Dynamic Visualization of DNA Methylation in Cell Cycle Genes during iPSC Cardiac Differentiation

Ning Li[1,2], Ba Thong Nguyen[3,4*], Zhenhe Zhang[1*], W. Robb MacLellan,[1] Yiqiang Zhang[1,3,4¶]

[1] Division of Cardiology, Department of Medicine, University of Washington, Seattle, WA 98109, USA

[2] State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China

[3] Department of Anatomy, Biochemistry and Physiology, John A. Burns School of Medicine, University of Hawaii at Manoa, Honolulu, HI 96813, USA.

[4] Center for Cardiovascular Research, John A. Burns School of Medicine, University of Hawaii at Manoa, Honolulu, HI 96813, USA.

* Equal contribution.

¶ Author for Correspondence: Yiqiang Zhang, PhD (yiqiang.zhang@hawaii.edu).

ABSTRACT

Background: Epigenetic DNA methylation is an essential mechanism controlling gene expression and cellular function. Existing analyses with conventional assays have generated significant insights into static states of DNA methylation, but were unable to visualize the dynamics of epigenetic regulation. **Aim:** We utilized a genomic DNA methylation reporter (GMR) system to track changes in DNA methylation during cardiac differentiation. **Methods and Results:** The promoter region of *Cdk1* (Cyclin-dependent kinase 1) or *Sox2* (SRY-Box Transcription Factor 2) gene was cloned upstream of the small nuclear ribonucleoprotein polypeptide N (Snrpn) minimal promoter followed by a fluorescent reporter gene. Mouse induced pluripotent stem cells (iPSCs) carrying Sox2 GMR rapidly lost fluorescent reporter signal upon the induction of differentiation. Cdk1 GMR reporter signal was strong in undifferentiated iPSCs, and gradually decreased during directed cardiomyocyte (CM) differentiation. RT-qPCR and pyrosequencing demonstrated that the reduction of *Sox2* and *Cdk1* was regulated by hypermethylation of their CpG regions during cardiac differentiation. The present study demonstrated the dynamic DNA methylation along the course of cell cycle withdrawal during CM differentiation. **Conclusion:** The GMR reporter system can be a useful tool to monitor real-time epigenetic DNA modification at single-cell resolution.

KEYWORDS: cardiac differentiation; cell cycle; DNA methylation; epigenetics; GMR (genomic DNA methylation reporter); pyrosequencing; iPSC (induced pluripotent stem cell); single-cell imaging

36 **INTRODUCTION**

37 Epigenetic DNA methylation is highly associated with gene expression in development and
38 cellular functions such as cell cycle progression and differentiation. Methylation status of gDNA
39 can be assessed by a number of available methods, such as gene-specific analysis using
40 Pyrosequencing and PCR quantification after bisulfite conversion, or digestion-based assay
41 followed by PCR or qPCR, or whole-genome sequencing on similarly processed samples.[1] DNA
42 methylation studies have generated significant insights into static states of DNA methylation but
43 could not provide data on epigenetic perturbations in a real-time manner. A live-cell gDNA
44 methylation reporter system is essential in visualizing dynamic genomic methylation states, such
45 as in cardiac differentiation of induced pluripotent stem cells (iPSCs).

46 Mouse iPSCs have been widely used to study lineage differentiation, which is often associated
47 with a perturbation in the cell cycle activity, but the exact temporal dynamics remained unclear
48 for various lineages. Cardiomyocytes (CMs) withdraw from activity cell cycling shortly after
49 birth,[2] but dynamic epigenetic DNA methylation during cardiac differentiation remained to be
50 visualized.

51 The small nuclear ribonucleoprotein polypeptide N (*Snrpn*) gene is a maternally imprinted gene
52 that is sensitive to DNA methylation of adjacent genomic regions.[3] *Snrpn* mini promoter region
53 contains the conserved elements between human and mouse, with the endogenous imprinted
54 differentially-methylated region (DMR). The *Snrpn* mini promoter can be used as a methylation
55 sensor for real-time reporting of the DNA methylation state of adjacent sequences, such as CpG
56 regions of a particular gene.[4] In this study, we adopted this Snrpn mini-promoter-based
57 Genomics DNA Methylation Reporter (GMR) to track DNA methylation states of stemness gene

58 (Sox2; SRY-Box Transcription Factor 2) and cell cycle gene (Cdk1; cyclin-dependent kinase 1)
59 during CM differentiation from mouse iPSCs. Sox2 is a transcription factor that regulates the
60 pluripotency of stem cells. Its promoter region has a high density of CpG island (186 CpG sites;
61 1530 C+G counts), while the super enhancer region has a low density of CpG sequence. The
62 enhancer region is important for Sox2 mRNA and protein expression and the pluripotency of
63 embryonic stem cells.[5] In addition, developmental cardiomyocytes have a proliferative phase
64 followed by cell cycle withdrawal and terminal differentiation, which is in part dependent on
65 Cdk1. There are 29 CpGs in Cdk1's promoter region. Our study showed dynamic signals of
66 DNA methylation reporter in Cdk1 and Sox2 during mouse iPSC cardiac differentiation.
67

68 **MATERIALS AND METHODS**

69 **Mouse iPSC Cell Culture**

70 Mouse iPSCs were maintained in ESC medium with 2iL [MEK inhibitor (MEKi), GSK3
71 inhibitor (CHIR), and leukemia inhibitory factor (LIF)]. Stem Cell Medium was made of DMEM
72 (Gibco; # 11995065) supplemented with 20% FBS (Fetal Bovine Serum; Gibco; #10082147),
73 1% penicillin/streptomycin (Gibco; #15070063), 1% NEAA (Non-Essential Amino Acid; Gibco;
74 #11140050) and 0.1 mM beta-mercaptoethanol (Sigma-Aldrich). The medium was filtered
75 through a Millipore SteriCup 0.22 mm filtration system, and then added with 2iL [10^3 Units/mL
76 LIF (Millipore; #ESG1107), 1.0 μM MEKi (Stemgent; #PD0325901) and 1.5 μM CHIR (Selleck
77 Chemicals; #CHIR-99021)] before used in mouse iPSCs. All medium was prepared under sterile
78 conditions, and the cells were cultured in an incubator at 37°C and 5% CO_2.

79 **Construction of Cell Cycle GMR, and Generation of Reporter iPSC Cell Lines**

80 Sox2se-CPG-TV-dTomatto and Gapdh-CpG-TV-GFP plasmids (Addgene plasmid # 70155, and
81 70148) were a gift from Dr. Rudolf Jaenisch.[4] The promoter of mouse *Cdk1* gene that includes
82 29 CpG was cloned from wildtype C56/Bl6j mouse gDNA, using a primer pair: Cdk1-pro-F (5'-
83 ACG*GGTACC*AGCAATAGCCAGGTGGTGGTG-3') and Cdk1-pro-R (5'-
84 GAC*CAATTG*CATAAGGGATCCCGGACTTAG-3'). Then the PCR amplicon of *Cdk1* CpG
85 was digested by KpnI and MfeI (New England Biolabs) to remove the overhangs. The resultant
86 fragment was ligated to the Snrpn-dTomato backbone from Sox2se-CPG-TV, to generate the
87 Cdk1-Snrpn-dTomato plasmid, which was then amplified in MAX Efficiency DH5α Competent
88 Cells (Invitrogen).

89 To generate *Sox2* and *Cdk1* GMR reporter cell lines, we transfected Sox2se-CPG-TV-dTomato
90 and Cdk1-Snrpn-dTomato plasmids into mouse iPSCs using Lipofectamine 2000 Transfection
91 Reagent (Invitrogen) according to the provider's protocol. Twenty-four hours following
92 transfection, cells were treated with puromycin (0.75 μg/ml) for 3 days. Then the puromycin-
93 resistant cells carrying GMR genes were harvested and sorted with a BD Aria II cell sorter based
94 on single-cell tdTomato expression. Cells were cultured in 96-well plates containing irradiated
95 mouse embryonic fibroblasts (iMEFs) feeder layer. Single colonies were passaged and used in
96 subsequent analyses.

97 **Directed Cardiomyocyte Differentiation from Mouse iPSCs**

98 For cardiac differentiation assay, we adopted Yashiro's method with some modifications.[6] Mouse
99 Sox2 and Cdk1 GMR iPSC lines were maintained in Stem Cell Medium with 2iL for three days,
100 then harvested by trypsin dissociation and suspended in 2iL Stem Cell Medium, followed by
101 incubation in an incubator (5% CO_2, 37 °C) for 30 minutes to remove iMEFs. Cells were washed

102 twice with DPBS (Dulbecco's phosphate buffered saline, Gibco) before medium replacement.
103 The iPSCs were re-seeded into gelatin-coated 24-well plates at a density of 120,000 cells/well in
104 2iL ESC medium. After 8 hours, medium was changed to Serum-free (SF) differentiation
105 medium containing the followings: 75% Iscove's modified Dulbecco's medium (IMDM, Gibco),
106 25% Ham's F12 Nutrient Mix (Gibco), supplemented with 0.5% N-2 supplement (Gibco), 0.5%
107 B-27 supplement (Gibco), 0.05% bovine serum albumin (BSA, Fisher Scientific), 1% Pen-Strep
108 (Gibco), 2 mM L-glutamine (Gibco), 0.5 mM L-ascorbic acid (Sigma), and 0.45 mM 1-
109 Thioglycerol (Sigma); and cells were cultured for 2 days. It was counted as Day 0 when the
110 medium was changed to SF medium with 8 ng/mL recombinant Activin A (R&D Systems), 5
111 ng/mL recombinant human VEGF 165 Protein (R&D Systems) and 0.25 ng/mL recombinant
112 human BMP-4 Protein (R&D Systems). Cells were cultured for 28 hours; and then the medium
113 was changed to StemPro-34 Serum-free Medium (SFM; Gibco) supplemented with 2 mM L-
114 glutamine, 0.5 mM L-ascorbic acid (Sigma), 5 ng/ml recombinant human VEGF 165 protein
115 (R&D Systems), 10 ng/ml human bFGF (Invitrogen), and 50 ng/ml recombinant human FGF-10
116 protein, and cells were cultured for another 3.5 days. Subsequently, cells were harvested and
117 passaged into gelatin-coated 24-well plates in 500k cells/well density in StemPro-34 SF
118 supplemented medium. After 2 days, RPMI 1640 medium (with L-glutamine; Gibco) with B-27
119 Supplement (minus vitamin A; Gibco) was used for further cell culture of the differentiated
120 myocytes.

121 **Flow Cytometry**

122 Mouse iPSCs and differentiated cells in tissue cultures were dissociated by incubation with
123 Trypsin-EDTA (0.25%; Gibco) and then fixed with 4% paraformaldehyde and 0.2% saponin in
124 PBS (Gibco). The fixed cells could be stored in 10% DMSO in FBS at -80°C for future use.

125 Mouse iPSCs were stained with Oct4-AF488 (Millipore; # MAB4419A4) to verify the stemness
126 gene. The cells harvested on different days of cardiac differentiation were stained with mouse
127 anti-cardiac Troponin T (cTnT) antibody (Thermo Fisher Scientific; #MS-295-P; 1:200) and then
128 with Alexa Fluor 647-conjugated donkey anti-mouse IgG (H+L) secondary antibody (Thermo
129 Fisher; A31571; 1:1000). Immunostained cells were analyzed on a FACScan Canto RUO flow
130 cytometer (BD) and the data were analyzed with FlowJo software (FlowJo; Ashland, OR).[7-9]

131 **RT-qPCR**

132 To evaluate gene expression levels during iPSC differentiation, total RNA in frozen cells was
133 isolated using a RNeasy mini kit (Qiagen). The Maxima H-minus cDNA synthesis kit (Thermo
134 Fisher Scientific) with dT18 primer and random hexamer primer option for reverse transcription
135 was used to synthesize first strain cDNA from total RNA. SYBR Select Real-Time PCR Master
136 Mix (Thermo Fisher Scientific) was used in qPCR reactions that were performed on a
137 QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems). Raw data was collected,
138 processed, and exported using the accompanying QuantStudio 12K Flex software suite. Ct values
139 were normalized to that of Gapdh, and a comparative $2^{-\Delta\Delta Ct}$ method was used to evaluate relative
140 gene expression in differentiated cells compared to iPSCs. Relative Expression Software Tool
141 (REST; version 2.0; Qiagen) was used to assess the changes in gene expression. The primer
142 sequences for genes of interest are the followings: Gapdh (5'-
143 CAACTCCCACTCTTCCACCTTC-3' and 5'-TTGCTGTAGCCGTATTCATTGTC-3'); Cdk1
144 (5'-GGCGAGTTCTTCACAGAGACTTG-3' and 5'-CCCTATACTCCAGATGTCAACCGG-
145 3'); and Tnnt2 (5'-AGAGGACACCAAACCCAAGC-3' and 5'-
146 CGACGCTTTTCGATCCTGTC-3').

147 **Bisulfite Conversion of gDNA, and DNA Methylation (CpG) Analysis by Pyrosequencing**

148 Genomic DNA from flash-frozen cells was isolated using a Blood and Tissue DNeasy kit
149 (Qiagen). About 500 ng gDNA was used for CT bisulfite conversion with an EZ DNA
150 Methylation-Gold kit (Zymo Research; #D5005), following the manufacturer's recommended
151 protocol.

152 DNA methylation was measured by pyrosequencing on PCR products from bisulfite-treated gDNA.
153 PyroMark Assay Design (version 2.0.1; Qiagen) was used for PCR primer pairs and
154 pyrosequencing primer design against CpG regions around the super enhancer of Sox2 and the
155 promoter of Cdk1. The sequence of primers are followings: for Sox2 gene: forward primer: 5'
156 TGATTATAGGGAYGTGGGAGAATTTT 3', reverse primer: Biotin-5'
157 AAAAAACCCTCAAACCTAATAAAATATCTA 3', pyrosequencing primer: 5'
158 GGGAGAATTTTTTTTGGAG 3'); and for Cdk1: forward primer: 5'
159 TGGAAGGAAAATAGAGTTTAAGAGTTAG 3', reverse primer: Biotin-5'
160 ACTCACRCCATACCTCTCTATCCC 3', pyrosequencing primer: 5'
161 AGTTTTGATTGGTTTTTTTGA 3'). After primer quality verification, targeted CpG regions
162 were amplified with a ZymoTaq Premix kit (Zymo; #E2003). Subsequently, PCR amplicons were
163 sequenced with the pyroseq primers using a PyroMark Q24 DNA sequencer (Qiagen). The
164 accompanied PyroMark Q24 software (version 2.0.8; Qiagen) was used to assess the CpG
165 methylation level (percentage of the relative light unit (RLU) of the C/(C+T) peaks (C, methylated
166 cytosine; T, unmethylated cytosine). CpG sites with reliable CpG methylation measurement were
167 included in the calculation for averaged DNA methylation level for the sequenced CpG regions.

168 **Statistics**

169 After the derivation of GMR reporter clones, experiments were carried out with at least
170 biological replications, unless otherwise specified. Group data are expressed as Mean± SEM
171 unless otherwise specified. Difference between groups was assessed by t-test, with a P value
172 <0.05 considered a significant difference. ANOVA analysis were performed using GraphPad
173 Prism (version 10.2; GraphPad Software).

174 **RESULTS**

175 **Mouse iPSC with GMR for Sox2 and Cdk1 –** To test the utility of Snrpn mini promoter's
176 DNA methylation sensing function, we generated mouse iPSC lines stably carrying the Sox2-
177 GMR construct that is comprised of the Sox2 super enhancer CpG island region fused with the
178 Snrpn minimal promoter, followed by dTomato reporter (**Figure 1A**). iPSCs transfected with
179 Sox2-GMR were subjected to puromycin-resistant selection. Cells with high expression of
180 dTomato reporter signals were collected by fluorescence-activated cell sorting (FACS), and
181 single clones were expanded, among which clone C7 was used for subsequent experiments
182 (**Figure 1B**). Similar approaches were used to generate mouse Cdk1-GMR iPSC lines, which
183 stably carry Cdk1 promoter's CpG region, Snrpn minimal promoter and dTomato reporter.
184 Among the stable Cdk1-GMR iPSC lines, clone B9 was used for subsequent analyses. As
185 assessed by immunostaining and flow cytometry, the expression of stemness proteins such as
186 Oct4, Nanog, or Sox2 in GMR lines remained at levels comparable to wild-type mouse iPSC.
187 Fluorescent reporter signals in both Sox2 and Cdk1 GMR iPSCs were readily detected with
188 epifluorescent microscopy imaging.

189 **Diminished reporter signals in cells differentiated from Cdk1 and Sox2 GMR iPSCs –** Cell
190 differentiation is accompanied by epigenetic reprogramming that sets the landscape for

191 transcriptional activity for lineage specification, cell identity and cell-specific functions. As a
192 proof of concept for the utility of GMR, we examined the reporter signal and cardiac gene
193 expression in cells differentiated from the GMR iPSCs lines. By day 2.5 of directed cardiac
194 differentiation, dTomato reporter signals in both Sox2 GMR and Cdk1 GMR cells decreased
195 substantially (**Figure 2A**). By day 6.5 of differentiation, dTomato signal was minimally detected
196 in ~2.08% in Sox2 GMR cells, consistent with the loss of stemness in differentiated cells
197 assessed by flow cytometry on the gene expression of Sox2 and Oct4. dTomato signal in Cdk1
198 GMR cells also reduced significantly in differentiated cells (6.5 days) compared to iPSCs
199 (64.3%±7.1% compared to 95.5%±1.5%; n=3; $p<0.01$). By 3 weeks, there was barely detectably
200 dTomato signal from Sox2 GMR or Cdk1 GMR-derived cells that were mostly contracting
201 myocytes (**Supplemental Figure and Videos**) expressing cTnT as detected by immunostaining
202 and flow cytometry (**Figure 2B, C**).

203 **Correlations between GMR signals, gene expression, and DNA methylation –** To further
204 verify that the GMR system can be used to visualize epigenetic regulation of genes by DNA
205 methylation, we examined the correlations between GMR reporter signal, transcript expression,
206 and CpG methylation of stemness and cell cycle genes. We performed RT-qPCR on cells
207 differentiated from Cdk1 GMR iPSCs. *Tnnt2* was expressed and increased in myocytes at 13.5
208 days of differentiation as compared to earlier timepoints. On the other hand, the transcript *Cdk1*
209 increased in 4.5 day differentiated cells compared to Cdk1 GMR iPSC, then decreased: *Cdk1* in
210 differentiated CM at day 13.5 was reduced to only 25% level of that in undifferentiated iPSCs or
211 CMs at differentiation data 6.5 (**Figure 3A**). Pyrosequencing showed significantly upregulated
212 CpG DNA methylation levels (hypermethylation) for stemness gene Sox2 and cell cycle gene
213 Cdk1 in the Cdk1-GMR reporter cells subject to cardiac differentiation. Sox2 and Cdk1 DNA

214 methylation was at 10.1% and 2.1% in undifferentiated iPSCs, and became 51.8% and 10.6%,
215 respectively, after induction of cardiac differentiation (at 4.5 day); and reached to 62.4% and
216 12.3% at 2 weeks of cardiac differentiation (**Figure 3B**). These results demonstrated DNA
217 methylation during stem cell differentiation, reflective of the reporter profiles of GMR system
218 and the accompanying gene expression.

219 **DISCUSSION**

220 Epigenetic DNA and histone modifications, and subsequently the changes in chromatin
221 accessibility, are the critical mechanisms controlling gene expression, thereby affecting cellular
222 phenotype and function, such as differentiation and proliferation.[7,10-16] Several approaches can be
223 used to reveal the status of DNA methylation; for example, fluorescent proteins in conjugation
224 with methyl-CpG-binding domain (MBD) or Synthetic-molecule/protein hybrid probes,[17]
225 modular fluorescence complementation-based epigenetic biosensors that combine engineered
226 DNA-binding proteins with domains recognizing defined epigenetic marks, both fused to non-
227 fluorescent fragments of a fluorescent protein.[18] The live-cell reporter system leveraging the
228 mini-promoter of imprinted *Snrpn* gene to visualize the CpG island regions of a target gene
229 represents a unique and highly specific system with detailed temporal and spatial resolutions
230 critical in the study of DNA methylation dynamics.[4]

231 In this study, we confirmed the fidelity of the CpG-Snrpn-based GMR approach in mouse iPSC
232 and differentiated myocytes. It is important to note that the methylation status of genes that have
233 too short no CpG regions may not be reflective in this GMR system. C2c12 cell lines carrying
234 *Gapdh* GMR (GFP) reporter and Sox2SE GMR (tdTomato) were also used to verify the expected
235 GMR signals. In addition, there was no reporter signal in cells transfected with pre-methylated

236 Sox2 or Gapdh GMR gene constructs (Data not shown). Live cell imaging data showed that the
237 positive GMR reporter signal reflects well the transcript expression of the corresponding gene.
238 Differentiated cells, like cardiomyocytes, have slower proliferation, and the cell cycle can be
239 arrested shortly after birth.[2] Our data show that during early differentiation (4.5 days), there was
240 an increased expression of *Cdk1,* then reduced significantly in more mature cardiomyocytes
241 (e.g., 13.5 days). It may be plausible that there was a fast proliferative phase right after the
242 transition to cardiac mesoderm lineage, leading to the higher *Cdk1* transcript expression, and the
243 changes in transcription activity may be delayed behind the GMR reporter signal for that short
244 period.[19] A caveat of using this GMR is that it is a "negative-positive" system in which the
245 reporter signal represents hypomethylation rather than hypermethylation. Nonetheless, the GMR
246 system is still informative as the change in reporter profile is consistent with epigenetic
247 mechanism in which an increase in DNA methylation leads to lower transcript expression, as
248 verified in our integrated transcriptional-epigenetic analysis (Figure 3). Lastly, we recognized
249 that while cell cycle gene *Cdk1* is reduced, accompanied by significantly upregulation of CpG
250 methylation in the promoter, the percentage methylation is much lower than Sox2. This could in
251 part due to the fewer CpG sites in mouse Cdk1 gene compared to Sox2 super enhancer and
252 promoter. Indeed, when a gene has too short CpG region, like mouse Tnnt2, its sequence will not
253 elicit well GMR-reporter dynamics as confirmed in our separated experiments (data not shown).

254 We also postulate that some genes conserved for various stage of cell functions in different cell
255 types have multiple regulatory mechanisms to fine tune gene expression, such as by microRNA,[9]
256 and epigenetic histone modifications.[20,21] It is worth noting that Cdk1 itself can affect histone
257 epigenetic landscape as shown in mouse embryonic stem cells.[22] The GMR reporter system can
258 be used with both live cell reporters to decipher the intricate cellular processes such as lineage

259 specification and tracking (e.g., cardiomyocyte) and phenotype (e.g., myocyte maturity or cell
260 cycle/proliferation activities) and functions (e.g., myocyte electrical and contractile activities).

261 In summary, the *Snrpn* mini-promoter-based GMR system, such as for *Cdk1* and other cell cycle
262 genes, can be instrumental in reporting dynamic DNA methylation states during cell
263 differentiation and cell cycling. Combining the new GMR system with other visualization
264 technologies will likely further delineate the epigenetic-transcriptional regulations of various
265 cellular phenotypes and functions.

266 **Declaration of Competing Interest**

267 The authors declare no competing financial interests.

268

269 **AUTHOR CONTRIBUTIONS**

270 Y.Z. conceived and conceptualized the study. Y.Z. and N.L. designed and performed
271 experiments, collected, and analyzed data. B.T.N. contributed to gene expression analysis. Z.Z.
272 conducted experiments on cell culture. W.R.M. helped analyze the results and discussion. N.L.
273 and Y.Z. wrote the manuscript. All co-authors provided their edits to the manuscript.

274

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Ning Li[1,2], Ba Thong Nguyen[3,4*], Zhenhe Zhang[1*], W. Robb MacLellan,[1] Yiqiang Zhang[1,3,4¶]

[1] Division of Cardiology, Department of Medicine, University of Washington, Seattle, WA 98109, USA

[2] State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China

[3] Department of Anatomy, Biochemistry and Physiology, John A. Burns School of Medicine, University of Hawaii at Manoa, Honolulu, HI 96813, USA.

[4] Center for Cardiovascular Research, John A. Burns School of Medicine, University of Hawaii at Manoa, Honolulu, HI 96813, USA.

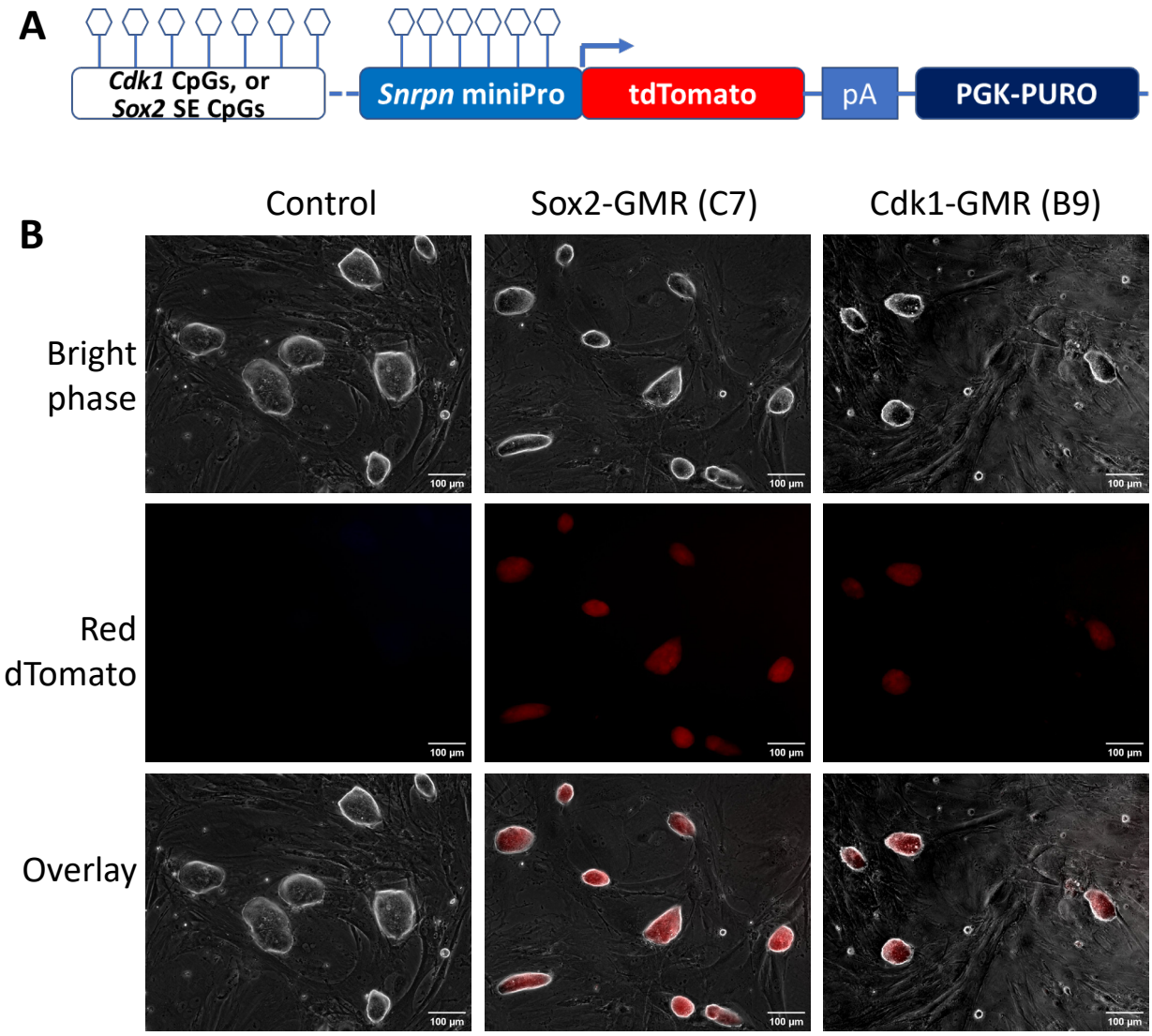
* Equal contribution.

¶ Author for Correspondence: Yiqiang Zhang, PhD (yiqiang.zhang@hawaii.edu).

Figures (3)

17 **Figures and Figure Legends**

18 **Figure 1.**



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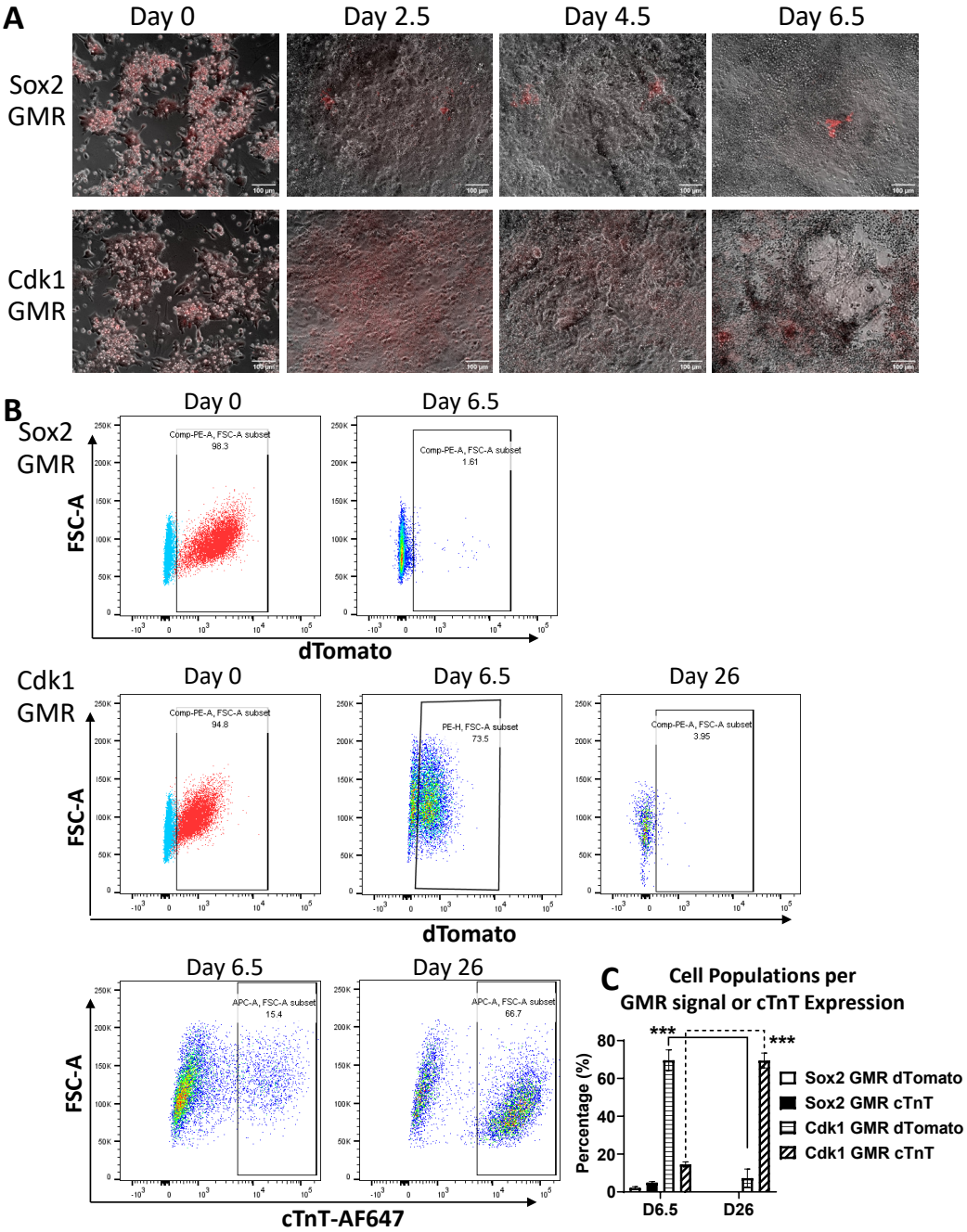
20 **Figure 1. Generation of mouse GMR iPSC lines. A**, Gene construction of Snrpn mini-
21 promoter based genomic DNA methylation reporter system. **B,** Microscopy images of wildtype
22 iPSC (control) and GMR iPSC lines for carrying GMR for Sox2 super enhancer or Cdk1
23 promoter's CpG regions.

24

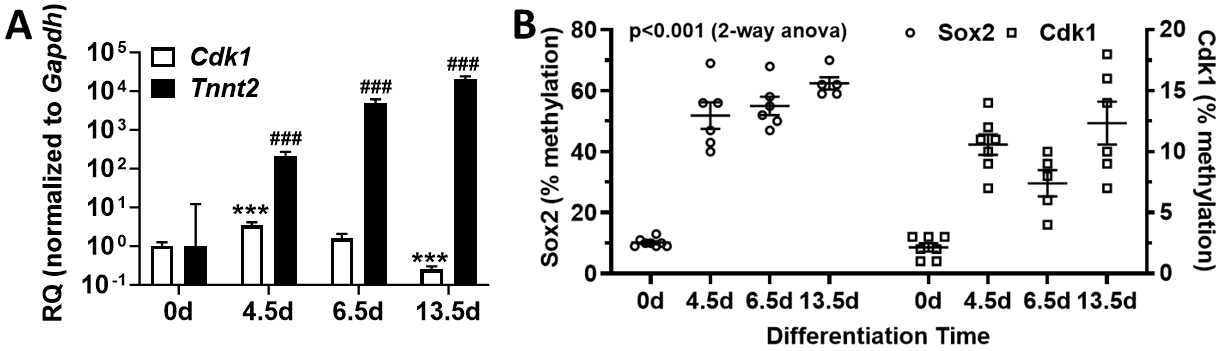
25 **Figure 2.**



26

27 **Figure 2. Dynamic reporter signal during cardiac differentiation of GMR iPSCs. A**,
28 Microscopy images of Sox2-GMR and Cdk1-GMR iPSCs (day 0) and differentiated cells. **B,**
29 Flow cytometry dot plots showing the signal of GMR reporter dTomato and cardiomyocyte
30 marker cTnT (cardiac troponin T). **C**, Cell population quantified by the expression of GMR
31 signal dTomato of cardiac myocyte marker cTnT.

32 **Figure 3.**



33

34 **Figure 3. Gene expression and DNA methylation during cardiac differentiation from Cdk1**
35 **GMR iPSCs. A**, Relative quantification of *Cdk1* and *Tnnt2* in undifferentiated iPSCs and
36 cardiac differentiation day 4.5, 6.5, and 13.5; as normalized to *Gapdh.* *** and ###, p<0.05 vs 0d
37 For *Cdk1* and *Tnnt2*, respectively; n=3. **B**, CpG DNA methylation in Sox2 and Cdk1 CpG
38 regions in Cdk1 GMR iPSCs (0d) or cells from directed cardiac differentiation for 4.5, 6.5, and
39 13.5 days. Data points are % methylation of CpG sites assessed with pyrosequencing assays.
40 p<0.001 (2-way ANOVA).