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Ryanodine receptor stabilization therapy suppresses Ca^{2+}- based arrhythmias in a novel model of metabolic HFpEF

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ABSTRACT

Heart Failure with preserved ejection fraction (HFpEF) has a high rate of sudden cardiac death (SCD) and empirical treatment is ineffective. We developed a novel preclinical model of metabolic HFpEF that presents with stress-induced ventricular tachycardia (VT). Mechanistically, we discovered arrhythmogenic changes in intracellular Ca^{2+} handling distinct from the changes pathognomonic for heart failure with reduced ejection fraction. We further show that dantrolene, a stabilizer of the ryanodine receptor Ca^{2+} channel, attenuates HFpEF-associated arrhythmogenic Ca^{2+} handling *in vitro* and suppresses stress-induced VT *in vivo*. We propose ryanodine receptor stabilization as a mechanistic approach to mitigation of malignant VT in metabolic HFpEF.

Heart failure (HF) with preserved ejection fraction (HFpEF), the most prevalent form of HF worldwide, is associated with significant morbidity and high mortality [1]. Initially considered a disorder of diastolic dysfunction related to hypertensive left-ventricular remodeling, HFpEF is now understood as a heterogeneous disease widely seen in patients with significant metabolic stress driven by underlying comorbidities of obesity, dyslipidemia, hypertension and decreased insulin sensitivity [1,2]. HFpEF is characterized by a high rate of sudden cardiac death (SCD; ~30–40%) with VT and/or ventricular fibrillation (VF) as underlying mechanisms [3,4]. Evidence that empiric anti-arrhythmic treatment is ineffective in HFpEF suggests that ventricular arrhythmias in metabolic stress-related HFpEF may arise via a distinct and as yet unidentified mechanism [5].

1. Results and discussion

To investigate arrhythmia mechanisms pertaining to the large clinical population of metabolic stress related HFpEF, we developed a preclinical model in wildtype mice (C57BL6/J). The model is based solely on mimicking the most common stressors (hypercaloric Western diet, hypertension, obesity) identified in patients with metabolic stress related HFpEF, without relying on genetically altered animals. To achieve this, we added high dietary fructose (20% of dietary calories), an important driver of metabolic stress in the heart [6], to the well-established murine HFpEF model produced by high fat diet and hypertension-inducing nitric oxide synthase inhibition (with L-NAME) [7]. After 5 months, this **f**ructose- **m**etabolic HFpEF model (FM-HFpEF) demonstrated key *in vivo* aspects of the clinical human HFpEF phenotype, including obesity, sarcopenia, decreased insulin sensitivity, high serum levels of brain natriuretic peptide (BNP), decreased global longitudinal strain (GLS) with normal ejection fraction (EF) and, importantly, stress-induced ventricular tachycardia (Fig. 1A-E).

Ca^{2+} "leaking" from its stores in the sarcoplasmic reticulum (SR) is the key mechanism underlying Ca^{2+} based arrhythmogenesis in heart failure with reduced ejection fraction (HFrEF) [8,9]. Here we report a massive arrhythmogenic intracellular Ca^{2+} leak from the SR in FM-HFpEF ventricular myocytes, much larger than in HFrEF [9], evidenced by a ~ 10-fold increase in spontaneous Ca^{2+} spark frequency and ~ 9-fold increase in spark mediated SR Ca^{2+} leak (Fig. 1F). As a critical driver of this huge SR Ca^{2+} leak we identified a highly significant

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increase (~ 28%) in SR Ca^{2+} load in FM-HFpEF myocytes compared to control cells (Fig. 1G). Consistent with high Ca^{2+} spark frequency and SR Ca^{2+} load in FM-HFpEF was a dramatic increase in spontaneous Ca^{2+} wave activity (~7-fold, Fig. 1H), the cellular trigger for Ca^{2+} based arrhythmias [8,9]. Dantrolene (1 μmol/L), a stabilizing agent for the ryanodine receptor Ca^{2+} release channels in the SR [10], effectively reduced the frequency of spontaneous Ca^{2+} waves in FM-HFpEF myocytes to control levels (Fig. 1H).

Aligned with exercise induced arrythmia seen in clinical HFpEF, we show β-adrenergic challenge ("stress") *in vivo* elicits VTs in FM-HFpEF mice (Fig. 1E). The Ca^{2+} basis for this arrhythmogenic activity was evidenced *in vitro* by demonstrating that β-adrenergic challenge (isoproterenol 50 nmol/L) further amplified the frequency of arrhythmogenic Ca^{2+} waves in FM-HFpEF myocytes (Fig. 1H). Importantly, the high degree of intracellular Ca^{2+} mobilization *in vitro*, together with the inducibility of sustained VTs *in vivo*, demonstrates an intact and highly sensitive β-adrenergic response in FM-HFpEF.

A crucial underlying mechanism of Ca^{2+} based arrhythmogenesis in

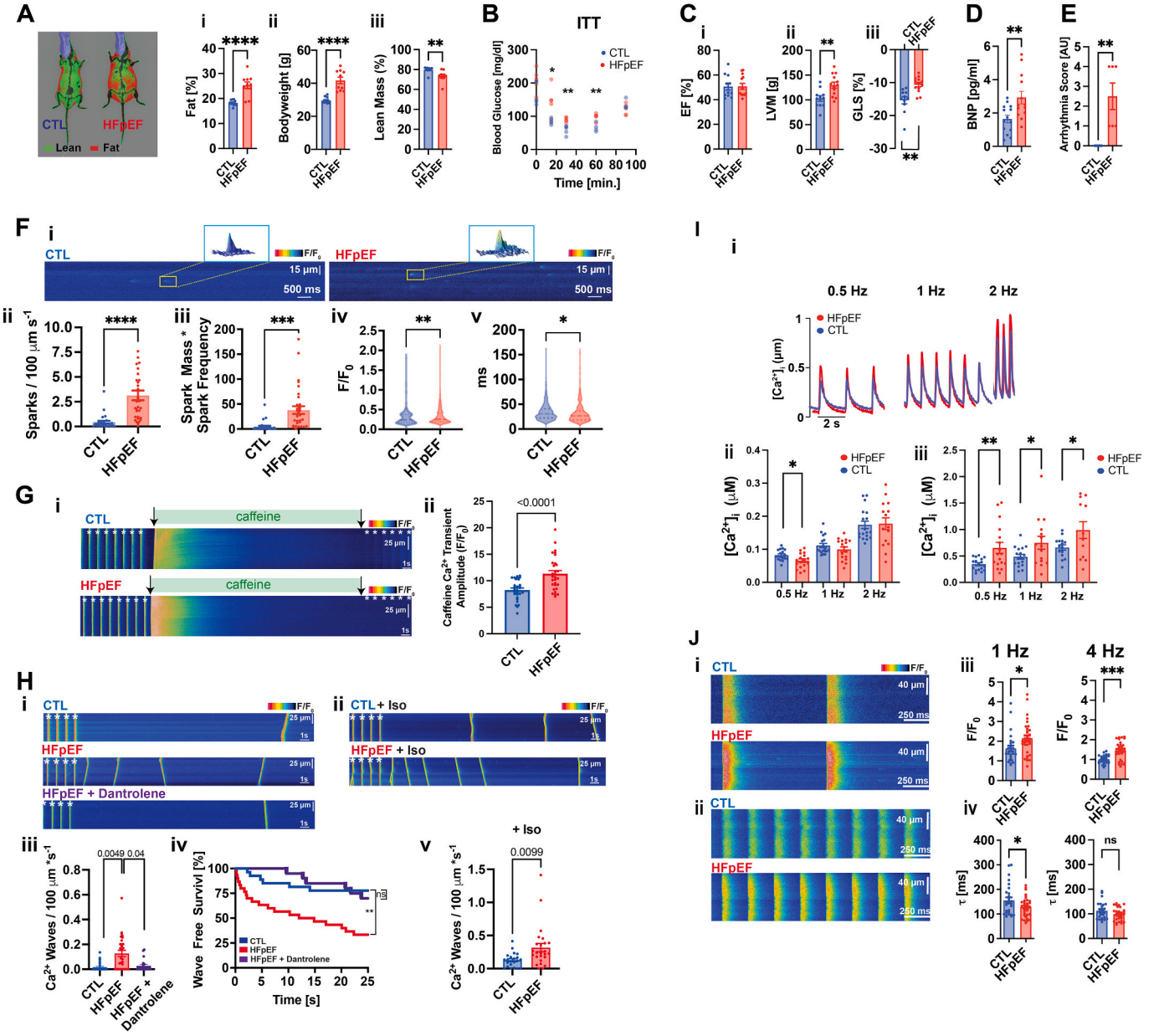

Fig. 1. Arrhythmogenic Ca^{2+} signaling in FM-HFpEF. Characterization of the metabolic FM-HFpEF model in mice. **A.** Body composition: **i.** Fat, **ii.** Bodyweight and **iii.** Lean Mass. **B.** Insulin tolerance test (ITT) **C.** Transthoracic echocardiography **i.** Ejection fraction (EF), **ii.** Left ventricular mass (LVM) **iii.** Global longitudinal strain (GLS), **: $p < 0.01$. **D.** Serum levels of B-type natriuretic peptide (BNP). **E.** EKGs and arrhythmia score. **: $p < 0.01$. Intracellular calcium imaging in control ($n = 4$) and FM-HFpEF (n = 4) mice. **F.** Ca^{2+} sparks. **i.** Exemplars of Ca^{2+} sparks. Summary data for **ii.** Ca^{2+} spark frequency, **iii.** Ca^{2+} spark-mediated SR Ca^{2+} leak, **iv.** Ca^{2+} spark amplitude, **v.** Ca^{2+} spark duration as full duration half maximum (FDHM). ****: $p < 0.0001$; ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$. **G.** SR Ca^{2+} load. **i** Exemplars of SR Ca^{2+} load elicited by application of caffeine (10 mmol/L) in a control and a FM-HFpEF myocyte. **ii** Summary data. **H.** Ca^{2+} waves. **i.** Exemplars of spontaneous Ca^{2+} waves. **ii.** Exemplars of spontaneous Ca^{2+} waves after isoproterenol treatment. *denotes stimulated beat. **iii.** Summary data of Ca^{2+} wave frequency **iv.** Ca^{2+} wave free survival **v.** Summary data of Ca^{2+} wave frequency (+ isoproterenol). ***: $p < 0.001$; **: $p < 0.01$. **I.** $[Ca^{2+}]_i$ in HFpEF myocytes. **i.** Exemplars of stimulated Ca^{2+} transients (0.5–2 Hz). Summary data of **ii.** diastolic $[Ca^{2+}]_i$, **iii.** Systolic $[Ca^{2+}]_i$. **J. i, ii.** Exemplars of stimulated Ca^{2+} transients (1, 4 Hz). Summary data of **iii.** Ca^{2+} transient amplitude and **iv.** Ca^{2+} transient decay. Statistical Analysis was performed using either Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparisons test (Fig. Hiii,iv). Data are shown as mean ± SEM.

HFrEF is increased diastolic Ca^{2+} concentration in the cytosol ($[Ca^{2+}]_i$), which sensitizes the cardiac ryanodine receptors (RyR2) to release Ca^{2+} [11]. Surprisingly, calibrated measurements of $[Ca^{2+}]_i$ in single myocytes electrically paced at physiological frequencies showed that diastolic $[Ca^{2+}]_i$ was not increased in FM-HFpEF myocytes (2 Hz, Fig. 1Ii,ii) despite an increase in the magnitude of systolic $[Ca^{2+}]_i$ (Fig. 1Ii-iii, Fig. 1Ji-iii). In addition, Ca^{2+} transient decay in FM-HFpEF mice was faster or unchanged from controls at physiological stimulation rates of 1–4 Hz (Fig. 1Ji,ii,iv), another striking difference from HFrEF where Ca^{2+} transients are prolonged [8,9]. To define the underlying mechanisms of this gain-of-function Ca^{2+} phenotype in FM-HFpEF, we first examined the ultrastructure of the transverse and axial tubular system (TATS) of living, single cardiomyocytes. Sub-diffraction Airy scan imaging revealed no changes in TATS density, organization or orientation in FM-HFpEF (Fig. 2A). These results are contrary to the severe reduction and disorganization of TATS pathognomonic for HFrEF, where the structural disruption of the cardiac dyad, the close apposition (~20 nm) of L-type Ca^{2+} channels ($Ca_v1.2$) with RyR2, causes arrhythmogenic SR

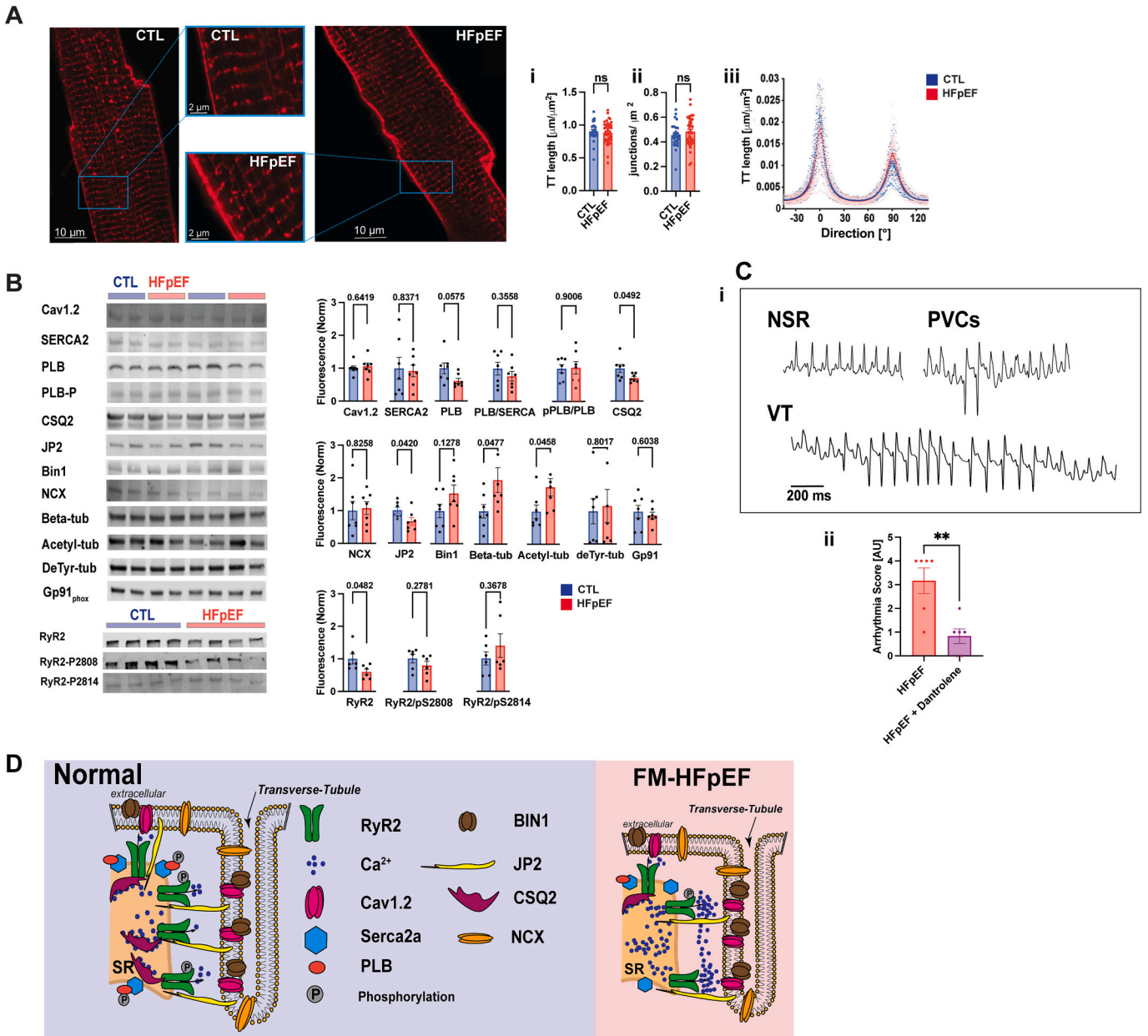

Fig. 2. Transverse-Axial Tubule system, protein expression levels and arrhythmia treatment in FM-HFpEF. A. Transverse-Axial Tubule system (TATS). AiryScan sub-diffraction images of living ventricular myocytes stained with the membrane dye di-8-ANEPPS (5 µmol/L) to label the TATS. Control mice ($n = 3$), FM-HFpEF mice (n = 4). **ii.** Total length of the TATS. **iii.** Number of junctions that connect intersecting perpendicular tubules. **iv.** The length of the TATS at each direction within the cells. **B.** Protein expression levels of key Ca^{2+} handling and structural proteins. Control mice ($n = 6–7$), FM-HFpEF mice (n = 6–7). $Ca_V1.2$: α1c subunit of the L-type Ca^{2+} channel, SERCA: SR Ca^{2+} ATPase, PLB: phospholamban, PLB-P: phosphorylated-PLB (Serine16), CSQ2: calsequestrin 2, NCX: Na^+/Ca^{2+} exchanger, JP2: junctophilin 2, BIN1: Bridging integrator 1, Beta-tub: Beta-tubulin, Acetyl-tub: Acetylated tubulin, deTyr-tub: Detyrosinated tubulin, gp91: gp91phox, RyR2: Ryanodine receptor type 2, RyR2-pS2808: phosphorylated RyR2 (Serine2808), RyR2-pS2814: phosphorylated RyR2 (Serine2814) **C.** *in vivo* dantrolene treatment of FM-HFpEF mice. **i.** examples of EKG traces: NSR: normal sinus rhythm, PVC: premature ventricular complex, VT: sustained ventricular tachycardia **ii.** Summary data showing arrhythmia index in FM-HFpEF mice (n = 6) pretreated with i.p. dantrolene (40 mg/kg i.p; 30 min) compared to untreated FM-HFpEF mice (n = 6). **: $p < 0.01$. **D.** Schematic depicting organization of the dyadic Ca^{2+} release-apparatus in normal and FM-HFpEF cardiac myocytes. Statistical Analysis was performed using Student's t-test. Data are shown as mean ± SEM.

Ca^{2+} release [12].

We next quantified the expression and post-translational modification (PTM) of structural and SR Ca^{2+} handling proteins within the dyad (Fig. 2B). We found the expression of $Ca_v1.2$, Na^+/Ca^{2+} exchanger (NCX) and BIN1, a TATS stabilizing and ion channel trafficking protein, unchanged in ventricles from HFpEF mice. In addition, we found no change in the SR Ca^{2+} ATPase (SERCA) and despite finding its regulatory protein phospholamban (PLB) in deficit, the ratio of PLB to SERCA was not impacted and the phosphorylation of PLB was unchanged in FM-HFpEF. In contrast, we found significant deficits in calsequestrin 2 (CSQ2), the major intra-SR Ca^{2+} buffering protein, and junctophilin2 (JP2), an important stabilizer of RyR2, in FM-HFpEF (Fig. 2B). Previous work linking similar deficits in CSQ2 and JP2 to malignant Ca^{2+} based arrhythmia [13,14] supports their contribution to the arrythmia identified here. Surprisingly, RyR2 expression was significantly reduced (Fig. 2B), while RyR2 phosphorylation at S2808 and S2814 was unchanged.

HFpEF hearts are adapted to maintain a normal EF in the face of high afterload (*i.e.*, increased systemic resistance) [15]. On the cellular level, our data show that increased SR Ca^{2+} load is central to this adaptation in FM-HFpEF myocytes. Intriguingly we also found a significant increase in tubulin protein and its modification by acetylation in FM-HFpEF, a finding aligned with a recent report in two disparate murine HFpEF models reporting global changes in protein acetylation linked to hypertrophic signaling [16]. We showed previously that similar tubulin changes are sufficient to increase mechano-transduction-dependent sarcolemmal Ca^{2+} influx and RyR2 Ca^{2+} release [17]. Additional work reported a background of Ca^{2+} influx *via* TRPC6 responsible for SR Ca^{2+} overload and arrhythmogenic Ca^{2+} waves in a sheep model of pacing induced HF [18]. While increased SR Ca^{2+} fluxes would be an adaptation to increase SR load and contractile activation necessary to maintain ejection fraction in HFpEF, these changes would also exacerbate the RyR2 Ca^{2+} leak arising from the significant remodeling of the cardiac dyad at the junctional SR. Future work will directly test this hypothesis.

Finally, we extended our *in vitro* dantrolene experiments *in vivo*, testing if RyR2 stabilization would be anti-arrhythmic in FM-HFpEF mice. In proof-of-concept experiments, acute *in vivo* dantrolene (40 mg/kg i.p.) significantly reduced arrhythmia burden and ablated stress-induced VTs in FM-HFpEF mice (Fig. 2C).

In summary, we report FM-HFpEF mice effectively model the complex cardiometabolic features and stress-induced ventricular tachycardias seen in clinical HFpEF (Fig. 1A-E) [1,2]. We show here for the first time that metabolic stress induced HFpEF produces Ca^{2+}-based arrhythmogenesis distinct from the "Ca^{2+} phenotype" pathognomonic for HFrEF [9]. Specifically, a massive intracellular Ca^{2+} leak, driven by high $[Ca^{2+}]_{SR}$, an unchanged diastolic $[Ca^{2+}]_i$ and increased systolic $[Ca^{2+}]_i$ underlie arrhythmogenic Ca^{2+} signaling in HFpEF. In addition, reduced expression of RyR2, its stabilizer JP2, and the intra-SR Ca^{2+} binding protein CSQ2, facilitate SR Ca^{2+} leak in FM-HFpEF myocytes. While the TATS structure and BIN1 expression are unchanged, changes in tubulin portend increased Ca^{2+} fluxes that would further exacerbate impaired Ca^{2+} handling. A schematic of the changes at the cardiac dyad in FM-HFpEF mice compared to normal physiology is shown in Fig. 2D. Importantly, these changes are also distinct from a form of genetic hypertension related HFpEF (Dahl salt-sensitive rats) that progresses to HFrEF, where the ß-adrenergic responsiveness of intracellular Ca^{2+} mobilization was blunted [19].

We show that dantrolene, a well characterized RyR stabilizer [10], normalizes arrhythmogenic Ca^{2+} waves in FM-HFpEF myocytes and suppresses VTs in FM-HFpEF mice. Future work will be needed to elucidate the long-term effects and clinical treatment potential of ryanodine receptor stabilization therapy in metabolic stress related HFpEF.

2. Materials and methods

Male C57BL6/J mice were used in this study. Cardiac myocyte isolation, Ca^{2+} imaging and sub-diffraction microscopy were performed as previously described [20]. Detailed descriptions are provided in the Supporting Information.

CRediT authorship contribution statement

Aaron D. Kaplan: Writing – review & editing, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Liron Boyman:** Writing – review & editing, Software, Methodology, Data curation. **Christopher W. Ward:** Writing – review & editing, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **W. Jonathan Lederer:** Writing – review & editing, Resources, Funding acquisition. **Maura Greiser:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

AI-assisted technology was not used in the preparation of this work (except grammar and spell checking).

Declaration of competing interest

None.

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