Stress-activated kinase MKK7 governs epigenetics of cardiac repolarization for arrhythmia prevention

MKK7 deficiency induces ventricular arrhythmias

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Abstract

**Background**—Ventricular arrhythmia is a leading cause of cardiac mortality. Most antiarrhythmics present paradoxical pro-arrhythmic side effects, culminating in a greater risk of sudden death.

**Methods**—We describe a new regulatory mechanism linking mitogen-activated kinase kinase-7 (MKK7) deficiency with increased arrhythmia vulnerability in hypertrophied and failing hearts using mouse models harbouring MKK7 knockout or overexpression. The human relevance of this arrhythmogenic mechanism is evaluated in human induced pluripotent stem cells-derived cardiomyocytes (iPSC-CMs). Therapeutic potentials by targeting this mechanism are explored in the mouse models and human iPSC-CMs.

**Results**—Mechanistically, hypertrophic stress dampens expression and phosphorylation of MKK7. Such MKK7 deficiency leaves histone deacetylase-2 (HDAC2) unphosphorylated and filamin-A (FLNA) accumulated in the nucleus to form a complex with Krüppel-like factor-4 (KLF4). This complex leads to KLF4 disassociation from the promoter regions of multiple key potassium channel genes (Kv4.2, KChIP2, Kv1.5, ERG1 and Kir6.2) and reduction of their transcript levels. Consequent repolarization delays result in ventricular arrhythmias. Therapeutically, targeting the repressive function of the KLF4/HDAC2/FLNA complex with the HDAC2 inhibitor Valproic acid (VPA) restores K^+^ channel expression and alleviates ventricular arrhythmias in pathologically remodelled hearts.

**Conclusions**—Our findings unveil this new gene regulatory avenue as a new anti-arrhythmic target where repurposing of anti-epileptic drug VPA as an antiarrhythmic is supported.

**Keywords:** Arrhythmias, Heart failure, Gene regulation, Drug repurposing
Clinical perspective

1. What is new?

- We discover a new mechanism linking mitogen-activated kinase kinase-7 (MKK7) deficiency with increased arrhythmia vulnerability in pathologically remodelled hearts.
- Mechanistically, MKK7 deficiency in the hypertrophied heart leaves histone deacetylase-2 (HDAC2) unphosphorylated and filamin-A (FLNA) accumulated in the nucleus, which forms an association with Krüppel-like factor-4 (KLF4), preventing its transcriptional regulation of Kchip2, Kcnd2, Kcnh2, Kcna5 and Kcnj11.
- Diminished K+ channel reserve causes repolarization delays, resulting in ventricular arrhythmias.
- Targeting the KLF4/HDAC2/FLNA complex with the HDAC2 inhibitor Valproic acid (VPA) restores K+ channel expression, therefore alleviating ventricular arrhythmias.

2. What are the clinical implications?

- Supported by functional characterization and human relevant data, our study not only discovers a unique mechanism underlying arrhythmogenesis, but also demonstrates a repurposing possibility of an anti-epileptic drug VPA for anti-arrhythmic application.
- Wider pursuit of existing drugs for new therapeutic indications is anticipated to encounter a low regulatory hurdle and rapid progression to patient benefits, which is eagerly sought by healthcare systems.
- Furthermore, our work provides exciting insights in developing a new class of anti-arrhythmics specifically targeting signal transduction cascades to replenish repolarization reserve for the treatment of ventricular arrhythmias, a leading cause of cardiac mortality.
**Introduction**

Sudden cardiac arrest from ventricular arrhythmias accounts for approximately 1 in 5 deaths worldwide. A common mechanism underlying fatal ventricular arrhythmias is prolonged repolarization, measured on the ECG as increased QT interval\(^1\). Excessive lengthening of repolarization can incite early after-depolarisations (EADs). On a suitable substrate, this triggers action potentials that propagate aberrantly through re-entry circuits producing arrhythmias\(^2\).

Prolonged repolarization is common in congenital mutations of genes coding for ion-flux proteins (e.g. long QT syndrome)\(^3\)\(^–\)\(^5\), in adverse responses for various drugs (e.g. anti-arrhythmics, antipsychotics, antibiotics, antihistamines)\(^6\)\(^,\)\(^7\) and most prevalently, in acquired conditions like cardiac hypertrophy and heart failure\(^8\)\(^–\)\(^11\). In the last case, substantially impaired ‘repolarization reserve’ due to widespread ion-channel remodeling places the heart at a greater risk of life-threatening rhythm disorders.

Ventricular arrhythmia is difficult to cure, treat or even prevent. Current treatment choices include defibrillation, which is immensely useful for acute restoration of normal rhythm; catheter ablation, which is becoming more commonplace; and anti-arrhythmic drugs for adjunctive, maintenance, or preventive use depending on the clinical diagnosis\(^12\). Implantable defibrillators or ablation, despite significantly reducing mortality in indicated cases, come with inherent risks ranging from infection to invasive hazards, defibrillator storm, heart perforation, or even exacerbation of heart failure\(^13\)\(^,\)\(^14\). On the other hand, the outcome of anti-arrhythmic drug treatment is disappointing too, including in patients with failing hearts\(^15\)\(^,\)\(^16\). Apart from beta-blockers, most available anti-arrhythmics are primarily ion-channel blockers having paradoxical pro-arrhythmic side-effects that can be terminal for failing hearts. These pro-arrhythmic effects of anti-arrhythmics are difficult to control since they often
result from direct alteration of ion channel activities, the very mechanism of their therapeutic purpose\textsuperscript{16}.

Acknowledging this serious situation, enormous effort has been devoted to understanding the mechanisms underlying ion-channel expression and function, including transcriptional regulation, epigenetic make-up, mRNA processing, protein quality control and trafficking, and subunit assembly into macromolecular complexes\textsuperscript{17}. A new concept emerging is the targeting regulatory mechanisms to replenish repolarization reserve, which may hold the key to developing a completely new class of anti-arrhythmics with improved efficacy and a reliable safety profile.

We describe here a new regulatory mechanism linking MKK7 stress signaling with repression of cardiac potassium (K\textsuperscript{+}) channel expression and increased arrhythmia susceptibility. Targeting this signal transduction pathway with the class-I HDAC inhibitor Valproic acid (VPA) shows therapeutic benefit in alleviation of ventricular arrhythmias in animal models. Thus, we propose this new gene regulatory avenue as an anti-arrhythmic target and provide evidence in support of repurposing the anti-epileptic drug VPA as a novel anti-arrhythmic approach.

**Methods**

Detailed methods are available in the online Data Supplement.

**Animal models**

Mice and rats in this study were maintained in a pathogen-free facility at the University of Manchester. Animal studies were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and were approved by the University of Manchester Ethics Committee. The experimental protocol for non-human primate was approved by the Institutional Animal Care and Use Committee of Peking University and was in accordance with the principles of laboratory animal care of China National Academy of Sciences/National Research Council.
Valproic acid (VPA) treatment

VPA (200mg/kg/day, Sigma Aldrich P4543) or equal volume of water (vehicle) for 3 days along with transverse aortic constriction (TAC), or 4 days followed 3 days TAC, or 2 weeks followed 3 weeks TAC were applied to various mouse models, thereafter the mice were subjected to a range of assessments.

Single-cell electrophysiological study

Patch pipettes (Harvard apparatus) had resistances of 1.1-2.3mΩ for mouse cardiomyocyte or 5.5-6.5mΩ for human induced pluripotent stem cells-derived cardiomyocytes (iPSC-CMs) when filled with respective pipette solution. Current- and voltage-clamping were achieved in the whole cell configuration, and data were analyzed using pClamp software (version 10.6, Axon Instruments Inc.). Action potentials (APs) were elicited in current-clamp by injecting square pulses of 1-2ms width and 1.5 times of threshold current at 1Hz using current clamp. 100 consecutive APs were recorded at 37°C. Liquid junction potential of −12 mV was corrected offline in the AP data analysis.

Immunoprecipitation

To investigate the association of KLF4, HDAC2 and FLNA, immunoprecipitations were performed with Pierce Protein A/G Magnetic Beads (Thermo Scientific) following the manufacturer’s instruction.

Experimental design and statistical analysis

Sample sizes were calculated based on available comparable data to achieve 80% power at a 5% significance level. Data are expressed as mean ± S.E.M. and analyzed using Student’s t-test for comparisons between two groups or one- or two-way ANOVA with Bonferroni post-hoc tests for comparisons among multiple groups. P values p<0.05 were considered statistically significant. When measuring 19 ion channel genes, Bonferroni correction for multiple testing was applied.
Results

MKK7 deficiency is associated with stress-induced ventricular arrhythmias

Our previous work identified MKK7 as a critical signal transducer to prevent hypertrophic remodeling\textsuperscript{18}. In the current study we observed that MKK7 phosphorylation was increased along with acute stress by pressure overload (from 1 day to 7 days), and then its expression and phosphorylation level rescinded in chronic (5 weeks) pressure-overloaded mouse hearts. In a consistent observation, MKK7 abundance and phosphorylation were decreased in hypertrophied primate hearts (Supplementary Fig. 1), which clearly indicates MKK7 deficiency underlying pathological hypertrophy. We then tested whether its deficiency is involved in ventricular arrhythmias commonly harboured in hypertrophied and failing hearts. Cardiac MKK7-deficient (MKK7-CKO) mice were used to mimic the MKK7 deficiency of pathologically remodelled hearts. 8-10 week old male MKK7-CKO mice and their littermate controls (MKK7-Flox) were subjected to pressure overload by transverse aortic constriction (TAC) to investigate primary electrophysiological remodeling events preceding the development of overwhelming hypertrophy. After 3-day TAC, the MKK7-CKO hearts were enlarged but without overt functional failure (Supplementary Fig. 2). Interestingly, MKK7-CKO mice displayed prolonged rate-corrected QT intervals (QTc) in surface ECGs (Fig. 1A, Supplementary Fig. 3). Next, we examined whether such prolonged QTc correlated with arrhythmia vulnerability using programmed electrical stimulation (PES) applied to in-vivo and ex-vivo preparations of MKK7-CKO and MKK7-Flox hearts. In contrast to controls (0 of 10 mice), markedly high incidences of ventricular tachycardia (VT), often preceded by early after-depolarisations (EADs), were seen in MKK7-CKO hearts (5 out of 10 mice) (Fig. 1B-1C). Consistently, monophasic action potentials (MAPs) recorded from the ex-vivo Langendorff-perfused hearts of these mice showed prolonged
repolarisation at 90% action potential durations (APD$_{90}$) (Fig. 1D). APDs recorded from isolated single cardiomyocytes confirmed this prolongation (Fig. 1E). Furthermore, arrhythmia susceptibility was examined in response to adrenergic stimulation. MKK7-CKO and MKK7-Flox mice were challenged by mini-pump delivery of Isoprenaline (ISO, 10mg/kg/day) for 2 weeks. Prolonged APD$_{90}$ and frequent arrhythmic events induced by the S1S2 extrastimulus-pacing programme were recorded in Langendorff-perfused MKK7-CKO hearts (5 out of 6 hearts). Conversely, no MKK7-Flox hearts displayed frequent arrhythmia episodes and their MAP durations were in the normal range (Supplementary Fig. 4).

A gain-of-function model of cardiac-specific MKK7 transgenic (MKK7-Tg) mice was brought in to further assess the link between MKK7 and arrhythmogenesis. As anticipated, none of the MKK7-Tg mice displayed arrhythmic episodes after 3-day or 7-day TAC stress (Supplementary Fig. 5A). Their MAP durations and cardiac function remained within the normal range (Supplementary Fig. 5B-5E). We then stressed MKK7-Tg mice more aggressively to evaluate the protective role of MKK7 against hypertrophy and arrhythmia by 5 weeks of TAC. In contrast to wild-type control mice, which developed considerably hypertrophic remodeling attendant with impaired cardiac function and increased arrhythmia sensitivity, MKK7-Tg mice manifested strong resilience against pathological hypertrophic remodeling with preserved contractility (Supplementary Fig. 5F-5G). MKK7-Tg mice exhibited no sign of ventricular tachycardia and action potentials remained normal (Supplementary Fig. 5A).

The cumulative evidence suggested a pro-arrhythmic affiliation of MKK7 deficiency and its protective role against arrhythmias, which led us to study the upstream signaling pathways underlying the acquired ventricular arrhythmia susceptibility of remodelled hearts.
MKK7 controls KLF4-HDAC2 signaling regulation of K⁺ channel expression

To discern the signaling mechanism responsible for delayed repolarization, we measured a wide range of ion-channels and found significant down-regulation of *Kcnip2* (coding for KChIP2), *Kcnd2* (coding for Kv4.2), *Kcnh2* (coding for ERG1), *Kcna5* (coding for Kv1.5) and *Kcnj11* (coding for Kir6.2) in the TAC stressed or ISO challenged MKK7-CKO myocardium but not in the MKK7-Tg counterpart (Fig. 2A and Supplementary Fig. 6A-6B). In addition, reduced mRNA levels of the K⁺ channels were also observed in the C57BL/6N hearts after 5-week TAC, as well as in the hypertrophied primate hearts (Supplementary Fig. 6C-6D). Furthermore, expression and phosphorylation of MKK7 were examined in 3-day TAC stressed MKK7-Flox hearts versus MKK7-CKO hearts (Supplementary Fig. 7). Meanwhile KChIP2 was chosen as a candidate to evaluate its protein expression, and we found a reduced protein level correlated with its decreased mRNA expression (Supplementary Fig. 7). Importantly, transcript levels of other ion transporters crucial for cardiac action potential and Ca²⁺ cycling remained unchanged (Supplementary Fig. 8). The reduction in the selective K⁺ channel genes was recapitulated in neonatal rat cardiomyocytes (NRCMs) as a consequence of stress following MKK7 knockdown (Supplementary Fig. 9). In-silico analysis revealed a cluster of consensus Krüppel-like factor (KLF) binding sites on the proximal promoter regions of the 5 K⁺ channel coding genes (Supplementary Fig. 10) and we found that the stress-responsive KLF isoform 4 (KLF4) influenced their transcript levels. Shown in Fig. 2B, knockdown of KLF4 caused a down-regulation of the K⁺ channel expression in NRCMs stressed by ISO (10µM). Consistently, a similar reduction in expression of the K⁺ channels was detected in KLF4 cardiac-deleted (KLF-CKO) mouse hearts after 3-day TAC (Fig. 2C). As a consequence, QTc was 33% prolonger in the KLF4-CKO hearts (111.94 ± 12.76 ms) in comparison with the αMHC-CRE controls (75.12
± 3.23 ms) (Fig. 2D). To ascertain the transcriptional role of KLF4 on these 5 K+ channels, we chose Kcnip2 and Kcnh2 for quantitative chromatin immunoprecipitation (ChIP) assays. ChIP analyses of ventricular extracts showed considerably reduced binding of KLF4 at the promoter regions of Kcnip2 and Kcnh2 of the MKK7-CKO hearts following 3-day TAC (Fig. 2E). Meanwhile, we also discovered that local H3 acetylation (H3ac) in the promoter regions of Kcnip2 and Kcnh2 was markedly decreased in TAC-stressed MKK7-CKO hearts (Fig. 2E). This reduction in H3ac suggested a gene repression mechanism involving histone deacetylases (HDACs). Given the pro-hypertrophic effects of class-I HDACs19–21, we probed for the prospective involvement of HDAC1 and/or HDAC2. Subsequent immunoprecipitation experiments clearly demonstrated that endogenous HDAC2, but not HDAC1, was associated with KLF4 in both 3-day TAC-stressed MKK7-CKO myocardium and ISO-treated MKK7-deficient NRCMs (Fig. 2F). Meanwhile we did not find HDAC2 association with KLF15, another cardiac-active KLF isoform (Fig. 2F). Of note, HDAC2 binding at the promoter regions of Kcnip2 and Kcnh2 in the MKK7-CKO hearts after 3-day TAC was not changed by ChIP (Fig. 2G). Interestingly, HDAC2 phosphorylation status regulated its interaction with KLF4. We also observed that phosphorylation of JNK1/2 and HDAC2 was increased following hypertrophic stimuli (Supplementary Fig. 11). Furthermore, with over-expression of JNK1 in NRCMs, HDAC2 became profoundly phosphorylated, thereby disrupting its association with KLF4 regardless of the absence of MKK7 (Fig. 2H). Collectively, these data illustrate that MKK7 deficiency leads to loss of HDAC2 phosphorylation. Unphosphorylated HDAC2 then associates with KLF4 for leading to reduced K+ channel transcript levels. HACD2 per se does not directly bind with the promoter regions of the 5 K+ channel genes.
Filamin A as scaffold for KLF4-HDAC2 association

In hypertrophied hearts, structural remodeling caused by inflammation, apoptosis and fibrosis can contribute toward arrhythmogenesis. We next investigated the molecular extent of myocardial remodeling and screened for possible alterations in cytoskeleton proteins, such as actin, actinin, tubulin and filamin A (FLNA); fibrotic marker α-smooth muscle actin (SMA); apoptotic markers such as Bcl2, Bax, and Bad; and inflammation markers TGFβ1 and Smad2 (Fig. 3A). Intriguingly, we found that protein expression of FLNA, but not its transcript, was substantially higher in MKK7-CKO hearts of 3-day TAC, and that this increase in FLNA protein was in the nuclear fraction (Fig. 3A-3C). This prompted us to test whether increased FLNA might be involved in the association of KLF4 and HDCA2. Indeed, we found FLNA interacted with both KLF4 and HDAC2 by co-immunoprecipitation and proximity ligation assay (Fig. 3D and Supplementary Fig. 12). Of note, such an interaction of FLNA with KLF4 and HDAC2 was not detected in myocardium of MKK7-Tg mice after 3-day or 7-day TAC (Supplementary Fig. 13). When we knocked down FLNA in MKK7-deficient NRCMs, HDAC2-KLF4 failed to associate despite unchanged protein levels (Fig. 3E). Strikingly, transcript levels of the 5 K⁺ channel genes were increased in the context of FLNA knockdown (Fig. 3F). Double knockdown of FLNA and HDAC2 did not give additive effects on restoring the 5 K⁺ channel expression (Fig. 3F), consistent with them acting through the same pathway. We explored further if the MKK7/JNK pathway is involved in FLNA protein turnover with over-expression of JNK1 in MKK7-deficient NRCMs. We observed that JNK1 over-expression led to a reduction of accumulated FLNA expression (Fig. 3G). Cysteine protease inhibitor (E-64D), but not proteasome inhibitor (MG132), prevented such a reduction in presence of protein biosynthesis inhibitor Cycloheximide (Fig. 3H). Furthermore, we performed immunoprecipitation with a series of FLNA fragments to locate FLNA direct binding
with KLF4 and HDAC2, respectively, and found that FLNA (aa2196-aa2436) was required for binding with KLF4, whereas fragment (aa1716-aa1955) was in association with HDAC2 (Fig. 3I). These results suggest that FLNA protein turnover is subject to MKK7/JNK1 regulation via a proteolytic mechanism, and that in the absence of MKK7, FLNA accumulates in the nucleus and acts as scaffold for HDAC2-KLF4 association to repress K\(^+\) channel expression.

**The HDAC2 inhibitor Valproic acid alleviated ventricular arrhythmias**

Finally, we attempted translation of the above-described signaling mechanism to derive therapeutic anti-arrhythmic effects. We reviewed the available cluster of class-I HDAC inhibitors, and were attracted to Valproic acid (VPA), a widely used anti-epileptic drug with a sound safety record showing substantial HDAC inhibitory effect\(^{22,23}\). VPA not only inhibited HDAC enzyme activity by 34%, but also significantly reduced HDAC2 expression, but not HDAC1, 3 and 8 expression in NRCMs and myocardium (Fig. 4A-4B). In-vitro attempts to restore the K\(^+\) channel mRNA levels in NRCMs were promising as they were up-regulated by VPA at an optimal dose of 20mM in MKK7-deficient NRCMs under ISO stimulation (Fig. 4C). Of note, VPA treatment did not introduce an additional increase in the K\(^+\) channel expression when both HDAC2 and MKK7 were knocked down in NRCMs, suggesting VPA effect on the restoration of the K\(^+\) channel expression is likely through the inhibition of HDAC2 (Fig. 4C). Interestingly, in KLF4 knockdown-NRCMs, VPA did not affect transcript levels of the 5 K\(^+\) channels (Fig. 4D).

We then moved to test the in-vivo efficacy of VPA in our arrhythmia models. MKK7-CKO mice, stressed by 3-day TAC, received intra-peritoneal VPA injections (200 mg/kg/day) for 3 days. At the end of the treatment, ChIP assays showed increased KLF4 occupancy at the promoter regions of *Kcnip2, Kcnd2, Kcnh2, Kcna5* and *Kcnj11*, concomitant with an enhanced H3ac level in the VPA-treated group.
compared to the vehicle group (Supplementary Fig. 14). Consistently, mRNA levels of the 5 K⁺ channels returned back to their normal levels with no significant changes in other critical ion-channels, including Scn5a and Cacna1c, or other key morphometric and functional parameters (Fig. 4E and Supplementary Fig. 15). Furthermore, no FLNA association with KLF4 and HDAC2 was detected in VPA-treated groups (Fig. 4F). More convincingly, whole-cell patch-clamp recordings of peak outward K⁺ currents in isolated cardiomyocytes, primarily consisting of transient outward current Iₒ, revealed an improved density profile in the VPA-treated group (Fig. 5A-5B). MAP durations at 50%-90% of repolarisations were reduced following VPA treatment while dV/dtₘₐₓ remained comparable throughout groups, and ex-vivo PES could not induce arrhythmic events in the VPA-treated group (Fig. 5C-5D).

To explore beneficial effects of VPA in exacerbated hypertrophy/heart failure and associated arrhythmias, we challenged MKK7-CKO mice for 7-day TAC and C57BL/6N mice for 5-week TAC. In both models, expression of cJun, KLF4 and HDAC2 remained unchanged (Supplementary Fig. 16), but we observed FLNA association with KLF4 and HDAC2 in their hearts (Fig. 6A), which experienced aggravated hypertrophic remodeling and significantly prolonged action potentials (Fig. 6B-6C and Supplementary Fig. 17-18). Sustained TAC stress predisposed MKK7-CKO (6 out of 7 hearts) and C57BL/6N (5 out of 6 hearts) mice to develop frequent VTs under ex-vivo PES stimulation. We then treated MKK7-CKO mice for 4 days of VPA (200 mg/kg/day) commencing on the third day after TAC, and C57BL/6N mice undergoing TAC for 3 weeks were then administered VPA for 2 weeks. VPA treatment potently restored the K⁺ channel expression and noticeably shortened APD₉₀ (Fig. 6B-6E). Notably, no KLF4/HDAC2/FLNA complex was detected in VPA-treated groups (Fig. 6A). Furthermore, VPA ameliorated the widespread hypertrophic remodeling by reducing hypertrophic growth and fibrosis.
(Supplementary Fig. 17-18), paralleled by an improvement of cardiac function (Supplementary Fig. 19-20), which is in line with previously described anti-hypertrophic effects of VPA\textsuperscript{19,24,25}.

Finally, we evaluated human relevance of the proposed K$^+$ channel regulatory mechanism and beneficent effects of VPA in human iPSC-CMs (Fig. 7A). Knockdown of MKK7 in iPSC-CMs significantly lowered mRNA levels of $Kcnip2$, $Kcnh2$, $Kcna5$ and $Kcnj11$ in the condition of ISO stimulation, whereas VPA treatment markedly restored the expression of the K$^+$ channels (Fig. 7B). Consistently, associations of FLNA with KLF4 and HDAC2 were detected in iPSC-CMs deficient of MKK7 under ISO stress; however, VPA treatment disrupted these associations (Fig. 7C). Furthermore, functional outcomes of VPA effects were measured using whole-cell patch-clamp recordings of APD$_{90}$ in isolated iPSC-CMs. APD$_{90}$ of MKK7-deficient iPSC-CMs was noticeably longer when stressed by ISO, this APD$_{90}$ prolongation (402.31±40.49ms) was significantly shortened to 313.5±18.96ms by VPA treatment (Fig 7D).

Together, our evidence from mouse models to human iPSC-CMs supports the proposed mechanism of KLF4/HDAC2/FLNA signaling for repression of K$^+$ channel expression. Anti-arrhythmic therapeutic potential of VPA is evident by disrupting the KLF4/HDAC2/FLNA complex, which allows expression of the 5 K$^+$ channel genes being restored and alleviate stress-induced ventricular arrhythmias (Fig. 8).

**Discussion**

Pathological remodeling in hypertrophy and heart failure is often associated with prolonged repolarization due to suppression of K$^+$ currents\textsuperscript{26–29}, which triggers ventricular arrhythmias responsible for 80-95% of sudden cardiac deaths\textsuperscript{30}. Following observation of transcript-level down-regulation of the K$^+$ channels in TAC-stressed MKK7-CKO mice, our molecular and cellular data identified KLF4 as a key
transcription factor regulating the expression of the K$^+$ channels. KLF4 is a cardiac-active KLF isoform, whose functional importance in the heart was demonstrated by a highly TAC-sensitive detrimental phenotype\textsuperscript{31}. In a very consistent manner, 3-day TAC stressed KLF4-CKO hearts displayed prolonged QTc intervals and altered expression of a group of ion channels, including down-regulation of the 5 K$^+$ channels, which was reminiscent of electrical remodeling in MKK7-CKO hearts after 3-days of TAC. This finding strengthens the notion that KLF4 plays an important role in regulating cardiac K$^+$ channels. Our data further show that KLF4-based regulation is elicited through the involvement of HDAC2. HDAC2 is a member of class-I HDACs notable for their pro-hypertrophic functions\textsuperscript{19–21}. In an early study, cardiac-specific double deletion of HDAC1 and HDAC2 caused mice to die during the postnatal stage\textsuperscript{32}. Prior to death mice were predisposed to arrhythmias and cardiomyopathy with an up-regulation of \textit{Cacna1h} and \textit{Cacna2d2} transcript expression\textsuperscript{32}. Notably, expression of other important Ca$^{2+}$ handling proteins remained unaltered, while \textit{Kcnj2} was down-regulated in these post-natal hearts\textsuperscript{32}. Interestingly, neither HDAC1- nor HDAC2-specific deletion in mouse hearts produced noticeable changes in cardiac function\textsuperscript{32}. Our data shows that HDAC2 knockdown or VPA treatment is able to restore the expression of the 5 K$^+$ channels, including \textit{Kcnj2}. This incongruity in the K$^+$ channel expression may be related to the fact that the transcriptome of post-natal hearts is different to the adult ones. For example, \textit{Cacna1h} is expressed differentially in embryonic hearts versus in adults\textsuperscript{32}. In this study, we unveiled a regulatory role of cytoskeletal protein FLNA by mapping out its distinct binding sites with KLF4 and HDAC2. The evidence that KLF4 cannot associate with HDAC2 when FLNA is knocked down implicates FLNA as a scaffold for KLF4 and HDAC2. Further data showing unchanged HDAC2 binding at the \textit{Kcnip2} and \textit{Kcnh2} promoter and knockdown of FLNA and HDAC2 giving no additive
effects on restoring the K⁺ channel expression indicates that HDAC2 does not
directly bind with the promoter regions and the effect of HDAC2 on promoter
chromatin is exerted through the KLF4/HDAC2/FLNA complex. On a separate note,
an adverse effect of excessive FLNA in MKK7-CKO hearts was similarly
documented in an animal model of periventricular heterotopia, in which M KK7-
activator MEKK4 deficiency resulted in accumulation of FLNA that impeded neuronal
migration. We reason that M KK7 regulation of FLNA turnover by a protease-
mediated mechanism may occur in multiple organs, like heart and brain.
MKK7 is a stress-activated MAP kinase. Facing a stress challenge, MKK7 rapidly
becomes activated, which enables it to prevent the formation of the
KLF4/HDAC2/FLNA complex, as we observed in Flox mice undergoing TAC stress
(3 days to 1 week). Evidence from MKK7-Tg mice also supports this notion that
MKK7 overexpression and increased JNK activation allowed MKK7-Tg hearts to be
resilient against pathological hypertrophy and associated arrhythmias under 5-week
TAC. However, sustained stress like that imposed by 5 weeks of TAC on C57BL/6N
mouse hearts, dampened endogenous MKK7 expression and activation, therefore
the KLF4/HDAC2/FLNA complex was formed. To establish a direct link between
MKK7 deficiency and cardiac electrical remodeling, we used an experimental model
of M KK7-CKO mice. The lack of MKK7 in the heart predisposed mice to ventricular
arrhythmias following TAC insult or adrenergic stress. However, M KK7 deficiency did
not affect the 5 K⁺ channel expression and repolarisation duration under the sham
condition, reiterating that its role is confined to the response to stress stimulation. It
is likely that other transcriptional regulation mechanisms for K⁺ channel expression
predominate in the absence of stress.
Benefiting from the discovery of the KLF4/HDAC2/FLNA regulation complex, we
made attempts to explore therapeutic values of VPA in various TAC-induced
arrhythmia models. VPA is a unique drug candidate because it induces proteasomal degradation of HDAC2, alongside inhibiting class-I HDAC catalytic activity. In our 3-day TAC model, we revealed an unambiguous anti-arrhythmic effect of VPA in MKK7-CKO mice based on an array of functional analyses. The expression of the K⁺ channels restored by VPA treatment are functionally critical for replenishing the depleted ‘repolarization reserve’ of overload-stressed hearts. In our prolonged TAC models (MKK7-CKO mice of 1-week TAC and C57BL/6N mice of 5-week TAC) with exacerbated hypertrophy, VPA exerted salutary effects by alleviating arrhythmia occurrences and hypertrophic remodeling, which demonstrates the therapeutic value of VPA in a more clinically-relevant setting. By investigation of VPA mechanism of action, we believe that HDAC2 degradation by VPA treatment would cause a disruption of the association of KLF4 and FLNA, which allows KLF4 to access the promoter regions of the K⁺ channels. Meanwhile, VPA-induced reduction in HDAC2 activity could indirectly increase H3ac level of the promoter regions. These effects in combination lead to restored expression of the 5 K⁺ channels and salient anti-arrhythmia benefits. Worthy of noting, VPA did not introduce an additional increase in the 5 K⁺ channel expression when HDAC2 was knocked down in MKK7-deficient NRCMs, suggesting VPA action is likely through the inhibition of HDAC2. Remarkably, we realize that VPA is already a first-line drug for various neuropsychiatric conditions without instance of significant cardiac side-effects. Although epidemiological data detailing cardiac effects of VPA exclusive of confounding conditions is lacking, a clinical study amongst bipolar patients revealed that VPA significantly reduces QT dispersion, implying a possible beneficial effect on human hearts. Given its reliable safety profile and potential in alleviating both arrhythmias and hypertrophy, repurposing VPA for cardiac applications seems an attractive prospect that merits rapid and prioritized scrutiny.
The physiological differences and similarities between rodents and humans have been widely studied. Mouse models contribute enormously to our current understanding of mammalian ion-channel functions, and are still the choice for investigating molecular mechanisms. All of the $K^+$ channels down-regulated in MKK7-CKO hearts have cardinal roles in human hearts too: $I_{b}$ (Kv4 and KChIP2) is crucial for myocyte repolarization in many mammalian hearts. $I_{Kr}$ (ERG1) is essential for human cardiac repolarization, but less so in murine hearts despite abundant transcript expression. $I_{Kur}$ (Kv1.5) is an important repolarizing current in human atrium, and it is also present in rodent hearts. $I_{KATP}$ (Kir6.2) is particularly involved in ischaemia-response in both species. The consensus KLF binding sites on the promoters of the above-listed genes are evolutionarily conserved. As such, it is very likely that MKK7 deficiency-induced transcription repression of $K^+$ channel expression is conversed across species. Indeed, we took human iPSC-CMs as a translational model, which provides a series of molecular and functional evidence, comprising of mRNA expression profile of $K^+$ channels, formation of KLF4/HDAC2/FLNA complex and action potential recordings that clearly demonstrate human relevance of this regulatory mechanism and beneficent effects of VPA.

In the current study, we did not investigate phosphorylation or hyper-oxidation of ion channels, so cannot exclude the possibility of posttranslational modifications altering function in the channels whose transcripts did not change. Indeed, as MKK7-CKO is a stress-activated pro-arrhythmic model we would expect phosphorylation of various excitation-contraction coupling proteins. In future work we plan on investigating the possible role of $Ca^{2+}$ cycling proteins (in particular the SERCA and the RyR2) in the contractile and arrhythmogenic phenotype of our MKK7 models. However, the lack of a difference in upstroke velocity of the action potential in isolated cardiomyocytes
is in keeping with the lack of significant posttranslational alterations in Na-channels. Furthermore, proteomic studies elucidating the KLF4/HDAC2/FLNA complex could uncover any additional members of the complex. A better understanding would be imperative for designing ideal small-molecule inhibitors to exclusively disrupt the complex formation without inhibiting any enzymatic activity or altering protein expression, therefore evading unpredictable side effects. Hopefully, a safe and effective new class of anti-arrhythmics specifically targeting molecular signal transduction is not far off.

Acknowledgment

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Disclosures

None.

References


and the Prevention of Sudden Cardiac Death) Developed in collaboration with the European Heart Rhythm Association and the Heart Rhythm Society. 

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**Legends**

Figure 1. MKK7-CKO mice are vulnerable to stress-induced ventricular arrhythmias. 

(A) Single surface-ECG beat (left) and mean rate-corrected QT intervals (right, Mitchell QTc) demonstrate QT prolongation following 3-day TAC (n=10 mice/group).

(B) In-vivo PES protocol comprising of eight serial supra-capture-threshold stimuli (S1, at 100ms interval) followed by an extra-stimulus (S2) delivered at progressively shortened S1S2 delay was applied. Induced ventricular arrhythmias were observed in 50% of the knockouts after 3-day TAC but none of the controls (n=10 mice/group).

(C) Ex-vivo PES traces show early-afterdepolarizations (EADs) and overt arrhythmias, observed in 67% of the MKK7-CKO/3d-TAC (7 out of 9 hearts) but none of the MKK7-Flox/3d-TAC heart preparations (n=9 mice/group). 

(D) Mean MAP
durations at 90% (APD<sub>90</sub>) of repolarizations recorded from ex-vivo Langendorff perfused hearts (n=9 mice/group). (E) Action potential traces of ventricular cardiomyocytes isolated from MKK7-CKO/3d-TAC and MKK7-Flox/3d-TAC hearts by whole-cell patch clamping at 37°C (left panel), with APD<sub>50</sub>, APD<sub>90</sub>, dv/dt<sub>max</sub> and maximum repolarization velocity during phase 1 of the action potential, which is determined by the transient outward current in the right panel (15 cells/group). All bar graphs express mean ± S.E.M values, ns = not significant.

Figure 2. KLF4-HDAC2 signaling regulates K<sup>+</sup> channel expression. (A) Quantitative real-time PCR (qPCR) analyses of <i>Kcnip2</i>, <i>Kcnd2</i>, <i>Kcnh2</i>, <i>Kcna5</i> and <i>Kcnj11</i> in myocardium. Bonferroni multiple testing correction was applied for 19 ion channel genes, and two-way ANOVA with Bonferroni correction for post-hoc comparisons were used to analyze mRNA expression of the 5 K<sup>+</sup> channel genes (n=12 mice/group, normalized to Gapdh content). (B) qPCR analyses show down-regulation of the K<sup>+</sup> channels upon KLF4 knockdown in NRCMs (n=4 independent experiments). (C) qPCR analyses of ion channel profiles in KLF4-CKO/3d-TAC hearts (n=5 mice/group, *P<0.05 KLF-CKO/TAC versus αMHC-Cre/TAC). (D) ECG analyses show 3-day TAC causing longer QTc in KLF4-CKO mice compared with that in αMHC-CRE controls (n=5 mice/group). (E) Chromatin immunoprecipitation (ChIP) reveals reduced KLF4 binding and H3ac level at the promoter regions of <i>Kcnip2</i> and <i>Kcnh2</i> in the MKK7-CKO/3d-TAC ventricles (n=4 mice/group, data normalized to % input DNA and expressed as fold change relative to control). (F) Association of endogenous HDAC2, but not HDAC1, with KLF4 determined by immunoprecipitation from myocardium and NRCMs, respectively. Meanwhile, HDAC2 did not associate with KLF15. Quantitative measurements reflecting the association intensity in myocardium samples are presented as bar graphs (n=3
independent experiments). (G) ChIP failed to detect change in HDAC2 binding at the promoter regions of *Kcnip2* and *Kcnh2* in the MKK7-CKO/3-day TAC ventricles (n=4 mice/group). (H) JNK1 overexpression disrupted the HDAC2-KLF4 association in ISO-treated NRCMs. JNK1 increased phosphorylated-HDAC2 that failed to bind with KLF4. IgG used as control for nonspecific interaction and GAPDH used as loading control (n=3 independent experiments).

Figure 3. FLNA as a scaffold for KLF4/HDAC2 regulation of K\(^+\) channels. (A) Immunoblots exhibit augmented FLNA expression (quantified in bar graph) in whole heart lysates from MKK7-CKO/3d-TAC mice, whilst other cytoskeleton proteins, fibrotic marker, apoptotic markers, or inflammation markers remains comparable (n=5 mice/group). (B) qPCR analyses demonstrate comparable transcript levels of FLNA between genotypes (n=8 mice/group, data normalised to Gapdh content). (C) Immunoblots on the nuclear fractions of myocardium or NRCMs illustrate elevated FLNA level. Histone-3 (H3) used as loading control (n=5). (D) FLNA association with KLF4 and HDAC2 detected in MKK7-deficient NRCMs after ISO stimulation. IgG used as control for nonspecific interaction, GAPDH as loading control (n=3). (E) FLNA knockdown disrupted KLF4-HDAC2 interaction in ISO-treated MKK7-deficient NRCMs, whereas expression of KLF4 and HDAC2 remained unchanged (n=3). (F) Transcript levels of the 5 K\(^+\) channels measured following knockdown of FLNA with or without HDAC2 (n=4 independent experiments). (G) Immunoblots show increased FLNA level in the nuclear fractions of ISO-treated MKK7-deficient NRCMs; while JNK1 overexpression reduced this augmented FLNA. H3 used as loading control for the nuclear fractions (n=3). (H) Cysteine protease inhibitor E-64D (5µM, 16 hours), but not proteasome inhibitor MG132 (2.5µM, 16 hours), inhibited this FLNA reduction. Pre-incubation with Cycloheximide (CHX, 10µg/ml for 4 hours)
demonstrates that JNK1-induced FLNA reduction is via proteolysis (n=3). (I) Schematic diagram of flag-tagged FLNA fragments used in immunoprecipitation experiments (upper panel), ABD: actin binding domain, DD: dimerization domain. Immunoprecipitation experiments demonstrate the distinct region of FLNA required for association with KLF4 or HDAC2 (lower panel), n=3. All bar graphs present mean ± S.E.M. values, ns = not significant.

Figure 4. Valproic acid restores K+ channel expressions by targeting HDAC2. (A) In-vitro HDAC activity was blunted (34% reduction) by VPA (4mM) in NRCMs (n=5 independent experiments). Pan-HDAC inhibitor (Trichostatin-A) was used as control for HDAC inhibitor activity. (B) Immunoblots show reduced expression of HDAC2 but not HDAC1, 3 and 8 upon VPA (20mM) in myocardium and NRCMs (n=5, GAPDH used as loading control). (C) qPCR analyses reveal increased transcript expression of the 5 K+ channels by VPA treatment (20mM) in MKK7-knockdown NRCMs under ISO stimulation (n=5 independent experiments). (D) qPCR analyses of transcript levels of the 5 K+ channels in KLF4 knockdown-NRCMs upon VPA treatment (n=4). (E) qPCR analyses demonstrate restored myocardial transcript expression of the 5 K+ channels by VPA treatment (n=8 mice/group). (F) Immunoblotting detection of FLNA association with KLF4 and HDAC2 in myocardium of MKK7-CKO/3d-TAC mice with VPA or vehicle treatment (n=4 mice/group). All bar graphs present mean ± S.E.M. values, ns = not significant.

Figure 5. Valproic acid prevents ventricular arrhythmias. (A) Representative peak (arrows) outward K+ current traces recorded from isolated adult cardiomyocytes. (B) Current-voltage relationship curves of peak outward K+ currents. Combination of Tetrodotoxin (TTX) and CoCl2 inhibited Na+ and Ca2+ channel activities, respectively,
and isolation of K⁺ currents subsequently confirmed by 4-aminopyridine (4-AP), n=14 cells/group. *Flox/3d-TAC/veh versus CKO/3d-TAC/veh, #CKO/3d-TAC/veh versus CKO/3d-TAC/VPA, p<0.05. (C) MAP durations at 50% (APD₅₀) and 90% (APD₉₀) of repolarizations and maximum depolarisation velocity recorded from ex-vivo Langendorff perfused hearts (n=9 mice/group). (D) Ex-vivo PES traces show the absence of arrhythmic events in MKK7-CKO/3d-TAC mice following VPA treatment (n=9 mice/group). Data presented as mean ± S.E.M, ns= not significant.

Figure 6. Valproic acid alleviates ventricular arrhythmias in heart failure. (A) Immunoblotting detection of FLNA association with KLF4 and HDAC2 in MKK7-CKO/7d-TAC hearts or C57BL/6N-5w-TAC hearts with or without VPA treatment (n=4 mice/group). (B) Ex-vivo MAP durations at 50% (APD₅₀) and 90% (APD₉₀) of repolarizations were markedly shortened by VPA treatment in MKK7-CKO/7d-TAC hearts (n=7 mice/group). (C) APD₅₀ and APD₉₀ of ex-vivo heart preparations were significantly shortened by VPA treatment in C57BL/6N/5w-TAC hearts (n=6 mice/group). (D) qPCR analyses show VPA treatment increasing transcript expression of Kcnip2, Kcnd2, Kcnh2, Kcna5 and Kcnj11 in myocardium of MKK7-CKO/7d-TAC mice (n=7 mice/group, normalized to Gapdh content). (E) qPCR analyses show enhanced transcript expression of the K⁺ channels by VPA treatment in C57BL/6N/5w-TAC hearts (n=6 mice/group). Data presented as mean ± S.E.M values.

Figure 7. VPA effects on K⁺ channel expression, KLF4/HDAC2/FLNA complex and action potentials in iPSC-cardiomyocytes. (A) Representative immunocytochemistry images confirm nature of iPSC-cardiomyocytes using alpha-actinin (red), Cx43 (green), and Alexa Fluor 555-Phalloidin (blue) (scale bar: 50µm). (B) qPCR analyses
of Kcnip2, Kcnh2, Kcna5 and Kcnj11 in iPSC-cardiomyocytes with MKK7 knockdown followed by ISO (5µM) stimulation with or without VPA treatment (4mM) (n=4 independent experiments performed in triplicates, normalized to Gapdh). (C) Immunoblotting detection of FLNA association with KLF4 and HDAC2 in iPSC-cardiomyocytes with MKK7 knockdown following ISO stimulation with or without VPA treatment (n=3). (D) Representative action potential traces of isolated single iPSC-cardiomyocyte recorded by whole-cell patch clamping at 37°C (15 cells/group, upper panel), and 90% action potential durations (APD$_{90}$) quantified in graph bar (lower panel). All bar graphs express mean ± S.E.M values.

Figure 8. The new regulatory mechanism linking MKK7-deficiency with increased arrhythmia susceptibility and therapeutic potential of VPA. KLF4 is involved in regulating the 5 K$^+$ channel expression in the healthy heart under stress. Loss of MKK7 in the hypertrophied heart (phenocopied by MKK7-CKO mice) leaves HDAC2 unphosphorylated and FLNA accumulated in the nucleus, where they form an association with KLF4. This complex leads to KLF4 dissociation from the promoter regions of the K$^+$ channels and consequently reduced transcript levels. Diminished K$^+$ channel reserves fail to cope with states of increased demand; resulting in repolarization delays and arrhythmias. Disrupting the repressive function of KLF4/HDAC2/FLNA complex by the HDAC2 inhibitor VPA not only restores K$^+$ channel expression, but also confers therapeutic benefits of normalizing cardiac repolarization and reducing arrhythmia susceptibility in pathologically remodelled hearts. Ac: Acetyl.
**A**

- P Q R T S
- CKO/3d-TAC

**B**

- MKK7-Flox
  - 3d-Sham (0 out of 10)
  - 3d-TAC (0 out of 10)

- MKK7-CKO
  - 3d-Sham (0 out of 10)
  - 3d-TAC (5 out of 10)

**C**

- s1 s2
- EAD
- Ventricular arrhythmia

- Flox/3d-TAC
- CKO/3d-TAC

**Rate-corrected QT interval (ms)**

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p < 0.001

**Flox CKO 3d-Sham 3d-TAC**

**Flox CKO 3d-Sham (0 out of 10)**

**Flox CKO 3d-TAC (5 out of 10)**

**MKK7-CKO MKK7-Flox**

**30 10 20 30 40**

**100ms**
Figure D shows a bar graph comparing APD90 (ms) between Flox/3d-TAC, Flox/3d-Sham, CKO/3d-TAC, and Flox/3d-TAC groups. The graph indicates a statistically significant difference between the Flox/3d-TAC and CKO/3d-TAC groups with a p-value of <0.001.

Figure E displays electrocardiogram (ECG) waveforms for Flox/3d-TAC (black line) and CKO/3d-TAC (red line) groups. The graph on the right shows a comparison of APD (ms) for APD50 and APD90 between the same groups. The APD90 for CKO/3d-TAC is significantly higher than that of Flox/3d-TAC with a p-value of 0.008. The graph on the left shows the maximum repolarization velocity (V/s) for the two groups, with a statistically significant difference at the 0.01 level.
A

B

C

D

3d-TAC/Veh

3d-TAC/VPA

3d-TAC/Veh

3d-TAC/VPA

3d-TAC/Veh

3d-TAC/VPA

39
A

IP: FLNA
IB: HDAC2
IP: FLNA
IB: KLF4
Input GAPDH

CKO/7d-TAC
Veh VPA
C57/5w-TAC
Veh VPA

B

![Bar chart showing APD50 and APD90 with p-values](chart.png)

- Flox/Veh
- Flox/VPA
- CKO/Veh
- CKO/VPA

C

![Bar chart showing APD50 and APD90 with p-values](chart.png)

- 5w-Sham
- 3w-Sham+2w-VPA
- 5w-TAC
- 3w-TAC+2w-VPA

p = 0.05
p = 0.01
p = 0.018
p = 0.03
D

- **Kcnip2**: p = 0.043
- **Kcnd2**: p = 0.012

E

- **Kcnh2**: p = 0.002
- **Kcna5**: p = 0.007
- **Kcnj11**: p = 0.007
**A**

**B**

**Kcnip2**

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**Kcnh2**

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**C**

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**p-values**

- **Kcnip2**: p=0.021, p=0.041
- **Kcnh2**: p=0.017, p=0.038
- **Kcna5**: p=0.033, p=0.046
- **Kcnj11**: p=0.047, p=0.042