Cardiac excitation-contraction coupling requires dyads, the nanoscopic microdomains formed adjacent to Z-lines by apposition of transverse tubules and junctional sarcoplasmic reticulum. Disruption of dyad architecture and function are common features of diseased cardiomyocytes. However, little is known about the mechanisms that modulate dyad organization during cardiac development, homeostasis, and disease. Here, we use proximity proteomics in intact, living hearts to identify proteins enriched near dyads. Among these proteins is CMYA5, an under-studied striated muscle protein that co-localizes with Z-lines, junctional sarcoplasmic reticulum proteins, and transverse tubules in mature cardiomyocytes. During cardiac development, CMYA5 positioning adjacent to Z-lines precedes junctional sarcoplasmic reticulum positioning or transverse tubule formation. CMYA5 ablation disrupts dyad architecture, dyad positioning at Z-lines, and junctional sarcoplasmic reticulum Ca\textsuperscript{2+} release, leading to cardiac dysfunction and inability to tolerate pressure overload. These data provide mechanistic insights into cardiomyopathy pathogenesis by demonstrating that CMYA5 anchors junctional sarcoplasmic reticulum to Z-lines, establishes dyad architecture, and regulates dyad Ca\textsuperscript{2+} release.
Cardiomyocytes exemplify the integration of form and function, in which the precise positioning of nanoscale structures enables efficient, coordinated cycles of contraction and relaxation. Sarcomeres, concatenated end-to-end at structures known as Z-lines to form myofibrils, drive cardiomyocyte contraction. Forceful cardiomyocyte contraction requires coordinated activation of individual sarcomeres throughout the cell. This is achieved through excitation–contraction (E-C) coupling. The initiating signal for cardiomyocyte contraction is the action potential, which propagates rapidly along the plasma membrane. In mature cardiomyocytes, the plasma membrane forms highly ordered tubular invaginations, T-tubules, that penetrate into the interior of cardiomyocytes and allow rapid transmission of the action potential throughout the cardiomyocyte interior. At structures termed dyads, T-tubules are juxtaposed to the junctional sarcoplasmic reticulum (jSR), a specialization of the sarcoplasmic reticulum (SR). The L-type Ca\(^{2+}\) channel, housed within T-tubule membranes, opens upon membrane depolarization and releases extracellular Ca\(^{2+}\) into the dyadic cleft or junction, the narrow (~12 nm) cytoplasmic space between the T-tubules and the jSR. This localized increase in Ca\(^{2+}\) activates RYR2, the major intracellular Ca\(^{2+}\) release channel of cardiomyocytes. Located on the jSR, activated RYR2 releases Ca\(^{2+}\) from the jSR lumen into the dyadic cleft, from which it subsequently diffuses to stimulate contraction of adjacent sarcomeres. Because of the relatively slow speed of diffusion, the positioning of dyads adjacent to Z-lines is important for coordinated contraction.

How sarcomeres, jSR, and T-tubules attain their intricate nanoscale organization within the cardiomyocyte and with respect to each other is a fundamental mystery in cardiac biology. Studies have focused on factors that regulate T-tubule formation and organization. cBIN1 (cardiac bridging integrator 1), a membrane-binding protein, JPH2 (junctophilin 2), a protein that interacts with both the cell membrane and jSR, and RYR2 have been found to be essential for the formation or maintenance of T-tubules. However, factors responsible for jSR organization and positioning have not been identified.

Disruption of dyad architecture and impaired E-C coupling are common features of failing cardiomyocytes. In rodent pressure overload, T-tubule disorganization preceded ventricular dysfunction. T-tubules were less closely associated with jSR, a lower fraction of Z-lines had associated dyads, and the distance from dyads to Z-lines increased. These changes were associated with impaired E-C coupling. Similar changes to T-tubules, jSR, and dyads were found in human dilated and ischemic cardiomyopathy.

CMYA5 (cardiomyopathy-associated protein 5), also known as myospryn, is an under-studied ~450 kDa protein that is selectively expressed in cardiac and skeletal muscle. It is a member of the tripartite motif-containing super-family (TRIM), which contain four protein-protein interaction domains (RING, BBox1, BBox2, and coiled-coiled) in a conserved order and generally functions as part of large protein complexes. Based on its co-expression with known cardiomyopathy genes, CMYA5 initially was linked hypothetically to cardiomyopathy. This link gained empirical support when a CMYA5 coding single nucleotide polymorphism was associated with left ventricular wall thickness and diastolic dysfunction. CMYA5 was previously reported to interact with multiple muscle proteins, including RYR2, the Z-line protein ACTN2 (α-actinin), desmin, titin, and PKA (protein kinase A). However, little has been reported about the in vivo function of CMYA5. A recent study published while this manuscript was in preparation demonstrated that CMYA5 knockout causes cardiac dysfunction and mislocalization of RYR2. However, the effect of CMYA5 knockout on dyad formation, structure, and function was not investigated in detail.

Here we performed a proximity proteomics screen for components of dyads and identified CMYA5. We found that CMYA5 is required to efficiently position jSR adjacent to Z-lines, an early and essential step in dyad assembly. Ablation of CMYA5 disrupted dyad architecture, dysregulated RYR2 channel activity, and impaired the fidelity of E-C coupling. Finally, CMYA5 protected heart function and dyad structure from pressure overload stress.

Results

Proximity proteomics identifies CMYA5 as a dyadic protein.

We developed a system to identify proteins that localize in the vicinity of dyads using BioID, a proximity labeling technique with an estimated labeling radius of ~10 nm, which matches the approximate diameter of the dyadic cleft. We fused BirA, an enzyme that releases short-lived biotin-free radicals, to the N-terminus of either junction (gene symbols: ASPH, BirA*-ASPH) or triadin (BirA*-TRDN), transmembrane jSR proteins that form a complex with RYR2. By localizing BirA* to the dyadic cleft, we anticipated that dyadic proteins would become biotinylated, so that they could be purified on streptavidin and subsequently identified by mass spectrometry. We expressed these dyad-targeted proteome biosensors in vivo in murine hearts using adeno-associated virus serotype 9 (AAV9) and the cardiac troponin T (Tnt2) promoter (Fig. 1a). We administered AAV to P1 mice, supplied biotin from P21 to P28, and collected hearts at P28 (Fig. 1b). Histological analysis confirmed efficient AAV transduction of cardiomyocytes (70 and 78% for BirA*-ASPH and BirA*-TRDN, respectively; Fig. 1c) and punctate staining of the BioID sensors overlying T-tubules, marked by CAV3 (caveolin 3) (Fig. 1d), consistent with appropriate expression and localization of the biosensors to dyads without perturbation of their overall organization. Probing protein lysates with streptavidin conjugated to hors eradish peroxidase confirmed biotin- and BirA*-dependent biotinylation of protein complexes (Fig. 1e). Biotinylated proteins were purified using immobilized streptavidin and analyzed by mass spectrometry (Fig. 1f). We ranked genes by the ratio of the average protein signal in the BirA*-ASPH and BirA*-TRDN groups to that in the control group. Among the proteins highly enriched in the BioID groups were RYR2, JPH2, ASPH, and TRDN (Fig. 1f and Supplementary Data 1). Recovery of these known dyadic proteins validated our experimental strategy.

We prioritized proteins found in both BioID groups that lacked signal in the control group. Among these proteins, one of the most highly enriched in the BioID groups was CMYA5, a protein expressed in striated muscle and neurons. Within the heart, CMYA5 is selectively expressed in cardiomyocytes (Supplementary Fig. 1). CMYA5 has been reported to interact with multiple cardiomyocyte proteins (e.g., RYR2, ACTN2, desmin, titin, and PKA) that have distinct localization patterns. In mature cardiomyocytes, we confirmed that CMYA5 co-localizes with jSR protein RYR2 in a striated pattern, with periodic peaks every 2 µm (Fig. 1g). We further tested the in situ proximity of CMYA5 and RYR2 using the proximity ligation assay (PLA), which detects proteins in close proximity (< 40 nm). Consistent with the reported interaction of CMYA5 with RYR2, we observed bright, punctate PLA signals in a striated pattern throughout adult cardiomyocytes, marking loci where CMYA5 and RYR2 are in close proximity (Fig. 1h). These results indicate that CMYA5 is a dyadic protein that is closely associated with RYR2.
**Fig. 1 BioID identification of dyadic proteins.**

- **a** Proximity proteomics strategy. BirA* and myc epitopes were fused to ASPH and TRDN, which interact with RYR2 in jSR of dyads. N-terminal fusion positions BirA* in the dyadic cleft. The fusion protein was expressed in vivo in cardiomyocytes using AAV9 and the cardiomyocyte-selective troponin T (Tnnt2) promoter. GFP was co-expressed using a P2A sequence.

- **b** Experimental timeline. Artwork from biorender.com.

- **c** Expression of BirA* dyadic biosensors in myocardium. Heart sections were stained for the myc epitope tag. Most cardiomyocytes were immunoreactive. Bar = 20 µm. Representative of 3 independent experiments.

- **d** Mature ventricular cardiomyocytes. In the left GFP + cell, myc immunoreactive signal co-localized with CAV3, a T-tubule marker. Bar = 10 µm. Representative of three independent experiments.

- **e** Protein lysates from mice expressing the indicated BirA*-fused biosensors or GFPs were analyzed by western blotting. AAV dose and treatment with exogenous biotin are indicated. M, middle dose (2 × 10¹⁰ vg/g). F, full dose (5.5 × 10¹⁰ vg/g). Ponceau S, total protein. SA-HRP labeled biotinylated proteins. Representative of two independent experiments.

- **f** Summary of mass spectrometry data. Red and blue symbols indicate protein signals from BirA* biosensors. Gray symbols show control (AAV-GFP) signals. One replicate pooled from three hearts was performed for each sensor and for control. Proteins were ranked by the ratio of the average signal of the two different BirA* biosensors to the control signal. Proteins lacking control signal grouped to the left. Only proteins with average/control ratio >5 are shown.

- **g** Co-localization of CMYA5 and jSR marker RYR2. Immunostained adult cardiomyocytes were imaged with a confocal microscope. Spatial profiles to the right demonstrate co-localization in a striated pattern with 2 µm spacing. Bar = 10 µm.

- **h** Proximity ligation assay. Punctate red signal, indicating close proximity of RYR2 and CMYA5, was observed when both RYR2 and CMYA5 antibodies were included, but not with either in isolation. Signal intensity is quantified at the right. n, number of cardiomyocytes. Two-sided Kruskal-Wallis with Dunn’s multiple comparison test vs. RYR2 + CMYA5: ***, P < 0.001; ****, P < 0.0001. Data are presented as mean ± SD. Bar = 10 µm.
**Fig. 2** Characterization of hearts lacking CMYA5. **a** Genomic structure of Cmya5 wild-type and Δ alleles. Cas9-mediated deletion of 9281 bp of exon 2 causes a frameshift (fs) after the 55th amino acid residue. RT-qPCR and genotyping amplicons are indicated. Representative of five independent experiments. **b** Cmya5 cardiac mRNA levels. RT-qPCR amplicons 1 and 2 represent the deleted region and the 3' end of the transcript, respectively. ANOVA with Dunnett's multiple comparison test vs. WT of the same amplicon. **c** Cardiac protein lysates were analyzed by western blotting. KO samples lacked CMYA5 immunoreactivity. Representative of five independent experiments. **d** Gross morphology of WT and KO hearts. Bar = 2.5 mm. Representative of three independent experiments. **e** Heart weight normalized to body weight, at the indicated ages. ANOVA with Dunnett’s multiple comparison test vs. WT at the same time point. **f** Heart weight to body weight (HW/BW) ratio. **g** Echocardiographic measurement of systolic heart function. FS fractional shortening. n, number of hearts. Data are presented as mean ± SD. **h** Cardiac protein lysates from WT and KO hearts were analyzed by western blotting. KO samples lacked CMYA5 immunoreactivity. Representative of five independent experiments. **i** Transmission electron microscopy of WT or KO ventricular myocardium. *, T-tubule. The WT T-tubule micrograph is enlarged and labeled. The WT T-tubule parameters, de novo synthesis, and coupling were measured from Supplementary Fig. 2a. **j** Isolated ventricular WT or KO cardiomyocytes immunostained for T-tubule (TT: CAV3) and jSR (RYR2, CASQ2, JPH2) markers, as well as FSD2. Bar = 200 nm. Representative of three independent experiments. **k** Quantification of T-tubule parameters, defined in Supplementary Fig. 2a. **l** Isolated WT or KO atrial cardiomyocytes immunostained for RYR2 or FSD2. NS, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Statistical tests were two-sided. Data are presented as mean ± SD.

**CMYA5 is required for normal heart function.** The in vivo function of CMYA5 has only been described in one study, which found that its ablation causes mild cardiac dysfunction, mislocalization of RYR2, and ultrastructural abnormalities of the SR and T-tubules19. However, the contribution of CMYA5 to dyad development, organization, and function was not investigated. We obtained an independent Cmya5 loss-of-function mouse model in which CRISPR/Cas9 and two guide RNAs (gRNAs) were used to delete 9281 bp in exon 2 (Fig. 2a; “Methods”). The mutant allele, Cmya5Δ, is predicted to cause a frameshift mutation after amino acid residue 55. We confirmed the Cmya5 genomic deletion (Fig. 2b) and its resultant depletion of Cmya5 transcript (Fig. 2c) and protein (Fig. 2d). Gross examination of Cmya5Δ/Δ (KO) hearts revealed substantial cardiac remodeling, including an increase in heart size (Fig. 2e) and weight, normalized to body weight, at 3 months of age (Fig. 2f). Echocardiographic analysis revealed diminished systolic ventricular function in KO mice, consistent with a recent study of an independently generated null allele19. Heterozygotes (Het) had normal heart weight and ventricular function (Fig. 2f, g). Diminished systolic function was accompanied by ventricular dilatation (Supplementary Fig. 2). These data indicate that Cmya5 is essential for normal heart function.
CMYA5 establishes normal dyad architecture and positioning adjacent to Z-lines. The architecture of cardiac dyads is critical for efficient E-C coupling. To assess the effect of CMYA5 ablation on dyad organization and structure, we first examined the T-tubule system by optically sectioning hearts stained with the plasma membrane dye MM 4-64[2]. In KO cardiomyocytes, we observed dramatic disruption of T-tubules (Fig. 2h). Quantitative image analysis[8] confirmed significantly reduced transverse fraction and increased longitudinal fraction in KO compared to control (Fig. 2h). Next, we imaged T-tubules and jSR by immunostaining component proteins in isolated adult cardiomyocytes. Both T-tubule (CAV3) and jSR (Ryr2, Casq2) (calsequestrin 2), JPH2 markers were disorganized in KO (Fig. 2i). FSD2 (fibronectin type III and SPRY domain containing 2), a protein closely related to the C-terminus of CMYA5[28] (Supplementary Fig. 3a), also lost its jSR localization pattern[12] (Fig. 2i). To confirm that CMYA5 ablation cell autonomously disrupts T-tubule organization in the absence of cardiac dysfunction, we performed mosaic Cmya5 inactivation using Cas9 and AAV-mediated somatic mutagenesis[7] (CassAv; Supplementary Fig. 3b). By administering low-dose AAV-gRNA[Cmya5], we ablated Cmya5 in a minority of cardiomyocytes without impairing heart systolic function (Supplementary Fig. 3c, d). Consistent with the Cmya5a/- results, the CASAAV-transduced cardiomyocytes, marked by GFP, exhibited T-tubule disorganization (Supplementary Fig. 3e-g). Together, these data confirm that CMYA5 is cell autonomously required for T-tubule organization and underscore the importance of CMYA5 for normal cardiac function.

To evaluate dyad architecture with greater resolution, we analyzed cardiomyocytes by transmission electron microscopy (TEM). T-tubules, jSR, and their intimate relationship with each other were all perturbed in KO (Fig. 2j, k and Supplementary Fig. 4a). These abnormalities were quantified by measuring T-tubule circularity, T-tubule:jSR coupling ratio (the fraction of each T-tubule juxtaposed to jSR), and Junction-Z-line distance (perpendicular distance from Z-line to jSR-T-tubule junctions), as defined in Supplementary Fig. 4a and Zhang et al.[10]. KO T-tubules were less circular, had reduced spatial coupling, and had greater and more heterogeneous Junction-Z-line distance (Fig. 2l). Together, these changes to dyad architecture and positioning would be expected to make E-C coupling weaker and more variable. While jSR morphology was abnormal in KO, overall Z-line and endoplasmic reticulum (ER) organization were unaffected, as revealed by the immunofluorescent patterns of SR protein Serca2a (sarco/endoplasmic reticulum Ca2+ -ATPase 2a) and Z-line protein Actn2 and by AAV-mediated expression of an ER-targeted fluorescent protein (Supplementary Fig. 4b-e). Although Cmya5 has been reported to interact with PKA and titin, we did not observe altered localization of PKA (Supplementary Fig. 5a) or titin (Supplementary Fig. 5b). Moreover, measurement of nuclear and cytoplasmic Nfat3 did not support the activation of the hypertrophic calcineurin pathway (Supplementary Fig. 5c, d), which is downstream of calcineurin. Although Cmya5 KO was reported to cause abnormal cardiac mitochondria[19], we did not detect abnormalities in the overall mitochondrial organization or mitochondrial morphology (Supplementary Fig. e-g).

Collectively, these data indicate that CMYA5 is required for dyad architecture and positioning with respect to Z-lines.

CMYA5 regulates the hierarchical assembly of dyads. To further dissect the inter-relationships between T-tubules, jSR, and Z-lines in normal and Cmya5 KO cardiomyocytes, we analyzed adult murine atrial cardiomyocytes, which have a paucity of T-tubules[28]. Ryr2-containing SR domains can be found just under the sarcolemma (peripheral couplings) or localized adjacent to Z-lines (corbular SR), as demonstrated in Fig. 2m and Supplementary Fig. 4f-g. These observations indicate that Ryr2 positioning adjacent to Z-lines does not require T-tubules. In Cmya5 KO atrial cardiomyocytes, Ryr2, Fsd2, and Casq2 lost their characteristic co-localization with Z-lines (Fig. 2m and Supplementary Fig. 6a), demonstrating that CMYA5 is also required in atrial cardiomyocytes to tether corbular SR to Z-lines. Notably, Ryr2 and Jph2 localization in peripheral couplings near the sarcolemma was preserved in KO atrial cardiomyocytes (Fig. 2m and Supplementary Fig. 6a), indicating a selective role for CMYA5 localization of the subset of Ryr2-containing SR domains normally positioned adjacent to Z-lines.

We further examined the organization of Z-lines, jSR, and T-tubules in control and KO ventricular cardiomyocytes at different developmental stages. In P7 cardiomyocytes, T-tubules, marked by CAV3, have not yet organized[38], but Ryr2, Cmya5, and Fsd2 were already co-localized in a striated pattern that corresponds to sarcomere Z-lines (Fig. 3a-c). Cmya5 ablation, however, disrupted the Z-line distribution of Ryr2 (Fig. 3c). These data further demonstrate that CMYA5 is required and T-tubules dispensable for the positioning of Ryr2 and jSr to Z-lines. CMYA5 deficiency resulted in reduced levels of jSR proteins Ryr2, Fsd2, and Cbin1 (Supplementary Fig. 6b), likely due to their degradation because of impaired jSR localization and assembly. We gained further insights by examining E15.5 cardiomyocytes, whose actively assembling subcellular structures are in different states of organization. Confocal imaging of Ryr2-GFP[31] knockout sections revealed subsets of Z-lines co-localized with both Cmya5 and Ryr2 (yellow arrowheads, Fig. 3d and Supplementary Fig. 6c), Cmya5 only (red arrowheads, Fig. 3d and Supplementary Fig. 6c), or neither Cmya5 nor Ryr2 (white arrowheads, Fig. 3d and Supplementary Fig. 6c). Z-lines that co-localized with Ryr2 but not Cmya5 were rare. Together, these data show that CMYA5 localization at Z-lines does not require co-localization of jSR/Ryr2 or T-tubules, whereas efficient T-tubule and jSR/Ryr2 localization at Z-lines does require CMYA5.

To further test this organizational hierarchy, in which CMYA5 reads Z-line positional information to properly localize jSR and T-tubules, we used CassAv7 to perform mosaic ablation of sarcomeres or Ryr2. Ryr2 mosaic somatic deletion by CassAv7 (Supplementary Fig. 6d) did not perturb the normal striated staining pattern of Cmya5 and Fsd2 (Fig. 3e, f). In contrast, CassAvA-mediated mosaic deletion of sarcomere protein Mhy6[32] (Supplementary Fig. 6e) disrupted the organization of Z-lines, Cmya5, and jSR, marked by Jph2 (Fig. 3g, h and Supplementary Fig. 6f-i). Taken together, these results demonstrate that dyads are built on scaffolding provided by sarcomeres. CMYA5 localizes to Z-lines, and subsequently contributes to tethering jSR adjacent to these structures. T-tubules subsequently form and co-localize with jSR, yielding organized, properly positioned dyads.

To investigate the features of CMYA5 required for its activity, we adopted a rescue strategy in which we used AAV to express CMYA5-related protein fragments. At 11.2 kb, the Cmya5 coding region exceeds the cargo capacity of AAV. All known protein-protein interactions involving CMYA5 map to its C-terminal end[12,23], contained within a fragment named MD9 (amino acids 2731–3739) that was found to bind and cluster Ryr2 in a heterologous expression system (Supplementary Fig. 3a)[12]. Furthermore, FSD2 shares 45.5% protein sequence similarity with the C-terminus of CMYA5 and retains the same domain architecture and order (Supplementary Fig. 3a)[12]. For these reasons, we assayed Cmya5MD9 and Fsd2 for their ability to rescue Cmya5 KO hearts. Cmya5MD9 and Fsd2 expressed from AAV vectors localized in a striated pattern in WT cardiomyocytes.
but not in Cmya5 KO (Supplementary Fig. 7). Neither CMYA5MD9 nor FSD2 rescued Cmya5 KO T-tubule organization (Supplementary Fig. 7a) or heart systolic function (Supplementary Fig. 7b), suggesting that portions of CMYA5 within the N-terminal portion of the protein contribute to CMYA5 localization. With the exception of amino acids 78-319, CMYA5's N-terminus is poorly conserved. When expressed in cardiomyocytes in vivo, CMYA51-450 and CMYA51-1200, which share the conserved region, co-localized well with Z-line marker ACTN2 (Supplementary Fig. 7c). These data indicate that the N-terminal portion of CMYA5 likely promotes its localization at Z-lines and its role in maintaining heart function.

CMYA5 regulates normal dyad Ca\(^{2+}\) dynamics. Dyads play a central role in E-C coupling, and Cmya5 KO disrupts dyad structure and positioning. To assess the functional consequence of Cmya5 KO on cardiomyocyte Ca\(^{2+}\) signaling, we visualized Ca\(^{2+}\) dynamics at individual dyads in contracting cardiomyocytes using a nanospark sensor localized to the dyadic cleft (GCaMP6f-junctin; abbreviated ASPH-G6f). After AAV-mediated expression of ASPH-G6f in adult heart, we measured spontaneous jSR Ca\(^{2+}\) release events in freshly isolated mature cardiomyocytes. KO cardiomyocytes had elevated Ca\(^{2+}\) spark frequency, which was strongly augmented by β-agonist (100 nM isoproterenol; ISO; Fig. 4a, b). Next, we examined junctional Ca\(^{2+}\) dynamics during
**Fig. 4** Altered dyadic Ca\(^{2+}\) release in CMYA5 KO cardiomyocytes. Ca\(^{2+}\) release in mature CMYA5 KO or WT ventricular cardiomyocytes was assessed using a dyad-localized Ca\(^{2+}\) nanosensor, GCaMP6f-Junctin (ASPH-G6f) and detected by confocal line scan imaging. **a, b** Surface plots of Ca\(^{2+}\) sparks in basal and isoproterenol (ISO)-stimulated (100 nM) cardiomyocytes. Quantification is shown in (b). Mann-Whitney test within basal or ISO conditions. **c-f** Ca\(^{2+}\) release during electrical pacing (red lines) under basal conditions or ISO stimulation. Dotted line, leading edge of the evoked Ca\(^{2+}\) transient. Arrowheads, aberrant Ca\(^{2+}\) release outside of the initial evoked Ca\(^{2+}\) transient, without (white) or with (red) wave-like propagation. **d** Representative Ca\(^{2+}\) transients under basal (top) or ISO (bottom) conditions. **e, f** Quantification (mean ± SD) of Ca\(^{2+}\) transient amplitude and Ca\(^{2+}\) spark frequency. Mann-Whitney test within basal or ISO conditions. **g-i** Measurement of SR Ca\(^{2+}\) stores using low-affinity Ca\(^{2+}\)-sensitive dye FluoSN and AAV-mediated expression of SR-targeted esterase, which trapped Fluo5N in SR. **g** FluoroSN distribution. **h, i** SR Ca\(^{2+}\) release induced by 10 mM caffeine. **g-i** are representative of three independent experiments. Caffeine-induced change in FluoroSN signal yielded an estimate of SR Ca\(^{2+}\) stores (mean ± SD). t test. Bar = 10 μm. **j** Ca\(^{2+}\) release at dyads of electrically paced cardiomyocytes was recorded using the ASPH-G6f dyad-targeted Ca\(^{2+}\) nanosensor. Gray arrowheads, consistently coupled dyads (activated with each electrical pacing event). Red arrowheads, inconsistently coupled dyads (activated with some pacing events but not others). **k** Fluorescence intensity profiles over time of yellow outlined dyads in j, numbered 1-4. Dyad 2 was consistently coupled, whereas dyads 1, 3, and 4 were inconsistently coupled. Black arrows, lack of activation with electrical pacing. Blue arrow, evoked Ca\(^{2+}\) spark not coordinated with the overall Ca\(^{2+}\) transient. Frequency of inconsistently coupled dyads in WT and KO cardiomyocytes, with and without ISO stimulation. The number of pacing events without dyadic Ca\(^{2+}\) release over three consecutive calcium transients was normalized to line scan length. n, number of cardiomyocytes. Mann-Whitney test within basal or ISO conditions. *, P < 0.05; **, P < 0.01; ****, P < 0.0001. Two-sided statistical tests were used. Data are presented as mean ± SD.

Electrically evoked E-C coupling. Representative results in Fig. 4c–l illustrate several abnormalities in KO cardiomyocytes. First, KO CMs exhibited reduced Ca\(^{2+}\) transient amplitude under both normal and ISO-treated conditions (Fig. 4c–e). This reduction is largely attributable to ~25% reduced SR Ca\(^{2+}\) content, measured in intact CMs by sequestering a low-affinity Ca\(^{2+}\)-sensitive dye in SR through AAV-mediated expression of an Sr-targeted esterase35 (Fig. 4g–i). Second, the spatiotemporal coordination of dyadic Ca\(^{2+}\) transients was impaired in KO CMs. In control cardiomyocytes, dyad Ca\(^{2+}\) release during full-fledged Ca\(^{2+}\) transients was temporally synchronized across adjacent dyads (Fig. 4c, WT: note smooth leading edge marked by dotted line). In contrast, Ca\(^{2+}\) release in KO cardiomyocytes had reduced synchronization across adjacent dyads (Fig. 4c, KO: note ragged leading edge marked by dotted line). Ca\(^{2+}\) release at some dyads was delayed relative to the overall initiation of the Ca\(^{2+}\) transient, making the transient less abrupt. This variability in dyad Ca\(^{2+}\) release is consistent with the architectural abnormalities that we observed in KO dyads. Third, the dyadic Ca\(^{2+}\) nanosensor revealed that a subset of CMYA5 KO dyads became intermittently uncoupled, i.e. some KO dyads activated inconsistently during electrical pacing (Fig. 4j, k). Quantitative analysis confirmed that CMYA5 KO dyads were significantly more likely to exhibit inconsistent coupling (Fig. 4l). This finding agrees with reduced Ca\(^{2+}\) release synchronization (Fig. 4c) and the abnormal and more heterogeneous dyad architecture (Fig. 2j–l). Finally, during electrical pacing, we observed more frequent evoked Ca\(^{2+}\) sparks interspersed between fully activated Ca\(^{2+}\) transients in KO cardiomyocytes (Fig. 4c, top arrowheads; Fig. 4k, blue arrow; quantified in Fig. 4l). Under ISO stimulation, these Ca\(^{2+}\) sparks became even more frequent and turned into wave-like propagations (Fig. 4c, bottom; arrowheads; Fig. 4f) that could increase the
propensity for Ca\textsuperscript{2+}-dependent arrhythmias. Collectively, these results demonstrate that CMYA5 is required to coordinate E-C coupling and regulate RYR2 activity.

**CMYA5 stabilizes dyad structure and function to biomechanical stress.** Biomechanical stress on the heart promotes T-tubule and dyad disorganization\textsuperscript{4,10}. Therefore we tested the hypothesis that CMYA5 stabilizes dyads in the context of biomechanical stress by analyzing the response of Cmya5 KO hearts to pressure overload, induced by surgical transverse aortic constriction (TAC). WT, Het, and KO mice were subjected to TAC or Sham operation, followed by weekly echocardiography. Physiological and histological studies were performed 4 weeks after TAC. After TAC, WT mice developed mild ventricular dysfunction which remained stable between weeks 1–4. In contrast, KO + TAC mice developed more severe ventricular dysfunction at 1 week, which became progressively more severe in subsequent weeks (Fig. 5a). This ventricular dysfunction was accompanied by adverse remodeling, manifested as progressive ventricular dilatation (Supplementary Fig. 8a, b). At necropsy, KO + TAC hearts appeared hypertrophied (Fig. 5b), and this was confirmed by gravimetric measurements (Fig. 5c). In Sham-operated mice, the area of fibrotic tissue was comparable between genotypes (Supplementary Fig. 8c). TAC induced fibrosis in all genotypes compared to Sham (Supplementary Fig. 8d); KO mice developed 2.8-fold more fibrosis than wild type (P < 0.0001), and Het mice also had a significantly greater fibrotic area (P < 0.01, 1.3-fold greater than WT). Consistent with increased fibrosis, we also detected increased apoptosis after TAC in both Het and KO compared to WT (Supplementary Fig. 8e, f).

To assess the effect of TAC and CMYA5 ablation on dyad architecture and positioning, we examined cardiomyocyte ultrastructure using TEM. Dyads in KO + TAC cardiomyocytes were highly abnormal (Fig. 5d). In terms of quantitative metrics, TAC induced abnormal T-tubule circulation in all genotypes, with KO and Het being more severely affected than WT (Fig. 5e). TAC significantly increased function-to-Z-line distance in KO compared to WT, and significantly reduced the coupling ratio to a comparable level across all genotypes (Fig. 5f, g).

We assessed dyad function by measuring dyadic Ca\textsuperscript{2+} release in cardiomyocytes expressing the ASPH-G6f dyadic Ca\textsuperscript{2+} nanosensor. The nanosensor adopted the expected striated localization in WT + Sham, which was less organized in WT + TAC, consistent with the known disruptive effect of TAC on dyads\textsuperscript{9} (Fig. 5h). KO + Sham dyads were also disorganized, and this disarray was further exacerbated by TAC (Fig. 5h). Ca\textsuperscript{2+} sparks, recorded using the ASPH-G6f nanosensor, were more frequent in KO than WT after TAC (Fig. 5i). ISO further amplified the deleterious effects of KO on Ca\textsuperscript{2+} spark frequency. Taken together, we conclude that CMYA5 is required to organize both jSR and T-tubular components of dyads and is essential for cardiomyocyte structural and functional integrity in the face of biomechanical stress.

**Discussion**

Cardiac dyads, nanodomains formed by the close apposition of T-tubules and jSR membranes, are critical to E-C coupling\textsuperscript{1-3}. The mechanisms that establish dyad architecture and that position dyads at sarcomere Z-lines have been an unsolved mystery. Given the importance of these structures for E-C coupling, efficient cardiac contraction, and cardiac rhythm, this puzzle has critical implications for cardiac homeostasis and disease. Here we revealed that CMYA5 plays an important role in a hierarchical process of dyad assembly, in which CMYA5 localizes to sarcomere Z-lines, even in fetal cardiomyocytes that lack T-tubules or jSR. In neonatal cardiomyocytes, jSR marked by RYR2 colocalizes with CMYA5 at Z-lines in the absence of T-tubules, which develop later. These results, and prior experiments demonstrating that CMYA5 forms a protein complex with RYR2 and influences its intracellular clustering\textsuperscript{12}, indicate that CMYA5-RYR2 interaction tethers jSR to Z-lines. When T-tubules form in juvenile cardiomyocytes, they co-localize with jSR through incompletely understood mechanisms involving JPH2\textsuperscript{26} and RYR2\textsuperscript{1}, forming dyads. Thus, our studies provide an integrated developmental time course of dyad assembly and identify CMYA5 as a core component of the mechanisms that establish the subcellular localization of dyads at Z-lines.

Functionally, in keeping with its key role in organizing dyad architecture and localization, we demonstrate that CMYA5 is critical for regulating cardiomyocyte Ca\textsuperscript{2+} release. CMYA5 ablation reduced the synchronization and amplitude of dyadic Ca\textsuperscript{2+} release, which likely contributed to the impaired ventricular function of Cmya5 KO hearts. We also observed more frequent spontaneous Ca\textsuperscript{2+} release in Cmya5 KO hearts, especially with \( \beta \)-adrenergic stimulation. These data indicate that CMYA5 regulates RYR2 activity, through direct physical interaction\textsuperscript{12}, by governing RYR2 clustering\textsuperscript{36}, or by recruiting proteins that modulate RYR2 activity, such as Protein Kinase A\textsuperscript{37,38}.

Despite the strong effects of CMYA5 ablation on dyad architecture and organization within cardiomyocytes, Cmya5 KO hearts had only mildly impaired baseline contractile function. This is unlikely to be due to redundancy with the structurally related FSD2, since FSD2 overexpression did not rescue Cmya5 KO hearts and FSD2 was unable to properly localize in the absence of CMYA5. Rather, it likely reflects the considerable contractile reserve of the mammalian heart and genetic compensation for chronic Cmya5 ablation. Stressing the heart by aortic constriction unmasked the profound effect of CMYA5 ablation. CMYA5 KO hearts developed severe, progressive ventricular dysfunction and underwent adverse remodeling. These organ level findings were linked to greater disarray of dyads in Cmya5 KO induced by TAC. Particularly notable was the marked increase in Ca\textsuperscript{2+} sparks, especially with \( \beta \)-adrenergic stimulation, which may be related to the propensity of failing hearts to develop ventricular arrhythmias. Together these data show that CMYA5 is important for the structural and functional integrity of cardiac dyads, particularly under stress. These findings suggest that CMYA5-dependent mechanisms may become dysregulated in diseased hearts, leading to loss of dyad architecture and function.

While this manuscript was in preparation, Tsoupri et al. reported the cardiac phenotype of an independently generated Cmya5 knockout mouse line\textsuperscript{19}. Consistent with our study, Tsoupri et al. found that Cmya5 knockout caused mild cardiac dysfunction, mislocalization of RYR2, and abnormal T-tubule and jSR morphology. Our study makes several conceptual advances beyond the work of Tsoupri et al. Our detailed studies of CMYA5 reveal its significant contribution to a hierarchical and developmentally ordered process of dyad assembly, in which CMYA5 tethers jSR to Z-lines, and with the subsequent addition of T-tubules. In addition to its critical role in establishing normal dyad architecture, CMYA5 regulated dyad function by enhancing the fidelity of E-C coupling and by limiting spontaneous RYR2 Ca\textsuperscript{2+} release. Finally, we show that CMYA5 protects dyad structure and function from biomechanical stress. Future investigations are warranted to determine how CMYA5 coordinates with other proteins to direct the formation, maintenance, and positioning of dyads, and how CMYA5 regulates Ca\textsuperscript{2+} release from RYR2.

**Methods**

**Animal.** Experiments were conducted compliant with all relevant ethical regulations. All animal experiments were performed under protocols approved by the
Fig. 5 CMYA5 protects the heart and dyads from the deleterious effects of pressure overload. Cmya5 KO and WT mice underwent baseline echocardiography (week 0) and then TAC or Sham surgery. After 4 weekly echocardiograms, necropsy was performed. a Systolic heart function. FS fractional shortening. Repeated-measures two-way ANOVA was performed for TAC cohort, with a comparison to WT-TAC (∧) at each time point. n, number of mice. b Gross cardiac morphology. Bar, 2.5 mm. c Heart weight normalized to body weight. Kruskal-Wallis with Dunn’s multiple comparison test vs. WT within Sham (*) or TAC (∧) cohorts. n, number of mice. d–g Dyad architecture analyzed by transmission electron microscopy. Boxed regions are magnified in the bottom row. Bar, 500 nm. Quantification of T-tubule circularity, jSR-to-T-tubule coupling ratio, and Junction-Z-line distance. Kruskal-Wallis with Dunn’s multiple testing correction vs. WT Sham (*) or TAC (∧). n, number of dyads per group, from at least 10 cardiomyocytes from 3 different mice. h, i Cardiomyocytes were transduced with dyad-localized ASPH-G6f Ca^{2+} nanosensor. Images of nanosensor distribution indicate dyad disorganization induced by TAC and Cmya5 KO (h). Quantification of Ca^{2+} sparks by confocal line scan imaging of nanosensor. Kruskal-Wallis with Dunn’s multiple testing correction within Sham (*) or TAC (∧). n, number of cardiomyocytes per group. ∧, P = 0.054; ∧ or *, P < 0.05; ∧ or **, P < 0.01; ∧∧ or ***, P < 0.001; ∧∧∧ or ****, P < 0.0001. Two-sided statistical tests were used. Data are presented as mean ± SD.
Boston Children’s Hospital Institutional Animal Care and Use Committee. Cmya5/ΔA (C57BL/6NJ-Cmya5fl/J; stock no. 032826) and Rosa 26SrfGFP/+Cag-Amp (C57BL/6; stock no. 026176) were obtained from the Jackson Laboratory. Ryr2-GFP knockin mice were described previously31. Mice were housed in a specific pathogen-free room with 12 h/12 h light/dark cycle, temperature (22 ± 2 °C), and relative humidity (45–65%). Mice were genotyped by PCR primers shown in Supplementary Table 1.

Echocardiography. Echocardiography was performed on a VisualSonics Vevo 2100 instrument with Vevostrain software by an investigator blinded to genotype or treatment group. Animals were awake during the procedure and held in a standard handgrip. The echocardiographer was blinded to group assignment.

Transverse aortic constriction. Aortic banding was performed on male mice between 25 and 30 g using a previously described protocol30. Mice were anesthetized with isoflurane, intubated, and mechanically ventilated. The chest cavity was entered through an incision in the left second intercostal space. The transverse aorta was dissected from the surrounding tissues. A suture knot was passed under the aorta and ligated against a 27 gauge needle between the brachiocephalic trunk and the left common carotid artery. The needle was then removed, resulting in a ligature with a fixed diameter constricting the aorta. The chest cavity, muscles, and skin were closed layer by layer. The sham operation was identical except that the aorta was not manipulated. The surgery was blinded to genotype.

Viral production and adenovirus-associated (AAV) injection. For overexpression experiments, genes of interest were subcloned into AAV9-cardiac tropinon I promoter vector41 (Addgene, #69915). myc-BirA was amplified from Addgene (#35700); EGFP was amplified from Addgene (#53225). HEK293T cell lysate containing AAV was layered on top of the density gradient and centrifuged at 45000 g for 2 h. AAV was recovered from the density gradient and titered by qPCR. AAV was then injected into P1 (postnatal day 1) pups subcutaneously in a total volume of 30 μL at a mosaic dose of 1 x 106 viral genomes per gram body weight (vg/g), or at a full dose of 5.5 x 1011 vg/g.

BioID proximity proteomics. In all, 0.5 mg protein extracts were immunoprecipitated using streptavidin Dynabeads (Invitrogen, #A28104) and were washed five times with 100 μL 0.1% Triton X-100/HEPES 20, KCl 4.9, MgCl2 1.2, NaCl 0.9, taurine 5, and 2,3-Butanedione monoxime (DBM) 10, centrifuged to between a one and five fold excess, and eluted in two volumes of streptavidin Dynabeads (Invitrogen, #M280). The beads were then removed using a magnet, and the supernatant was dried in a speed-vac. The eluate was spiked in, and the samples were incubated at 37 °C overnight. The beads were washed five times with RIPA lysis buffer (Santa Cruz, #sc-24948) and stored in PBS at −80 °C. The total protein was measured using bicinchoninic acid (Pierce, #23225) and 50 μL samples were used as input for reverse transcription using SuperScript III First-strand Synthesis Mix (Thermo Fisher Scientific, #18080400). Gene expression was analyzed by qPCR using Power SYBR Green PCR Master Mix (Applied Biosystems, #4367659) and a Bio-Rad CFX96 touch thermocycler, using qPCR primers listed in Supplementary Table 1.

Western blotting. In all, 50 μg cell lysates or nuclear extracts47 were separated by 4–12% SDS-PAGE (Invitrogen, #NW04120BOX) and transferred to Immobilon-P PVDF membranes (Merck Millipore, #IPVH0010). The membranes were blocked with 5% nonfat dry milk and incubated with primary antibody (Supplementary Table 2) overnight at 4 °C, followed by incubation with secondary antibody for 1 h at room temperature. Western blot signals were captured using a Fujifilm LAS-3000 imager and quantified using Fiji.

In situ confocal imaging of cardiomyocyte T-tubule structure in intact hearts. For in situ T-tube imaging27, intact mouse hearts underwent Langendorff perfusion at room temperature with 0 Ca2+ Tyrode solution (in mM, NaCl 137, glucose 15, HEPES 20, KCl 4.9, MgCl2 1.2, NaH2PO4 1.2), and quickly mounted on a Langendorff perfusion system. HEK293T cells were passaged at an oxygenated Ca2+ Tyrode (pH 7.4) and Ca2+-concentrated Ca2+ Tyrode (pH 7.3) buffer (pH 7.0, 5% CO2, 5% O2). 30 min later, the hearts were placed in a perfusion chamber mounted on the stage of an Olympus FX3000RS confocal microscope and imaged in situ with x60 (NA = 1.4) oil immersion lens. The optical pinhole was set to 1 airy disc (≤1 μm axial resolution). Excitation for M44-64 was 488 nm, and emission was 680–780 nm.

Cardiomyocyte isolation. Single ventricular or atrial myocytes were enzymatically isolated from mouse hearts. Hearts were excised from isoflurane-anesthetized animals, rinsed in cold perfusion buffer (in mM, NaCl 137, glucose 15, HEPES 20, KCl 4.9, MgCl2 1.2, NaH2PO4 1.2, taurine 5, and 2,3-Butanediol monoxime (DBM) 10), and quickly mounted on a Langendorff perfusion system. HEK293T cells were passaged at an oxygenated Ca2+-concentrated Ca2+ Tyrode (pH 7.2) buffer (pH 7.0, 5%) until blood was completely cleared (about 5 min). Solution was then switched to the perfusion solution (PB containing 0.7 mg/mL Type II Collagenase (Worthington, #LS004176), 0.1 mg/mL Type XIV Protease (Sigma-Aldrich, #F5147), 1 mg/mL bovine serum albumin (BSA, Sigma-Aldrich, #A93912) and 50 μM Ca2+). Once hearts became soft (about 15–30 min), perfusion was stopped and ventricles or atria were gently minced into small pieces and agitated by blunt-tipped transfer pipettes in PB containing 1 mg/mL BSA and 50 μM Ca2+.

Cover slips were pre-coated with 5 μg/mL laminin (Sigma-Aldrich, #L2020) for at least 30 min at 37 °C. Freshly isolated myocytes were plated onto the coverslips for 30 min in the incubator. Attached mouse CMs were cultured in MEM medium (Sigma-Aldrich, #M2279) supplemented with 10 mM BDM.

Ca2+ dye loading and live-cell imaging. To simultaneously record cytosolic and SR Ca2+ signals35, freshly isolated CMs from AAV9-Tnnt2-srCES2 injected mice were incubated with low-affinity Ca2+ indicator Fluo-3/AM (5 μM/L, Invitrogen, #F14204) at 37 °C for 10 min, washed once, and incubated with Rhod2-AM (5 μM/L, Invitrogen, #R1244) at 37 °C for 8 min, and then gently washed twice. Dye loading, washing, and Ca2+ imaging were conducted in Tyrode solution containing 1 mM Ca2+.

Confocal imaging was performed with an Olympus FX3000RS microscope with a x60 1.4 NA oil immersion objective and a line scan speed of 3.78 μm/s. The pinhole was set for a nominal 1 μm optical section. For single-channel measurement of ASPH-G66, excitation was at 488 nm, and fluorescence emissions were collected at between 490 and 540 nm. For simultaneous measurement of Fluo3 and Rhod2, excitation was at 488 and 543 nm, and fluorescence emissions were collected at between 490 and 520 nm and > 650 nm, respectively. For Ca2+ transients recording, CMs were perfused with Tyrode solution containing 10 mM butanedione monoxime to avoid motion artifacts, and field stimulation was applied at 1 Hz.

Histologic and immunofluorescent assays. Hearts were fixed in 4% paraformaldehyde (PFA) overnight and allowed to sink in 30% sucrose (typically 3–4 h) prior to freezing in tissue freezing medium (TFM, General Diagnostics). 10 μm thick cryosections were affixed to slides. Heat sections were permeabilized with 0.5% Triton X-100 for 20 min and blocked with 10% normal donkey serum for 1 h. Seeded isolated cells were fixed in 2% PFA for 15 min at room temperature, washed 3 times with PBS, permeabilized with 0.1% Triton X-100 for 10 min, then rinsed 3 times with PBS, and blocked with 1% BSA for 1 h. The sections or cells were then incubated with primary antibody (Supplementary Table 2) overnight at 4 °C, washed with PBS 3 times, incubated with Alexa fluor dyes-conjugated donkey secondary antibody (Invitrogen, #L2000) for 2 h at room temperature, washed with PBS 3 times, and then incubated with 1 mg/mL 4', 6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature. Immunofluorescence staining was visualized using a confocal microscope at 405 nm (DAPI), 488 nm (Alexa fluor 488), 543 nm (Alexa fluor 555) and 647 nm (Alexa fluor 647) excitation, and 488 nm (Alexa fluor 488), 520 nm > 650 nm (561 nm). For proximity ligation assay (PLA), PFA (4%)-fixed CMs were permeabilized with PBS containing 0.1% Triton X-100. Dualink PLA was carried out using the
DNA sequencing (RNA-seq). Single-cell RNA-seq data on normal human hearts were obtained from GSE109816 using the provided unique molecular identifier matrix.

Image processing and analysis. T-tubule and Ca$^{2+}$ imaging data were analyzed using custom code written in Interactive Data Language (ITT, New York, NY). The code is provided in the Supplementary Materials. EM images were analyzed to determine T-tubule circularity, coupling ratio, and Junction-Z-line distance, as described in Supplementary Fig. 4a and ref. 10.

Statistics and reproducibility. Each experiment was repeated independently with similar results at least two times, or as specified in the figure legends. Measurements were made blinded to group assignment. Two-sided Student’s t-test and analysis of variance were used for normally distributed data, and two-sided P < 0.05 was considered statistically significant. Statistical analysis was performed using Graphpad Prism 9. Results are displayed as mean ± standard deviation. Sample sizes indicate independent biological replicates.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Data for this manuscript are provided in the figures, Supplementary Figures, Tables, Data, and code. The mass spectrometry proteomics data generated in this study have been deposited in the PRIDE database under accession code PXD028960. The sequencing data used in this study are available in the GEO database under accession code GSE109816. Source data are provided with this paper.

Code availability. Custom code used for image processing is provided in the Supplementary Materials.

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**Author contributions**

F.L. designed experiments, performed experiments, analyzed data, and wrote the manuscript. Q.M. performed surgeries and echocardiograms. W.X. contributed to data analysis. D.Z., M.E.S., B.D.J., Y.G., and F.N., and H.C. contributed ideas, samples, and reagents. C.L.L. assisted in data collection. W.T.P. oversaw the overall project, analyzed data, and edited the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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