RBM20-KO rats	*J. Clin. Invest.* 2014	doi: 10.1172/JCI74523.
Srobs1	MBNL1-KO mice	*Sci. Rep.* 2015	doi: 10.1038/srep09042.
	RBFOX2-KO mice	*Cell Rep.* 2015	doi: 10.1016/j.celrep.2015.02.013.
	RBM20-KO rats	*Nat. Med.* 2012	doi: 10.1038/nm.2693.
Ttn	RBM20-KO rats	*Nat. Med.* 2012	doi: 10.1038/nm.2693.
	hnRNPU-KO mice	*Proc. Natl. Acad. Sci. U. S. A.* 2015	doi: 10.1073/pnas.1508461112.

Table S2 (related to Figure 3). Summary of known cardiac alternative splicing events that were investigated as illustrated in Figure 3.

Feature ID	P-value	Relative RPKM	Relative RPKM	WT Means	Relative RPKM	Relative RPKM	KO Means	Ratio
Sptan1_11	7.86E-03	3.41E-04	1.84E-04	2.16E-03	0.28	0.21	4.63	0.00107
Acp5_1	5.00E-04	0.04	0	4.41E-03	1	1	7.02	0.02000
Ppfibp1_3	4.95E-04	0.03	3.17E-03	0.08	0.57	0.83	6.87	0.02369
Ndrg4_6	9.88E-04	6.17E-03	0	0.07	0.1	0.13	6.29	0.02683
Fbxo40_3	2.81E-04	0.02	0.02	0.28	0.21	0.42	8.87	0.06349
Fam198b_1	0.04	0.09	0	0.14	0.53	0.67	4.8	0.07500
Smdt1_2	6.43E-03	0	0.06	0.47	0.18	0.56	6.37	0.08108
Nrap_5	1.52E-03	0.05	0.09	0.89	0.71	1	10.33	0.08187
Tnnt2_4	0.04	1.18E-03	0	0.3	5.25E-03	7.28E-03	4.29	0.09417
1500017E21Rik_4	0.02	0.06	0.12	0.1	0.7	1	4.03	0.10588
Tpm1_12	5.94E-13	0.01	9.71E-03	14.67	0.1	0.07	108.86	0.11594
Gsn_4	4.53E-03	2.51E-03	9.42E-03	0.92	0.03	0.06	8.97	0.13256
Atpif1_1	6.61E-05	0	0.18	3.1	0.34	1	26.8	0.13433
Nptn_4	7.57E-03	0.16	0.01	0.87	0.9	0.29	7.26	0.14286
Synpo2l_3	9.91E-04	0.16	0.16	0.36	1	1	8.01	0.16000
Ttn_14	1.36E-03	0.13	0.15	14.01	0.76	0.95	40.95	0.16374
Clu_3	5.51E-07	0.01	4.87E-03	0.56	0.03	0.06	16.15	0.16522
Cxcl12_1	0.05	0.16	0.2	1.62	1	1	7.54	0.18000
Vmp1_2	0.04	0.08	0.12	0.34	0.59	0.5	4.31	0.18349
Fhl1_2	1.34E-10	0.19	0.18	1.45	1	1	29.04	0.18500
H19_5	0.02	0.05	5.56E-03	0.06	0.15	0.14	4	0.19159
Pkm_1	3.25E-05	0.04	0.05	3.24	0.28	0.18	21.89	0.19565
Pdlim5_5	7.81E-03	0.05	0.04	3.88	0.23	0.22	15.37	0.20000
Tnnt2_1	0.04	3.55E-03	5.55E-04	1.07	9.82E-03	0.01	6.96	0.20711
Sptan1_9	0.01	0.12	0.04	0.68	0.47	0.26	6.74	0.21918
Casq2_1	0.01	4.13E-03	0.05	2.16	0.15	0.09	10.31	0.22554
Popdc2_1	0.02	0.08	0.06	2.62	0.21	0.29	11.48	0.28000
Sorbs2_7	2.27E-04	0.08	0.09	0.68	0.3	0.28	11.05	0.29310
Rhod_2	0.02	0.29	0.37	0.18	1	1	5.41	0.33000
Lamp2_3	2.82E-04	0.33	0.34	5.13	1	1	22.74	0.33500
Postn_5	6.56E-09	0.09	0.31	0.75	0.64	0.54	22.36	0.33898
Ldb3_5	2.77E-03	0.31	0.37	18.39	1	1	11.19	0.34000
Strn_3	0.04	0.1	0.32	0.34	0.6	0.62	4.62	0.34426
Fez2_1	0.02	0.33	0.36	2.42	1	1	10.51	0.34500
Aldoa_9	4.99E-11	0.16	0.24	44.49	0.52	0.55	136.5	0.37383
Ptpn11_2	1.36E-03	0.36	0.29	2.59	0.83	0.76	14.73	0.40881
Myl4_7	9.86E-03	0.17	0	8.84	0.2	0.21	32.27	0.41463
Mybpc3_1	0.02	7.13E-03	6.19E-03	1.27	0.02	0.01	7.54	0.44400
Ctsd_4	2.21E-03	0.05	0.04	2.83	0.14	0.05	17.84	0.47368
P4hb_1	0.02	0.05	0.08	1.17	0.18	0.09	7.36	0.48148
Tnnt2_7	8.29E-03	0.11	0.05	43.03	0.17	0.16	114.35	0.48485
Rtn4_6	3.46E-03	0	0.06	0.15	0.07	0.05	7.05	0.50000
Tfpi_10	0.01	0.37	0.33	1.66	0.76	0.64	8.93	0.50000
Ccdc141_9	7.63E-03	1	0.95	6.42	0.63	0.34	1.93	2.01031
Smtn_5	0.03	1	1	2.78	0.48	0.51	0.39	2.02020
Coro6_6	7.86E-03	0.33	0.36	3.59	0	0.34	0.32	2.02941
Hint2_1	0.04	0.44	0.35	3.46	0.17	0.2	0.87	2.13514
Osgepl1_1	0.04	1	0.92	3.38	0.68	0.21	0.76	2.15730
Kcnj3_1	0.02	1	1	2.9	0.54	0.34	0.28	2.27273
Ivns1abp_2	2.80E-03	0.21	0.19	18.34	0.06	0.11	10.89	2.35294
Adck3_11	0.02	0.07	0.12	3.15	0	0.08	0.22	2.37500
Mrpl51_2	0.04	0.2	0.26	3.38	0.13	0.06	0.77	2.42105
Echs1_5	0.03	0.05	0.05	2.49	0.02	0.02	0.37	2.50000
Oxct1_3	8.95E-06	0.12	0.16	12.2	0.09	0.02	2.28	2.54545
Bsg_1	8.32E-06	0.31	0.35	43.58	0.04	0.21	12.22	2.64000
Ctgf_2	0.01	0.19	0.18	2.09	0.07	0.07	10.39	2.64286
Myl2_8	7.47E-03	2.38E-03	1.59E-03	5.89	2.67E-04	1.18E-03	1.12	2.74361
Obscn_6	1.52E-03	1	1	7.84	0.53	0.18	2.18	2.81690
Myl2_12	1.25E-04	3.32E-03	2.78E-03	9.03	5.44E-04	1.58E-03	1.66	2.87194
Cdh13_3	0.04	0.27	0.38	3.72	0.16	0.06	0.78	2.95455
Nudt7_5	6.41E-03	0.34	0.83	4.94	0.35	0.03	0.52	3.07895
Ndufs1_2	1.24E-04	0.19	0.18	8.86	0.06	0.06	1.22	3.08333
Ttn_19	3.71E-05	0.1	0.1	9.84	0.03	0.03	1.41	3.33333
Ttn_6	3.45E-03	0.05	0.05	5.17	0.01	0.02	0.85	3.33333
Lmo7_16	2.65E-03	0.57	0.68	8.32	0.19	0.18	2.63	3.37838
Mtch2_6	0.01	0.33	0.46	5.17	0.17	0.06	1.21	3.43478
Chpt1_5	4.45E-07	0.92	1	14.35	0.35	0.19	2.25	3.55556
Asb2_2	1.51E-05	1	1	14.6	0.35	0.2	3.89	3.63636
Eif4a2_17	5.02E-05	0.64	0.8	19.4	0.23	0.16	8.44	3.69231
D4Wsu53e_3	0.02	0.29	0.19	3.83	0.07	0.05	0.76	4.00000
Ech1_5	7.92E-09	0.06	0.07	12.41	8.63E-03	0.02	0.52	4.54069
Narf_2	0.04	0.39	0.39	3.57	0.05	0.12	0.5	4.58824
Smdt1_3	5.01E-04	0.48	0.52	8.9	0.17	0	1.81	5.88235
Slc25a39_14	0.04	0.79	0.78	3.32	0.06	0.18	0.97	6.54167
Ttll1_4	7.86E-03	1	1	3.74	0.29	0	0.2	6.89655
Sgca_2	3.45E-03	0.73	1	5.41	0.07	0.17	0.73	7.20833
Gpcpd1_13	0.03	0.09	0.14	2.65	0.02	0.01	0.2	7.66667
Obscn_5	1.97E-03	0.5	0.59	4.26	0.05	0.06	0.36	9.90909
Acot7_6	0.02	1	0.43	3.12	0.08	0.04	0.27	11.91667
Mff_10	3.94E-03	0.52	1	4.19	0.05	3.25E-03	0.18	28.54460
Ift81_2	3.93E-03	1	1	3.88	0	0.03	0.02	66.66667
Ndufv1_1	0.03	0.02	0.06	2.67	0	4.97E-04	9.19E-03	160.96579

Table S3 (related to Figure 4). Candidate genes exhibiting aberrant alternative splicing in PRMT1-cKO mice.

Transparent Methods

Animal experiments

PRMT1^{KI} mice that carry *Prmt1*$^{tm1a(EUCOMM)Wtsi}$ allele and *Prmt1*$^{flox/flox}$ mice were obtained and generated as previously described (Hashimoto et al., 2016). To create PRMT1-cKO mice, *Prmt1*$^{flox/flox}$ mice were mated with αMHC-cre transgenic mice (strain name: B6.FVB-Tg(Myh6-cre)2182Mds/J, stock number: 011038, The Jackson Laboratory). C57BL/6J mice were purchased from CLEA Japan for isolation of primary cardiomyocytes and non-myocytes. Genotype of PRMT1-cKO mice was determined by PCR and agarose gel electrophoresis. Briefly, a small piece of tail was removed from 10-14-day-old mice, and direct PCR was performed using HelixAmp Direct PCR 3G (NanoHelix #DPR200) in accordance with the manufacturer's instructions. The primer sequences were as follows: *Prmt1* (5'-GTGCTTGCCATACAAGAGATCC-3' and 5'-GTGAAACATGGAGTTGCGGTAT-3'); αMHC-cre transgene (5'-ATGACAGACAGATCCCTCCTATCTCC-3' and 5'-CTCATCACTCGTTGCATCATCGAC-3'). To assess cardiac contractile function, echocardiography was performed as described previously (Murata et al., 2013). Male mice were used for all experiments except X-gal staining.

All animal experiments were performed in a humane manner and approved by the Institutional Animal Experiment Committee of the University of Tsukuba. Experiments were conducted in accordance with the Regulations for Animal Experiments of the University of Tsukuba and the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Histological analysis

Hearts were embedded in paraffin and sectioned as previously described (Murata et al., 2013). Deparaffinized cardiac sections (5 and 3 μm thick) were stained with hematoxylin-eosin (HE) and Masson's trichrome reagents. Images were obtained using a BX53 microscope and DP21 digital camera (Olympus).

For immunohistochemistry, deparaffinized cardiac sections (5 μm thick) were placed in 10 mM sodium citrate buffer (pH 6.0), boiled 20 seconds in a microwave oven, and cooled at room temperature for 20 minutes. To inactivate endogenous peroxidase, sections were incubated with 3% H_2O_2 for 30 minutes. After incubation with TSA blocking reagent (Perkin Elmer, #FP1020)

for 30 minutes, sections were incubated with anti-PRMT1 antibody (1:200, rabbit-monoclonal, abcam, #ab92299) at room temperature for 60 minutes. Sections were then reacted with biotinylated anti-rabbit antibody (1:500, Vector, #BA1000) for 30 minutes at room temperature. Secondary antibodies were detected using the tyramide signal amplification (TSA) Plus Fluorescein System (Perkin Elmer, #NEL756001KT). For double-staining, sections were incubated with anti-cardiac troponin I antibody (1:50, rabbit-polyclonal, abcam, #ab47003) for 60 minutes at room temperature. Primary antibodies were visualized using Alexa Fluor 568 conjugated secondary antibody (Thermo Fisher Scientific, A11011). CF640R conjugated wheat germ agglutinin (WGA, Biotium, #29026) and Hoechst 33258 were used to stain cellular membranes and nuclei. Fluorescent images were obtained using a FluoView FV10i confocal laser-scanning microscope (Olympus).

X-gal staining

For X-gal staining, 8-week-old mice were perfused with 4% paraformaldehyde (PFA), then the heart tissues were dissected, following immersion fixation in 4% PFA for 15 minutes. The hearts from E19 embryos were harvested and fixed with 4% PFA for 15 minutes. Tissues were then cryoprotected, embedded in O.C.T. compound, and cryostat sectioned. Sections were stained in 1xPBS containing 1 mg/ml X-gal, 5 mM $K_4Fe(CN)_6/3H_2O$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 0.05% sodium deoxycholate, and 0.02% NP-40 at 37˚C overnight. Slides were then counter-stained with eosin. Data images were obtained using a BX53 microscope and DP21 digital camera (Olympus).

Western blotting

Immunoblot analysis was performed as previously described (Murata et al., 2016). The primary antibodies were as follows: anti-PRMT1 (1:500-1:1000, Millipore, #07-404), anti-cardiac troponin I (1:250, Santa Cruz Biotechnology, #sc-31655), anti-vimentin (1:1000, Cell Signaling Technology, #5741), anti-GAPDH (1:2000-1:10000, American Research Products, #05-50118).

Cardiomyocyte isolation

Ventricles harvested from neonatal mice were minced and enzymatically digested in HBSS (with Mg^{2+}) containing 0.45 mg/ml collagenase type II (Worthington, #CLS2) and 0.11 mg/ml pancreatin (GIBCO, #002-0036DG) at 37ºC with gentle shaking and intermittent pipetting. Cell suspension was collected and filtered through a cell strainer (40 μm nylon mesh). The cells

were collected by centrifugation, resuspended in cardiomyocyte (CM) growth medium (DMEM/M199 (4:1) with 10% horse serum (HYCLONE, #SH30074.03), 5% fetal bovine serum (FBS), 1.6 mM L-glutamine (Life Technologies, #25030-081), 80 μM non-essential amino acid (GIBCO, #11140-050), and penicillin-streptomycin (PS)), and plated on non-coated plastic dishes. After 2.5 hours incubation with 5% CO_2 at 37ºC, the non-adhesive cells (cardiomyocytes) were collected, resuspended in CM growth medium containing 25 μM cytosine β-D-arabinofuranoside (Ara-C, SIGMA, #C1768), and plated on gelatin-coated dishes. The remaining adhesive cells (non-myocytes) were grown in DMEM supplemented with 10% FBS and PS.

RNA experiments

Cardiac RNA was extracted using mirVana miRNA Isolation Kit (Thermo Fisher Scientific, #AM1560) in accordance with the manufacturer's instructions. Large RNA fraction containing mRNA was used for gene expression assay. Reverse transcription and quantitative real-time-PCR was performed as described previously (Murata et al., 2013, Murata et al., 2016).

To validate alternative splicing events, RT-PCR was performed using AmpliTaq Gold DNA Polymerase (Thermo Fisher Scientific, #4398881) and PrimeSTAR Max DNA Polymerase (for detecting *Fbxo40* isoforms, TAKARA, #R045A). PCR products were separated by 8.5% acrylamyde gel electrophoresis and stained with ethidium bromide. Images were acquired using an LAS-3000 imaging system (GE healthcare) and analyzed by ImageJ software (National Institutes of Health). Percent spliced in (PSI) value was calculated from the band intensity, as previously described (Giudice et al., 2014). The primers for RT-PCR were as follows:

Tmed2-forward (5'-GTACACATTTGCAGCCCACA-3')
Tmed2-reverse (5'-CTCCTGTTCGTGCTTTACGG-3')
Snap23-forward (5'-GTGTTGTGGCCTCTGCATCT-3')
Snap23-reverse (5'-TGGCTGCTCCTGTAGTTTGCT-3')
Mef2a-forward (5'-AACTCAAGGGCCTCTCCAAA-3')
Mef2a-reverse (5'-CACTACAGGCGTGGCAAGAG-3')
Ktn1-forward (5'-GATCCATGAGAAAGATGGACAGA-3')
Ktn1-reverse (5'-GCAGCTCCTGGACTTGAGAA-3')
Lmo7-forward (5'-TGAGCCAAAGTCAGCTCTCC-3')
Lmo7-reverse (5'-ACTTGCTCCCATTCTCACCA-3')
Sorbs1-forward (5'-AGACCTTCGTCTGCCTACCC-3')

Sorbs1-reverse (5'-GCATCCTTTGCCCTTCTCTC-3')

Ttn (exon 47-48)-forward (5'-CCAGGCCGAGTTGACCATCA-3')

Ttn (exon 47-48)-reverse (5'-GCAATGGAGGCGTGCTGATT-3')

Ttn (exon 48-50)-forward (5'-CAGCACGCCTCCATTGCAAG-3')

Ttn (exon 48-50)-reverse (5'-CAGGGATGGTCAAGACGGCA-3')

Ttn (exon 132-134)-forward (5'-TCGGGAGGAGGAGTATGAGG-3')

Ttn (exon 132-134)-reverse (5'-GGGTGGCAGAGGTTTGAGTT-3')

Ttn (exon 147-150)-forward (5'-GCTCCCACTCCTGTCCCTAA-3')

Ttn (exon 147-150)-reverse (5'-CACCCTGATGAACTCCTCCAC-3')

Asb2 (exon 6-7)-forward (5'-CAGCATCACCCCTTTGTTTG-3')

Asb2 (exon 6-7)-reverse (5'-TCATTCTTGCTGGCCTCGT-3')

Asb2 (extended ex6)-forward (5'-CAGCATCACCCCTTTGTTTG-3')

Asb2 (extended ex6)-reverse (5'-CCTGGCCTAAGAGTCCTCCA-3')

Fbxo40-forward (5'-AGAAGCCAGAAGCAGGGAAG-3')

Fbxo40-reverse (5'-GCTCCACGATGTTGAAAGGA-3')

Nrap-forward (5'-TGTTGCTTCTCCCTGTCTCC-3')

Nrap-reverse (5'-GGCTGCCACAGACTTCTTCA-3')

Eif4a2-forward (5'-TGACGTGCAACAAGTGTCCT-3')

Eif4a2-reverse (5'-CCACACCTTTCCTCCCAAA-3')

RNA sequencing

For the preparation of cDNA library, total RNA was extracted from the heart of 6 weeks-old mice (n = 2) using ISOGEN II (Nippon Gene, #311-07361). Ribosomal RNA (rRNA) was removed using RiboMinus Eukaryote Kit (Thermo Fisher Scientific, #A1083708). Depletion of rRNA was confirmed using the Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA libraries were then prepared using SOLiD total RNA-Seq kit (Thermo Fisher Scientific, #4445374) in accordance with the manufacturer's instructions. Briefly, rRNA-depleted RNA was fragmented by RNase III, and subsequently incubated with T4 polynucleotide kinase. After purification of fragmented RNA, the size of fragmented RNA was determined using the Agilent 2100 Bioanalyzer. Fragmented RNA was ligated to SOLiD adaptor, and reverse transcribed by ArrayScript reverse transcriptase (Thermo Fisher Scientific). The cDNA was purified using Agencourt AMPure XP (Beckman Coulter), and amplified by 15 cycles of PCR using SOLiD barcoded 3' primers. Amplified cDNA was purified and size-selected using Agencourt AMPure

XP. The quality of the cDNA library was checked by the Agilent 2100 Bioanalyzer, and quantified by TaqMan qPCR. RNA sequencing was carried out using the 5500xl SOLiD system (Thermo Fisher Scientific) in the single-end sequencing mode (75 bp reads) in accordance with the manufacturer's instructions.

The data were imported into CLC Genomics Workbench software (v9.5.3, Qiagen), and mapped to the mouse genome (GRCm38) using RNA-sequencing analysis tool. Transcript expression was analyzed against 83,260 transcript models downloaded from the CLC website. Quantification value for each transcript was obtained in RPKM (reads per kilobase per million reads), and used for empirical analysis of DGE in CLC Genomics Workbench. RNA isoforms were filtered based on significant expression changes ($p < 0.05$) between control and PRMT1-cKO mice samples. Relative expression of isoforms between two groups was then compared by calculating isoform ratio in the following manner:

Relative RPKM = transcript-specific RPKM / total RPKM from transcript isoforms

(means of N=2, respectively)

Isoform ratio = Relative RPKM in WT / Relative RPKM in cKO

By filtering for changes at least 2.0-fold, 82 transcripts (74 genes) were selected as candidate splicing regulation events. To visually confirm these splicing events, BAM files were exported from CLC Genomics Workbench, sorted, imported to into the Integrative Genomics Viewer (IGV), and Sashimi plot images were created.

Data and software availability

The accession number for the data reported in this study is GEO: GSE112938.

Gene ontology (GO) analysis

GO overrepresentation test was performed using the PANTHER web tool (http://pantherdb.org). The gene list (74 genes) was uploaded and analyzed by statistical overrepresentation test (Fisher's Exact with FDR multiple test correction; Annotation Version and Release Date: GO Ontology database Released 2018-07-03).

Cell culture

C2C12 mouse myoblasts were grown in DMEM supplemented with 10% FBS and PS (growth medium). Cell differentiation was induced by switching from growth medium to DMEM containing 2% horse serum and PS. At three days after induction of differentiation, cells were

collected and used for mRNA experiment.

Transfection and immunoprecipitation assay

To investigate the function of eIF4A2 isoforms, mouse eIF4A2 isoforms were amplified by PCR from heart cDNA and inserted into pHA-C1 vector.

The primers for cDNA cloning were as follows:

XhoI-eIF4A2-forward (5'-CCGCTCGAGCTATGTCTGGTGGCTCCGCGGA-3')

eIF4A2-wt-BamHI-reverse (5'-CGCGGATCCTTAAATTAGGTCAGCCACAT-3')

eIF4A2-tr-BamHI-reverse (5'-CGCGGATCCCTATCGACTCCTGTGAATAT-3')

For detecting ubiquitination, plasmids were transfected into C2C12 cells using Lipofectamine LTX (Thermo Fisher Scientific, #5338100) in accordance with the manufacturer's instructions. After 16 hours incubation, cells were treated with MG132 (10 μM final concentration, Enzo Life Sciences, #BML-PI102-0005) for 8 hours. Cells were lysed in ice-cold TNE buffer containing 0.1% Triton X-100. After centrifugation, the supernatants were incubated overnight with anti-HA antibody (Roche, #11867423001) at 4ºC. To collect the immunocomplex, SureBeads Protein G Magnetic Beads (Bio-Rad, #1614023) were added to samples. After incubation for 1 hour at 4ºC, the beads were washed three times with lysis buffer, 2xSDS sample buffer was added, and incubated for 5 minutes at 99°C. Immunoblot was performed using following primary antibodies: anti-HA (1:2000, mouse-monoclonal, Wako, #014-21881), anti-ubiquitin (1:500, mouse-monoclonal, Santa Cruz Biotechnology, #sc-8017), anti-β-actin (1:2000, rabbit-polyclonal, Medical & Biological Laboratory, #PM053).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Prism Software). The data were analyzed using Student's *t*-test and Mann Whitney test, and two-way ANOVA followed by Bonferroni multiple comparison test. Significant differences were defined as $p < 0.05$.

Supplemental references

Mi, H., Muruganujan, A., Casagrande, J.T., and Thomas, P.D. (2013). Large-scale gene function analysis with the PANTHER classification system. Nat. Protoc. *8*, 1551-1566.

Murata, K., Ishida, J., Ishimaru, T., Mizukami, H., Hamada, J., Saito, C., and Fukamizu, A. (2016). Lactation Is a Risk Factor of Postpartum Heart Failure in Mice with Cardiomyocyte-specific Apelin Receptor (APJ) Overexpression. J. Biol. Chem. *291*, 11241-11251.

Murata, K., Saito, C., Ishida, J., Hamada, J., Sugiyama, F., Yagami, K., and Fukamizu, A. (2013). Effect of lactation on postpartum cardiac function of pregnancy-associated hypertensive mice. Endocrinology *154*, 597-602.