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Converse role of class I and class IIa HDACs in the progression of atrial fibrillation

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ABSTRACT

Atrial fibrillation (AF), the most common persistent clinical tachyarrhythmia, is associated with altered gene transcription which underlies cardiomyocyte dysfunction, AF susceptibility and progression. Recent research showed class I and class IIa histone deacetylases (HDACs) to regulate pathological and fetal gene expression, and thereby induce hypertrophy and cardiac contractile dysfunction. Whether class I and class IIa HDACs are involved in AF promotion is unknown. We aim to elucidate the role of class I and class IIa HDACs in tachypacing-induced contractile dysfunction in experimental model systems for AF and clinical AF.

Methods and results: Class I and IIa HDACs were overexpressed in HL-1 cardiomyocytes followed by calcium transient (CaT) measurements. Overexpression of class I HDACs, HDAC1 or HDAC3, significantly reduced CaT amplitude in control normal-paced (1 Hz) cardiomyocytes, which was further reduced by tachypacing (5 Hz) in HDAC3 overexpressing cardiomyocytes. HDAC3 inhibition by shRNA or by the specific inhibitor, RGFP966, prevented contractile dysfunction in both tachypaced HL-1 cardiomyocytes and *Drosophila* prepupae. Conversely, overexpression of class IIa HDACs (HDAC4, HDAC5, HDAC7 or HDAC9) did not affect CaT in controls, with HDAC5 and HDAC7 overexpression even protecting against tachypacing-induced CaT loss. Notably, the protective effect of HDAC5 and HDAC7 was abolished in cardiomyocytes overexpressing a dominant negative HDAC5 or HDAC7 mutant, bearing a mutation in the binding domain for myosin enhancer factor 2 (MEF2). Furthermore, tachypacing induced phosphorylation of HDAC5 and promoted its translocation from the nucleus to cytoplasm, leading to up-regulation of MEF2-related fetal gene expression (β-MHC, BNP). In accord, boosting nuclear localization of HDAC5 by MC1568 or Go6983 attenuated CaT loss in tachypaced HL-1 cardiomyocytes and preserved contractile function in *Drosophila* prepupae. Findings were expanded to clinical AF. Here, patients with AF showed a significant increase in expression levels and activity of HDAC3, phosphorylated HDAC5 and fetal genes (β-MHC, BNP) in atrial tissue compared to controls in sinus rhythm.

Conclusion: Class I and class IIa HDACs display converse roles in AF progression. Whereas overexpression of Class I HDAC3 induces cardiomyocyte dysfunction, class IIa HDAC5 overexpression reveals protective properties. Accordingly, HDAC3 inhibitors and HDAC5 nuclear boosters show protection from tachypacing-induced changes and therefore may represent interesting therapeutic options in clinical AF.

1. Introduction

Atrial fibrillation (AF) is the most common sustained and progressive clinical tachycardia which contributes to cardiovascular morbidity and mortality [1]. AF is characterized by specific electrical, transcriptional and structural changes in the cardiomyocyte, commonly denoted as remodeling [2]. Cardiomyocyte remodeling underlies contractile dysfunction and the progression of AF. Therefore, it is of great interest to dissect the molecular mechanisms underlying cardiomyocyte remodeling, with the aim to identify novel druggable targets which

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attenuate remodeling and AF progression.

Previous research identified that (re)activation of pathological and fetal gene program in cardiomyocytes promotes AF onset and progression [3,4]. Ausma et al. showed upregulation of two proteins of the fetal program in the goat model for AF, i.e. the slow-contracting beta-myosin heavy chain isoform (β-MHC) and smooth muscle α-actin (α-SMA) [5–8]. In persistent AF patients, numerous fetal/neonatal variants of the titin protein were observed in cardiac myofibrils, and atrial re-expression of TnI-skeletal-slow-twitch (ssTnI) was found in patients with paroxysmal AF [9]. In addition, persistent AF was associated with higher cardiac mRNA expression of brain natriuretic peptide (BNP) [10]. Interestingly, pathological and fetal gene expression is under control of epigenetic regulation [11–15]. Hence, epigenetic regulation has been identified as an important mechanism underlying the progression of cardiac diseases [11–15]. Epigenetic regulation refers to processes that influence the packaging or processing of nuclear DNA, thus controlling the on/off states of multiple genes with discrete switches. The packaging of chromatin is largely dependent on the acetylation status of histones, which is controlled by histone acetyl transferases and histone deacetylases (HDACs) [11–15]. HDACs are an ancient family of enzymes that catalyze the removal of acetyl groups from the ε-amino group of specific acetyl lysine residues within their protein substrates. In general, deacetylation of histones in nucleosomes induces chromatin condensation, which inhibits binding of transcription factors and other components of the transcriptional machinery to gene promoter and enhancer regions, ultimately resulting in transcriptional repression. As such, histone deacetylation serves as an important regulator of gene expression.

The zinc-dependent HDACs are classified into four groups based on their structure, complex formation, and expression pattern: class I (HDAC1, HDAC2, HDAC3, and HDAC8), class IIa (HDAC4, HDAC5, HDAC7, and HDAC9), class IIb (HDAC6 and HDAC10), and class IV (HDAC11) [16]. We recently reported on a cytosolic member of HDAC class IIb, HDAC6, and its prominent role in AF progression [17]. HDAC6 deacetylates α-tubulin, which causes disruption of microtubule structure, contractile dysfunction and AF progression [17]. However, whether the other HDAC classes are involved in AF progression is unknown. Of the four classes, class I and IIa are well studied regarding their role in pathological gene expression, structural changes and the development of hypertrophy and heart failure [18–21]. Class I HDACs reveal high HDAC activity in cardiomyocytes, but findings on their role in cardiac disease development are conflicting [18]. In recent years, class IIa HDACs, especially HDAC4, HDAC5 and HDAC9, have attracted considerable attention as regulators of transcriptional reprogramming especially in cardiac diseases. Under normal circumstances, class IIa HDACs localize in the nucleus and suppress cardiomyocyte hypertrophy by repressing the activity of pro-hypertrophic transcription factors, such as members of the myocyte enhancer factor-2 (MEF2) family [19,20]. In response to stress signals, class IIa HDACs are phosphorylated and exported from the nucleus, thereby activating transcriptional reprogramming and the induction of hypertrophic gene expression resulting in cardiac disease [18–21].

Although previously findings indicate a role for transcriptional remodeling in AF progression [3], and our previous study revealed a prominent role for the cytosolic member of HDAC class IIb, HDAC6, in AF progression [17], the involvement of class I and class IIa HDACs in AF is still unknown. Therefore, we examined the role of class I and IIa HDACs on contractile function in tachypaced HL-1 cardiomyocytes and *Drosophila*, followed by exploration of the downstream pathway. Experimental findings were confirmed in AF patients. Here, we show that HDAC3 overexpression causes contractile dysfunction in HL-1 cardiomyocytes. Both pharmacological and genetic inhibition of HDAC3 prevents tachypacing-induced contractile dysfunction in experimental models for AF progression. In contrast, class IIa HDAC5 and HDAC7 overexpression protects against tachypacing-induced contractile dysfunction, possibly via prevention of MEF2 related fetal gene expression, including β-MHC and BNP expression [19,20]. In line, HDAC5 nuclear boosters attenuated tachypacing-induced contractile dysfunction in experimental models for AF. Finally, findings for HDAC3 and HDAC5 were confirmed in atrial tissue biopsies from patients with AF compared to control patients in sinus rhythm (SR), indicating activation of HDAC class I and IIa in patients with AF.

2. Materials and methods

2.1. Tachypacing of HL-1 cardiomyocytes and calcium transient measurements

HL-1 cardiomyocytes were subjected to tachypacing (TP, 5 Hz, 40 V, pulse duration of 20 ms) with a 4-well C-pace system from IonOptix for 12 h except specifically stated for time-course pacing, followed by calcium transient measurements as described before [17]. See Supplemental Information section of detailed information.

2.2. Plasmids

Retroviral constructs of HDACs and HDACs mutants (HDACm) were generated in the lab of Dr. Miguel A. Esteban [22]. To facilitate the detection of HDAC overexpression by Western blot analyses, the constructs contained a FLAG tag in the carboxyl terminal. DNA mutagenesis of HDAC5 and HDAC7 in the MEF2 binding domain was produced using suitable oligos and a PCR-based method. All new plasmids were verified by sequencing before use [22]. GFP-HDAC5 constructs were a generous gift from Dr. Johannes Backs' lab [18].

2.3. Retroviral infection of HL-1 cardiomyocytes

Generation of retro-virus and infection of HL-1 cardiomyocytes were performed as previously described [22]. See Supplemental Information section of detailed information.

2.4. Drosophila stocks, tachypacing, and heart wall contraction assays

The *Drosophila* wild-type W1118 strain was used for all drug screening experiments. Information on *Drosophila* stocks [23], crossing, tachypacing (TP, 4 Hz, 20 V, pulse duration of 5 ms) and heart wall contraction assays are described in the Supplemental Information section.

2.5. Real time PCR

Total RNA from HL-1 cardiomyocytes, *Drosophila* and human tissue was isolated by use of TRIzol reagent (Invitrogen) and 0.5 μg RNA per sample was used for synthesizing cDNA by utilizing the Reverse Transcriptional kit (Invitrogen) following the manufacturer's instructions. Gene expression was determined by quantitative real time PCR on a Bio-Rad CFX384 real time system using SYBR green dye (Invitrogen). Gene expression was corrected for levels of the reference gene GAPDH. Primer sequences are listed in Supplemental Information section Table S1.

2.6. Protein extraction and Western blot analysis

HL-1 cardiomyocytes or human tissue samples were used for protein extraction and Western blot analyses as described in the Supplemental Information section.

2.7. Immunofluorescence

Detailed information on immunofluoresent staining procedures can be found in Supplemental Information section.

Table 1
Baseline demographic and clinical characteristics of patients with PeAF and control patients in SR.

	SR	PeAF
N	12	12
RAA (n)	12	12
LAA (n)	7	7
Age (mean, std)	60 ± 17	69 ± 5
Months of AF (median, range)	–	22 (4–244)
Persistent AF	-	5
Long-standing persistent AF	-	7
Underlying heart disease (n)/surgical procedure		
MVD/MV replacement or repair	8 (67%)	11 (92%)
CABG	4 (33%)	1 (8%)
AVD	1 (8%)	1 (8%)
Medication (*n*)		
ACE / ARB	7 (58%)	10 (83%)
Digoxin	0 (0%)	4 (33%)
Ca^{2+} channel blocker	0 (0%)	2 (17%)
β-blocker	5 (42%)	8 (67%)
Statin	7 (58%)	3 (25%)

Maze: atrial arrhythmia surgery; MVD: mitral valve disease; AVD: aortic valve disease; ACE: angiotensin-converting enzyme; CABG: coronary artery bypass surgery; ARB: angiotensin receptor blockers; β-Blocker: beta-adrenergic antagonists.

2.8. Patients

Before surgery, one investigator assessed patient characteristics (Table 1), as described before [24]. Right atria appendages (RAA) and left atria appendages (LAA) were obtained from patients with (long standing) persistent AF (PeAF) and control patients in SR. After excision, atrial appendages were immediately snap-frozen in liquid nitrogen and stored at −80 °C. The study conforms to the principles of the Declaration of Helsinki. The Institutional Review Board approved the study, and patients gave written informed consent. Tissues were used to perform real time PCR and Western blot experiments.

2.9. Statistical analysis

Results are expressed as mean ± SEM. Biochemical analyses were performed at least in duplicate. Individual group mean differences were evaluated with the Students *t*-test. Categorical data differences were evaluated with Pearson's chi-squared test. All *P* values were 2-sided. Values of $P < 0.05$ were considered statistically significant. SPSS version 22 was used for all statistical evaluations.

3. Results

3.1. Screening role of class I and IIa HDACs on tachypacing-induced remodeling in HL-1 cardiomyocytes

To study the role of class I and IIa HDACs on contractile function in cardiomyocytes, various members of the HDAC classes were overexpressed in HL-1 cardiomyocytes by retroviral infection (Supplemental Fig. S1A, B). Control DsRED retroviral infected HL-1 cardiomyocytes revealed normal CaT amplitudes, which were significantly reduced after tachypacing (Fig. 1A, B, Supplemental Fig. S2). Of the examined class I HDACs, HDAC1 or HDAC3 overexpression resulted in CaT loss in normal paced cardiomyocytes which was further reduced by tachypacing in HDAC3 but not in HDAC1 overexpressing cardiomyocytes (Fig. 1B). This result indicates a detrimental effect of overexpression of class I HDACs, especially HDAC3, on contractile function in HL-1 cardiomyocytes.

Overexpression of class IIa HDACs, i.e. HDAC4, HDAC5, HDAC7 and HDAC9, rendered mixed results. None of the class IIa HDACs caused CaT changes in control HL-1 cardiomyocytes (Fig. 1A, C). Overexpression of HDAC5 and HDAC7 significantly precluded tachypacing-induced decline in CaT in HL-1 cardiomyocytes, whereas overexpression of HDAC4 or HDAC9 were not protective (Fig. 1A, C). Under normal circumstances, HDAC5 and HDAC7 are localized in the nucleus and bind to MEF2, via their MEF2 binding domain, resulting in repression MEF2 activity [19,20]. In response to stress signals, HDAC5 and HDAC7 are phosphorylated and exported from the nucleus, thereby activating MEF2 and permitting the induction of pathological fetal gene expression [19,20]. To test whether the protective effect of HDAC5 and HDAC7 is via binding to MEF2, HL-1 cardiomyocytes were transfected with mutant HDAC5m or HDAC7m with a dysfunction MEF2 binding domain. Notably, loss of MEF2 binding capacity abrogated their protection from tachypacing-induced CaT loss (Fig. 1C), suggesting that binding of HDAC5 and HDAC7 to MEF2 prevents cardiomyocyte remodeling.

Consequently, our findings suggest converse roles of class I and class IIa HDACs in tachypacing-induced remodeling in HL-1 cardiomyocytes. Overexpression of class I HDACs, especially HDAC3, causes contractile dysfunction, while overexpression of IIa HDAC5 and HDAC7 protects against tachypacing-induced CaT loss, possibly via nuclear binding to MEF2 transcription factor and thus limiting downstream pathological reprogramming.

3.2. Knockdown of HDAC3 protects against tachypacing-induced contractile dysfunction in experiment models for AF

To study the role of class I HDAC3 in contractile dysfunction in more detail and test whether HDAC3 may represent a druggable target in AF, HDAC3 expression and activity levels were measured in tachypaced HL-1 cardiomyocytes and in RAA and LAA of patients with PeAF and control patients in SR. In HL-1 cardiomyocytes, tachypacing significantly increased HDAC3 protein levels as confirmed by immunofluorescent staining (Fig. 2A, B), and HDAC3 activity levels as indicated by increased deacetylation of its target acetyl histone 4 at lysine 5 (AcH4k5) [25] (Fig. 2C, D). In line with tachypaced HL-1 cardiomyocytes, PeAF patients revealed significant higher levels of HDAC3 expression and activity, as indicated by increased deacetylation of its target AcH4k5, in left and right atrial appendages compared to control patients in SR (Fig. 3A-C).

To examine whether HDAC3 knockdown improves contractile function in HL-cardiomyocytes, HDAC3 was suppressed by retro-viral infection of HDAC3 shRNA in HL-1 cardiomyocytes (Fig. 4A). As expected, HDAC3 knockdown significantly protected against tachypacing-induced CaT loss in HL-1 cardiomyocytes, while HDAC1 knockdown did not reveal an effect (Fig. 4B, C). Consistently, HDAC3 knockdown in the *Drosophila* heart also protected against tachypacing-induced contractile dysfunction, including heart rate reduction and increased arrhythmicity (Fig. 4E-G). Taken together, these data suggest that HDAC3 contributes to AF remodeling and progression and thus inhibition of HDAC3 may represent a druggable target in AF.

3.3. Tachypacing induces HDAC5 phosphorylation and its nuclear export in HL-1 cardiomyocytes

Conversely to HDAC3, class IIa HDAC5 and HDAC7 protected against tachypacing-induced CaT loss in HL-1 cardiomyocytes. Of these two protective class IIa HDACs, HDAC5 represents an interesting candidate, because its expression is abundant in the heart in contrast to HDAC7 [20,26,27], and its function is regulated by calpain [28], which was previously found to induce structural remodeling in AF via degradation of HDAC6-deacetylated microtubules [17]. Therefore, we determined the role of HDAC5 in cardiomyocytes in more detail. Upon stress, HDAC5 gets phosphorylated, resulting in dissociation of HDAC5 from MEF2, and its nuclear export [26,28–30]. Firstly, we studied whether tachypacing induces HDAC5 phosphorylation in HL-1

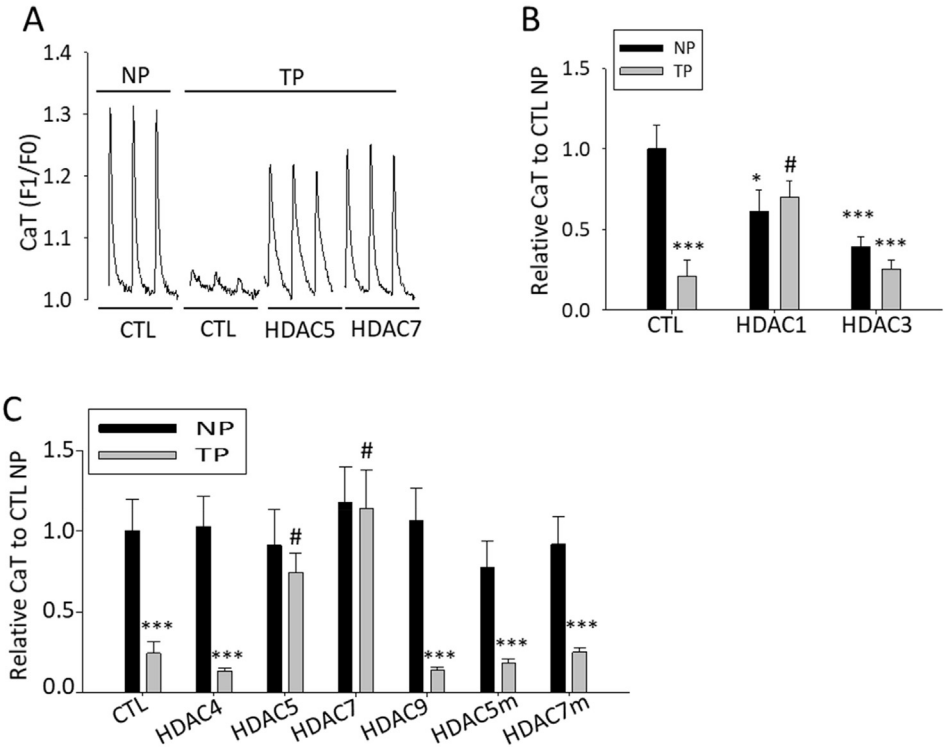

Fig. 1. Converse role of Class I and IIa HDACs in regulation of contractile function in HL-1 cardiomyocytes. A) Representative CaT traces of HL-1 cardiomyocytes, showing that cardiomyocytes overexpressing HDAC5 or HDAC7 are protected against tachypacing-induced CaT reduction. B,C) Quantified data showing relative CaT amplitudes of non-paced (NP) and tachypaced (TP) cardiomyocytes, each from groups as indicated. Cardiomyocytes were infected with control plasmid DsRED retrovirus (CTL), HDAC1, HDAC3, HDAC4, HDAC5, HDAC7,HDAC9, HDAC5 mutant (HDAC5m) or HDAC7 mutant (HDAC7m) retrovirus. HDAC5m, HDAC7m have mutations in MEF2 binding domains and therefore cannot find to MEF2. $^{*}P < 0.05$ vs CTL NP, $^{***}P < 0.001$ vs CTL NP, $^{\#}P < 0.01$ HDAC TP vs CTL TP. N ≥ 8 for each group.

cardiomyocytes. As expected, tachypacing gradually and significantly increased phosphorylation of HDAC5 in HL-1 cardiomyocytes (Fig. 5A, B). Secondly, we determined the localization of HDAC5 in the HL-1 cardiomyocytes both by transfection of GFP-HDAC5 and immunostaining of endogenous HDAC5. In control cardiomyocytes (0 h TP), over 50% of GFP-HDAC5 was found exclusively in the nucleus (Fig. 5C, D). During tachypacing, the percentage of nuclear GFP-HDAC5 decreased gradually, and after 12 h tachypacing, < 10% of the

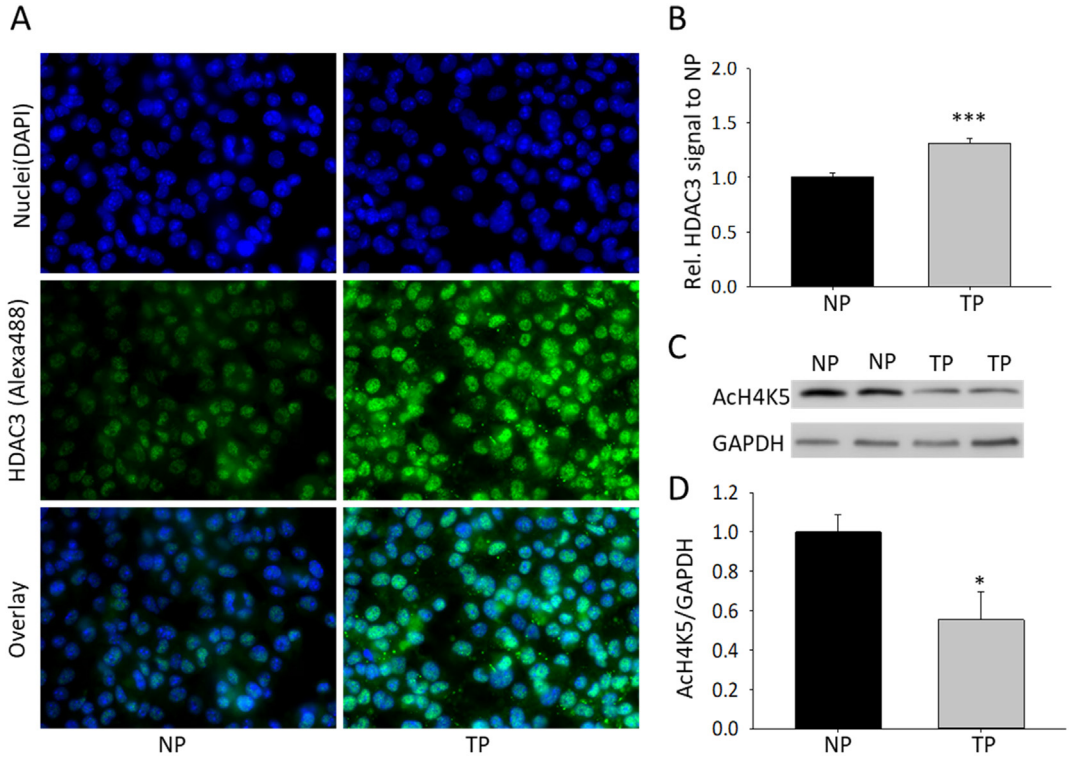

Fig. 2. Tachypacing increases HDAC3 protein and activity levels in HL-1 cardiomyocytes. A, B) Representative immunofluoresent staining and quantified data of HDAC3 in non-paced (NP) and tachypaced (TP) HL-1 cardiomyocytes. N = 24 images for NP, N = 49 images for TP, from over 1000 cardiomyoyctes. $^{***}P < 0.001$ vs NP. C, D) Representative Western blot and quantified data of acetyl-Histone H4 lysine 5 (AcH4K5), an HDAC3 substrate, indicating increased HDAC3 activity after TP. N = 4 experiments per group. $^{*}P < 0.05$ vs NP.

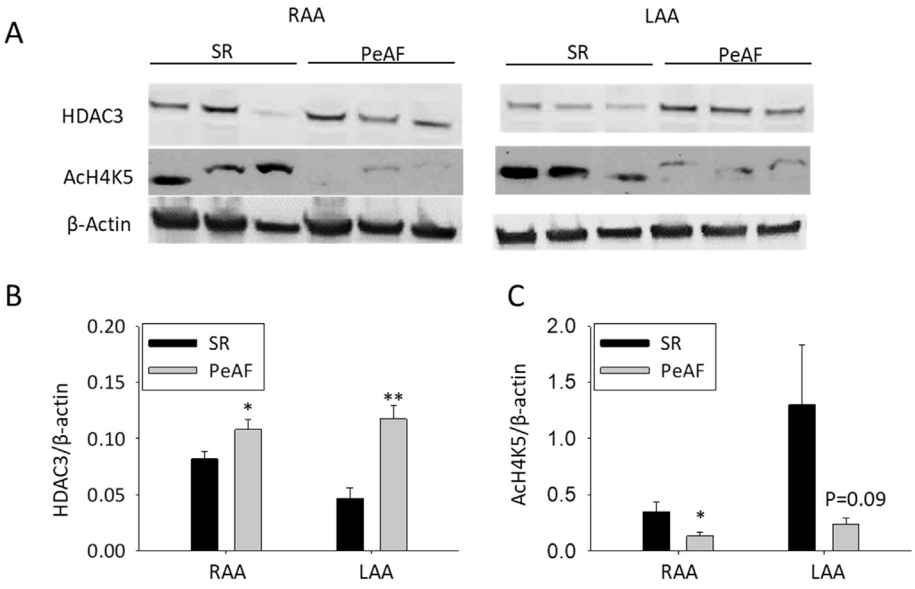

Fig. 3. HDAC3 protein and activity levels are increased in PeAF patients compared to SR. A, B) Representative Western blot and quantified data of HDAC3 in patients with SR and PeAF. *$P < 0.05$ SR RAA vs AF RAA, **$P < 0.01$ SR LAA vs PeAF LAA. N = 7 for SR RAA, N = 8 for PeAF RAA, N = 5 for SR LAA and N = 5 for PeAF LAA. C) Quantified data of AcH4K5 levels for the groups as indicated, revealing significant increased HDAC3 activity in PeAF patients. *$P < 0.05$ SR RAA vs PeAF RAA. N = 9 for SR RAA, N = 9 for PeAF RAA, N = 4 for SR LAA and N = 4 for PeAF LAA.

cardiomyocytes revealed exclusively nuclear staining of HDAC5 (Fig. 5C, D). Similar findings were observed for endogenous HDAC5, which was localized in the nucleus in control conditions and exported to the cytoplasm following tachypacing (Supplemental Fig. 3A, B). Together, these results demonstrate tachypacing to induce phosphorylation and nuclear export of HDAC5 in HL-1 cardiomyocytes.

3.4. MEF2 regulates fetal gene expression in tachypaced HL-1 cardiomyocytes

Upon tachypacing, nuclear HDAC5 is phosphorylated, released from MEF2 and exported to the cytosol [26,28–30], which allows the histone acetyltransferase p300 to associate with MEF2 via the HDAC docking site, thereby converting MEF2 from a transcriptional repressor to a transcriptional activator of fetal genes, including β-MHC and BNP [19,20]. To test whether tachypacing induces the expression of β-MHC, HL-1 cardiomyocytes were tachypaced and mRNA levels of β-MHC and α-MHC levels were determined by quantitative PCR with reverse transcription (RT–PCR). The ratio of β-MHC to α-MHC increased significantly after 12 h tachypacing in HL-1 cardiomyocytes (Fig. 6A). Interestingly, tachypacing induced a gradual and significant induction of BNP mRNA in HL-1 cardiomyocytes (Fig. 6B), as was previously found in persistent AF [10]. In addition, tachypacing-induced BNP levels, correlate significantly with the phosphorylated HDAC5 levels (Fig. 6C). These results reveal that tachypacing induces the expression of fetal genes in HL-1 cardiomyocytes, which correlates partly with HDAC5 phosphorylation.

3.5. HDAC5 phosphorylation and fetal gene expression are induced in persistent AF patients

To investigate whether a similar modulating role of HDAC5 is found in patients with AF, the amount of phosphorylated HDAC5 was measured in LAA and RAA of patients with PeAF and controls in normal SR. A significant increase in the level of phosphorylated HDAC5 was observed in the LAA of patients with PeAF compared to SR controls (Fig. 7A, B). Moreover, the gene expression levels of β-MHC, α-MHC and BNP were determined. Comparable to tachypaced HL-1 cardiomyocytes, a significant induction in the ratio of β-MHC/α-MHC in the RAA and increased expression of BNP in the LAA was observed in patients with PeAF compared to control SR patients (Fig. 7C, D). Again the induction of BNP correlated significantly with phosphorylated HDAC5 levels (Fig. 7E), consistent with the results in tachypaced HL-1 cardiomyocyte (Fig. 6C). Our findings in patients with PeAF indicate that phosphorylation of HDAC5 and subsequent activation of MEF2-related fetal gene expression underlies cardiomyocyte remodeling and AF progression, especially in the LAA.

3.6. HDAC3 inhibitor and HDAC5 nuclear boosters protect against tachypacing-induced contractile dysfunction in HL-1 cardiomyocytes and in Drosophila

Given that HDAC3 knockdown protects against contractile dysfunction in both cardiomyocyte and *Drosophila* models for AF, we expect the specific HDAC3 inhibitor, RGFP966, to protect against tachypacing-induced contractile dysfunction in experimental models for AF. As expected, RGFP966 pretreatment significantly prevented tachypacing-induced CaT loss in HL-1 cardiomyocytes (Fig. 8A, B and Supplemental Fig. S4A). RGFP966 also significantly protected against tachypacing-induced heart rate reduction and increased arrhythmicity index in the *Drosophila* model for AF (Fig. 8C-E).

Our results also suggest that HDAC5 protects against AF-remodeling and may represent an interesting therapeutic target to prevent pathological fetal gene expression, functional loss and AF progression. Consequently, compounds boosting HDAC5 nuclear localization and thereby inhibiting the release of HDAC5 from MEF2, may have a therapeutic potential for treatment of AF and it is of interest to test these compounds in experimental AF. One such compound is MC1568, since it inhibits the activity of HDAC4 and HDAC5, thereby leaving MEF2-HDAC complexes in a repressed state [31]. The other compound is the PKC inhibitor Go6983, which has been reported to block HDAC5 nuclear export [28,32]. We tested MC1568 and Go6983 in tachypaced HL-1 cardiomyocytes and *Drosophila* prepupae. Both compounds increased the nuclear HDAC5 level and protected against the CaT loss in tachypaced HL-1 cardiomyocytes (Fig. 9A, B; Supplemental Figs. S4B-F and S5A, B). They also protected against the reduction in heart rate and increased arrhythmicity in tachypaced *Drosophila* prepupae (Fig. 9C-E, Supplemental Fig. S5C-E).

The findings indicate that both HDAC3 inhibitors and HDAC5 nuclear boosters may represent promising novel compounds to prevent AF promotion.

4. Discussion

In the current study, we evaluated the role of class I and class IIa HDACs in tachypacing-induced cardiomyocyte remodeling. We found

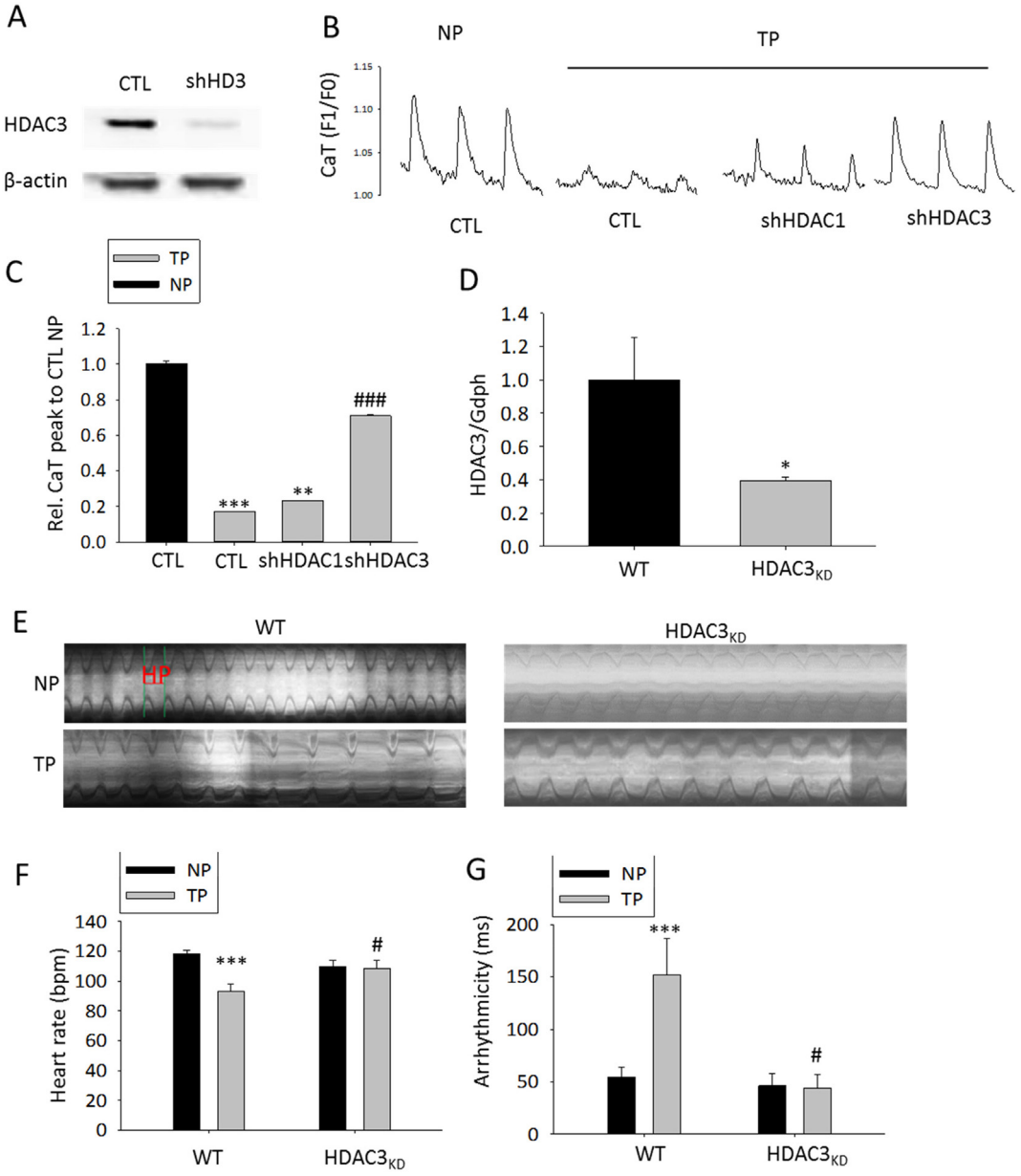

Fig. 4. HDAC3 knockdown protects against tachypacing-induced contractile dysfunction in both HL-1 cardiomyocytes and *Drosophila*. A) Western blot showing successful knockdown of HDAC3 with retro viral HDAC3 shRNA compared to Luciferase shRNA (CTL) in HL-1 cardiomyocytes. B,C) Representative CaT traces and quantified data showing that knockdown of HDAC3 protects against tachypacing-induced CaT loss in HL-1 cardiomyocytes. $^{**}P < 0.01$, $^{***}P < 0.001$ vs CTL NP, $^{\#\#\#}P < 0.001$ vs CTL TP. N = 10 cardiomyocytes per group. D) Representative qPCR showing significant knockdown of HDAC3 in *Drosophila*. $^{*}P < 0.05$ HDAC3 knockdown ($HDAC3_{KD}$) vs wild type control (WT). N = 2 experiments for WT, N = 3 for $HDAC3_{KD}$. E) Representative heart wall traces (10 s) prepared from high-speed movies of *Drosophila* prepupa. Movies were made in non-tachypaced (NP) and after tachypacing (TP) of *Drosophila* prepupa. Heart period (HP) is the time interval between two beats. Heart rate (BPM: beats per minute) is calculated by mean of HP divided by 1 min. Arrhythmicity index is calculated as the standard deviation of HP in milliseconds (ms). F, G) Quantified heart rate and arrhythmicity index of NP and TP in WT and $HDAC3_{KD}$*Drosophila*, showing HDAC3 knockdown significantly protected against tachypacing-induced heart rate loss and increased arrhythmicity index. $^{***}P < 0.001$ vs WT NP, $^{\#}P < 0.5$ vs WT TP. N ≥ 11 *Drosophila* per group.

that overexpression of class I members, HDAC1 and HDAC3, results in detrimental effects on contractile function in HL-1 cardiomyocytes. Also, HDAC3 expression and activity levels were increased in atrial tissue from PeAF patients compared to controls in SR, indicating a role for HDAC3 in clinical AF. In line, genetic and pharmacological inhibition of HDAC3 protected against tachypacing-induced contractile dysfunction in both HL-1 cardiomyocytes and *Drosophila*, suggesting that HDAC3 inhibition protects against AF remodeling.

In contrast, overexpression of class IIa HDAC5 and HDAC7 revealed protective effects against tachypacing-induced contractile dysfunction via binding to MEF2, thereby possibly preventing fetal gene expression. In addition, tachypacing resulted in phosphorylation of HDAC5, nuclear export and downstream fetal gene activation in HL-1 cardiomyocytes. The experimental findings were confirmed in atrial appendages of patients with PeAF. In line, compounds which boost nuclear HDAC5 attenuated tachypacing-induced contractile dysfunction in both HL-1 cardiomyocytes and *Drosophila*.

Taken together, the findings suggest that HDAC3 and HDAC5 are interesting therapeutic targets in AF.

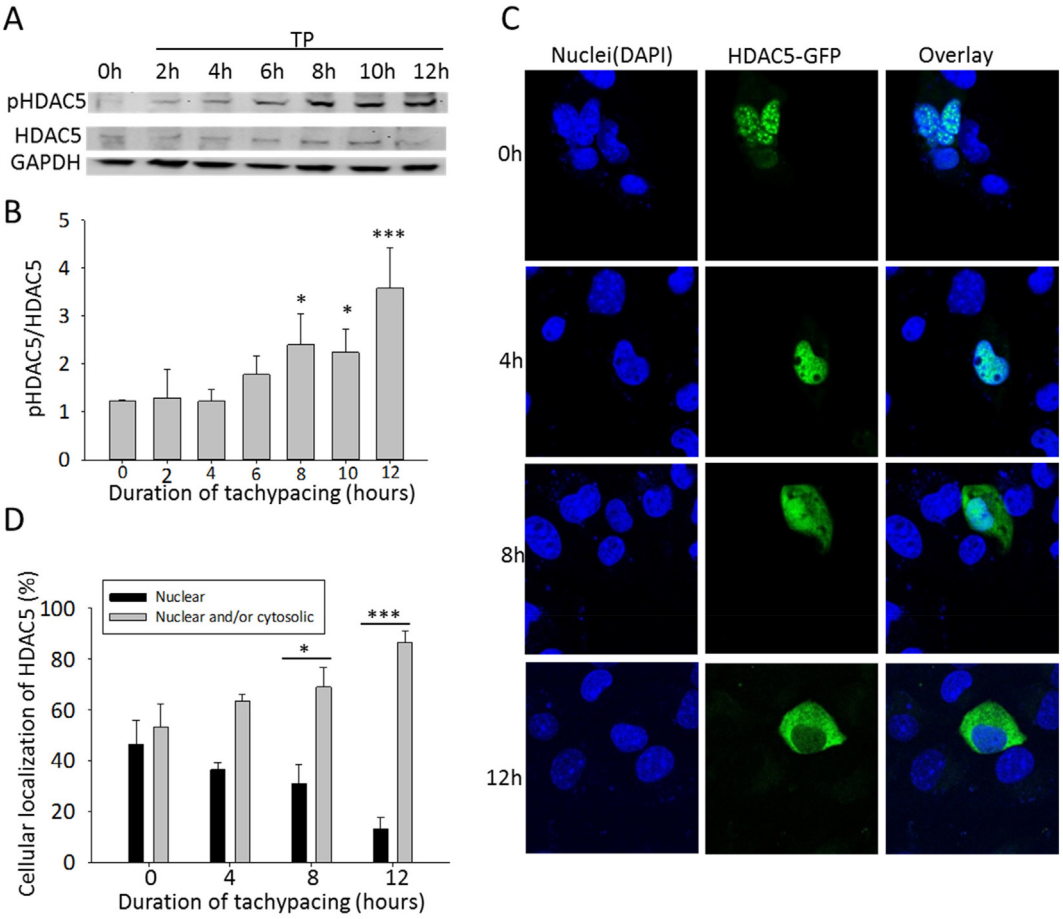

Fig. 5. Tachypacing incudes HDAC5 phosphorylation and nuclear export. A) Representative Western blot of phosphorylated HDAC5 (pHDAC5), HDAC5 and GAPDH in HL-1 cardiomyocytes tachypaced for the duration as indicated. B) Quantified ratio of pHDAC5 to HDAC5 from at least 3 independent experiments. $^{*}P < 0.05$ vs 0 h, $^{***}P < 0.001$ vs 0 h. C) Representative confocal images of cardiomyocytes transfected with HDAC5-GFP construct showing the localization of HDAC5 in HL-1 cardiomyocytes tachypaced from 0 h to 12 h. D) Quantified data of cellular localization of HDAC5, showing significant decrease in nuclear localization and significant increase in cytosolic localization of HDAC5 after 12 h TP. $^{***}P < 0.001$ vs 0 h chi-square test. N ≥ 22 cardiomyocytes per group.

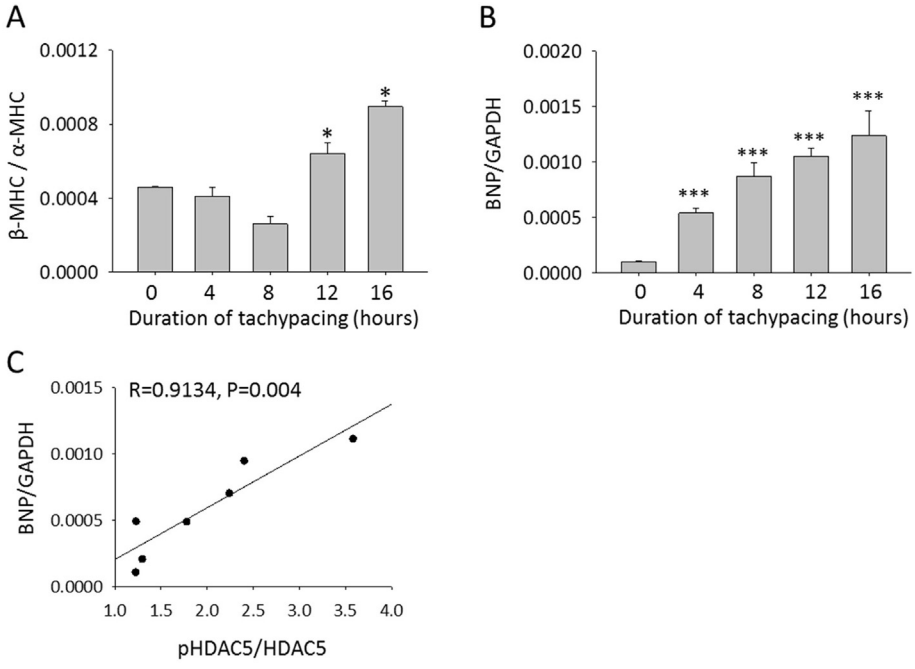

Fig. 6. Activation of fetal gene program in tachypaced HL-1 cardiomyocytes. A) Time course of TP-induced increase in gene expression ratio of β-MHC (MHY7) to α-MHC (MHY6). $^{*}P < 0.05$, $^{***}P < 0.001$ vs 0 h. B) BNP gene expression is significantly increased during TP. $^{***}P < 0.01$ vs 0 h. C) BNP gene expression level correlates significantly with level of pHDAC5 during time-course of TP.

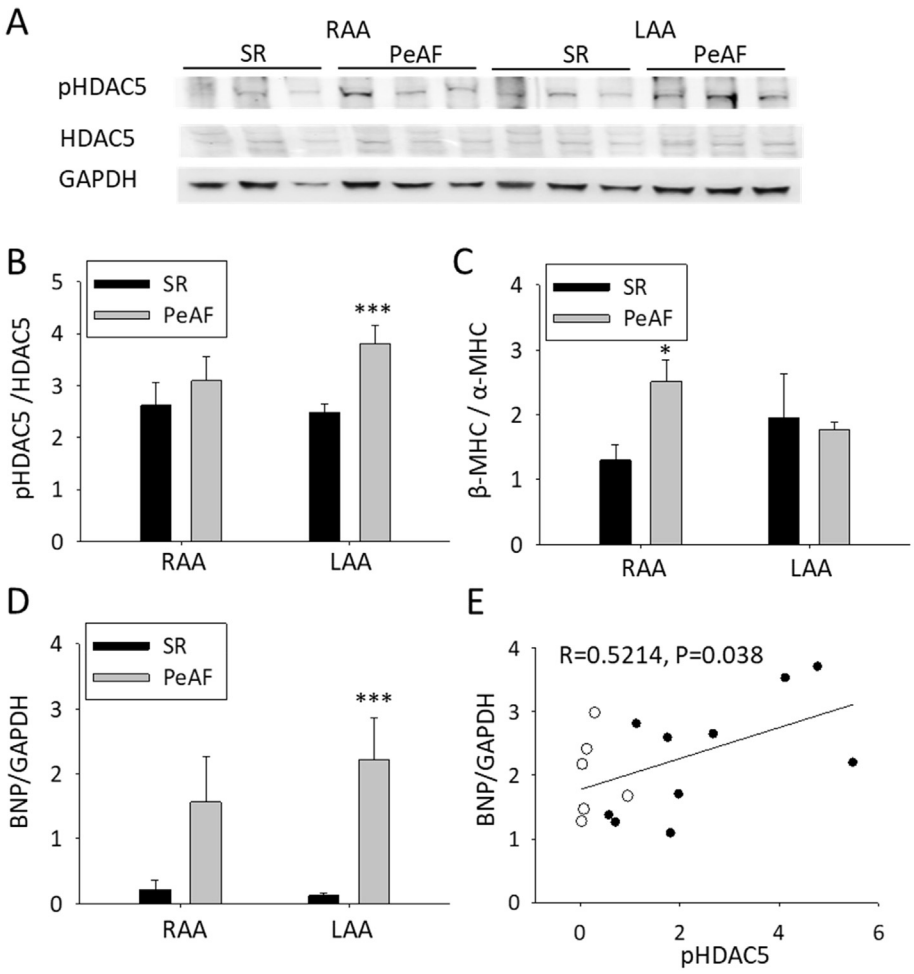

Fig. 7. pHDAC5 levels correlate with BNP gene expression levels in LAA of AF patients. A) Representative Western blot of phosphorylated HDAC5 (pHDAC5), HDAC5 and GAPDH in patients with PeAF. B) Quantified ratio of pHDAC5 to HDAC5 in patients. N = 5 from each group. ***$P < 0.001$ PeAF vs SR. C) The gene expression ratio of β-MHC (MHY7) to α-MHC (MHY6) is significantly increased in PeAF patients. *$P < 0.05$ vs SR RAA. N = 6 for SR, N = 7 for PeAF. D) BNP gene expression is significantly increased in PeAF patients. ***$P < 0.001$ vs SR LAA. N = 6 for SR, N = 7 for PeAF. E) BNP gene expression level correlates significantly with pHDAC5 levels in patients (SR N = 3 for LAA/RAA, PeAF N = 5 for LAA/RAA). Open circles: SR, filled circles: PeAF.

4.1. Role of class I HDACs in cardiac growth and disease

In the present study, overexpression of members of the class I HDAC family, HDAC1 and HDAC3 show detrimental effects on normal contractile function of HL-1 cardiomyocytes. Class I HDACs control expression of a vast array of genes involved in core cellular activities, such as cell proliferation and death [33]. All class I HDAC members are ubiquitously expressed in the heart, localize predominantly in the nucleus and display high enzymatic activity toward histone substrates [33]. Redundant functions of HDAC1 and HDAC2 have been described by Olson's lab, i.e. cardiac deletion of either HDAC1 or HDAC2 does not substantially interfere with normal heart development, whereas cardiac deletion of both HDAC1 and HDAC2 results in neonatal lethality, accompanied by cardiac arrhythmias, dilated cardiomyopathy, and upregulation of genes encoding skeletal muscle-specific contractile proteins and calcium channels in the heart [34]. Trivedi et al. found that in HDAC2-deficient mice exposed to hypertrophic stimuli, cardiac hypertrophy and fibrosis were attenuated and that cardiac-specific overexpression of HDAC2 resulted in cardiac hypertrophy [35]. The recent finding that HDAC1 and HDAC2 play a major role in autophagy driven by α-adrenergic stimulation in cultured cardiomyocytes provides another indication that HDAC1 and HDAC2 may act as a driver of adverse cardiac remodeling [36]. Although the roles of the class I HDACs, HDAC1 and HDAC2, in cardiac hyperplasia, growth, and hypertrophic responsiveness have been reported, the role of HDAC3 in the heart has been less well explored. Cardiomyocyte-specific overexpression of HDAC3 in mice results in cardiac abnormalities at birth and increased cardiac wall thickness due to increased cardiomyocyte hyperplasia [37]. Consistent with its detrimental role in heart development and contraction, we observed HDAC3 to play a role in AF promotion. HDAC3 expression and activity are increased in atrial tissue of AF patients and in tachypaced HL-1 cardiomyocytes, suggesting HDAC3 to contribute to AF progression, possibly via changing the expression of ion-channels and contractile proteins, as have been observed for HDAC1 and HDAC3 [34]. In line, HDAC3 knockdown or HDAC3 inhibition prevented against contractile dysfunction in tachypaced HL-1 and *Drosophila* models for AF, suggesting HDAC3 as a potential druggable target in AF.

4.2. Key role for class IIa HDAC5 in AF

In the present study, we identified a key role for tachypacing-induced activation, nuclear export of HDAC5, and consequently transcriptional reprogramming in cardiomyocytes. In general, class IIa HDACs (HDAC4, HDAC5, HDAC7 and HDAC9) all have large N-terminal extensions with conserved binding sites for the transcription factor MEF2 and the chaperone protein 14-3-3 (Supplemental Fig. S6A). Binding of MEF2 to these sites results in suppression of transcriptional activity [19,38]. Following phosphorylation by kinases, such as calcium/calmodulin-dependent protein kinase (CaMK), which is activated in AF [39], class IIa HDACs bind 14–3-3 and shuttle from the nucleus to the cytoplasm [19,38,40]. The dissociation of class IIa HDACs from MEF2 allows the histone acetyltransferase p300 to associate with MEF2 via the HDAC docking site, thereby converting MEF2 from a transcriptional repressor (MEF2-HDAC complex) to a transcriptional activator (MEF2-p300 complex) of fetal gene program [19,38,40]. In response to injury, MEF2 is activated resulting in pathological cardiac hypertrophy and heart failure [41].

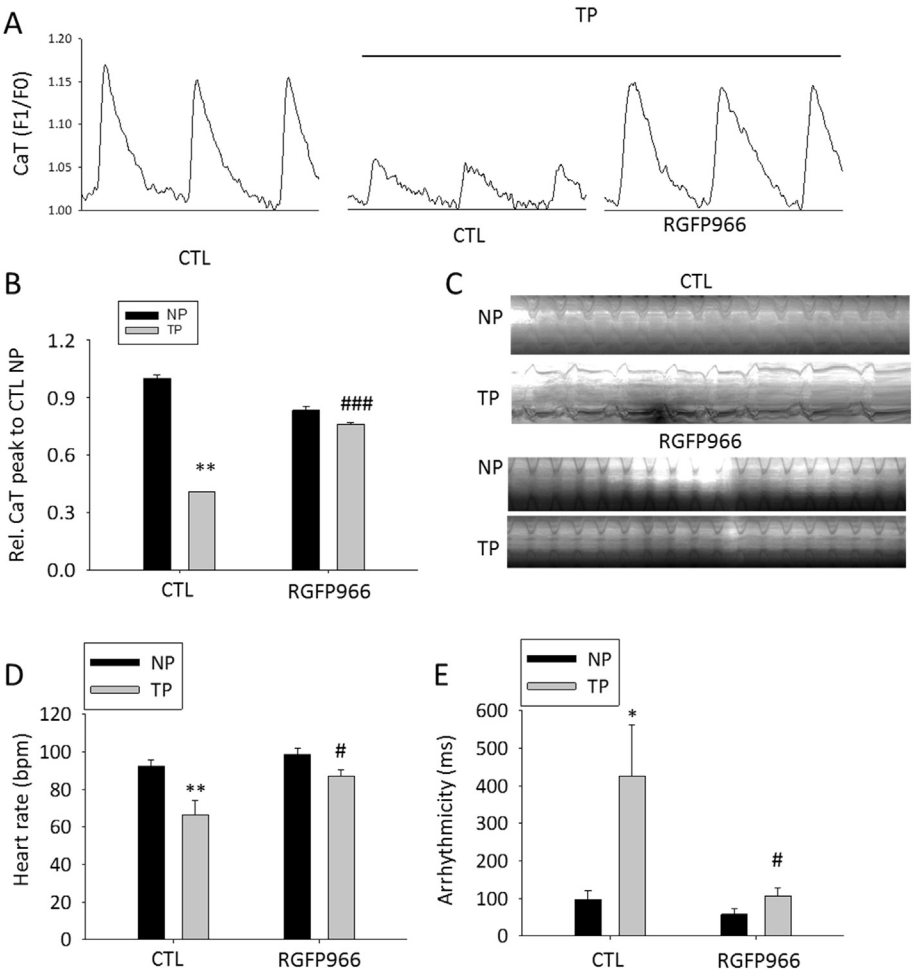

Fig. 8. HDAC3 inhibitor protects against tachypacing-induced contractile dysfunction in HL-1 cardiomyocytes and *Drosophila*. A, B) Representative CaT traces and quantified data showing specific HDAC3 inhibitor RGFP966 (5 μM) protects against tachypacing-induced CaT loss in HL-1 cardiomyocytes. Cardiomyocytes are treated with either DMSO (CTL) or 5 μM RGFP966 before tachypacing. $^{**}P < 0.01$ vs CTL NP, $^{\#\#\#}P < 0.001$ vs CTL TP. N = 10 cardiomyocytes per group, two independent experiments. C-E) Representative heart wall traces and quantified data showing specific HDAC3 inhibitor RGFP966 (50 μM) protects against tachypacing-induced heart rate reduction and increased arrhythmicity in *Drosophila* prepupa. $^{*}P < 0.05$ vs CTL NP, $^{**}P < 0.01$ vs CTL NP, $^{\#}P < 0.05$ vs CTL TP. N = 11 *Drosophila* per group.

Although all members of Class IIa HDACs can bind to MEF2 to suppress fetal gene expression, hypertrophy and heart failure, in the current study we observed a protective role for HDAC5 and HDAC7, but not HDAC4 and HDAC9, in tachypacing-induced remodeling (Supplemental Fig. S6B). This discrepancy may be explained by tachypacing-induced activation of particular upstream kinases which only induce phosphorylation of specific members of HDAC class IIa. Furthermore, specific HDAC class IIa members may affect different downstream targets that affect cardiac remodeling. An example of the latter is the recently described non-transcriptional function for HDAC4 in the heart comprising its association with cardiac sarcomeres and lowering of myofilament calcium sensitivity [42]. In our study, a possible protective role of HDAC4 to suppress MEF2 related fetal gene expression may have been offset by the HDAC4 detrimental effect on contractile function. Moreover, HDAC4 binds constitutively to 14-3-3 in yeast and mammalian cells, whereas HDAC5 binding to 14-3-3 is dependent on CaMK signaling. In addition, 14-3-3 binding to HDAC5 is required for CaMK-dependent disruption of MEF2-HDAC complexes and nuclear export of HDAC5 [43,44]. Since CaMK signaling is involved in AF progression, the role of specific upstream kinases seems plausible. The involvement of HDAC7 in heart diseases has not been studied well. However, there are indications for HDAC7 to regulate the function of MEF2 proteins in heart and muscle tissue [45], suggesting a similar role as HDAC5.

HDAC5 and HDAC9 have redundant roles in the suppression of cardiac growth in response to stress signaling, and both are highly enriched in the heart compared to HDAC7 [26,27]. Despite sharing common upstream kinases, which stimulate the translocation of HDAC5 and HDAC9, only translocation of HDAC5 is activated by calpain [26–28]. Interestingly, calpain was found to be activated in AF and underlies structural remodeling and contractile dysfunction in AF [24]. Thus, calpain-induced HDAC5 activation may be involved in transcriptional reprogramming and cardiomyocyte remodeling as observed in AF.

4.3. Therapeutic implications

In the current study, we observed opposite key roles for class I HDAC3 and class IIa HDAC5 in AF remodeling. Class I HDAC3 overexpression caused contractile dysfunction in HL-1 cardiomyocyte model for AF. Also, HDAC3 protein and activity are both increased in tachypaced HL-1 cardiomyocytes and in AF patients. Both pharmacological and genetic inhibition of HDAC3 prevented contractile dysfunction in HL-1 and *Drosophila* model for AF. Specific HDAC3 inhibitor, RGFP966, thus represents an interesting compound to be tested in big animal model for AF and ultimately in clinical AF. On the contrary, class IIa HDAC5 protected against AF remodeling via binding to MEF2 and suppression of MEF2-related fetal gene program. Consequently, compounds inhibiting the release of HDAC5 from MEF2 might have a therapeutic potential for treatment of AF. One such compound, MC1568 [31], protected against tachypacing-induced contractile dysfunction in both HL-1 and *Drosophila* models for AF. Also, inhibitors of upstream kinases, which phosphorylate HDAC5 and thus initiate derepression of MEF2, would also be of interest. An example is inhibition of CaMK or PKC, since these represent two main kinases involved in HDAC5 phosphorylation and nuclear export in cardiomyocytes [44]. Indeed, CaMK inhibitors have been reported to prevent AF [39]. Furthermore, the PKC inhibitor Go6983 has been reported to block HDAC5

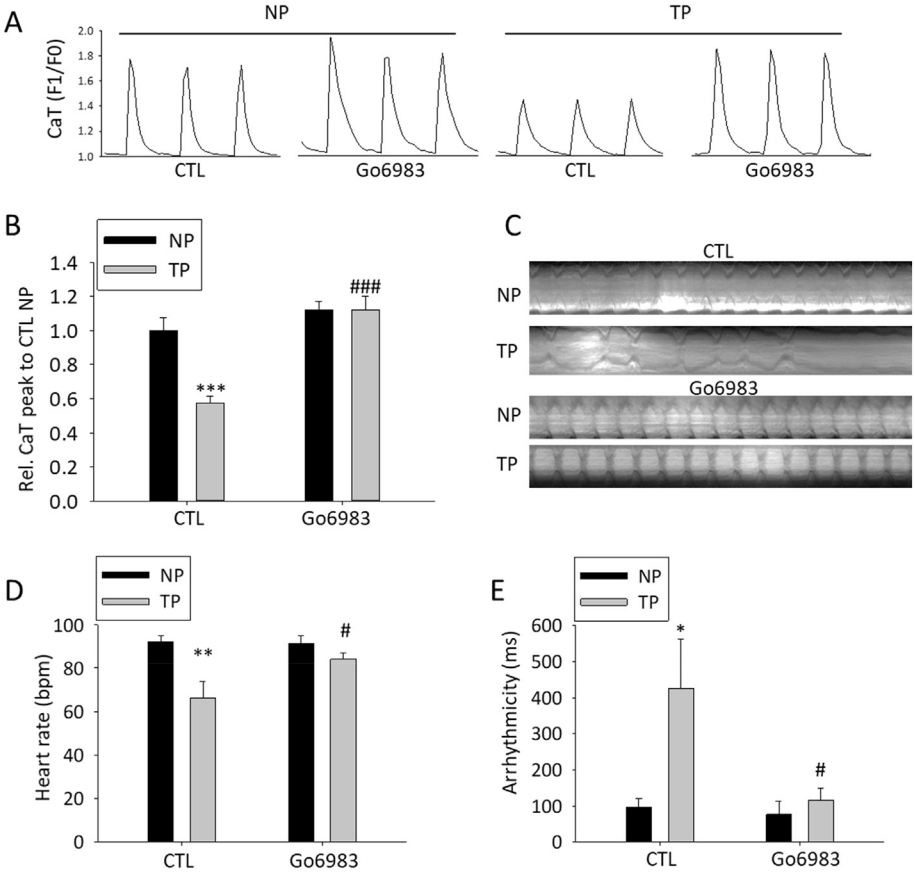

Fig. 9. HDAC5 nuclear booster Go6983 protects against tachypacing-induced contractile dysfunction in HL-1 cardiomyocytes and Drosophila. A, B) Representative CaT traces and quantified data showing HDAC5 nuclear booster, Go6983 (5 μM), protects against tachypacing-induced CaT loss in HL-1 cardiomyocytes. Control cardiomyocytes are treated with vehicle DMSO (CTL). $^{***}P < 0.001$ vs CTL NP, $^{\#\#\#}P < 0.001$ vs CTL TP. N = 9–11 movies from around 50 cardiomyocytes per group. C-E) Representative M-mode heart wall traces and quantified data showing Go6983 (50 μM) protects against tachypacing-induced heart rate reduction and increased arrhythmicity in *Drosophila* prepupae. $^{**}P < 0.01$ vs CTL NP, $^{\#}P < 0.05$ vs CTL TP. N = 11–12 *Drosophila* per group, two independent experiments.

nuclear export and also α-tubulin deacetylation after nerve injury [28,32]. We found Go6983 to prevent tachypacing-induced remodeling in HL-1 cardiomyocytes and *Drosophila* models for AF. Thus, our study suggests HDAC5 repression of MEF2 responsive genes to be involved in AF. Therefore, HDAC5 nuclear boosters, which prevent stress-induced HDAC5 release from MEF2, such as MC1568 and PKC inhibitor Go6983, might represent novel therapeutic approaches to attenuate AF progression.

4.4. Limitations of the study

We describe the role of class I and IIa HDACs on cardiomyocyte dysfunction in AF by utilizing tachypaced HL-1 cardiomyocyte (mouse atrial tumor cells) and *Drosophila* model systems for AF related remodeling, because both systems have merit to identify potential signaling pathways involved in AF remodeling. As a result, findings have been confirmed repeatedly in the tachypaced dog model for AF and clinical AF [17,46,47]. Nevertheless, caution must be taken in extrapolating findings from the model systems to clinical AF. In addition, the current study provides evidence for a role of HDAC3 and HDAC5 activation in AF. Future studies should elucidate the upstream pathways involved in the activation of HDAC3 or HDAC5.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2018.09.010.

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