



# Sarcomere integrated biosensor detects myofilament-activating ligands in real time during twitch contractions in live cardiac muscle

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## ABSTRACT

The sarcomere is the functional unit of cardiac muscle, essential for normal heart function. To date, it has not been possible to study, in real time, thin filament-based activation dynamics in live cardiac muscle. We report here results from a cardiac troponin C (TnC) FRET-based biosensor integrated into the cardiac sarcomere via stoichiometric replacement of endogenous TnC. The TnC biosensor provides, for the first time, evidence of multiple thin filament activating ligands, including troponin I interfacing with TnC and cycling myosin, during a cardiac twitch. Results show that the TnC FRET biosensor transient significantly precedes that of peak twitch force. Using small molecules and genetic modifiers known to alter sarcomere activation, independently of the intracellular  $\text{Ca}^{2+}$  transient, the data show that the TnC biosensor detects significant effects of the troponin I switch domain as a sarcomere-activating ligand. Interestingly, the TnC biosensor also detected the effects of load-dependent altered myosin cycling, as shown by a significant delay in TnC biosensor transient inactivation during the isometric twitch. In addition, the TnC biosensor detected the effects of myosin as an activating ligand during the twitch by using a small molecule that directly alters cross-bridge cycling, independently of the intracellular  $\text{Ca}^{2+}$  transient. Collectively, these results aid in illuminating the basis of cardiac muscle contractile activation with implications for gene, protein, and small molecule-based strategies designed to target the sarcomere in regulating beat-to-beat heart performance in health and disease.

## 1. Introduction

The cardiac sarcomere is a multimeric contractile apparatus consisting of a thin myofilament-based allosteric regulatory complex together with the myosin-based thick myofilament that generates force [1]. Interlacing myofilaments operate in synchrony to regulate and generate the forces necessary for heart performance. Beat-to-beat control of cardiac sarcomere activation refers to the status of the thin filament regulatory system in controlling the degree to which contraction is turned on and off during a twitch. Sarcomere activation control mechanisms involve a dynamic interplay between thin filament-localized  $\text{Ca}^{2+}$  in concert with regulatory elements in the thick and thin myofilaments, including troponin, tropomyosin, and myosin [1,2]. Sarcomere activation can be dramatically altered by factors intrinsic to myofilaments, including posttranslational modifications, the environmental milieu (ischemia/hypoxia), and by inherited mutations in

sarcomeric proteins [3–5]. Despite great efforts, it has not been possible to directly investigate and monitor sarcomere activation in the physiological setting of live cardiac muscle during a twitch.

The thin filament regulatory system, which is at the center of sarcomere activation dynamics and its beat-to-beat regulation during heart contraction, is composed of actin, tropomyosin (Tm), and the troponin complex (Tn) [1,6]. Regulation of sarcomere activation refers to the processes that control Tm's position on the thin filament [1,2]. Cardiac troponin is a heterotrimeric complex comprised of the Tm binding subunit, troponin T (cTnT), the calcium binding subunit, troponin C (cTnC), and the inhibitory subunit, troponin I (cTnI). Cardiac TnI (cTnI) is the molecular switch of the thin filament regulatory system [6,7]. Prior to contraction, the C-terminus of cTnI is strongly bound to actin, inhibiting Tm from rotating to a position favorable for strong myosin binding to actin. Upon  $\text{Ca}^{2+}$  binding to cTnC, cTnI rapidly switches from strong binding to actin to strong binding to cTnC. This cTnI switch

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mechanism is essential for opening the TnC hydrophobic patch, and then permitting Tm to translocate on the thin filament for active contraction to commence [2,8,9].

The prevailing model of sarcomere activation posits that  $\text{Ca}^{2+}$ , cTnI switching [9], and strong myosin binding serve as activating ligands to regulate Tm's position on actin from an inhibiting to a disinhibiting position [1]. A wealth of biochemical studies indicate that  $\text{Ca}^{2+}$ , cTnI, and myosin are the key effectors in orchestrating the sequential blocked, closed, and open state transitions in the myofilament regulatory system, as governed by their effects on Tm displacement [10,11]. A central feature of this model is that strong myosin binding is required to displace Tm to form the open state of thin filament regulatory apparatus in initiating contraction [1,11,12]. While highly informative, it is presently unknown whether this model holds in the dynamic physiological setting of live, intact muscle.

Well established approaches are in place to quantitatively detect and monitor the electrical and intracellular  $\text{Ca}^{2+}$  handling properties of live cardiac muscle [13]. However, to date, it has not been possible to investigate thin filament activation dynamics in real time under physiological conditions of intact excitation-contraction coupling and under load, all of which dramatically affect overall contractile performance [1]. To address this, we implement here a live cardiac muscle thin filament activation biosensor. The FRET-based biosensor was genetically engineered into full length human cTnC and incorporated into the myofilaments. The rationale is based on extensive *in vitro* works documenting that cTnC's global conformation changes upon  $\text{Ca}^{2+}$  activation *in vitro*. Data show that cTnC becomes more compact, with the N and C terminal lobes of TnC coming closer together, going from the apo to  $\text{Ca}^{2+}$  activated state [14–17]. Our premise of engineering a TnC conformation-dependent FRET-based biosensor integrated into the sarcomere of intact cardiac muscle derives from these studies. We use this TnC probe with the aim to detect global TnC conformational changes during a single cardiac twitch contraction.

In addition, the premise that TnC's conformation provides a nexus point in responding to and integrating near and long-range thin filament activating ligands, including  $\text{Ca}^{2+}$  binding to TnC, cTnI binding to TnC, Tm and myosin, is predicated on extensive *in vitro* works, as has been shown during steady-state conditions [1,2,6,18–20]. In permeabilized cardiac muscle reconstituted with cTnC conformational state-sensitive fluorescent probes, it has been shown that myosin cross-bridge binding to actin can induce TnC conformational changes under steady-state conditions, even in the absence of  $\text{Ca}^{2+}$  [19]. This is evidence that myosin binding causes Tm displacement and that this can be detected by TnC conformational changes, at least under steady-state conditions [1,19]. More recently, *in vitro* demonstration of FRET-based probes on TnC to detect  $\text{Ca}^{2+}$ -dependent TnC conformational changes have been established using the isolated troponin complex and in reconstituted permeabilized preparations under steady-state activating conditions, with recordings on the tens of seconds to minutes time-scale [17]. It is presently unknown whether these foundational steady-state results will translate to transient conditions in intact cardiac muscle in real time, which operates on the milliseconds time-scale. We tested here the hypothesis that TnC's conformation can serve as a real-time biosensor and report the effects of multiple thin filament activating ligands during a single cardiac twitch contraction in live muscle.

## 2. Material and methods

### 2.1. Animal work

The procedures used in this study were approved under guidelines of the University of Minnesota Committee on the Use and Care of Animals. Adult ventricular cardiac myocytes were isolated from adult female Sprague Dawley rats or adult C57/Bl6 mice of both sexes.

### 2.2. FRET pair, gene construct, recombinant vectors, and transgenesis

An intramolecular Clover/mRuby2 FRET pair was engineered into cardiac troponin C (details below). The Clover and mRuby2 FRET pair was used because, at the time of study design, this FRET pair was superior to any previous FRET pair in terms of fluorescence intensity brightness and photostability, with the highest Förster radius of any ratiometric FRET pair yet described [17,21,22]. Clover ( $\lambda_{\text{excitation max}}/\lambda_{\text{emission max}} = 505/515 \text{ nm}$ ) and mRuby2 ( $\lambda_{\text{excitation max}}/\lambda_{\text{emission max}} = 559/600 \text{ nm}$ ) were used as mRuby2 is an outstanding FRET partner for Clover because of its high extinction coefficient, extensive excitation overlap with Clover emission, high quantum yield, large Stokes shift, fast maturation, and high photostability [21,22]. Excellent overlap between donor emission and acceptor absorbance yields a Clover-mRuby2  $R_0$  of 6.3 nm—the highest  $R_0$  of any previous FRET pair (at that time of study initiation). Thus, the Clover-mRuby2 pair allows FRET imaging with less phototoxicity and a higher signal/background fluorescence ratio than any previous CFP and YFP at the time of study [21,22].

The 480 bp full-length (less the starting methionine) human cardiac Troponin C (hcTnC) cDNA was synthesized *de novo* and engineered to be flanked on the 5' end by a 19 amino acid flexible linker peptide enriched in leucine, glycine, serine, and alanine; and flanked on the 3' end by an 8 amino acid flexible linker enriched in leucine and alanine. Linker design was based in part on previous works showing a range of linker lengths attaching CFP/YFP fluors to cTnC not affecting the  $\text{Ca}^{2+}/\text{Mg}^{2+}$  binding characteristics of isolated TnC [17]. The 19 amino acid linker is inserted between a *PasI* restriction site at the 5' end and an *EcoRV* restriction site at the 3' end. The 8 amino acid linker is inserted between a *PfoI* restriction site at the 5' end and a *BclI* restriction site at the 3' end. On the 5' end of the 19 amino acid flexible linker is the 684 bp cDNA of the green fluorescent protein “Clover”. The Clover cDNA is inserted between a *SalI* restriction site at the 5' end and the aforementioned *PasI* restriction site at the 3' end. On the 3' end of the 8 amino acid flexible linker is the 714 bp cDNA of the red fluorescent protein “mRuby2”. The mRuby2 cDNA is inserted between the aforementioned *BclI* restriction site at the 5' end and an *FseI* restriction site at the 3' end.

The human cardiac TnC biosensor gene construct was inserted in a DNA vector downstream of an alpha myosin heavy chain ( $\alpha\text{MHC}$ ) promoter element for robust and cardiac specific expression in a mammalian system. Human cardiac TnC is highly conserved between mammalian species and used to aid in comparing to other studies that used human cTnC [17]. This  $\alpha\text{MHC}$  TnC biosensor vector was inserted by pronuclear injection into donor egg cells and implanted in C57/Bl6 murine surrogate mothers. Live pups were genotyped using the PCR sense primer located in mRuby2: 5'-CAACATCAAGATGCCCGGCA-3', and the PCR antisense primer located in the SV40 PolyA region: 5'-CACTGCATTCTAGTTGTGGTTTGTCC-3' with an annealing temperature of 62 °C. Of the live pups, 5 founders carrying the transgene were identified and the line with greatest breeding vigor was selected for further validation.

For recombinant adenovirus, a similar DNA constructed was synthesized *de novo* but containing *HindIII* and *EcoRI* at the 5' and 3' ends respectively for insertion into a pDC316 recombinant adenovirus production vector. pDC316-TnC biosensor and an adenovirus genome cosmid were co-transfected by calcium phosphate into confluent HEK293 cells. Noble agar was overlaid after transfection and clonal viral plaques were amplified. High-titer plaque-purified stocks were produced and viral aliquots were stored at -80 °C [23].

### 2.3. Heart morphology and echocardiography

After initial weigh-in, eight to twenty week old NTG and TG mice of both sexes were euthanized by sodium pentobarbital injection. Hearts were excised and rinsed of excess blood and weighed. Hindlimbs were

degloved and the tibia was exposed by blunt dissection prior to measurement.

For Sirius red/fast green staining, fresh frozen slides were fixed with chilled acetone then washed in 70% ethanol followed by water. Slides were then stained with Sirius red/fast green for 15 min and rinsed in water. After rinsing Sirius red/fast green stain, slides were dehydrated with 70% and 100% ethanol and then Formula 83 (CBG Biotech cat# CH0104).

Eight to twenty week old Ntg and TnC biosensor Tg mice of both sexes were lightly anesthetized by 2.5% Isoflurane and prepared for echocardiography. M-mode imaging was performed with a Vevo 2100 (Visual Sonics) at 1000fps with the MS550D 22–55 MHz transducer. The parasternal short axis view was used to assess systolic and diastolic ventricle size. M-mode imaging was also used to calculate fractional shortening.

## 2.4. Myocyte isolation and culture

Eight to twelve week old mice of both sexes were injected with 0.25 cc 10,000 U/ml heparin and anesthetized 10 min later with 0.15 cc 50 mg/ml sodium pentobarbital. The heart was rapidly excised and placed in ice cold Krebs-Henseleit buffer modified with 25 mM HEPES, 10 mM 2,3-Butanedione monoxime, 30 mM Taurine and the aorta was cannulated. The mounted heart was placed on a perfusion system at 37 °C. The heart was perfused with calcium free modified Krebs-Henseleit buffer with 2 mg/ml crudely purified collagenase for 10 min. The heart was then removed from the cannula, the atria were removed and the ventricles were dissected into pieces approximately 2 mm in diameter. The dissected ventricular tissue was then gently mechanically triturated in collagenase to liberate isolated myocytes. After brief low-speed centrifugation, isolated myocytes were resuspended in nominal calcium and .025 mg/ml bovine serum albumin in a modified Krebs-Henseleit buffer. After the final trituration step, calcium was added back to the buffer step wise until reaching a final concentration of 2 mM  $\text{CaCl}_2$ . Myocytes were plated on laminin-coated (ThermoFisher cat# 23017015) glass coverslips in M199 media (Gibco cat# 31100–035) containing 5% fetal bovine serum and incubated for one hour at 37 °C with 5%  $\text{CO}_2$ . After the one-hour incubation, the media was changed to serum free M199 media and the myocytes were used for experiments within 5 h. In a subset of studies, cardiac myocytes were also isolated from adult rats and transduced with recombinant adenovirus containing TnC FRET biosensor expression cassette (driven by CMV), using methods detailed previously [3,24].

## 2.5. Small molecules

For the small molecule studies in isolated myocytes, sarcomere length and fluorescence were first recorded under baseline conditions as outlined above. Following baseline recordings, the myocytes were then treated with one of the following small molecules for 10 min for pairwise analysis: Isoproterenol, a  $\beta$  adrenoreceptor agonist [13] (50 nM, Sigma), W7, a TnC-TnI uncoupling drug [25,26] (10  $\mu\text{M}$ , Sigma), Pimobendan, a troponin  $\text{Ca}^{2+}$  sensitizer and positive inotrope [27,28] (10  $\mu\text{M}$ , Sigma), Omecamtiv mecarbil, a selective cardiac myosin modulator [29,30] (250 nM, Selleckchem), or para-Nitro-blebbistatin, a myosin II inhibitor [31] (10  $\mu\text{M}$ ; kind gift from A. Málnási-Csizmadia). After treatment, sarcomere length and fluorescence were recorded again.

## 2.6. Cardiac papillary isolation

Eight to twelve week old mice of both sexes were injected with 0.15 cc 10,000 U/ml heparin and then euthanized by cervical dislocation. The hearts were rapidly excised and rinsed in Krebs–Henseleit Buffer (KHB) supplemented with 40 mM BDM. Intact anterior and posterior papillary muscles were carefully dissected from the left

ventricle in oxygenated (95%  $\text{O}_2$ /5%  $\text{CO}_2$ ) BDM–KHB pH 7.4 at 4 °C with minimal cutting and stretching injury. Papillary muscles were attached to platinum foil ( $0.001 \times 0.02''$ , 4 N) clips in the shape of an  $\Omega$  using 7–0 silk suture. Papillary muscles were then mounted by the platinum clips in a flow chamber (IonOptix) filled with BDM–KHB between a hook connected to length-controlled micromanipulator and a force transducer (MyoTronic) via platinum foil ( $0.001 \times 0.02''$ , 4 N) clips. Preoxygenated (95%  $\text{O}_2$ , 5%  $\text{CO}_2$ ) KHB pH 7.4 with 2 mM  $\text{CaCl}_2$  was perfused into the chamber at a flow rate of about 2 mL/min. The temperature in the muscle chamber was set by ambient room temperature at 22 °C. After a 10–20 min BDM washout protocol, the muscle was placed under load and stretched to an optimum physiological length where the developed isometric twitch force was maximal. Stimulus was applied through platinum at 4 V with either 0.2 Hz or 1 Hz pacing frequency with an 8 ms pulse width. Force and fluorescence recording were then made concurrently using the IonOptix Calcium and Contractility System as described below.

## 2.7. Real-time calcium, contractility, and sarcomere biosensor measurements

For real-time simultaneous sarcomere length and TnC biosensor measurements, a modified Ionoptix Calcium and Contractility system was used. Sarcomere length changes were detected at 240 Hz sampling rate and fluorescence measurements were detected at 1000 Hz sampling rate with two photomultiplier tubes (Fig. S2). Measurements were made at 37 °C and a stimulation frequency of 0.2 Hz, unless specified otherwise. This pacing is used by many groups and allowed SL transient comparison to past works on isolated myocytes [3]. For TnC biosensor measurements, a Xenon arc lamp was used and filtered with a 500/10 nm hard-coated bandpass filter before entering the microscope turret. The microscope turret featured a 505 nm dichroic long-pass filter. Fluorescent light from the sample was shunted through microscope side port where it was split by a dichroic mirror with light above 650 nm going toward the Myocam for sarcomere length detection and light below 650 nm being further shunted to an additional filter cube. In this final filter cube, a 565 nm dichroic long-pass mirror split the light again. Light with wavelengths below 565 nm corresponding to the Clover protein was further refined by a 534/20 nm hard-coated band-pass filter before reaching the photomultiplier tube and light with wavelengths above 565 nm was further cleaned up by a 592/43 nm hard-coated bandpass filter before reaching the photomultiplier tube. For calcium fluorescence measurements, the mouse myocytes were pre-loaded for 20 min in M199 solution containing 1  $\mu\text{M}$  Fura-2 AM (ThermoFisher Cat #F1221), and a ten-minute period was allowed for deesterification. For calcium fluorescence measurements the 360/380 nm ratio was determined. For each myocyte, several contractions were measured, averaged, and analyzed using the IonWizard software.

For adenovirus transduced biosensor experiments, adult cardiac myocytes were isolated as previously described<sup>24</sup>. Briefly, the myocytes were isolated by collagenase digestion and mechanical trituration prior to plating on laminin coated coverslips. Myocytes were treated with AdTnC biosensor at a multiplicity of infection of 100 for 48 h prior to measurement with the aforementioned IonOptix calcium and contractility system.

For the Omecamtiv mecarbil (OM) studies in the intact papillary muscle, force and fluorescence were first recorded under baseline conditions as outlined above. Following the collection of baseline recordings, buffer, as described above, with a final concentration of 250 nM OM was perfused into the chamber for 10 min. After 10 min force and fluorescence were recorded again. For both single myocyte and papillary muscle experiments, a paired experimental design was used (each prep compared before and after) to evaluate small molecules and drugs which is a powerful experimental design to quantify statistically significant differences.

## 2.8. Steady-state force and TnC biosensor signal in permeabilized cardiac muscle

**Skinned Papillary Strips.** Papillary muscles were isolated as described above. Once the muscles were dissected from the left ventricle, they were cut in half longitudinally and platinum foil clips in the shape of an omega ( $\Omega$ ) were attached to each end of each individual strip using 7–0 silk suture. Papillary muscles were then incubated in skinning solution (100 mM KCl, 10 mM Imidazole, 2 mM EGTA, 1 mM  $\text{MgCl}_2$  (Fluka Analytical), 4 mM ATP, 50% glycerol, protease inhibitor (Pierce Protease Inhibitor Tablets A32963), 30 mM BDM, 1% w/v Triton X-100) at room temperature for 2 h. After incubation, papillary muscles were individually placed in 1.5 ml microcentrifuge tubes filled with approximately 1 ml of storage solution (100 mM KCl, 10 mM Imidazole, 2 mM EGTA, 1 mM  $\text{MgCl}_2$  (Fluka Analytical), 4 mM ATP, 50% glycerol, and protease inhibitor (Pierce Protease Inhibitor Tablets A32963)) and stored at  $-20^\circ\text{C}$  for no more than 7 days. At the time of the experiment, a papillary strip was mounted by the platinum clips in a flow chamber (Ionoptix) filled with relaxing solution (100 mM KCl, 10 mM Imidazole, 2 mM EGTA, 1 mM  $\text{MgCl}_2$  (Fluka Analytical), 4 mM ATP) between hooks connected to a length-controlled micromanipulator and a force transducer (MyoTronic). The temperature of the chamber was set by ambient room temperature at  $22^\circ\text{C}$ . The strip was stretched to an optimum physiological sarcomere length of approximately  $2.0\ \mu\text{m}$  as detected by a MyoCam (Ionoptix). Strips were calcium activated using solutions of pCa 9, pCa 7, pCa 6.5, pCa 6, pCa 5.7, pCa 5, and pCa 4 and changes in developed tension and fluorescence intensity were simultaneously recorded using the Ionoptix Calcium and Contractility system. Recorded forces and fluorescence intensities were normalized to pCa4 values for each individual preparation and then fit to the Hill equation:

$$F = \frac{1}{1 + (10^{(x-pCa50)})^{nHill}}$$

with pCa50 being the calcium concentration at half activation and nHill representing the Hill coefficient, using non-linear least squares regression in R statistical programming language.

**Permeabilized adult cardiac myocytes.** Cardiac myocytes were mechanically isolated from the ventricles of whole hearts from adult Tg and Ntg littermate mice, as described [32,33]. The experimental apparatus and the permeabilized single cardiac myocyte attachment and tension-pCa determination procedures are as detailed previously [32,33]. Relaxing and activating solutions contained (mM): 7 EGTA, 1 free  $\text{Mg}^{2+}$ , 4  $\text{MgATP}$ , 14–5 creatine phosphate, 20 imidazole, and sufficient KCl to yield a total ionic strength of 180 mM. Solution pH was adjusted to pH 7.00 using KOH/HCl at  $15^\circ\text{C}$ .

## 2.9. Western blotting and immunofluorescence

The primary antibodies used were to  $\alpha$ -actinin (immunofluorescence) (1:500, Abcam, EA-53), and cardiac troponin C (Western blot) (1:2500, Novus Biologicals, 1A2). The secondary antibodies were conjugated to Alexa Fluor 647 (ThermoFisher) and IRDye Alexa Fluor 680 (ThermoFisher) respectively. For Western blots signals were visualized and quantified using the Odyssey system (Licor). Laminin coated coverslips containing isolated myocytes were treated with 4% paraformaldehyde in phosphate buffered saline for five minutes followed by two washes in phosphate buffered saline. Samples were blocked with 5% bovine serum albumin with 0.5% Triton X-100 in phosphate buffered saline for at least one hour. Primary antibody was applied at room temperature for one hour in phosphate buffered saline with 0.5% Triton X-100 and 0.5% BSA. Several washes were performed in PBS with 0.5% Triton X-100 and 5% BSA, followed by one-hour incubation of the secondary antibody at room temperature in PBS with 0.5% Triton X-100 and 0.5% BSA. Coverslips were mounted on microscope slides with ProLong Diamond mounting medium. Myocytes were

imaged using a Nikon A1R confocal microscope with  $60\times$  water-immersion objective; myocytes were illuminated sequentially in line scanning mode by 488 nm, 561 nm, and 640 nm lasers.

## 2.10. Analysis and statistics

For contractility and calcium analysis, curve fitting was performed on unfiltered, averaged single myocyte or papillary transients (IonWizard 6.5; IonOptix) using a truncated Taylor series expansion of any analytical function to represent the signal within a phase of the transient, additional details on the fitting methodology is available from IonOptix.

To account for changes in the fluorophore concentration during papillary isometric twitches, ratiometric analyses were performed, taking advantage of the intramolecular nature of the FRET biosensor and its 1:1 stoichiometry.

$$\text{Ratio}_{\text{Fluorescence}} = \frac{I_{\text{mRuby2}}}{I_{\text{Clover}}}$$

Where  $I$  refers to the fluorophore intensity counted using the PMT. Here, the intensity modulation of the mRuby2 fluorophore signal is ratioed against the intensity of the Clover fluorophore signal. Increases in mRuby2 intensity greater than the proportional increase in Clover intensity are assumed to be due to an increase the degree of FRET between the two fluorophores. [34]

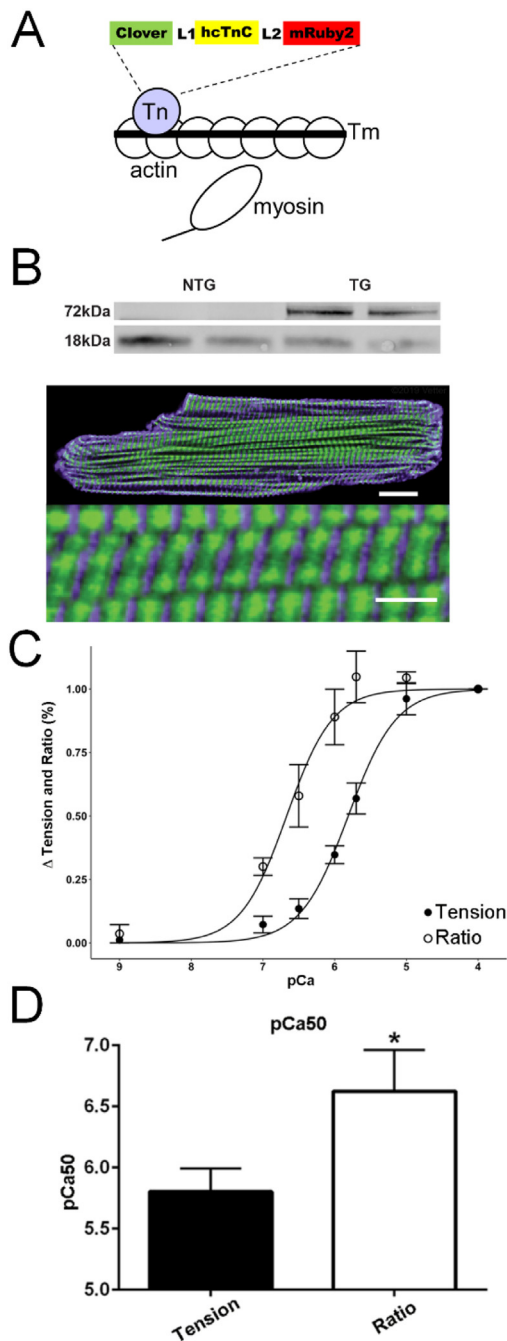
Mean  $\pm$  S.E.M. are presented in histogram summary figures. For isoproterenol, W7, pimobendan, omecamtiv mecarbil, and para-nitroblebbistatin studies, two-tailed paired  $t$ -tests were performed. For all other summary histograms, two-tailed unpaired  $t$ -tests were performed (GraphPad PRISM version 6.0; GraphPad Software Inc.). In Figs. 2, 3, and 4 the Clover intensity transient is overlaid with a LOWESS curve the aid visual representation of the single cell fluorescent traces [35]. No analyses were performed on these curves as they are only for ease of visualization [35]. The parameters of the LOWESS curve were  $Q = 20$  and  $\lambda = 1$  with a tri-cube weighting function of:  $\omega(x) = \frac{1}{(1 + |d|^3)^3}$  where  $d$  is the distance of a given data point from the point on the curve being fitted. Sample sizes were established on the basis of Power analysis, as informed by previous experience in the laboratory with respect to biological variability.  $P$  values  $< 0.05$  were considered statistically significant and indicated by asterisks in figures.

## 3. Results

### 3.1. Real-time detection of TnC-based thin filament activation in intact adult cardiac myocytes

As detailed below, the troponin C FRET-based biosensor probe was integrated into the sarcomere and shown to function normally in cardiac muscle, in terms of myofilament activation and contractile function from cell to whole animal. Data support that the well-known activation-dependent conformational changes in TnC (compacted global TnC structure during activation [15,17,36]) bring the Clover and mRuby2 FRET pair closer together (in the vicinity of the  $R_0$  of 6.3 nm) to increase FRET and produce a transient that is detected with millisecond time resolution during a single twitch (e.g., Fig. 1).

The TnC biosensor was composed of fluorescent proteins Clover (donor) and mRuby2 (acceptor) engineered at the N- and C-terminal ends of cardiac TnC respectively (see Methods for details), and cardiac-directed TnC biosensor transgenic mouse lines were then established (Methods; Figs. 1a; S1,2). In independent Tg lines, the TnC biosensor stoichiometrically replaced 47% of the endogenous cTnC (Fig. 1b). Immunofluorescence detection of the TnC biosensor in intact cardiac myocytes demonstrated correct localization of the TnC biosensor in the thin filaments between the  $\alpha$ -actinin labeled Z-lines (Fig. 1b, bottom). Similar findings were obtained in rat adult cardiac myocytes using



**Fig. 1.** Expression and function of the cardiac sarcomere incorporated TnC biosensor in intact cardiac myocytes. **A**, Model of the troponin C sarcomere biosensor, where FRET occurs by conformational changes in TnC during sarcomere activation. **B**, Western blot of cTnC expression probed with an anti-cTnC antibody in transgenic mouse hearts reveals stoichiometric replacement of endogenous cTnC by the higher molecular weight biosensor cTnC, blot is spliced horizontally to remove intervening non-specific bands. Full blot is in the Supplement. Below. Immunofluorescence with confocal microscopy images showing localization of Clover (green) is restricted between  $\alpha$ -actinin (purple) delineated Z lines, scale bar is 25  $\mu$ m, bottom of panel shows magnified detail of the same cell, Scale bar is 2.5  $\mu$ m. **C**, Measurements of the  $\text{Ca}^{2+}$ -sensitivity of steady-state isometric tension development and simultaneous FRET ratiometric fluorescence intensity changes in skinned papillary strips exposed to solutions of increasing  $\text{Ca}^{2+}$  concentration (FRET ratio defined in Methods). Tension was normalized to the pCa 4.0 value obtained in each preparation ( $n = 6$ ). **D**, Summary statistics of  $\text{Ca}^{2+}$  concentration at half maximal activation (pCa50) for tension and FRET fluorescence ratio demonstrate a significantly higher pCa50 for the fluorescence ratio than tension ( $n = 6$ ). Summary statistics of the Hill coefficient (nHill) for tension ( $1.33 \pm 0.11$ ) and fluorescence ratio ( $1.77 \pm 0.27$ ) show no significant difference ( $n = 6$ ). Mean  $\pm$  S.E.M are

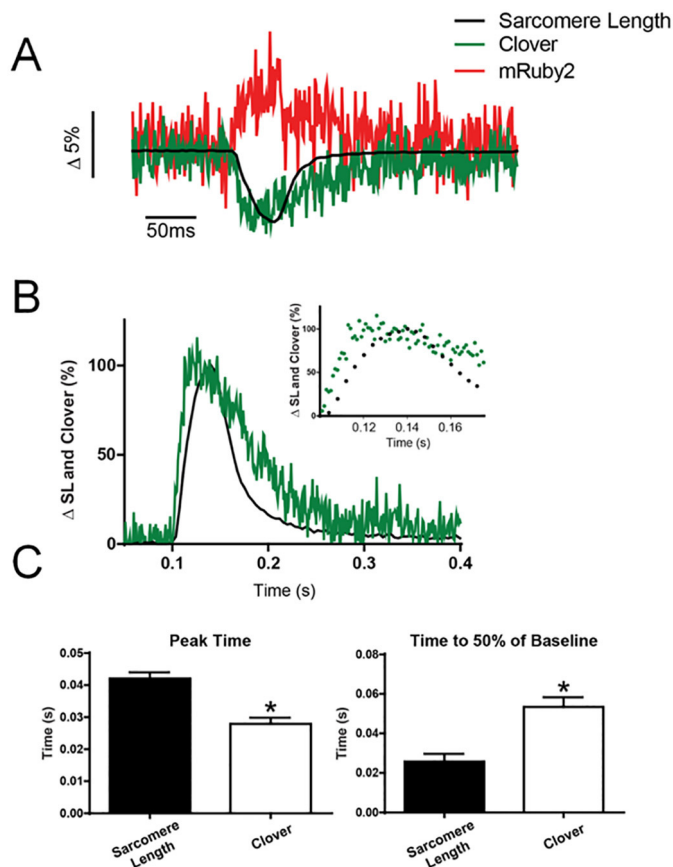
presented, Unpaired two-tailed  $t$ -test:  $*P < 0.05$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

acute gene transfer (Fig. S10). Thus, consistent with numerous past sarcomere-engineering studies, we find the TnC biosensor is incorporated correctly into the sarcomere without evidence of accumulation in the cytosol [3,23,37–41].

The troponin C biosensor derives from extensive in vitro studies showing steady-state  $\text{Ca}^{2+}$  responsive FRET upon TnC probe reconstitution into myofilaments [17]. To characterize the TnC biosensor we developed, biophysical analysis of  $\text{Ca}^{2+}$  titrations in permeabilized cardiac muscle preparations was conducted by simultaneously measuring force and TnC conformation-dependent FRET signals under steady-state  $\text{Ca}^{2+}$  activating conditions (Fig. 1C,D). Data show, qualitatively similar to that of Badr et al. [17], sigmoidal tension-pCa and TnC FRET ratio-pCa relationships, with the TnC FRET ratio-pCa curve shifted to the left of the tension-pCa curve, under steady state  $\text{Ca}^{2+}$  activating conditions. These biophysical data show that the sarcomere integrated TnC FRET biosensor detects  $\text{Ca}^{2+}$  – induced conformational changes in TnC in a  $\text{Ca}^{2+}$  concentration-dependent manner such that steady-state FRET increased as a function of increased activating levels of  $\text{Ca}^{2+}$  (Fig. 1C). In addition, data show that TnC biosensor-derived FRET was significantly more sensitive to  $\text{Ca}^{2+}$  than isometric tension (Fig. 1C,D).

In addition, as inherited mutations in the sarcomere can directly cause cardiomyopathy [42], we validated that the TnC biosensor itself had no significant effects on heart structure or function. Tension-pCa relationship studies in permeabilized single adult cardiac myocytes with (Tg) and without (Ntg littermate) the TnC biosensor incorporated into the sarcomeres were conducted (Fig. S3). Data show no change in pCa50, indicating that the design and sarcomere integration of the TnC biosensor had no effect on the steady state  $\text{Ca}^{2+}$  activating function of the muscle. This differs and significantly improves upon earlier studies in which the TnC FRET probe caused a significant alteration in skinned muscle pCa50 values [17]. Also, heart histology, heart morphology, or in vivo heart function were not different than controls, nor did the TnC biosensor have any effect on intact cardiac myocyte function (also confirmed by Ad5 acute gene transfer; Fig.S10), as compared to myocytes from non-transgenic littermates (Figs. S4–7). Because the cardiac muscle is highly sensitive to even small perturbations in sarcomere function, these are direct physiological evidence that the TnC biosensor confers normal TnC function, from the isolated myocyte to the intact heart level in vivo. These in vivo results are in agreement with in vitro work showing various CFP/YFP FRET pairs engineered cTnC having normal  $\text{Ca}^{2+}/\text{Mg}^{2+}$  binding properties [17]. Collectively, these data show the TnC biosensor integrated normally into the sarcomere and confers normal TnC sarcomeric function that is indistinguishable from control. The sarcomere-integrated TnC biosensor also differs significantly from other probes that were designed specifically as cytoplasmic or myofilament localized  $\text{Ca}^{2+}$  indicators [43,44].

We next sought to advance steady-state studies by us (Fig. 1), and others [16,17,36,45], to dynamic twitch contractions in live cardiac muscle. To enable this, we leveraged this TnC FRET pair, optimized for enhanced photostability and brightness [21,22]. In intact single adult cardiac myocytes, TnC biosensor Clover and mRuby2 signals were resolved in the millisecond time domain and were anti-correlated, indicating FRET (Fig. 1; Supplementary Video S1). This finding, together with work in reconstituted systems (Fig. 1), and with other FRET-based TnC pairs activated by direct  $\text{Ca}^{2+}$  titrations in vitro [17], is evidence that the TnC biosensor is capable of reporting in real-time the global conformational changes in TnC that occur during the cardiac twitch. Cardiac myocytes are small enough (lengths  $\sim 110$   $\mu$ m) for the optical system to collect all the emitted fluorescence across the entire contracting myocyte thus, with this technique, possible motion artifacts of



**Fig. 2.** TnC biosensor transient in unloaded intact single adult cardiac myocytes. A, Representative single cardiac myocyte traces of simultaneous sarcomere length, and the Clover, and mRuby2 fluorescence transients, with Clover fluorescence decreasing and simultaneously mRuby2 fluorescence increasing, indicating FRET. Scale bar represents a 5% change from baseline. B, Amplitude normalized Clover (green) and sarcomere length (black) dynamics from ensemble averaged myocytes (transients normalized from 0 to 100% to compare kinetics) show TnC biosensor Clover transient preceding sarcomere length shortening, inset shows the same data, highlighting the peak timing differences. C, Summary statistics from sarcomere length dynamics and Clover intensity show that peak activation of the TnC biosensor precedes sarcomere length change, and biosensor inactivation follows relaxation of the myocyte. Myocytes are unloaded and measured at 37 °C with 0.2 Hz stimulation,  $n = 14$  myocytes in each group. Mean  $\pm$  S.E.M. are presented, unpaired two-tailed  $t$ -test: \* $P < 0.05$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fluorophores moving in and out of the plane of detection, are highly mitigated, making it feasible to track only Clover. To validate Clover signal versus FRET pair ratio data, in a subset of experiments, FRET ratios (mRuby/Clover; defined in Methods) were determined for the isolated cells and shown to be no different than the Clover only transient.

### 3.2. Thin filament activating roles of cTnI and myosin in live cardiac myocytes

We investigated thin filament activation dynamics in intact and unloaded adult cardiac myocytes previously estimated from modeling studies [46]. Results showed that the peak amplitude of the TnC biosensor transient significantly preceded peak contraction (sarcomere length/SL)(Fig. 2, Fig. S8). The TnC biosensor transient was temperature-sensitive, paralleling the temperature dependence of contraction (Fig. S9), and, while temperature has myriad effects on muscle, this is consistent with the temperature dependence of the troponin-Tm

regulatory system [1].

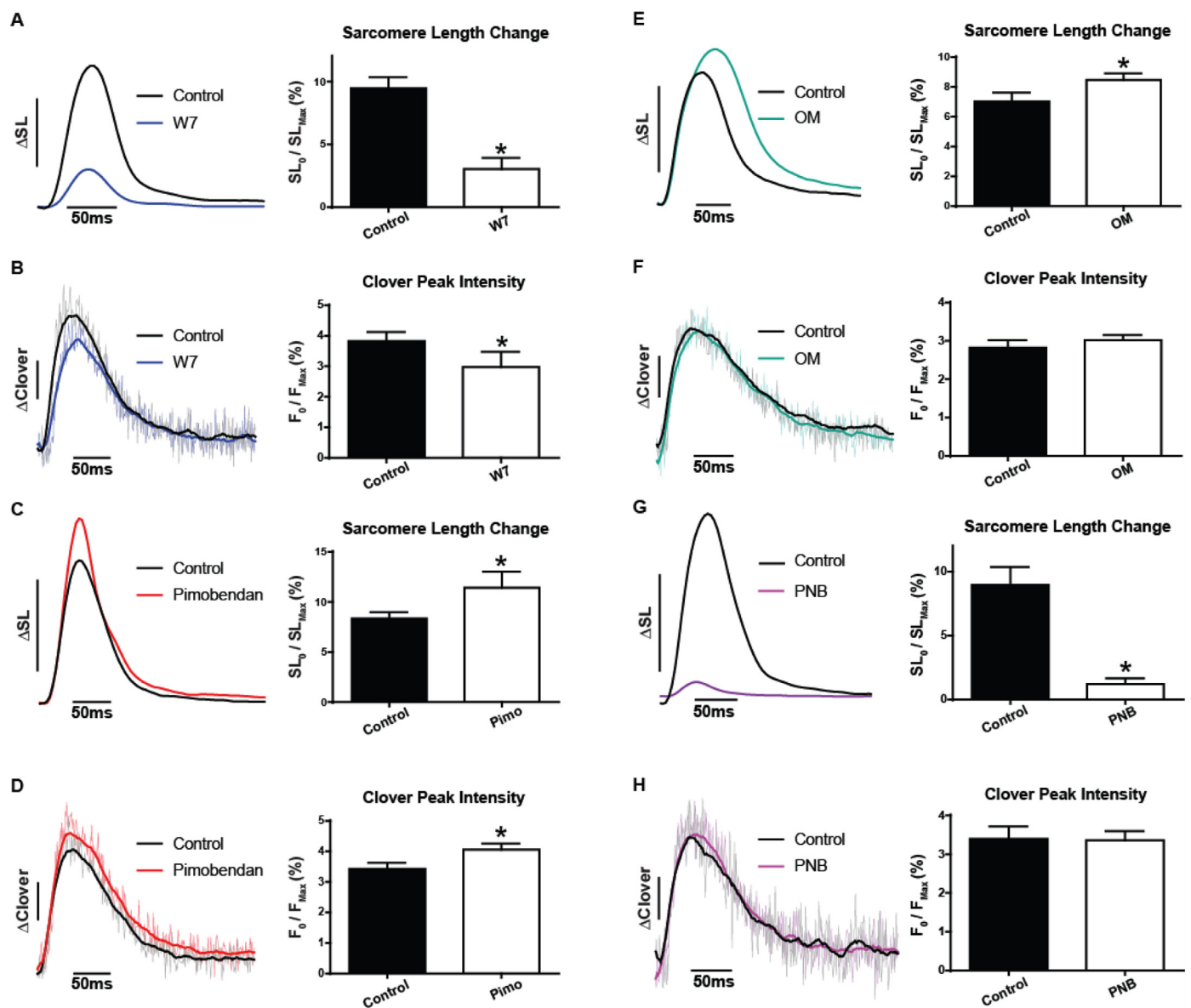
Next we tested the effects of enhanced cardiac myocyte excitation-contraction coupling on sarcomere activation via  $\beta$ -adrenergic stimulation (isoproterenol/Iso) [13]. Here, the TnC biosensor peak amplitude was increased and the TnC biosensor decay rate was significantly faster with Iso, paralleling the effects of Iso to increase contraction (positive inotropy) and to speed relaxation (positive lusitropy)(Fig. S8). The increased amplitude and faster decay rate of the TnC biosensor transient by Iso can be explained by the effects of Iso to increase the  $\text{Ca}^{2+}$  amplitude and by the combined effects of cTnI phosphorylation and faster  $\text{Ca}^{2+}$  transient decay to cause faster myofilament inactivation [47]. MyBP-C, titin, and multiple other molecules could also have roles, and it will be interesting to dissect these in future studies.

We examined the role of cTnI as an activating ligand [6,9]. In vitro studies have well demonstrated that the cTnI switch peptide binding to cTnC is required for the full activation of cTnC, unlike in skeletal muscle where  $\text{Ca}^{2+}$  binding alone appears sufficient for full TnC activation [9,48]. We first used N-aminoethyl-5-chloro-1-naphthalene-sulfonamide (W7), a small molecule, originally discovered in binding to calmodulin, that does bind directly to the cTnC hydrophobic patch and markedly decreases cTnI switch peptide binding to the N-terminus of cTnC [25]. Thus, W7 was used to test the effects of inhibiting cTnC-cTnI interaction and consequently this disrupts sarcomere activation in live myocytes [26]. Here, the TnC biosensor amplitude was significantly depressed by W7 (Fig. 3) accompanying the effect of W7 to reduce myocyte SL amplitude (Fig. 3a). Because the  $\text{Ca}^{2+}$  transient is not altered by W7 (Fig. S11), this is evidence that the TnC biosensor is not a myoplasmic  $\text{Ca}^{2+}$  sensor. In conjunction with the switch-peptide occlusion mechanism [9,48] and the effects of W7 on contraction [26], this is evidence that the TnC biosensor directly detects the key role of cTnC-cTnI interaction in live cells [1,2,9]. Next, we used Pimobendan, a small molecule positive inotrope [27] shown to directly enhance the affinity of cTnI for cTnC [28]. Here, Pimobendan caused a significant increase in the TnC biosensor FRET amplitude that paralleled the increase in SL amplitude (Fig. 3). Thus, the TnC biosensor reports the enhanced sarcomere activation elicited by increasing the affinity of cTnI for cTnC's hydrophobic cleft. Collectively, these findings, by titrating cTnC-cTnI interactions, demonstrate that cTnI engagement with cTnC is a key effector of cardiac sarcomere activation in the live myocyte.

Present models suggest that strong myosin binding is the essential ligand causing the open state of the myofilament regulatory system [1,10,11]. To test this theory in intact cardiac myocytes, we first used Omecamtiv mecarbil (OM), a myosin-modulating drug shown to increase the rate of cross-bridge cycling by accelerating phosphate release, with no effects on intracellular  $\text{Ca}^{2+}$  handling [29,30]. OM caused a significant increase in SL amplitude in adult cardiac myocytes but, surprisingly, had no effect on TnC biosensor amplitude or kinetics (Fig. 3). This result was unexpected, given the extensive literature on strong myosin binding as the required effector of sarcomere activation [1,11]. To test more thoroughly this hypothesis, we investigated whether fully blocking strong myosin binding would depress the TnC biosensor signal, as predicted by current theory [1,11]. TnC biosensor transgenic cardiac myocytes were exposed to para-nitroblebbistatin (PNB), a non-fluorescent small molecule that selectively disables myosin function by blocking myosin in an actin-detached state and rendering myosin incapable of producing force [31]. Surprisingly, whereas PNB effectively blocked unloaded myocyte contraction (Fig. 3), there was no effect of PNB on TnC biosensor transient amplitude or kinetics (Fig. 3).

### 3.3. Mechanical load and sarcomere activation

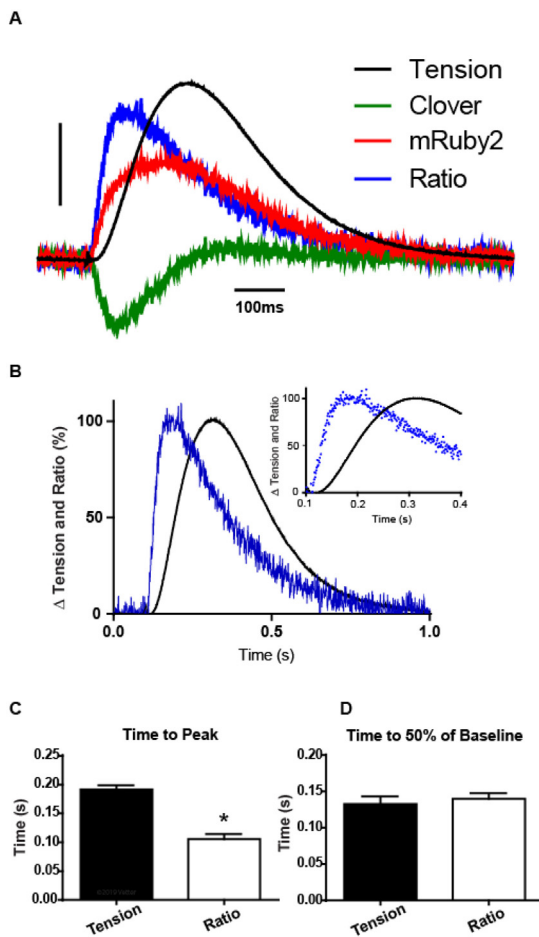
Mechanical load is well known to markedly alter myosin function, and load is predicted to have an impact on sarcomere activation in live muscle [1]. To compare to the results above using unloaded conditions,



**Fig. 3.** Myofilament-targeted small molecules probe function in live cardiac myocytes. TnC-TnI modulation. A, Ensemble average traces of SL shortening (left) in isolated myocytes before and after treatment with 10  $\mu M$  W7 and summary statistics (right) from sarcomere dynamics reveal decreased contractile amplitude ( $n = 8$  pairs). B, Ensemble average traces of Clover transient (left) in isolated myocytes before and after treatment with 10  $\mu M$  W7 and summary statistics (right) of Clover signal reveal decreased peak fluorescence intensity ( $n = 8$  pairs). C, Ensemble average traces of SL shortening (left) in isolated myocytes before and after treatment with 10  $\mu M$  Pimobendan with summary statistics (right) from sarcomere dynamics showing increased contractile amplitude ( $n = 11$  pairs). D, Ensemble average traces of Clover signal (left) in isolated myocytes before and after treatment with 10  $\mu M$  Pimobendan and summary statistics (right) of Clover intensity changes reveal increased peak fluorescence intensity ( $n = 11$  pairs). Myocytes measured at 37  $^{\circ}C$  with 0.2 Hz stimulation;  $\Delta SL$  bar represents a 5% change from baseline,  $\Delta Clover$  bar represents a 1% change from baseline. Mean  $\pm$  S.E.M are presented, Paired two-tailed  $t$ -test:  $*P < 0.05$ . Myosin targeted small molecules probe sarcomere activation in live cardiac myocytes. E, Ensemble average traces of SL shortening (left) in isolated myocytes before and after treatment with 250 nM Omecamtiv mecarbil and summary statistics (right) from sarcomere dynamics reveal increased contractile amplitude ( $n = 14$  pairs). F, Ensemble average traces of Clover signal (left) in isolated myocytes before and after treatment with 250 nM Omecamtiv mecarbil and summary statistics (right) of Clover intensity changes reveal no difference in peak fluorescence intensity ( $P = 0.2847$ ) ( $n = 17$  pairs). G, Ensemble average traces of SL shortening (left) in isolated myocytes before and after treatment with 10  $\mu M$  para-Nitroblebbistatin and summary statistics (right) from sarcomere dynamics reveal decreased contractile amplitude ( $n = 8$  pairs). H, Ensemble average traces of Clover signal (left) in isolated myocytes before and after treatment with 10  $\mu M$  para-Nitroblebbistatin and summary statistics (right) of Clover intensity changes reveal no difference in peak fluorescence intensity ( $P = 0.8930$ ) ( $n = 8$  pairs). Myocytes were measured at 37  $^{\circ}C$  with 0.2 Hz stimulation;  $\Delta SL$  bar represents a 5% change from baseline,  $\Delta Clover$  bar represents a 1% change from baseline. Mean  $\pm$  S.E.M are presented, Paired two-tailed  $t$ -test:  $*P < 0.05$ .

we next studied intact cardiac muscles under mechanical load. Cardiac papillary muscles were attached to a recording apparatus, and load was imposed via stepwise muscle length extension to set the optimal peak isometric twitch force amplitude for each preparation [1] (experimental apparatus shown in Fig. S2). Thus, for all experiments below, mindful of potential inhomogeneities, muscles were studied at the physiologically relevant optimal length that produced peak isometric twitch

contractions. Results showed that the TnC biosensor Clover and mRuby2 transients were anti-correlated, indicating FRET (Fig. 4). Cardiac muscle under mechanical load showed the TnC biosensor transient [herein, the mRuby2/Clover ratio to account for motion effects [34]] reached peak significantly before the development of peak isometric twitch force (Fig. 4). For these papillary experiments, mRuby2/Clover ratios are the necessary and well-validated approach to



**Fig. 4.** TnC biosensor transient in live intact cardiac muscle under physiological load. A, Representative single cardiac papillary muscle traces of simultaneous twitch force, Clover, mRuby2, and ratiometric calculation of fluorescent intensity. Scale bar represents a 10% force change or a 2% change in fluorescence intensity ratio. B, Representative single cardiac papillary of amplitude normalized FRET ratio (blue) and tension (black) dynamics at 22 °C. Inset shows the same data highlighting the peak timing difference. C, D, Summary statistics of tension and FRET ratio reveals that the TnC biosensor conformational change precedes peak tension development ( $n = 9$  pairs). Experiments were at room temperature and 1.0 Hz stimulation;  $\Delta$ Tension bar represents a 10% change from baseline,  $\Delta$ Clover bar represents a 2% change from baseline. Mean  $\pm$  S.E.M are presented, Unpaired two-tailed  $t$ -test:  $*P < 0.05$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

effectively address motion artifacts of the larger multicellular cardiac muscles [34]. In addition, motion artifacts are also mitigated because of the intramolecular FRET probe strategy we used, in that motion would change equally the numbers of Clover and mRuby2 fluorophores detected by the PMT. Interestingly, alignment of the loaded and unloaded TnC biosensor FRET ratio traces showed a prolongation of the loaded transient (by  $\sim 28\%$ ) that was due to a significant delay in the inactivation of the TnC biosensor transient (Fig. 5a,b).

To investigate mechanism, we probed sarcomere activation in the presence of the direct myosin modulatory small molecule OM [30]. Under load, OM caused a more rapid upstroke of the TnC biosensor transient, as quantified by the time to peak signal intensity being significantly faster with OM (Fig. 5e,f). In addition, OM decreased twitch force as reported by a significantly depressed peak amplitude of the TnC biosensor transient (Fig. 5c,d). These results, together with the loaded vs. unloaded findings above (Fig. 5a,b), provide direct evidence in live cardiac muscle that the troponin-located TnC biosensor is sensitive to detecting the effects of cycling myosin as a sarcomere activating ligand.

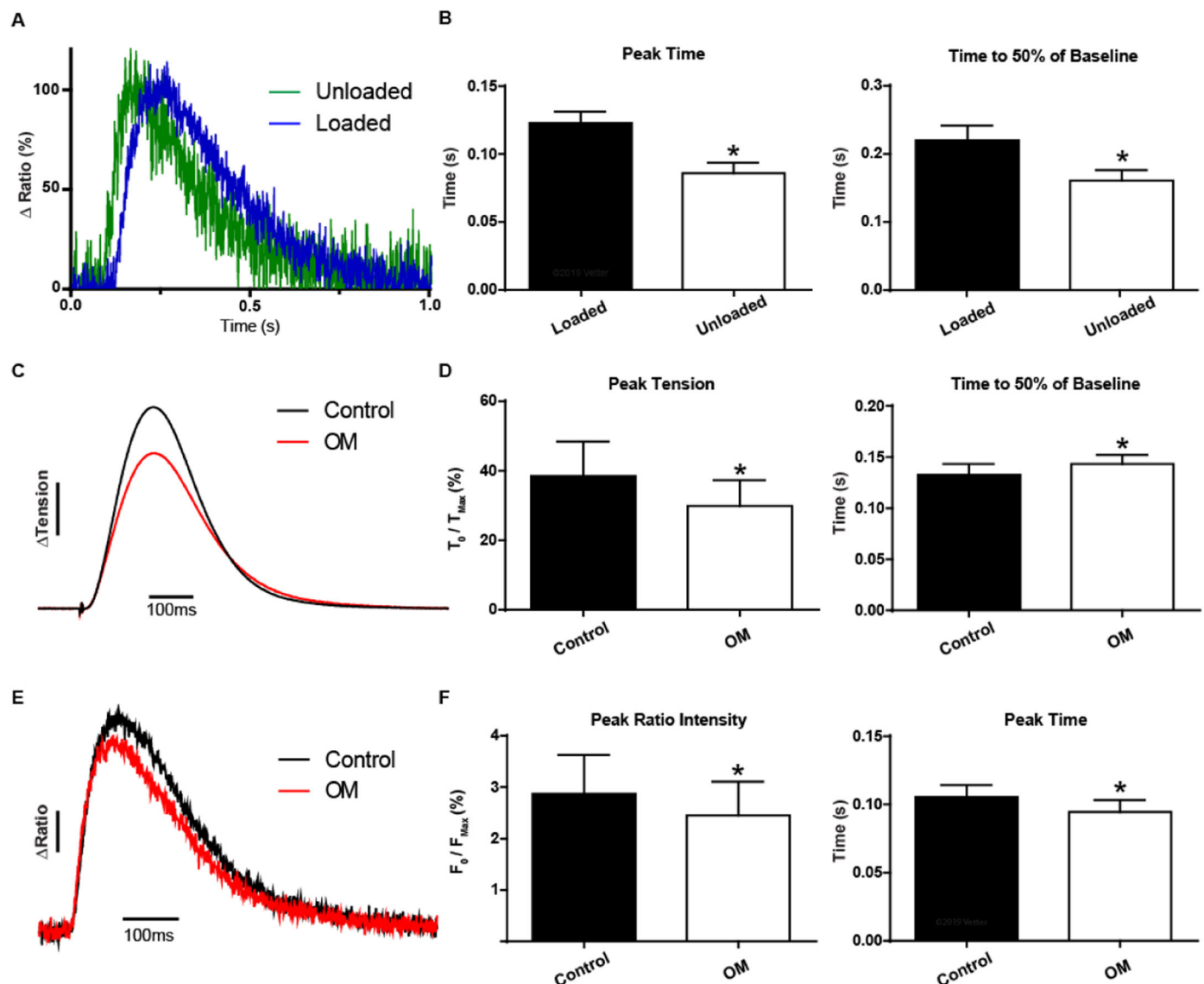
Next, we examined the effects on sarcomere activation due to an inherited sarcomeric mutation known to cause significant cardiomyopathy in patients [3]. The cTnI mutation R193H was used here as it has been shown to cause heightened myofilament  $\text{Ca}^{2+}$  sensitivity and organ-level diastolic dysfunction in animal models and in humans [24,40,49–51]. Here, cTnI R193H mice [40] were crossed with TnC biosensor mice to generate double transgenic lines. In loaded cardiac muscle from cTnI R193H Tg mice, results showed a significant delay in twitch force decay, and this was paralleled by a significant delay in TnC biosensor transient inactivation (Fig. 6). This finding, together with results showing no effect of cTnI R193H on the amplitude or kinetics of the intracellular  $\text{Ca}^{2+}$  transient [51], is evidence that cTnI R193H prolongs sarcomere activation by increasing the lifetime of cTnC-cTnI interaction. This prolonged cTnC-cTnI interaction is predicted to contribute to inappropriately increased myofilament-based  $\text{Ca}^{2+}$  buffering in the late phase of twitch contraction [42]. This sarcomere-based dysfunction, in turn, has been modeled as a key substrate for producing the fatal arrhythmias characteristic of the inherited cardiomyopathies [52].

#### 4. Discussion

Elucidating the physiological basis of thin filament activation in live cardiac muscle is essential to understanding beat-to-beat regulation of heart performance in vivo. We sought here to design and test a sarcomere-incorporated biosensor capable of detecting thin myofilament activation status in live cardiac muscle. Toward this goal, we implemented a cardiac TnC FRET-based biosensor to test the hypothesis that TnC's conformation can serve as a real-time biosensor and report the effects of multiple thin filament activating ligands during a single cardiac twitch contraction in live muscle.

Data show that the TnC biosensor is incorporated normally into the myofilaments by stoichiometric replacement of endogenous TnC, and that the biosensor itself has no untoward effects on cardiac myocyte or heart functional performance in vitro or in vivo. Because even slight changes in troponin  $\text{Ca}^{2+}$  binding kinetics cause marked alterations in cardiac muscle function, this is physiologically relevant functional evidence that the  $\text{Ca}^{2+}$  activating properties of the TnC biosensor functions as endogenous TnC. This is in agreement with in vitro work showing various CYP/YFP FRET pairs engineered in cTnC did not alter  $\text{Ca}^{2+}/\text{Mg}^{2+}$  binding properties of isolated TnC [17]. We therefore posit that this TnC biosensor detects, for the first time to our knowledge, activation-dependent changes in cardiac TnC's conformation, as determined by intramolecular FRET in real time during a twitch. Results show a well-delineated TnC conformation-dependent FRET transient, wherein the biosensor signal rises and reaches a peak prior to peak force during a twitch contraction. In addition, data derived from small molecule and genetic approaches, shown previously to alter thin filament activation [3,26], provide evidence that the TnC biosensor can detect the effects of multiple thin filament activating ligands, including effects due to  $\text{Ca}^{2+}$ , the TnI switch domain interfacing with TnC, and cycling cross-bridges altering TnC's conformation. This is a significant physiological advance, as it was not a foregone conclusion that past biophysical studies probing TnC and sarcomere function under steady-state activating conditions [15,16,36], which can take minutes to collect data, would translate to the physiological setting of intact excitation-contraction coupling in live cardiac muscle, wherein the dynamics of the contraction transient are on the millisecond time scale.

We interpret the biosensor FRET transient as reporting TnC's global conformational change, from extended to compact, during the twitch, as based on extensive in vitro work on TnC [17,36,53,54]. During a twitch, we posit that the TnC-tethered FRET probes Clover (donor) and mRuby2 (acceptor) move closer together, consistent with the increased FRET signal observed. Based on the Clover/mRuby2  $R_0$  of 6.3 nm, together with understanding of the useful FRET pair spacing range of 0.5–1.5  $R_0$  [21,22,55], we estimate that the Clover to mRuby2

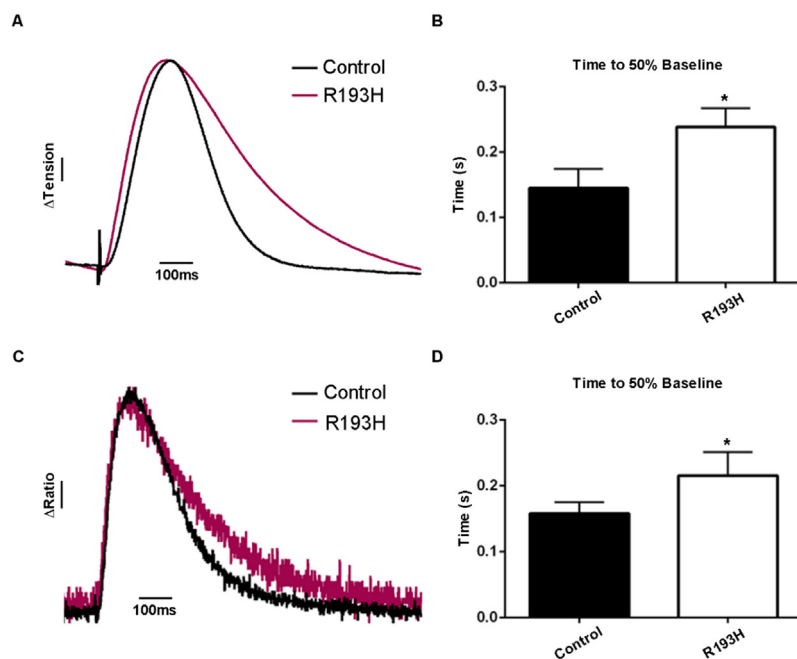


**Fig. 5.** Load-dependent effects of myosin on sarcomere activation in live cardiac muscle. **A**, Ensemble average of single myocytes (green) and papillaries (blue) reveals a load dependence to sarcomere activation (22 °C and 0.2 Hz). Note, FRET ratio-derived transients obtained in single myocytes were not different from Clover transient traces in the same single myocytes. **B**, Summary statistics of sarcomere biosensor kinetics reveals increasing load and physiological complexity significantly delays peak activation and time to inactivation ( $n = 8$  per group). **C**, Ensemble average traces of tension recordings (non-normalized) in papillaries before and after treatment with 250 nM Omecamtiv mecarbil shows alterations in tension development and kinetics,  $\Delta$ Tension bar represents a 10% change from baseline. **D**, Summary statistics of papillary twitch tension with 250 nM Omecamtiv mecarbil reduces the change in peak tension and slows the relaxation kinetics ( $n = 14$  pairs). **E**, Ensemble average traces of FRET ratio recordings (non-normalized) in papillaries before and after treatment with 250 nM Omecamtiv mecarbil shows alterations in peak intensity and kinetics.  $\Delta$ Ratio bar represents a 5% change in FRET from baseline. **F**, Summary statistics of ensemble traces from **e** show Omecamtiv mecarbil reduces the peak FRET ratio intensity change while shortening time to peak activation ( $n = 14$  pairs). **C–F**, Data obtained at 22 °C with 1.0 Hz stimulation. Mean  $\pm$  S.E.M are presented. Unpaired and paired two-tailed  $t$ -tests were used, as appropriate:  $^*P < 0.05$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fluorophore distance varies within a range of  $\sim 3$  nm (high FRET) to  $> \sim 9$  nm (no detectable FRET) during the twitch. Thus, with this biosensor probe, we report the presence and absence of FRET for this interprobe spacing range. In addition, given that each TnC biosensor is integrated into the sarcomere at a spacing no closer than every seven actin monomers, and together with the Clover/mRuby2  $R_0$ , we conclude that each TnC biosensor operates independently from neighboring biosensors, substantiating intramolecular FRET and not intermolecular FRET.

In addition, given the experimental limitations of the contracting myocyte, in which the number of fluorophores in the detected volume varies in time and from sample to sample, it is virtually impossible to measure FRET efficiency values, or the precise corresponding inter-

probe distances, accurately. This would require having simultaneous measurements of the fluorescence of donor-acceptor and donor-only samples [56], which is not feasible. However, there is enough information to show that: (a) the changes in FRET are within the expected range for this system (lobes of TnC) [36,53], (b) the fluorescence signals are detected with sufficient precision to detect subnanometer changes in distance, and therefore (c) the time dependence of the FRET signal change reflects accurately the time dependence of TnC structural changes during the myocyte twitch. Given uncertainties regarding the conformations and orientations of the tethered fluorescent proteins relative to the attached sites on TnC [55], the FRET values observed are consistent with distances within the sensitivity range of this donor-acceptor pair, which has an  $R_0$  value of 6.3 nm [21,22]. Thus, they are



**Fig. 6.** Altered sarcomere activation due to cardiomyopathic mutant sarcomeric protein expression in live cardiac muscle. **A**, Ensemble average traces of tension recordings in papillaries show restrictive cardiomyopathy cTnI mutant R193H have alterations in relaxation kinetics,  $\Delta$ Tension bar represents a 10% change from baseline. **B**, Summary statistics of papillary twitch tension shows R193H slows relaxation kinetics ( $n = 6$  for control and  $n = 5$  for R193H mutants). **C**, Ensemble average traces of FRET ratio recordings in papillaries show R193H mutants have slowed relaxation kinetics,  $\Delta$ Ratio bar represents a 10% change in FRET from baseline. **D**, Summary statistics of papillary FRET ratio shows R193H mutation slows relaxation kinetics ( $n = 6$  for control and  $n = 4$  for R193H mutants). **A–D**, Data obtained at 22 °C with 1.0 Hz stimulation. Mean  $\pm$  S.E.M are presented. Unpaired t-tests were used, as appropriate: \* $P < 0.05$ .

within the range of  $\text{Ca}^{2+}$ -dependent FRET values and FRET changes that have been previously reported by others for cardiac TnC [17].

There is strong rationale for proposing that TnC's conformation serves as a nexus in detecting the effects of multiple ligands that regulate thin filament activation. Extensive in vitro data demonstrate that TnC's conformation is sensitive to and can integrate near and far myofilament activating ligands across the sarcomere, including  $\text{Ca}^{2+}$  binding to TnC, cTnI binding to cTnC, and myosin binding to actin [1,12,18,19,36]. Studies in reconstituted myofilament systems show that fluorescent probes placed on skeletal TnC can detect the effects of cycling myosin to displace Tm and induce thin filament activation, when studied under steady-state activating conditions [18]. This effect was also seen in cardiac TnC conformational changes in reconstituted cardiac muscles [19]. In addition, recent in vitro studies have engineered FRET pairs into cardiac TnC to successfully monitor TnC conformational changes under steady-state  $\text{Ca}^{2+}$ -activating conditions [17]. While foundational, an important caveat with these previous studies is that data are collected under steady state conditions, operating on the seconds-to-minutes time scale. Because live cardiac muscle operates under dynamic conditions, with excitation-contraction coupling occurring on the 10s of millisecond timescale, it is unclear whether previous steady-state findings would translate to the physiological setting of live muscle. Accordingly, the findings reported here are significant in showing evidence of TnC-localized biosensing of multiple activating ligands, including cycling myosin, under conditions of physiological excitation-contraction coupling and mechanical load in live cardiac muscle.

Data shows that the TnC biosensor is capable of detecting the effects of the TnI switch domain as an activating ligand. Small-molecule and genetic approaches were used that specifically disrupt TnI's interaction with TnC, independent of effects on the  $\text{Ca}^{2+}$  transient. In addition, under physiological loading conditions, the TnC-localized biosensor detects the effects of small molecules that specifically alter myosin cross-bridge cycling rate. To our knowledge, these are the first data providing evidence in live cardiac muscle that TnI and cycling myosin cross-bridges can transduce thin filament activation, as detected by TnC conformational changes during a physiological twitch contraction. Based on extensive in vitro data that TnI-TnC and myosin transduce sarcomere-activating effects via Tm displacement [1,6,57,58], the present data suggest that the TnC biosensor transient reports effects of

Tm displacement in regulating thin filament activation. The potential implications of these findings, in further understanding the mechanism of sarcomere function in intact cardiac muscle, are discussed further below.

It is well established that tropomyosin's (Tm) position on the thin filament governs the overall activation status of the cardiac sarcomere [1,2]. In diastole, Tm blocks strong cross-bridge formation to actin, and this inhibition is relieved by a  $\text{Ca}^{2+}$ -dependent switch in troponin. Here, the C-terminus of TnI switches its strong binding to actin to strong binding to TnC [9], thus permitting Tm displacement and force production [8]. In addition, structural studies provide compelling evidence that strong myosin binding is required to fully displace Tm and accomplish complete activation of the thin filament regulatory system [57,58]. Thus, in cardiac muscle both TnI switching, from actin to TnC, and myosin binding to actin serve as activating ligands by orchestrating Tm displacement on the thin filament. The present findings provide new evidence of the dynamics of TnI and myosin activating ligands during a twitch, with evidence that cycling myosin effects are highly dependent upon mechanical loading in live cardiac muscle.

Our findings can be discussed in the context of current models of sarcomere activation. Sarcomere activation is defined as the functional status of the thin filament regulatory system, whereby the open state position of Tm is permissive for strong cross-bridge formation with actin [1,2]. It follows that regulation of sarcomere activation entails the processes that control Tm's position on the thin filament to control contraction. Sarcomere activation has been modeled by Tm orchestrating blocked, closed, and open states on the thin filament, and this formalism has guided the field for decades [1,11]. However, assigning the relative roles of cTnI and myosin as activating ligands, during the non-steady-state conditions of the cardiac twitch, has remained elusive. This is an important void to address, as sarcomere dysfunction underlies multiple forms of acquired and inherited heart disease [3,42]. Biochemical studies posit strong myosin binding to actin as the essential activating ligand required for the open state of the thin filament regulatory system [11]. However, recent cryo-EM studies suggest that the open state, as discerned by the disposition of Tm on the actin filament, is highly dependent on striated muscle-lineage [57]. Specifically, in cardiac muscle, structural-based results suggest that the  $\text{Ca}^{2+}$ -dependent cTnC-cTnI complex is the dominant activating ligand translocating Tm into the force-permissive open state, with little to no significant role

of myosin to acquire the open state [57]. Results from the present study find support from these structural findings. Under unloaded and loaded conditions, cTnI appears sufficient as an activating ligand. Results further suggest that under loaded conditions, myosin has a primary role to maintain Tm's position in the open state.

This study provides multiple lines of evidence that the cTnC-cTnI interface is a key sarcomere activating ligand during the cardiac twitch. Results with small molecules, which directly target modification of the cTnC-cTnI interface, [9] indicate that the initiation of the sarcomere open state during the cardiac twitch is due to the effects of the cTnC-cTnI as an activating ligand regulatory complex. Strengthening (pimobendan) or weakening (W7) the cTnC-cTnI interface [9,25,48], independently of  $\text{Ca}^{2+}$  handling, causes a corresponding increase or decrease in the TnC biosensor transient amplitude, respectively. In addition, the biosensor transient is significantly prolonged and decays more slowly due to a disease-causing cTnI R193H mutant, known to prolong cTnC-cTnI association and delay cardiac relaxation, all without altering the  $\text{Ca}^{2+}$  transient [3]. Because sarcomere-modifying small molecules (e.g., W7, OM) and mutant cTnI R193H do not alter the intracellular  $\text{Ca}^{2+}$  transient, these are evidence that the TnC biosensor transient is reflective of sarcomere-intrinsic regulatory processes and is not a myoplasmic  $\text{Ca}^{2+}$  sensor. This distinguishes the TnC biosensor from other genetically encoded sensors that specifically report  $\text{Ca}^{2+}$  content in cardiac myocytes, and not sarcomere regulatory dynamics per se [59]. This distinction is important because sarcomere performance can be altered dramatically in cardiac diseases independent of changes in the intracellular  $\text{Ca}^{2+}$  transient [3,51].

Because cTnI's switching mechanism, from actin to TnC, is well known from in vitro studies to control Tm's translocation on the actin filament [1,2], it follows that the troponin-localized biosensor is sensitive to, and provides an indirect measure of, Tm's disposition (e.g., Blocked/Closed to Open state) during the twitch. Upon muscle stimulation, data here support a model whereby the formation of the cTnC $\text{Ca}^{2+}$  state causes the regulatory switch peptide domain of cTnI to rapidly reverse its strong binding to actin and to then become strongly bound to cTnC. Here, formation of the cTnC - cTnI complex is stabilized, under either loaded or unloaded conditions, and this appears to dominate the initiation of the sarcomere-activating open state required for cycling myosin interactions with actin (Fig. S12). In the setting of the cardiac twitch, intracellular  $\text{Ca}^{2+}$  rises and significantly falls before peak force development [1], underscoring the key role of the proximity of the cTnI switch peptide to the N-terminal domain of cTnC to sustain Tm in a dis-inhibiting position, permitting the sarcomere activation open state [9]. This is supported by noting the TnC biosensor transient reaches its peak prior to development of peak force. Results using W7 or pimobendan further underscore how inhibiting or enhancing cTnC-cTnI interactions, respectively, ultimately affects the population of cTnI available to re-bind to actin and shift Tm's position from open to closed/blocked states.

The TnC biosensor detected the thin filament activating effects of cycling myosin cross-bridge under conditions of mechanical load. In 1972, Bremel and Weber first suggested the role of myosin as a key regulator of sarcomere activation in muscle [10]. In the following decades, numerous studies have supported the critical role of myosin as an actuator of the troponin-tropomyosin allosteric regulatory system to initiate sarcomere activation [1,2,6,11,20]. Compared to unloaded conditions, mechanical loading demonstrated prolonged sarcomere activation, as manifest by a significant delay in the inactivation of the TnC biosensor transient. Because mechanical loading causes a significant slowing in the cross-bridge cycling rate and increases the myosin duty cycle [1], this is evidence that the troponin-localized biosensor can detect, and is sensitive to, the load-dependent effect of altered myosin cycling on the TnC biosensor during a twitch, as predicted from in vitro studies [1,10,18,19]. The data further suggest that during the decay phase of the cardiac twitch, cycling myosin attachment to actin sustains Tm in the open state, which, in a reciprocal

manner, facilitates the maintaining of the cTnI switch peptide domain at or near the cTnC hydrophobic patch [9]. Therefore, we posit that cycling myosin effectively increases the lifetime of the cTnC-cTnI activating ligand complex during the relaxation phase of the twitch (Fig. S12). The TnC biosensor detected this effect of cycling myosin, demonstrating for the first time in live muscle, the dynamic interconnection between the thin filament and myosin in orchestrating sarcomere activation.

The TnC biosensor also detected the effects of modifying cycling myosin using a myosin-specific small molecule approach. The myosin-modulating drug OM depressed the TnC biosensor peak amplitude while the TnC biosensor transient had a faster time to peak. These findings are consistent with studies showing OM accelerating the phosphate release step from myosin while suppressing myosin's working stroke [29]. Myosin-targeted small-molecule effects on the TnC biosensor transient are of further interest as disease-causing myosin mutations can produce similar alterations in myosin function [3,5,42]. We posit that other sarcomere mutants, including those in myosin heavy and light chains, MyBP-C, titin, and others that modify myosin cycling [3], would affect sarcomere activation as well. In unloaded conditions, completely blocking myosin function did not affect the TnC biosensor transient. The force-velocity relationship dictates that under unloaded conditions the cross-bridge cycling rate increases and the number of strong binding cross-bridges significantly decreases, as compared to loaded contractions [1]. This can account for the lack of a detected effect of myosin on the TnC biosensor during unloaded contractions. It then follows that myosin's effect to activate the sarcomere is highly load dependent, as now shown here in live cardiac muscle via the TnC biosensor. Taken together, these data support a primary role for cycling myosin under baseline physiological conditions to sustain sarcomere activation, rather than to initiate sarcomere activation, most evident in the late phase of contraction under load, as supported by recent cryo-EM studies [57]. Collectively, cTnI and strong myosin binding each have roles in sarcomere activation/inactivation that are time and load dependent in the context of the cardiac twitch (Fig. S12).

Taken together, these findings have implications for understanding cardiac sarcomere activation mechanisms that govern beat-to-beat control of heart performance. For example, in several inherited and acquired cardiac diseases, sarcomeric dysfunction, and not  $\text{Ca}^{2+}$  mishandling, underlies disease initiation, and this is termed EC-uncoupling [42,51,60]. Here, disease severity couples closely with the degree of sarcomere-based EC-uncoupling. In acute ischemia, hypoxia, and acidosis, the intracellular  $\text{Ca}^{2+}$  transient is normal when sarcomere-based heart pump performance is severely depressed [60]. Furthermore, in inherited cardiomyopathies premature sudden cardiac death can result directly from sarcomeric mutations, uncoupled from alterations in the  $\text{Ca}^{2+}$  transient [42]. These underscore the critical role of sarcomere performance in cardiac health and disease. Advances here may provide further insights into the function of the sarcomere in health and disease, including elucidating the roles of new sarcomere-modifying drugs [27], myofilament post-translational modifications [47], and the effects of an expanding catalog of sarcomere mutations that cause human disease [3,42].

Future investigations of sarcomere regulation under varying physiological conditions including stress and across species will be of interest. In addition, this new biosensor should be of value in discerning mechanisms of inter-myofilament signaling, including new small molecules, disease models, and the roles of MyBP-C, titin, and others. Collectively, these advances further highlight the sarcomere as an excellent therapeutic target. Finally, it would be of interest to develop a TnC biosensor for live skeletal muscle study. We speculate, owing to the significant differing mechanism of troponin-based sarcomere regulation in heart versus skeletal muscle [48], that ligands of sarcomere activation will differ significantly between the striated muscle lineages when studied in the physiological context of intact muscle.

Supplementary data to this article can be found online at <https://>

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## Author contributions

ADV and JMM developed the initial study design and approach. ADV, AAM and JMM performed the experiments. ADV, BRT, AAM, DDT and JMM analyzed and interpreted the data. All authors contributed to writing the manuscript.

## Disclosures

None.

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