Inhibition of CDK5 Alleviates the Cardiac Phenotypes in Timothy Syndrome

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http://dx.doi.org/10.1016/j.stemcr.2017.05.028

SUMMARY

L-type calcium channel CaV1.2 plays an essential role in cardiac function. The gain-of-function mutations in CaV1.2 have been reported to be associated with Timothy syndrome, a disease characterized by QT prolongation and syndactyly. Previously we demonstrated that roscovitine, a cyclin-dependent kinase (CDK) inhibitor, could rescue the phenotypes in induced pluripotent stem cell-derived cardiomyocytes from Timothy syndrome patients. However, exactly how roscovitine rescued the phenotypes remained unclear. Here we report a mechanism potentially underlying the therapeutic effects of roscovitine on Timothy syndrome cardiomyocytes. Our results using roscovitine analogs and CDK inhibitors and constructs demonstrated that roscovitine exhibits its therapeutic effects in part by inhibiting CDK5. The outcomes of this study allowed us to identify a molecular mechanism whereby CaV1.2 channels are regulated by CDK5. This study provides insights into the regulation of cardiac calcium channels and the development of future therapeutics for Timothy syndrome patients.

INTRODUCTION

L-type calcium channel CaV1.2 plays an essential role in cardiac development and function (Flucher and Franzini-Armstrong, 1996; Seisenberger et al., 2000). The missense mutations in CACNA1C encoding CaV1.2 channel are associated with Timothy syndrome (TS), a multisystem disorder that features long-QT syndrome and syndactyly (Boczek et al., 2015; Hennessey et al., 2014; Papineau and Wilson, 2014; Splawski et al., 2004). TS patients are treated clinically with β-adrenergic blockers, calcium channel blockers, and sodium channel blockers (Jacobs et al., 2006; Shah et al., 2012). However, these regimens are insufficient to prevent lethal arrhythmias in TS patients (Corona-Rivera et al., 2015; Kawaida et al., 2016; Philipp and Rodriguez, 2016). Therefore, new therapeutics for TS are still needed. Previously, we found that roscovitine, a cyclin-dependent kinase (CDK) inhibitor, could rescue the phenotypes in human induced pluripotent stem cell (iPSC)-derived cardiomyocytes (CMs) and neurons from TS patients (Pasca et al., 2011; Song et al., 2015; Yazawa et al., 2011). However, the mechanisms whereby roscovitine restores the cardiac functions in TS CMs have not been fully elucidated. In this study, we sought to investigate the mechanisms underlying the beneficial effects of roscovitine on TS CMs and to identify additional therapeutic compounds for TS.

RESULTS

Roscovitine Analog and CDK Inhibitor Tests

To confirm the cause of this disease and obtain ideal controls for the TS iPSCs, we generated isogenic control iPSCs from the TS iPSCs using TALEN (transcription activator-like effector nuclease) technology, and characterized the isogenic control iPSCs (Figure S1). The isogenic control iPSCs demonstrated a normal karyotype and pluripotency, and the CMs derived from the isogenic control iPSCs showed regular calcium transients in calcium imaging and normal voltage-dependent inactivation percentage values in voltage-clamp recordings, which are comparable with the values in CMs derived from non-isogenic control iPSCs generated from skin fibroblasts of healthy donors (Figures S1A–S1J). To search for roscovitine analogs that are more potent or less toxic than roscovitine and explore the mechanisms underlying the effects of roscovitine on TS CMs, we tested 20 roscovitine analogs and four CDK inhibitors with different specificities against CDKs using a contraction assay with MATLAB-based analysis (Huebsch et al., 2015; Yazawa et al., 2011) and calcium imaging (Figure 1A). Two rounds of chemical test were conducted to examine the effects of the compounds. The first round of chemical testing was conducted using TS CM clusters isolated from the monolayer CMs to screen and identify the positive compounds that could increase the spontaneous beating rate and decrease the contraction irregularity of the TS CM clusters (Figures S2A–S2C and Table S1). The subsequent test was conducted using the intact monolayer CMs to validate the beneficial effects of the positive compounds on TS CMs and to eliminate the potential bias that could be caused by isolating the CMs from the original culture (Figures 1B–1D). From the chemical tests, we identified two roscovitine analogs, CR8 and Myoseverin-B, and two CDK inhibitors, PHA-793887 and DRF053, that had beneficial effects on TS CMs.
When we summarized the CDK targets of all positive compounds, it was found that four out of the five positive compounds have been reported to inhibit CDK5 (Bettayeb et al., 2008; Brasca et al., 2010; Meijer et al., 1997; Oumata et al., 2008) (Figures 1B, S2G, and S2H), suggesting that CDK5 could be involved in the molecular mechanisms underlying TS.

The Effects of CDK5 Inhibition on TS CMs

To examine whether CDK5 inhibition is beneficial for TS CMs, we first constructed a lentivirus containing the dominant negative (DN) mutant of CDK5. We used patch-clamp recordings and calcium imaging to assess the physiological properties of the TS CMs infected with the CDK5 DN lentivirus. The phenotypes of TS CMs include a delayed voltage-dependent inactivation of CaV1.2 channels, abnormal action potentials, and abnormal calcium transients (Song et al., 2015; Yazawa et al., 2011). The TS CMs with CDK5 DN expression demonstrated a significantly enhanced voltage-dependent inactivation of CaV1.2 channels compared with the CMs without CDK5 DN expression (Figures 2A–2C). Moreover, the expression of CDK5 DN significantly shortened the paced action potential duration and rescued the abnormal spontaneous action potentials in TS CMs (Figures 2D, 2E, S3A, and S3B; Table S3). In addition, we examined the effects of CDK5 DN expression on the calcium currents in TS CMs. CDK5 DN significantly reduced the late calcium currents in TS CMs (Figures S3C–S3E). Finally, CDK5 DN expression alleviated the abnormal spontaneous calcium transients and paced calcium transients, and significantly reduced the calcium transient duration and half decay time in the paced TS CMs (Figures 2F–2J and S3F–S3M). Overall, the results indicated that CDK5 DN expression could alleviate all the previously reported phenotypes in TS CMs.

Next, we examined the effect of roscovitine on the TS CMs infected with the CDK5 DN lentivirus, to investigate whether CDK5 inhibition partially accounts for the therapeutic effects of roscovitine on TS CMs. The results showed that roscovitine did not further enhance the voltage-dependent inactivation of CaV1.2 in TS CMs with CDK5 DN expression, indicating that CDK5 DN expression is sufficient to rescue the delayed voltage-dependent inactivation of CaV1.2 in TS CMs (Figures 2K and 2L). To validate our findings using another approach, we designed short hairpin RNA (shRNA) lentiviral constructs that target CDK5 and confirmed the knockdown efficiency of the constructs (Figures S4A–S4C). We then infected TS CMs with the CDK5 shRNA lentivirus and examined the effects of CDK5 shRNA expression on the reported phenotypes in TS CMs. CDK5 shRNA expression significantly enhanced the voltage-dependent inactivation of CaV1.2 in TS CMs (Figures 2M and 2N). In addition, CDK5 shRNA expression alleviated the abnormal spontaneous calcium transients in TS CMs (Figures S3M and S4D–S4F). The effects of CDK5 shRNA expression on TS CMs were thus

(Figures 1B–1D and S2; Table S1; Movie S1). When we summarized the CDK targets of all positive compounds, it was found that four out of the five positive compounds have been reported to inhibit CDK5 (Bettayeb et al., 2008; Brasca et al., 2010; Meijer et al., 1997; Oumata et al., 2008) (Figures 1B, S2G, and S2H), suggesting that CDK5 could be involved in the molecular mechanisms underlying TS.
similar to the effects of CDK5 DN expression on TS CMs, indicating that CDK5 inhibition is beneficial for TS CMs.

The Molecular Mechanism Underlying the Positive Effects of CDK5-Specific Inhibition on TS CMs

The positive effects of CDK5-specific inhibition on TS CMs prompted us to investigate its underlying mechanisms. CDK5 has been reported to phosphorylate serine or threonine in two consensus sequences, S/T-P-X-R/H/K and P-X-S/T-P (where X is any amino acid) (Dhariwala and Rajadhyaksha, 2008; Plattner et al., 2014). We examined the sequences of CaV1.2 channels and found five consensus sequences located at the II-III loop and the C terminus (C-term), which are conserved in both humans and rodents.
We generated plasmids containing FLAG-tagged full-length CaV1.2 and YFP-tagged CDK5 for a co-immunoprecipitation (coIP) assay. The coIP results demonstrated a binding of CDK5 with CaV1.2 (Figure 3B). Next, we generated FLAG-tagged II-III loop and FLAG-tagged C-term of CaV1.2 constructs to repeat the coIP assay, and validated the binding of CDK5 with the two fragments (Figure 3C).

To examine whether CDK5 phosphorylates CaV1.2, we designed an in vitro kinase assay. The wild-type II-III loop or the C-term of CaV1.2 was used as the substrates in this assay. We generated mutant II-III loop and mutant C-term constructs with substitutions of serine/threonine to glycine or alanine in all CDK5 consensus sequences as negative controls (Figures S4G and S4H). The phosphorylation of the substrates by CDK5 consumes ATP and produces ADP that is converted into luminescence. (E and F) Wild-type (WT) II-III loop and C terminus (C-term) were phosphorylated by CDK5. PHA-793887 (PHA) and the mutagenesis (II-III Mutant [MT]: S783G; C-term 4MT: S1742A/S1799A/S1882A/T1958A) blocked the phosphorylation (II-III: n = 3 for both PHA groups and n = 6 for WT and MT groups; C-term: n = 6 for both PHA groups and n = 9 for WT and MT groups). **p < 0.01, Student’s t test for WT versus MT/4MT; data are mean ± SEM.

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(Figure 3A). We generated plasmids containing FLAG-tagged full-length CaV1.2 and YFP-tagged CDK5 for a co-immunoprecipitation (coIP) assay. The coIP results demonstrated a binding of CDK5 with CaV1.2 (Figure 3B). Next, we generated FLAG-tagged II-III loop and FLAG-tagged C-term of CaV1.2 constructs to repeat the coIP assay, and validated the binding of CDK5 with the two fragments (Figure 3C).

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or C-term as the substrates. Moreover, the luminescence signal was significantly reduced when using mutant II-III loop or C-term as the substrates, compared with using wild-type II-III loop or C-term as the substrates in the kinase reactions. The results indicated the phosphorylation of the II-III loop and the C-term of CaV1.2 by CDK5 in vitro (Figures 3E and 3F). The remaining signals in the mutant II-III loop and C-term could result from the phosphorylation of p35 by CDK5 (Asada et al., 2012) and/or non-specific phosphorylation of some serine/threonine residues in the mutant II-III loop and C-term. To provide additional support for the in vitro biochemical results, we examined whether wild-type CDK5 overexpression alters CaV1.2 channel functions in control CMs. We observed that wild-type CDK5 overexpression significantly delayed the voltage-dependent inactivation of CaV1.2 (Figures 3G and 3H) and induced abnormal calcium transients in control CMs (Figure 3I). Taken together, the results demonstrated that CDK5 potentially affects CaV1.2 functions by direct binding and phosphorylation, and that CDK5 overexpression could result in delayed voltage-dependent inactivation of CaV1.2 in control CMs.

To further explore the signaling pathways underlying the effects of CDK5 inhibition on TS CMs, we measured the mRNA expression of CDK5 and its activator p35 (CDK5R1) and p39 (CDK5R2) in control and TS CMs. We found a significant increase in the mRNA expression of p35 (CDK5R1) in TS CMs compared with controls (Figures 4A and 4B). We next measured the expression of EGR1, a transcription factor that regulates p35 transcription (Harada et al., 2001; Shah and Lahiri, 2014), and observed a significant increase in EGR1 mRNA expression in TS CMs (Figure 4C). We found that the increased p35 (CDK5R1) mRNA expression led to an increased p35 protein expression and a hyperactivation of CDK5 in TS CMs (Figures 4D and S4I–S4K). We then examined the protein expression of ERK (Harada et al., 2001; Shah and Lahiri, 2014), the upstream regulator of EGR1. The results showed that the expression of phosphorylated ERK was increased in TS CMs, indicating an elevated ERK activity (Figures 4D and S4K). Previous reports have established a connection between an increased intracellular calcium concentration and ERK activation in CMs (Wheeler-Jones, 2005; Zarain-Herzberg et al., 2011). Therefore, one of the potential mechanisms in TS CMs is that excessive calcium influx through the mutant CaV1.2 channels causes an increase in ERK activity, resulting in a subsequent induction of EGR1 and an increase in p35 expression. The increased expression of p35 causes CDK5 hyperactivation, which enhances the delayed inactivation of the mutant CaV1.2 channels, leading to more severe phenotypes in the TS CMs. Thus, CDK5 inhibition using CDK5 inhibitors, DN, or shRNA alleviates the phenotypes in TS CMs (Figure 4E).

DISCUSSION

In this study, we used human CMs derived from the iPSCs generated from the skin fibroblasts of two TS patients to identify additional potential therapeutic compounds based on roscovitine, our previously reported lead compound for TS (Song et al., 2015; Yazawa et al., 2011). We tested the hypothesis that the beneficial effects of roscovitine on TS CMs are associated with its inhibitory effect on CDKs. We revealed a role of CDK5 in the pathogenesis of TS and a mechanism underlying the therapeutic effects of roscovitine on
TS CMs, whereby roscovitine exhibits its effects in part by inhibiting CDK5. Roscovitine has been reported to enhance the voltage-dependent inactivation of CaV1.2 with the TS mutation in a heterologous overexpression system (Yarotsky and Elmslie, 2007; Yarotsky et al., 2009, 2010). Compared with the heterologous system, using TS CMs derived from iPSCs allowed us to examine the effects of roscovitine in a more physiologically relevant cardiac environment. Patient-specific iPSCs provided us with the opportunity to identify key mediators such as CDK5, which is involved in the regulation of CaV1.2 channels in CMs. Although there are several recent updates that provide insights into the structural and functional changes in the CaV1.2 channels caused by the TS mutations (Dick et al., 2016; Li et al., 2016) and a few previous reports indicating the potential regulation of CaV1.2 by CDK5 in pancreatic β cells and a neuronal progenitor cell line (Furusawa et al., 2014; Wei et al., 2005), our findings demonstrate that CDK5 plays an important role in regulating CaV1.2 functions in CMs and that the inhibition of CDK5 is beneficial for TS CMs.

There are still some concerns in the studies using human iPSC-based disease models, one of which is that patient-specific iPSC-based models of long-QT syndrome demonstrate relatively prolonged action potentials (Itzhaki et al., 2011) relative to the patients’ QT intervals. Thus, the iPSC-derived CMs do not fully respond to physiologically relevant high pacing frequencies (1–2 Hz) in calcium imaging and action potential recordings. Another concern is that there might be a progression in the disease phenotypes in iPSC-based models of long-QT syndrome over time, and it would be difficult to identify the optimal time points to examine the effects of therapeutic candidates in the patient-specific CMs (See Supplemental Note).

Although we found the role of CDK5 in cardiac calcium channel regulation, exactly how CDK5 regulates CaV1.2 channels via phosphorylation in the II-III loop and C-term remains unclear. Therefore, further investigation is necessary to elucidate the molecular mechanisms by which CDK5 phosphorylates and regulates CaV1.2 channels in CMs. Moreover, based on the consensus sequences targeted by CDK5, CDK5 may have effects on the functions of other ion channels such as HCN4 and Nav1.5, which play important roles in cardiac function. This report provides insights into the molecular bases of cardiac calcium channel regulation and the development of future therapeutics for TS patients.

**EXPERIMENTAL PROCEDURES**

In brief, human iPSCs were cultured using a standard feeder-free protocol with Essential 8 medium and Getrex (Thermo-Fisher/Life Technologies) following the manufacturer’s instructions. Human control and patient iPSC lines were differentiated into CMs using the monolayer-based in vitro differentiation protocol (Song et al., 2015). Whole-cell patch-clamp recordings in single CM were conducted using standard methods with voltage and current-clamp modes for measuring L-type Ca²⁺/Ba²⁺ currents and action potentials, respectively. The statistics used for every figure are indicated in the corresponding figure legends. Student’s t test (paired or unpaired, two tailed) was conducted with the t test functions in Microsoft Excel software. One-way ANOVA with Bonferroni’s or Dunnett’s post hoc analyses for multiple comparisons was conducted with the GraphPad Prism software. All data meet the assumptions of the statistical tests. All samples used in this study were biological repeats, not technical repeats. We did not exclude any samples and results from the analyses in this study.

Additional experimental procedures and detailed methodology are described in Supplemental Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, Supplemental Note, four figures, three tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.05.028.

**AUTHOR CONTRIBUTIONS**

L.S. and M.Y. conceived of and designed the project, and analyzed all the data. L.S., S.E.P., Y.I., K.M., and M.Y. performed the experiments. L.S. and M.Y. wrote the manuscript. S.E.P., Y.I., and K.M. edited and proof-read the manuscript. All co-authors approved the submitted manuscript.

**ACKNOWLEDGMENTS**

We thank A.K. Rinderspacher for providing the chemical information of the Roscovitine analogs; and G. Pitt, R.S. Kass, R. Robinson, H. Colecraft, and S. Marx for helpful discussions. The flow cytometry experiment was performed in the CCTI Flow Cytometry Core at Columbia University, supported in part by the Office of the Director, NIH under awards S10OD020056. Financial support was provided by the Leona M. and Harry B. Helmsley Charitable Trust stem cell starter grant, NIH Pathway to Independent Award (R00HL11345), and startup funds from the Columbia Stem Cell Initiative and Department of Rehabilitation and Regenerative Medicine to M.Y. and the NIH Kirschstein-NRSA predoctoral fellowship (SF31HL131087) award to L.S.

Received: July 14, 2016
Revised: May 22, 2017
Accepted: May 23, 2017
Published: June 22, 2017

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Supplemental Information

Inhibition of CDK5 Alleviates the Cardiac Phenotypes in Timothy Syndrome

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Figure S1. Isogenic control iPSC generation and characterization, related to the result section named Roscovitine analog and CDK inhibitor tests. (A) The forward and reverse sequencing results confirmed the successful generation of isogenic control iPSC lines. (B) Phase contrast image of isogenic control iPSCs. Scale bar, 100μm. (C) A representative calcium transient trace from the single cardiomyocyte (CM) derived from the isogenic control iPSCs using the calcium indicator R-GECO1. The CMs demonstrated normal calcium transients. Y-axis, ΔF/F0 for R-GECO1. **Student’s t-test; n.s. not significant. (D) The current-voltage relationship and voltage-dependent inactivation percentage value for the non-isogenic control iPSCs or isogenic control iPSCs “1.0 (relative)” means that the data points were normalized to the corresponding peak current value to make the traces. (G, H) The current-voltage relationship and voltage-dependent inactivation percentage value quantification in CMs derived from isogenic control iPSCs and non-isogenic control iPSCs (n=12 for the isogenic control group from three lines and n=10 for the non-isogenic control group from two lines. Student’s t-test; n.s. not significant). (J) Teratoma formation with isogenic control clones. The hematoxylin and eosin staining demonstrated all three germ layers. Scale bar, 100μm. The replicates (n) are independent single cell biological replicates. See Table S2 for the detailed information of the iPSC lines used for each experiment.
Figure S2. A summary of the chemical tests of Roscovitine analogs and CDK inhibitors using Timothy syndrome cardiomyocytes, related to Figure 1. (A) Representative traces from the MATLAB-based analysis of contractions in Timothy syndrome (TS) cardiomyocyte (CM) clusters before treatment and 24 hours after the treatment of each positive compound. (B) The analysis of the beating rate of TS CM clusters before treatment and 24 hours after the treatment of each compound (n=24 for Ros, n=11 for CR8, n=12 for Myo-B, n=10 for PHA, n=11 for NSC43067 and n=17 for PD0332991 from one TS patient line. *P<0.05, ****P<0.005, ***P<0.001; Student’s t-test, paired). The beating rate value after treatment was normalized to the corresponding beating rate value before treatment for each sample in each group. Note that DRF053 compound was directly tested on monolayer TS CMs but not on TS CM clusters. (C) The analysis of the contraction irregularity of TS CMs before treatment and 24 hours after the treatment of each compound (n=16 for Ros, n=6 for CR8, n=10 for Myo-B, n=9 for PHA, n=10 for NSC43067 and n=6 for PD0332991 from one TS patient line. *P<0.05; Student’s t-test, paired). The irregularity value after treatment was normalized to the corresponding irregularity value before treatment for each sample in each group. Note that the DRF053 compound was directly tested on monolayer TS CMs but not on TS CM clusters. See also Figure 1D for additional tests of positive compounds on intact monolayer TS CMs. (D) Representative paced (0.5Hz) calcium transient traces of single TS CM before and after the treatment of PHA. 10μM PHA treatment for 30min alleviated the prolonged paced calcium transients in TS CMs. Blue dots indicate electrical pulses (2ms, bipolar pulse, 4 volts) and arrow heads indicate that the cell did not respond to a specific pulse. (E, F) The analysis of calcium transient duration and calcium transient half decay time in paced TS CMs before and 30 minutes after the treatment of PHA (n=36 from two lines). 10μM PHA treatment for 30min significantly reduced the calcium transient duration and calcium transient half decay time in the paced TS CMs (*P<0.05; Student’s t-test, paired; Data are mean ± s.e.m.). (G) The effects of the positive compounds on CDK5 activity. Ros, CR8, PHA and DRF053 reduced CDK5 activity in vitro at the dose of 50nM. Myo-B significantly inhibited CDK5 activity in vitro at 1μM (n=3 for Ros, CR8, PHA, Myo-B and DMSO control group, n=4 for DRF053; ***P<0.005, ****P<0.001; One-way ANOVA with Dunnett’s post-hoc analysis). (H) The effect of Myo-B on CDK5 activity at different doses. Myo-B exhibited an inhibitory effect on CDK5 at mM dose, but not μM dose (n=3 for each dose; Data are mean ± s.d.). Ros, Roscovitine. Myo-B, Myoseverin-B. PHA, PHA-793887. The replicates (n) for the data in A-F are independent biological replicates from multiple rounds of experiments. The replicates (n) for the data in G and H are independent sample replicates from multiple rounds of experiments. See Table S2 for the detailed information of the iPSC lines used for each experiment.
Figure S3. The expression of the dominant negative mutant of CDK5 alleviated the abnormal electrophysiological properties and the abnormal calcium transients in single Timothy syndrome cardiomyocyte, related to Figure 2. (A) Spontaneous action potentials in Timothy syndrome (TS) cardiomyocyte (CM) with and without CDK5 dominant negative (DN) expression. Arrowheads show putative delayed afterdepolarizations (DADs). Dashed lines show 0 mV. (B) Action potential duration quantification in TS CMs with (n=13) and without CDK5 DN expression (n=11). APD50 and APD90, action potential duration at 50% and 90% of repolarization. (C) Representative calcium current recordings in the control CM and the CDK5 DN lentivirus infected (+CDK5 DN) and uninfected TS CM. (D) Late calcium current percentage (defined by normalizing the calcium current intensity at 350ms from the peak to the peak current intensity) quantification in the control CMs (n=13 from three lines) and the TS CMs with (n=10 from two lines) and without CDK5 DN expression (n=14 from two lines; **P<0.01; n.s., not significant; One-way ANOVA with Bonferroni post-hoc). (E) The current-voltage relationships of the calcium current in the control CMs (Green, circle, n=13 from three lines) and the TS CMs with (Blue, square, n=10 from two lines) and without CDK5 DN expression (Red, triangle, n=14 from two lines) were statistically not significantly different. (F) Representative spontaneous calcium transient traces of single TS CM infected with the R-GECO1 lentivirus and the YFP lentivirus or the YFP-CDK5 DN (D144N, kinase dead) lentivirus. The expression of CDK5 DN alleviated the abnormal spontaneous calcium transients in TS CMs. Y-axis, ΔF/F0 for R-GECO1 transient traces of single TS CM infected with the R-GECO1 lentivirus and the YFP lentivirus or the YFP-CDK5 DN (D144N, kinase dead) DN expression (Red, triangle, n=10 from two lines) and without CDK5 DN expression (Green, circle, n=11 from three lines) and the TS CMs with (Blue, square, n=10 from two lines) and without CDK5 DN expression (Red, triangle, n=14 from two lines) were statistically not significantly different. (F) Representative spontaneous calcium transient traces of single TS CM infected with the R-GECO1 lentivirus and the YFP lentivirus or the YFP-CDK5 DN (D144N, kinase dead) lentivirus. The expression of CDK5 DN alleviated the abnormal spontaneous calcium transients in TS CMs. Y-axis, ΔF/F0 for R-GECO1 transient traces of single TS CM infected with the R-GECO1 lentivirus and the YFP lentivirus or the YFP-CDK5 DN (D144N, kinase dead) lentivirus. The expression of CDK5 DN alleviated the abnormal spontaneous calcium transients in TS CMs. Y-axis, ΔF/F0 for R-GECO1 transient traces of single TS CM infected with the R-GECO1 lentivirus and the YFP lentivirus or the YFP-CDK5 DN (D144N, kinase dead) lentivirus. The expression of CDK5 DN alleviated the abnormal spontaneous calcium transients in TS CMs. Y-axis, ΔF/F0 for R-GECO1 transient traces of single TS CM infected with the R-GECO1 lentivirus and the YFP lentivirus or the YFP-CDK5 DN (D144N, kinase dead) lentivirus. The expression of CDK5 DN alleviated the abnormal spontaneous calcium transients in TS CMs. Y-axis, ΔF/F0 for R-GECO1 transient traces of single TS CM infected with the R-GECO1 lentivirus and the YFP lentivirus or the YFP-CDK5 DN (D144N, kinase dead)}
Figure S4. The expression of CDK5 shRNA alleviated the abnormal calcium transients in single Timothy syndrome cardiomyocyte and the supplemental information for the in vitro kinase assay and western blot analysis, related to Figure 2, Figure 3 and Figure 4. (A) A scheme of the lentiviral CDK5 knockdown system. (B) qPCR results to confirm the CDK5 knockdown in HEK 293Ta cells (n=6 per group from three rounds of experiments; Data are mean ± s.e.m.). (C) Representative images of GFP-positive Timothy syndrome (TS) cardiomyocytes (CMs) with CDK5 shRNA in patch clamp recordings. Scale bar, 10μm. (D) Representative spontaneous calcium transient traces of single TS CM infected with the R-GECC01 lentivirus and the scrambled control shRNA lentivirus or the CDK5 shRNA lentivirus. The CDK5 shRNA expression alleviated the abnormal spontaneous calcium transients in TS CMs. Y-axis, AF/F0 for R-GECC01 (calcium fluorescent indicator). (E, F) The analysis of calcium transient half decay time and the percentage of cells with irregular timing of calcium transients in the TS CMs with scrambled shRNA expression (n=33 from two lines from three rounds of experiments) or CDK5 shRNA expression (n=31 from two lines from three rounds of experiments). The expression of CDK5 shRNA reduced the percentage of cells with irregular timing of calcium transients in three rounds of experiments and calcium transient half decay time in TS CMs (***P<0.01; Student’s t-test; Data are mean ± s.e.m.). (G) Co-immunoprecipitation (IP) was performed using FLAG antibody resins with HEK 293T cell lysates expressing YFP-CDK5 and Wild-type (WT) II-III loop or mutant (II-III MT: S783G) II-III loop or WT C-terminus (C-term) or mutant (4MT: S1742A/S1799A/S1882A/T1958A) C-term. Anti-(α-) human CDK5 and FLAG-tag antibodies were used for immuno-blotting (IB). Mutagenesis does not have any significant effect on the binding affinity of CDK5 to II-III loop and C-term. (H) The dose-response curve of PHA-793887 on the luminescence signal from the in vitro kinase assay with wild-type II-III loop as the substrates. The curve reached a plateau phase from the dose of 5μM PHA-793887 and there was a stable background luminescence signal in the kinase assay. Therefore, the luminescence values from the reactions using the wild-type substrates (II-III loop or C-term) with the addition of 5μM PHA-793887 were used to normalize the background in Figure 3E and Figure 3F. Data are mean ± s.d. (I) Endogenous CDK5 activity in CMs derived from control (Ctrl) iPSCs, embryonic stem cell line H9 and TS iPSCs. TS CMs demonstrated a significantly higher CDK5 activity compared with control CMs (n=11 for Ctrl, n=9 for H9 and n=9 for TS; *P<0.05, **P<0.01; n.s., not significant; One-way ANOVA with Bonferroni post-hoc; Data are mean ± s.e.m.). (J) 5 μM PHA-793887 (PHA) addition significantly reduced CDK5 activity in TS CM samples in the endogenous CDK5 activity assay (n=9 for TS group and n=8 for TS with PHA group; ***P<0.005; Student’s t-test; Data are mean ± s.e.m.). Data were normalized to the average value from TS CM samples without PHA addition. (K) The full western blot images of the cropped images showed in Figure 4D. The p35 band was determined according to the molecular weight and the band position of p35 in the previous pilot immunoblotting experiments. The molecular weight of the nonspecific band in this blotting of p35 was over 79 kDa and the band was not shown in the films with shorter exposure times. The replicates (n) are independent biological replicates from multiple rounds of experiments. See Table S2 for the detailed information of the iPSC lines used for each experiment.
**Table S1.** Summary of Roscovitine analog test, related to Figure 1 and Figure S2.

**Table S2.** Detailed information of the iPS cell lines used for each experiment, related to all Figures.

**Table S3.** Raw data from the paced action potential recordings from single TS cardiomyocyte with and without CDK5 DN expression, related to Figure 2.

**Movie S1.** Monolayer Timothy syndrome cardiomyocytes before the treatment of 2μM CR8 and two hours after the treatment of 2μM CR8. The movies were used for the contraction irregularity analysis in **Figure 1D.**
Table S2. Detailed information of the iPS cell lines used for each experiment, related to all Figures.

| Experiment                                      | Figure number | The information of the cell lines used for the experiment                                                                 |
|-------------------------------------------------|---------------|--------------------------------------------------------------------------------------------------------------------------|
| Contraction assay                               | Figure 1D     | TS monolayer cardiomyocytes were differentiated from clone TS1-E3-5.                                                         |
| Voltage-clamp recording & paced action potential | Figure 2A-2E & Figure 2K-2N | TS cardiomyocytes were differentiated from clone TS1-E3-5 and TS2-E7-1. Control cardiomyocytes were differentiated both from clone IM-E1-5 and NH-E5-4 and from an embryonic stem cell line H9. The samples were collected from three rounds of differentiation and viral infection. |
| Calcium imaging                                 | Figure 2F-2J & Figure S3I-S3L | TS cardiomyocytes were differentiated from clone TS1-E3-5 and TS2-E7-1. The samples were collected from three rounds of differentiation and viral infection. |
| Voltage-clamp recording                         | Figure 3G&3H  | The control cardiomyocytes differentiated from clone IM-E1-5 and NH-E5-4 were used. The samples were collected from four rounds of differentiation and viral infection. |
| Calcium imaging                                 | Figure 3I     | The control cardiomyocyte clusters differentiated from clone IM-E1-5 and NH-E1-1 were used. The samples were collected from two rounds of differentiation and viral infection. |
| Quantitative PCR                                 | Figure 4A-4C  | Control cardiomyocyte samples were collected from cardiomyocytes differentiated from non-isogenic iPS clone IM-E1-5 and NH-E1-1, and isogenic control iPS clone 1 and clone 2 that were generated from the TS iPS clones (TS1-E3-5 and TS2-E7-1). TS cardiomyocyte samples were differentiated from clone TS1-E3-5 and TS2-E7-1. |
| Western blot analysis                           | Figure 4D & Figure S4K | The experiments were repeated four times with different samples to examine p35 protein expression. The control cardiomyocyte samples were collected from clone IM-E1-5, NH-E5-4, and isogenic control clone 4. The experiments were repeated three times with different samples to examine ERK and phosphorylated ERK protein expression. The control cardiomyocyte samples were collected from clone IM-E1-5, NH-E5-4, and isogenic control clone 4. The samples were collected from four rounds of differentiation and viral infection. Consistent results were found in the experiments and representative images from one of the experiments were shown. |
| Calcium imaging                                 | Figure S1C-S1D | Control cardiomyocytes were differentiated from clone IM-E1-5 and NH-E5-4. Isogenic control cardiomyocytes were differentiated from isogenic control clone 1 and clone 2 that were generated from the TS iPSC clones (TS1-E3-5 and TS2-E7-1). TS cardiomyocyte samples were differentiated from clone TS1-E3-5 and TS2-E7-1. |
| Voltage-clamp recording                         | Figure S1F-S1H | Control cardiomyocytes were differentiated from clone IM-E1-5 and NH-E5-4. Isogenic control cardiomyocytes were differentiated from isogenic control clone 1, clone 2 and clone 4 that were generated from the TS iPSC clones (TS1-E3-5 and TS2-E7-1). TS cardiomyocyte samples were differentiated from clone TS1-E3-5 and TS2-E7-1. |
| Contraction assay                               | Figure S2A-S2C | TS cardiomyocyte clusters were differentiated from clone TS1-E3-5.                                                          |
| Calcium imaging                                 | Figure S2D-S2F | TS cardiomyocytes were differentiated from clone TS1-E3-5 and TS2-E7-1.                                                      |
| Spontaneous action potential recording & calcium | Figure S3A-S3B, Figure S3C-S3E | TS cardiomyocytes were differentiated from clone TS1-E3-5 and TS2-E7-1. Control cardiomyocytes were differentiated both from clone IM-E1-5 and NH-E5-4 and from an embryonic stem cell line H9. The samples were collected from three rounds of differentiation and viral infection. |
| Calcium imaging                                 | Figure S3F-S3M & Figure S3M | TS cardiomyocytes were differentiated from clone TS1-E3-5, TS2-E6-2 and TS2-E7-1. The samples were collected from three rounds of differentiation and viral infection. |
| Calcium imaging                                 | Figure S4D-S4F & Figure S3M | TS cardiomyocytes were differentiated from clone TS1-E3-5 and TS2-E7-1. The samples were collected from three rounds of differentiation and viral infection. |
| CDK5 activity assay                             | Figure S4I & S4J | TS cardiomyocytes were differentiated from clone TS1-E3-5 and TS2-E7-1. Control cardiomyocytes were differentiated both from clone IM-E1-5 and NH-E5-4 and from an embryonic stem cell line H9. The samples were collected from four rounds of differentiation and the experiments were conducted four times. |

Clone TS1-E3-5 was derived from one TS patient (TS1). Clone TS2-E6-2 and TS2-E7-1 were derived from another TS patient (TS2). Clone IM-E1-5 was derived from Human embryonic lung fibroblast IMR90 (ATCC). Clone NH-E1-1 and NH-E5-4 were derived from normal adult human dermal fibroblasts (NHDF, Life Technologies).

We confirmed that the data from clone TS1-E3-5 and TS2-E7-1 derived from two TS patients are homogeneous and the data from the two clones are combined as TS group in the experiments. We confirmed the data from the clone IM-E1-5, NH-E1-1 and NH-E5-4 derived from two commercially available healthy fibroblasts are homogeneous and the data from some of the clones are combined as control group in the experiments. We confirmed that cardiomyocytes derived from isogenic control iPSC clones were comparable to cardiomyocytes derived from non-isogenic control clones. In some experiments, the cardiomyocytes differentiated from isogenic control clones and non-isogenic control clones were both used as control group.

The detailed generation/characterization of the iPSC clones, the cardiac differentiation method and the use of the calcium indicator R-GECO1 for calcium imaging have been reported previously (Song et al., 2015). The names of each iPSK clones described here are the same with our previous publication.
Table S3. Raw data from the paced action potential recordings from single TS cardiomyocyte with and without CDK5 DN expression, related to Figure 2.

| Parameter                        | Group                                | Mean  | s.e.m. | n  |
|---------------------------------|--------------------------------------|-------|--------|----|
| Resting membrane potential (mV) | TS cardiomyocytes without CDK5 DN    | -50.6 | 3.57   | 8  |
|                                 | TS cardiomyocytes with CDK5 DN       | -53.0 | 4.08   | 10 |
| Peak amplitude (mV)             | TS cardiomyocytes without CDK5 DN    | 29.3  | 3.5    | 8  |
|                                 | TS cardiomyocytes with CDK5 DN       | 27.3  | 3.4    | 10 |
| APD90a (ms)                     | TS cardiomyocytes without CDK5 DN    | 1,624.4 | 182.0 | 8  |
|                                 | TS cardiomyocytes with CDK5 DN       | 801.3 | 93.7   | 10 |
| Time to peak (ms)               | TS cardiomyocytes without CDK5 DN    | 92.4  | 30.4   | 8  |
|                                 | TS cardiomyocytes with CDK5 DN       | 40.1  | 11.4   | 10 |
| Maximal upstroke velocity (mV/ms)| TS cardiomyocytes without CDK5 DN    | 42.4  | 1.84   | 8  |
|                                 | TS cardiomyocytes with CDK5 DN       | 40.0  | 2.25   | 10 |

aAPD90: Action Potential Duration at 90% of repolarization. The representative traces and APD90 quantification were shown in Figure 2D and 2E.
Supplemental experimental procedures:

Cell culture:
Control and Timothy syndrome induced pluripotent stem cells (iPSCs) were generated using a virus-free method from the skin fibroblasts and characterized as described previously (Song et al., 2015). Mycoplasma tests were conducted using MycoAlert (Lanza) when the iPSC lines were generated. Human embryonic stem cell (ESC) line H9 (#WA09, NIH cell line code #0062) was obtained from WiCell Research Institute, Inc. (August 8th, 2014, Agreement # 15-W0039) as an additional control human pluripotent stem cell line. iPSCs or the ESC line H9 were cultured with Essential 8 media with 100 unit/ml penicillin and 100 μg/ml streptomycin or plates or dishes (Corning) coated with Geltrex (#A14133-022, from Life Technologies) following the manufacturer's instruction. The iPSC lines or the ESC line H9 were maintained and passaged following the previously-reported protocol using dispase (Life technologies) (Song et al., 2015). Human iPSC-derived cardiomyocytes were maintained in EB5 media: DMEM/F12 medium with GlutaMAX (Life Technologies) containing 5% fetal bovine serum (FBS, Hyclone, Thermo Scientific), 1mM non-essential amino acids (Life Technologies), 0.1mM beta-mercaptoethanol (Sigma-Aldrich), 100 unit/ml penicillin and 100 μg/ml streptomycin. Human embryonic kidney (HEK) 293T cells (ATCC) and 293Ta cells (Lenti-Pac, GeneCopoeia) were cultured in Dulbecco’s Modified Eagle Media (DMEM) (Life Technologies) supplemented with 10% FBS, 2mM L-glutamine (Life Technologies), 100 unit/ml penicillin and 100 μg/ml streptomycin. All cells were cultured in HERAcell 150i (Thermo Scientific) incubators and culture procedures were conducted in the Biosafety Cabinet Class II type A2 tissue culture hoods (Labconco).

The generation of isogenic controls:
The transcription activator-like (TAL) sequences for the isogenic control generation were synthesized using the service from Life technologies. The ordered TAL binding sequences were TCCCTTCTACGGCATCAT corresponding to RVD (T)-HD-HD-HD-NG-HD-NG-HD-NI-HD-NN-HD-NI-HD-NG-HD-NI-NI-NI-HD-NG for hCACNA1C_TAL_R, and TGCCTCCTACCTATCATTC corresponding to RVD (T)-NN-HD-HD-HD-NI-HD-NG-NI-HD-NG-NI-NG-HD-NI-NG-NI-NI-NI-HD-NG-NI-NG-NI-NI-HD-NG-NI-NI-HD-NG-NI-NI-NI for hCACNA1C_TAL_L, where 'T' indicates the first binding repeat that is provided by the vector. The synthetic hCACNA1C_TAL_L and hCACNA1C_TAL_R were assembled from synthetic oligonucleotides and/or PCR products. Based on the manufacturer’s protocol, the fragments were cloned into TALtrunc_FokI using 100% sequence verified s ubfragments. The resulting plasmids were purified from transformed bacteria and quantified by UV spectroscopy (Nanovue Plus, G.E. Healthcare Life Sciences). The final constructs were confirmed by sequencing. The synthesized TAL sequences were released from the original vectors by digesting the vectors with NotI and HindIII. The isolated synthesized TAL sequences were sub-cloned into a pEF1a_MCS vector digested with NotI and HindIII. The pEF1α_MCS_TALEN_L and pEF1α_MCS_TALEN_R vectors were purified from transformed bacteria and quantified by UV spectroscopy. The final constructs were confirmed by sequencing. The donor DNA was cloned from the genomic DNA of a control iPSC line using the PCR reaction with Phusion polymerase (Thermo Scientific), a standard Phusion PCR protocol, and the primer set: Forward 5'-GTGAATGACAAGCTTACGTCAT-3' and Reverse 5'-GGAGGCTTTCAGGACTGTTG-3'. The PCR product (1.7 kb) was purified using the QIAquick PCR purification kit (Qiagen) and the sequence was confirmed by sequencing to match the normal CACNA1C gene sequence. The TALEN L plasmid, TALEN R plasmid, donor DNA and a green fluorescent protein (GFP) plasmid (pmaxGFP ® from Lonza or AAVS1-CAG-hrGFP, Addgene: #52344) were transduced into Timothy syndrome iPSCs cultured in 6 well plates at the ratio of 15:15:1:5 (3.6 μg DNA/well) using transfection with Lipofectamine LTX with an optimized protocol described previously (Song et al., 2015). The GFP positive cells were sorted using CFTI flow cytometry service at Columbia University and returned to culture in a hypoxia incubator (5% oxygen) for recovery. The recovered clones were expanded and stocked. Genomic DNA from each clone was isolated using DNeasy Blood and Tissue Kit (Qiagen) and the region encoding CACNA1C exon 8a was amplified using Phusion polymerase PCR and the primer set: Forward 5'-TACACTAATCATCATAGGGTTCAT-3' and Reverse 5'-TAGCGATTCCCAGTTTAGGTAC-3'. The PCR product (1.2 kb) was purified using the QIAquick PCR purification kit (Qiagen) and directly sequenced using the original forward primer and a new reverse primer (Ori-R-MY): 5'-CTCAAGGATAGACTTCTCTTGATGTT-3'. Positive clones from the screen were expanded and adapted to normoxia incubator (20% oxygen) for following experiments. For isogenic control generation, we used three Timothy
syndrome patient lines (clone#: TS1-E3-5, TS2-E6-2 and TS2-E7-1) derived from two independent patients and picked up 91 single cell clones for screening. We obtained 91 single cell clones using the protocol described above. Among the 91 clones, we identified 9 isogenic control clones in total from TS1-E3-5 and TS2-E7-1. No positive clone was identified from the 8 clones recovered from TS2-E6-2 following the protocol described above. Overall, the success rate is 9.9%. We used the isogenic control clone 1, 2, 3, 4 and 5 for gene expression profiling. We used clone 1, 2, 3 and 4 for karyotyping. We used the cardiomyocytes differentiated from clone 1 and clone 2 for calcium imaging and qPCR. We used the cardiomyocytes differentiated from clone 1, clone 2 and clone 4 for electrophysiological recordings. We used the cardiomyocytes differentiated from clone 1 and clone 4 for western blot experiments as described in Table S2.

The characterization of isogenic control iPSCs:
The karyotyping of isogenic control iPSCs was performed by WiCell (Wisconsin). For the RT–PCR of NANO G using the primer set (Forward 5'- CAGCCCCGATTCTTCCAACCAGTCCC-3' and Reverse 5'- CGGAAGATCCCCAGGCGGTACC-3'), RNA from the isogenic control iPSCs was prepared using the RNeasy Mini kit and RNase-Free DNase set (Qiagen). cDNA was synthesized from 1 μg RNA using the SuperScript III First-Strand Synthesis System for RT–PCR (Life Technologies). The cDNA (21 µl) was diluted with DNase-free water (Invitrogen) at 1:5 and 1 µl of the samples was used for conventional RT–PCR with Ex taq (Clontech/Takara Bio). The teratoma formation assays were performed following the previously-reported protocol (Song et al., 2015) and the H.E. staining was performed by the pathological staining service at Columbia University. For the teratoma formation assays, two 8-week-old severe combined immunodeficient (SCID) beige male mice (Charles River Laboratories) were used for each line and the teratoma formation was observed in all mice that were injected with the iPSCs. All animal protocols and handling for the teratoma formation assay were performed following the guidelines established by Columbia Institutional Animal Care and Use Committee under our approved protocol (for L.J.S. and M.Y., #AC-AAA5655). To examine the differentiation potentials of the isogenic control iPSCs, non-isogenic control iPSCs and Timothy syndrome iPSCs were imaged with the genetically encoded indicator R-GECO1 as reported previously (Song et al., 2015). The calcium transients of the cardiomyocytes derived from isogenic control iPSCs, non-isogenic control iPSCs and Timothy syndrome iPSCs were imaged with the genetically encoded indicator R-GECO1 as reported previously (Song et al., 2015). The Ba²⁺ current recordings were obtained from the cardiomyocytes derived from isogenic control iPSCs and non-isogenic control iPSCs with the procedure and the protocol described in the electrophysiology method section.

Preparation of chemical compounds:
(R)-Roscovitine (Purity: ≥ 98% (HPLC)) and the Roscovitine analogs (R)-CR8 (Purity: ≥ 95% (HPLC)), Myoseverin B (Purity: ≥ 97% (HPLC)), Purvalanol A (Purity: ≥ 98% (HPLC)), Purvalanol B (Purity: ≥ 97% (HPLC)), Olomoucine (Purity: ≥ 98% (HPLC)), N⁵-Isopropylolomoucine (Purity: ≥ 98% (HPLC)) and Bohemine (Purity: ≥ 95% (HPLC)) were purchased from Sigma Aldrich and all the rest of the Roscovitine analogs (13 in total) were purchased from ChemBridge Corp. The ChemBridge compounds with 7-digit name were from EXPRESS-Pick™ Collection for which the minimum purity was 90% and the identity was confirmed using 1H-NMR and/or LC-MS/ELSD. The ChemBridge compounds with 8-digit name were from the CORE Library for which the minimum purity was 85% and the identity was confirmed using LC-MS/ELSD. (R)-Roscovitine was dissolved in DMSO (dimethyl sulfoxide, Sigma-Aldrich, #276855-100ML). Except for Bohemine that was dissolved in methanol following manufacturer’s instruction, all the other Roscovitine analogs were dissolved in DMSO. For the cyclin-dependent kinase (CDK) inhibitors, PD0332991 (Purity: ≥ 98% (HPLC)) and NSC43067 (Purity: ≥ 98% (HPLC)) were purchased from Sigma Aldrich. (R)-DRF053 (Purity: ≥ 98% (HPLC)) was purchased from Abcam and PHA-793887 (Purity: ≥ 98% (HPLC)) was purchased from ApexBio. Except for PD0332991 that was dissolved in sterile distilled water, all the other CDK inhibitors were all dissolved in DMSO. The stock concentration for (R)-Roscovitine was 5mM and the stock concentration of all the other chemical compounds was 10mM. All the stock solutions were stored at -80°C and thawed only once after made.

The analysis of cardiomyocyte contractions for compound tests:
The working solution of each compound was made by diluting the stock solution in EB5 media to a final concentration of 5 μM except for (R)-CR8, which was diluted to a final concentration of 1 or 2 μM. 0.1% or less DMSO (or methanol) did not have any significant
effects on the phenotypes in Timothy syndrome cardiomyocytes. The contraction analysis was performed as reported previously (Yazawa et al., 2011). In brief, images were collected at a rate of 5 frames per second using the NIS-elements software (Nikon) and converted to multi-frame TIFF images for analysis using the NIS-elements viewer. The multiple-frame TIFF images were processed using the Image Processing Toolbox in MATLAB R2009b (Mathworks). Relative motion between successive frames was quantified by subtracting each frame from the preceding frame and summing across all pixels. Movement was calculated by plotting the relative motion over time. Contractions were detected as a peak of relative motion and a second, typically smaller peak, corresponded to the relaxation of the cardiomyocytes. Peaks were selected manually and the rate of contraction was measured. The length of time between contractions was also measured and contraction irregularity was measured by calculating the ratio of the standard deviation to the mean of the intervals between contractions. The Timothy syndrome cardiomyocytes were used for the tests at day 21-22 after cardiac differentiation. The movies were taken before the treatment, and 24 hours after the treatment of each compound from the Timothy syndrome cardiomyocyte clusters in the first round of test for Roscovitine analogs and CDK inhibitors. The movies were taken before the treatment, and 2 hours after the treatment of each positive compound from the intact monolayer Timothy syndrome cardiomyocytes for a second round of test to validate positive compounds. The compound DRF053 was directly tested in intact monolayer Timothy syndrome cardiomyocytes in the second round of test but it was not tested in the first round of test. The cardiomyocytes from one Timothy syndrome iPSC line (clone#: TS1-E3-5) were used mainly for the tests and the cardiomyocytes from another independent Timothy syndrome iPSC line (clone#: TS2-E7-1) were used for validation. The contraction rate and the irregularity of each sample before and after treatment were compared using paired Student’s t-test. The contraction rate and irregularity value of each sample after treatment were normalized to its respective value before treatment in the corresponding figures.

Plasmid construction and the preparation of lentiviruses:
The CDK5 cDNA was amplified from the cDNA samples of a control iPSC line using Phusion polymerase (Thermo Scientific) and with primer sets that allowed us to add restriction enzyme site NolI and Kozak sequence before the start codon and another site XhoI after the stop codon. The fragment was subcloned into a pcDNA3 vector (Invitrogen) that was digested with NolI and XhoI for the following generation of CDK5 WT and CDK5 dominant negative (DN) lentiviruses. For the generation of CDK5 dominant negative mutant (DN), the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent) was used to generate the mutation leading to the D144N mutation in CDK5 protein. The plasmid containing the CDK5 DN or CDK5 WT was used as the templates to amplify the CDK5 DN or CDK5 WT sequence using Phusion polymerase and the primer sets that allowed us to add restriction enzyme site EcoRI and Kozak sequence before the start codon and another site XhoI after the stop codon. The PCR products were subcloned into lentiviral vector that was prepared from LV-SD-Cre (Addgene, #12105, no longer available currently) digested with EcoRI and XhoI. XL-10 Gold competent cells (Agilent) transformed with the lentiviral LV-SD vectors were inoculated at 24-30 °C. The purified LV-SD vectors were transfected together with pCMV-dR8.2 dvpr and pCMV-VSV-G (Addgene #8455 & 8454) into HEK 293T cells for lentiviral production, following a protocol described previously (Song et al., 2015). The shRNA constructs for CDK5 were purchased from GeneCopoeia along with the Lenti-Pac FIV Expression Packaging Kit (FPK-LvTR-40). The knockdown efficiency of the shRNAs was examined and the scrambled shRNA (as a control) lentivirus and CDK5 shRNA lentivirus were prepared in the lentiviral 293Ta packaging cells (Lenti-Pac, #CLv-PK-01) purchased from GeneCopoeia, following the manufacturer’s instructions. The shRNA lentiviruses were concentrated 6 folds using the Lenti-X concentrator (Clontech) following the manufacturer’s instructions to infect the Timothy syndrome cardiomyocytes.
The FLAG-tagged full-length rat Cav1.2 plasmid, the FLAG-II-III loop plasmid and the FLAG-C-terminus plasmid were generated using conventional sub-cloning method using Phusion and PCR primers in pcDNA3 vector as described above. The QuikChange II XL Site-Directed Mutagenesis Kit was used to introduce the mutation(s) to the FLAG-II-III loop plasmid and the FLAG-C-terminus plasmid leading to S783G mutation in the II-III loop amino acid sequence and S1742A/S1799A/S1882A/T1958A mutations in the C-terminus amino acid sequence.

Electrophysiology:
The control and Timothy syndrome iPSCs were differentiated into cardiomyocytes following a protocol reported previously (Song et al., 2015). The cell clusters were isolated from the monolayer cardiomyocytes and re-plated into gelatin-coated wells in 6-well plates.
Calcium imaging and the analysis of calcium transient traces:

at day 16. For the experiments to examine the effects of CDK5 DN expression in Timothy syndrome cardiomyocytes, the cells were infected with the YFP-CDK5 DN lentivirus at day 19-21 after differentiation. For the experiments to examine the effects of CDK5 shRNA expression in Timothy syndrome cardiomyocytes, the cells were infected with CDK5 shRNA lentivirus at day 19-21 after differentiation. The CDK5 shRNA lentiviruses were concentrated using the lenti-X concentrator (Clontech) by 6 folds. The cells were dissociated into single cells for whole-cell patch clamp recordings at day 31 to 36 and plated into 35mm glass bottom culture dishes (7mm diameter No. 1.5 glass, MatTek, #P35G-1.5-7-C) coated with Geltrex. The patch-clamp recordings using cardiomyocytes infected with CDK5 DN construct were performed between day 35 and day 42 after differentiation while recordings using cardiomyocytes infected with CDK5 shRNA construct were conducted between day 40 and day 47. Whole-cell patch clamp recordings of iPSC-derived cardiomyocytes were conducted using a MultiClamp 700B patch-clamp amplifier (Molecular Devices) and an inverted microscope equipped with differential interface optics (Nikon, Ti-U). The glass pipettes were prepared using borosilicate glass (Sutter Instrument, BF150-110-10) using a micropipette puller (Sutter Instrument, Model P-97). Voltage-clamp measurements were conducted at room temperature (~24°C) using an extracellular solution consisting of 5mM BaCl2, 160mM TEA-Cl and 10mM HEPES (pH7.4 at 25 °C) and a pipette solution of 125mM CsCl, 0.1mM CaCl2, 10mM EGTA, 1mM MgCl2, 0.3mM TrisGTP and 10mM HEPES (pH 7.4 adjusted with CsOH at 25 °C). For calcium current recording, another extracellular solution was used: 5mM CaCl2, 137mM NMDG, 0.5 mM MgCl2, 25mM CsCl, 10mM TEA-Cl, 10mM glucose, and 10mM HEPES (pH7.4 adjusted with HCl at 25 °C). Two pulse protocols were used. One protocol was that cells were held at -90mV and then depolarized to -10mV for 400 ms at a rate of 0.1 Hz for the Ba2+ current recordings. The other protocol was that cells were held at -90mV and depolarized to -50mV for 2 seconds to eliminate the T-type current contamination, and then depolarized to +10/-10mV for 400 ms at a rate of 0.1 Hz for the Ca2+/Ba2+ current recordings to measure the L-type current; cells were held at -90 mV, stimulated with a 2-s family of pulses from -90 to +50/±20mV for the current-voltage relationship of the Ca2+/Ba2+ currents. The recordings were conducted under room temperature.

The voltage-dependent inactivation (percentage) is defined as the Ba2+ current intensity at 350ms from the peak divided by the peak current intensity in Figure 2B, Figure 2N, Figure 3H and Figure S1G. The late calcium current percentage is defined as the calcium current intensity at 350ms from the peak divided by the peak current intensity in Figure S3D. The data points to plot the representative traces in Figure 2A, Figure 2M, Figure 3G and Figure S1F were normalized to the respective peak current value of the trace and therefore labeled as “1.0 (relative)”. The values used to plot the current-voltage relationship of the Ba2+ current recordings (Figure 2C) and Ca2+ current recordings (Figure S3E) were normalized to their respective peak current value before being used for the grouped analysis. In Figure 2L, the voltage-dependent inactivation percentage values from the Timothy syndrome cardiomyocytes with CDK5 DN expression after the treatment with Roscovitine were normalized to the voltage-dependent inactivation percentage values from the Timothy syndrome cardiomyocytes with CDK5 DN expression before the treatment with Roscovitine, and therefore the Y-axis title was labeled as “Voltage-dependent inactivation (relative)”. (R)-Roscovitine stock solution was diluted with the extracellular solution into a working solution of 5μM and the same concentration of DMSO was used as a control.

Current-clamp recordings were conducted in normal Tyrode solution containing 140mM NaCl, 5.4mM KCl, 1mM MgCl2, 10mM glucose, 1.8mM CaCl2 and 10mM HEPES (pH7.4 with NaOH at 25 °C) using the pipette solution: 120mM K D-glucanote, 25mM KCl, 4mM MgATP, 2mM NaGTP, 4mM Na2-phospho-creatine, 10mM EGTA, 1mM CaCl2 and 10mM HEPES (pH 7.4 with HCl at 25 °C). The recordings were conducted at 37 °C. Cardiac action potentials were stimulated (5ms, 0.3nA) in current clamp mode at 37 °C (0.2 Hz). First, we paced the patient cardiomyocytes at 0.5 Hz or 1 Hz for action potential recordings and we found that the cardiomyocytes could not respond the pacing frequencies due to the prolonged action potential phenotype (>2 seconds). Therefore, we decided to use lower pacing frequency (0.2 Hz) and examined the effects of CDK5 DN expression on the paced action potentials in Timothy syndrome cardiomyocytes. Recorded action potentials were analyzed using Clampfit 10.4 (Axon Instruments). The spontaneous action potential duration (action potential duration at 50% of repolarization (APD50) and action potential duration at 90% of repolarization (APD90)) values from the Timothy syndrome cardiomyocytes with CDK5 DN expression in Figure S3B were normalized to the values from the Timothy syndrome cardiomyocytes without CDK5 DN expression, and therefore the Y-axis title was labeled as “Relative action potential duration”.

Calcium imaging and the analysis of calcium transient traces:
The Timothy syndrome iPSCs, non-isogenic control iPSCs and isogenic control iPSCs were differentiated into cardiomyocytes following a protocol reported previously (Song et al., 2015). The cell clusters were isolated from the monolayer cardiomyocytes and re-plated into gelatin-coated wells in 6-well plates at day 16 after differentiation. For the calcium imaging of the cardiomyocyte clusters derived from the non-isogenic control iPSCs, isogenic control iPSCs or Timothy syndrome iPSCs, and the cardiomyocyte clusters infected with the yellow fluorescent protein (YFP) lentivirus or the YFP-CDK5 WT lentivirus, the clusters were transferred to glass-bottom dishes (7mm diameter, No. 1.5 glass) coated with Geltrex at day 23 and the calcium imaging was conducted following the protocol described previously (Song et al., 2015) between day 25 to day 31. For the single cell imaging of the cardiomyocytes derived from the isogenic control iPSCs, the cell clusters were dissociated into single cells at day 40 and the imaging was conducted following the protocol described previously (Song et al., 2015) at day 43 and day 44. For the single cell imaging of the Timothy syndrome cardiomyocytes infected with the YFP lentivirus or the YFP-CDK5 DN lentivirus, the cells were infected with the lentiviruses of R-GECO1, a genetically encoded calcium indicator, and other corresponding lentiviral constructs at day 19-21 and dissociated into single cells at day 31 after differentiation. The calcium imaging was conducted following the protocol described previously (Song et al., 2015) between day 35 and day 42. For the single cell imaging of the Timothy syndrome cardiomyocytes infected with the R-GECO1 lentivirus and scrambled shRNA lentivirus or CDK5 shRNA lentivirus, the cells were infected with the R-GECO1 lentiviruses and other corresponding constructs at day 19-21 and dissociated into single cells at day 36 after differentiation. The scrambled shRNA or CDK5 shRNA lentiviruses were concentrated using the lenti-X concentrator (Clontech) by 6-fold and therefore it took one extra week for the cells to recover from the viral infection. The calcium imaging was conducted following the protocol described previously (Song et al., 2015) between day 40 and day 47. In brief, the Nikon Ti-U epi-fluorescent microscope connected to EMCCD Digital Monochrome camera (Evolve, EVO-512-M-FW-16-AC, 512x512, Photometrics) was used with LED light source (Lumencore Spectra X 6 line LED system, with Chroma excitation mounted filters, ET395/25x, ET470/30, ET550/15x, ET640/30x;) with dichroic mirror and emission filter (dichroic mirror, D/F/Cy3/Cy5pc; emission, ET430/36 513/44 595/41 719/105) operated by MetaFlour (Molecular Devices, acquisition: exposure time, 20 ms; gain 3 (6x); transfer speed, 5MHz, Image size: 512x512; binning, 1.0). The normal Tyrode solution (see “Electrophysiology”) with 10% FBS was used as bath solution. Temperature of the bath solution was maintained at approximately 37 °C using a digital temperature controller (TC-344B, Warner Instruments) for the duration of the experiment. Nikon objective lens 40x (CFI S Fluor, NA 0.90) for cell clusters and 60x (CFI Plan Apo Lambda H, NA 1.40) for single cells were used to capture the fluorescent signal from R-GECO1 (illumination intensity, 10 in 0-255, cy3) indicator. The calcium transient half (50%) decay time and the percentage of cells with irregular timing of calcium transients were analyzed. The calcium transient half decay time is defined as the average time interval between the point of the peak and the point at 50% decay of the peak in the group. The data for the percentage of cells with irregular timing of calcium transients was pooled from three rounds of experiment. The definition of a cell with irregular timing of calcium transients is that the time intervals between adjacent calcium transients from the cell were significantly different.

For the paced calcium transient recordings to examine the effects of CDK5 DN expression on the abnormal paced calcium transients in Timothy syndrome cardiomyocytes, the cardiomyocytes were prepared with the same experimental schedule as described above. The Nikon automatic microscope (Nikon Eclipse TiE with a motorized stage) connected to sCMOS camera (Andor Zyla sCMOS 4.2 MP) together with a stage top incubator (at 37°C, 5% CO₂ and 20% O₂, controlled by TOKAI HIT Hypoxia gas delivery system) were used for this experiment. Nikon objective lens 40x (Nikon CFI Plan Apo Lambda, NA 0.95) was used for single cell recordings and the normal Tyrode solution with 10% FBS was used as bath solution. A stimulus isolation unit (Warner instruments, SIU-102) and a perfusion insert with electric field stimulation for 35mm dish (Warner instruments, RC-37FS) were used for electrical pacing. The stimulus isolation unit was set at Bipolar pulse and 4 volts. The pulses were controlled by the Nikon NLS-element software and were given at a frequency of 0.5 Hz with a duration of 2ms. The parameters (Bipolar pulse, 4 volts, 2ms, 0.5 Hz) used for the experiments were first optimized using the control cardiomyocytes and control cardiomyocytes responded to the electrical pulses given with this set of parameters. Identical pacing parameters and experimental setting were used for the Timothy syndrome cardiomyocytes with YFP expression and the Timothy syndrome cardiomyocytes with YFP-CDK5 DN expression. The calcium transient duration, amplitude, integrated calcium transients and the calcium transient half (50%) decay time were analyzed. The calcium transient duration is defined as the average time interval between the starting point of the upstroke of the transient and the ending point by
which the transient decayed back to the baseline in the group. The amplitude of the transients was calculated as the percent change relative to the baseline fluorescence (Δ F/F0). Integrated calcium transients (average area under the curve (AUC) of the transients) were analyzed using the Graphpad prism software.

For the paced calcium transient recordings to examine the effects of PHA-793887 on the abnormal calcium transients in Timothy syndrome cardiomyocytes, the Timothy syndrome cardiomyocytes were isolated from the monolayer culture and re-plated into gelatin-coated wells in 6-well plates at day 16 after differentiation. The cells were dissociated at day 28 after differentiation and used for calcium imaging between day 31 to day 35. The Nikon automatic microscope with CO2 (5%) and temperature control (37°C) were used for this experiment. Nikon objective lens 40x was used for single cell recordings and the EB5 media was used as bath solution. The stimulus isolation unit and perfusion insert with electric field stimulation for 35mm dish were used for electrical pacing. The pacing parameters were Bipolar pulse, 4 volts, 2ms and 0.5Hz. The cells were loaded with Fluoforte calcium dye for calcium imaging following the manufacturer’s instruction (ENZ-51016, Enzo Life Sciences). A basal recording was acquired from each cell before the treatment of PHA-793887 and after the basal recording, the solution was changed to EB5 media with 10 μM PHA-793887. Thirty minutes after the treatment of PHA-793887, a second recording was acquired from the same cell. The calcium transient duration and the calcium transient half (50%) decay time from the recordings before and after treatment were analyzed. Paired student’s t-test was used for statistics.

Co-immunoprecipitation and western blot analysis:

Anti-ERK1/2 antibody (Mouse mAb, Catalog # 9107, Clone # 3A7, 1: 1,000 dilution, Cell Signaling), Anti-Phospho-ERK1/2 antibody (Rabbit mAb, Catalog # 4370, Clone # D13.14.4E, 1:2,000 dilution, Cell Signaling), Anti-p35 (Rabbit polyclonal Ab, Catalog # sc-820, Clone # C-19, 1: 1,000 dilution, Santa Cruz) and Anti-beta-Tubulin antibody (Mouse mAb, Catalog # T5201, Clone # TUB 2.1, 1:4,000 dilution, Sigma Aldrich) were used for Western blotting. Anti-FLAG antibody (Mouse mAb, Catalog # F3165, Clone # M2, 1:8,000 dilution, Sigma Aldrich) and Anti-CDK5 antibody (Rabbit mAb, Catalog # ab40773, Clone # EP716Y, 1: 1,000 dilution, Abcam) were used for the immunoblotting of the co-immunoprecipitation (IP) analysis.

For the co-IP, HEK 293T cells were plated at the density of 0.4 million/well in 6-well plates and transfected with 3.6 μg of the YFP-CaMKII plasmid and the plasmid of FLAG-tagged full length Cav1.2, wild-type (WT) II-III loop, mutant (MT) II-III loop, WT C-terminus or MT C-terminus (at the ratio of 1: 1) using Lipofectamine 2000 (Life technologies) following the manufacturer’s protocol 24 hours after plating. The cells were lysed 48 hours after the transfection with the cell lysis buffer containing 1% Triton X-100 (Sigma Aldrich), 50mM Tris-HCl, 150mM NaCl and 1X protease inhibitor cocktail (Catalog # P8340, Sigma-Aldrich), pH 7.4. The cell lysates were incubated with the Anti-FLAG M2 Affinity Gel (Catalog # A2220, Sigma-Aldrich) for 2 hours at 4°C for IP. The resins were then washed five times with TBS (Tris-based saline solution, 50mM TrisHCl, 150 mM NaCl, pH7.4) at 4°C and transferred to new tubes for immunoblotting. The sample buffer containing Urea (2x stock, 8M Urea, 40 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS), 10% beta-mercaptoethanol and 0.01% bromophenol blue) was added to the resins and the samples were boiled for 5 minutes at 95°C for denaturing the proteins. The samples were then loaded to Tris-HCl based SDS-PAGE gels with 5% stacking gel and 10% separation gel along with the ladder (Prestained SDS-PAGE standards, broad range, catalog # 161-0318, Biorad). The proteins were electro-transferred to PVDF-membranes (Invitronol™ PVDF, Catalog # LC2005, NOVEX by Life Technologies) using the XCell SureLock™ Mini-Cell system (Invitrogen) overnight at 4°C. Next day, the membranes were blocked with the SuperBlock Blocking Buffer in PBS (phosphate-buffered saline, Catalog # 27515, Thermo Fisher Scientific) for 30 minutes at room temperature (RT) and incubated with the primary antibody (diluted in the SuperBlock Blocking buffer) for 1 hour at RT followed by an incubation of the corresponding secondary antibody (Thermo Fisher Scientific, Pierce, anti-mouse #31430; anti-rabbit, #31460,1:8,000 dilution in the SuperBlock Blocking buffer) for 30 minutes at RT. The membranes were then incubated with the Pierce ECL western blotting substrate (Catalog # 32209, Thermo Fisher Scientific) followed by exposing to X-ray films (CL-X posure™ film, Catalog # 34091, Thermo Fisher Scientific) in a dark room. The co-IP experiments were repeated twice or three times to confirm the results and the representative images were shown in the corresponding figures.

For western blot analysis, the cardiomyocytes were collected at day 26 or day 27 after differentiation and lysed with cell lysis buffer containing 1% Triton X-100, 50mM Tris-HCl, 150mM NaCl, 250mM sucrose, 1x protease inhibitor cocktail, 1x phosphatase.
In vitro kinase assay:

To prepare the substrates, the HEK 293T cells were plated at the density of 2 million/dish in 100mm dishes and transfected with 18 µg of the plasmid containing the FLAG-tagged wild-type (WT), mutant (MT) II-III loop, WT or MT C-terminus using Lipofectamine 2000 following the manufacturer’s protocol 24 hours after plating. The cells were lysed 48 hours after the transfection with the cell lysis buffer containing 1% Triton X-100, 50mM Tris-HCl, 150mM NaCl and 1x protease inhibitor cocktail (Catalog # P8340, Sigma-Aldrich), pH 7.4, and then were incubated with the Anti-FLAG M2 Affinity Gel for 2 hours at 4°C. 35 µl packed resins were used for the cell lysate collected from one 100mm dish. After the incubation, the resins were washed three times with TBS at 4°C and twice with TBS at RT. The resins were distributed into multiple tubes and each tube contains 10 µl packed resins. For the kinase reactions, the 5X Reaction Buffer A, DTT (0.1M), CDK5/p35 (0.1µg/µl), ADP-Glo™ reagent, detection reagent, UltraPure ATP and ADP were purchased from Promega (CDK5/p35 kinase enzyme system, Catalog # V3271, ADP-Glo™ kinase assay, Catalog # V9101, Promega). The final kinase reaction mix contains 10 µl packed resins (substrate), 1X Reaction Buffer A, 50 µM DTT, 50 µM ATP, 0.1 µg CDK5/p35 in distilled water. The stock of PHA-793887 was diluted with DMSO and added to the corresponding samples with WT II-III loop or WT C-terminus as substrates in the PHA-793887 treated groups at the concentration of 0.5 or 5 µM. The same volume of DMSO was added to the rest of the samples to achieve the same concentration of DMSO in all the reactions. A series of samples for a standard curve were prepared based on the manufacturer’s instructions to determine the ATP-ADP conversion from the luminescence signals in every round of experiment. The kinase reaction tubes with the reaction mixes were incubated at 26-27°C for 60 minutes for the kinase reaction. The ADP-Glo™ reagent was then added to the reactions for an incubation of 40 minutes at 26-27°C to deplete the ATP in the reactions. Next the detection reagent was added to the reactions for an incubation of 45 minutes at 26-27°C. 20ul of the sample from each tube was then transferred into a 96 well microplate and the luminescence was measured with the GloMax® 96 Microplate Luminometer (Promega) with an integration time of 1.5s. The luminescence values were converted into the ATP-ADP conversion values based on the standard curve. All the ATP-ADP conversion values were normalized to the average ATP-ADP conversion value from the reactions with WT fragments as substrates and 5 µM PHA-793887 addition, in order to eliminate the background. The dose of PHA-793887 used to eliminate the background luminescence was determined based on the results from the pilot experiments using WT II-III loop as substrate to examine the background luminescence in the assay. Six doses (1 nM, 50 nM, 0.5 µM, 5 µM, 50 µM and 500 µM) of PHA-793887 were tested and a dose-response curve was shown in Figure S4H. The relative ATP-ADP conversion values (luminescence) that were normalized to the values from the reactions using WT fragments as substrates were used to make Figure 3E and Figure 3F. Two rounds of independent experiments were conducted for the data in Figure 3E and
three rounds of independent experiments were conducted for the data in Figure 3F.

**In vitro CDK5 activity assay:**

For the test of chemical compounds (Ros, CR8, PHA and Myo-B) on CDK5 activity, the CDK5 activity assay was conducted following the manufacturer’s protocol (CDK5/p35 kinase enzyme system, Catalog # V3271, ADP-Glo™ kinase assay, Catalog # V9101, Promega) with some modifications. The reaction mixes contain 1 μg Histone H1 (substrate), 1X Reaction Buffer A, 50 μM DTT, 50 μM ATP, 0.1 μg CDK5/p35 and 1ul compound solution or pure DMSO in distilled water. The compounds (Roscovitine, CR8, PHA-793887, DRF053 and Myoseverin B) were made and diluted as stock solutions in DMSO at different concentrations before testing. The final dose for Roscovitine, CR8, PHA-793887 and DRF053 in the reaction mix was 50nM and Myoseverin-B were tested at the dose of 50nM, 500nM, 1 μM, 10 μM, 100 μM, 1mM and 5mM. DMSO was added to the control (Ctrl) reaction mixes. The final concentration of DMSO for every reaction mix was 1%. A series of samples for a standard curve were prepared based on the manufacturer’s instructions to determine the ATP-ADP conversion from the luminescence signals in every round of experiment. The kinase reaction tubes with the reaction mixes were incubated at 26-27 °C for 60 minutes for the kinase reaction. The ADP-Glo™ reagent was then added to the reactions for an incubation of 40 minutes at 26-27 °C to deplete the ATP in the reactions. Next the detection reagent was added to the reactions for an incubation of 45 minutes at 26-27 °C. 20ul of the sample from each tube was then transferred into a 96 well microplate and the luminescence was measured with the GloMax® 96 Microplate Luminometer (Promega) with an integration time of 1.5s. The luminescence values were converted into the ATP-ADP conversion values based on the standard curve. The ATP-ADP conversion values normalized to control group (Luminescence) were used for data analysis in Figure S2G and Figure S2H.

To examine the endogenous CDK5 activity, an CDK5 activity assay was conducted following the previous reported protocols (Bu et al., 2002; Hallows et al., 2003) with some modifications. iPSC-derived cardiomyocytes differentiated from control iPSCs, embryonic stem cell line H9 and Timothy syndrome iPSCs were collected at day 26 or day 27 for the assay. Cardiomyocytes were isolated from monolayer culture and lysed with the cell lysis buffer containing 1% NP-40, 20mM Tris-HCl, 137mM NaCl, 1x protease inhibitor cocktail, 1x phosphatase inhibitor cocktail 3 and 1x phosphatase inhibitor cocktail, pH 7.4. The protein concentration in the samples was measured using a standard bicinchoninic acid (BCA) assay kit. 40 μg of proteins from each sample were aliquoted and used as one sample for CDK5 immunoprecipitation. The sample was incubated with CDK5 antibody-conjugated agarose beads (cdk5 (J-3) AC, catalog #:sc-6247 AC, Santa Cruz Biotechnology) for 2 hours at 4 °C (rocking continuously) for CDK5 immunoprecipitation. 5μl resuspended bead solution was used for the immunoprecipitation of CDK5 from each sample. After immunoprecipitation, the beads were washed three times with cold TBS and twice with room-temperature TBS. A reaction mix containing 1X Reaction Buffer A, 50 μM DTT, 50 μM ATP, 1 μg Histone H1 in distilled water was added to each sample for detecting CDK5 activity. The stock of PHA-793887 was diluted with DMSO and added to the corresponding samples in the PHA-793887 treated TS groups at the concentration of 5 μM. The same volume of DMSO was added to the rest of the samples to achieve the same concentration of DMSO in all the reactions. A series of samples for a standard curve were prepared based on the manufacturer’s instructions to determine the ATP-ADP conversion from the luminescence signals in every round of experiment. The kinase reaction tubes with the reaction mixes were incubated at 26-27 °C for 60 minutes for the kinase reaction. The ADP-Glo™ reagent was then added to the reactions for an incubation of 40 minutes at 26-27 °C to deplete the ATP in the reactions. Next the detection reagent was added to the reactions for an incubation of 45 minutes at 26-27 °C. 20ul of the sample from each tube was then transferred into a 96 well microplate and the luminescence was measured with the GloMax® 96 Microplate Luminometer (Promega) with an integration time of 1.5s. The luminescence values were converted into the ATP-ADP conversion values based on the standard curve. Four rounds of experiment were conducted. The values from each round of experiments were normalized to the average value of the control cardiomyocyte samples before being pooled together for the final analysis that is showed in Figure S4I. The values from each round of experiments were normalized to the average value of the Timothy syndrome cardiomyocyte samples without PHA addition before being pooled together for the final analysis that is showed in Figure S4J.

**Quantitative RT–PCR:**
RNA from the control and Timothy syndrome cardiomyocytes at day 19 after differentiation was prepared using the RNeasy Mini kit and RNase-Free DNase set (Qiagen). cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT–PCR (Life Technologies). The cDNA (21 μl) was diluted with DNase-free water (Invitrogen) at 1:1 and 1 μl of the samples was used for qPCR analysis. SYBR Advantage qPCR Premix (Clontech/TaKaRa Bio) and StepOnePlus real time PCR systems (Life Technologies) were used for qPCR. The primer sets for detecting the CDK5, CDK5R1 (p35), CDK5R2 (p39), EGR1 and GAPDH transcripts were as follows: CDK5 Forward 5’- GGCTTCAGGTCCCTGTGTAG-3’, Reverse 5’- ATGGTGACCTCGATCCTGAG-3’; CDK5R1 (p35), Forward 5’- ATGCATTGAATCCTTGAGCC-3’, Reverse 5’- CTTCTCCGACCTGAAGAACG-3’; CDK5R2 (p39), Forward 5’- GCACCTCAGTCGTCCAAAT-3’, Reverse 5’- CCACCTGTGTCAGCTTGCAT-3’; EGR1, Forward 5’- AGCCCTACGACCTGCAC-3’, Reverse 5’- GGAAAAGCGCCAGTATAGG-3’; GAPDH, Forward 5’- GATGACATCAAGAAGGTGGTA-3’, Reverse 5’- GTCTACATGGCAACTGTGAGG-3’. The C_T value of each sample at 50% of the amplification curve was used and GAPDH was used to normalize the expression of CDK5, CDK5R1 (p35), CDK5R2 (p39) and EGR1. All the samples are biological repeats from two to three rounds of independent cardiac differentiation.

Statistical analysis:
The statistics used for every figure have been indicated in the corresponding figure legends. The Student’s t-test (paired and unpaired) was conducted with the t-test function in Microsoft Excel software. F-test was conducted before the t-test to determine whether the samples have equal variance in Microsoft Excel software. The Student’s t-test was two tails. The One-way ANOVA with Bonferroni post-hoc or Dunnett’s post-hoc analyses for multiple comparisons were conducted with the Graphpad prism software. All the data meet the assumptions of the statistical tests. All the samples used in this study were biological repeats, not technical repeats. We did not exclude any samples or results from the analysis in this study.
Supplemental note:

The calcium imaging experiment to examine the effects of CDK5 shRNA expression on Timothy syndrome cardiomyocytes was conducted at later time points than the time points for the calcium imaging experiment to examine the effects of CDK5 DN expression on Timothy syndrome cardiomyocytes. This was because the infection efficiency of the scrambled shRNA lentiviruses and the CDK5 shRNA lentiviruses was moderate and we concentrated the scrambled shRNA lentiviruses and the CDK5 shRNA lentiviruses to infect Timothy syndrome cardiomyocytes for calcium imaging. Thus it took one extra week for the cardiomyocytes to recover from the viral infection with 6-fold concentrated lentiviruses. The calcium imaging of the Timothy syndrome cardiomyocytes with scrambled shRNA or CDK5 shRNA expression was therefore conducted one week later than the calcium imaging of the Timothy syndrome cardiomyocytes infected with YFP or YFP-CDK5 DN expression. We found that the disease phenotype progressed over time and there was an increase in the average calcium transient half-decay time and the percentage of single Timothy syndrome cardiomyocyte with very prolonged calcium transients in the scrambled shRNA group, compared with the YFP group, in Figure S3 and Figure S4. The beneficial effects of CDK5 inhibition and reduction on the abnormal calcium transients in Timothy syndrome cardiomyocytes were consistent, in spite of the different time points of the calcium imaging experiments.
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