

# Mitochondrial fusion dynamics is robust in the heart and depends on calcium oscillations and contractile activity

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Mitochondrial fusion is thought to be important for supporting cardiac contractility, but is hardly detectable in cultured cardiomyocytes and is difficult to directly evaluate in the heart. We overcame this obstacle through *in vivo* adenoviral transduction with matrix-targeted photoactivatable GFP and confocal microscopy. Imaging in whole rat hearts indicated mitochondrial network formation and fusion activity in ventricular cardiomyocytes. Promptly after isolation, cardiomyocytes showed extensive mitochondrial connectivity and fusion, which decayed in culture (at 24–48 h). Fusion manifested both as rapid content mixing events between adjacent organelles and slower events between both neighboring and distant mitochondria. Loss of fusion in culture likely results from the decline in calcium oscillations/contractile activity and mitofusin 1 (Mfn1), because (i) verapamil suppressed both contraction and mitochondrial fusion, (ii) after spontaneous contraction or short-term field stimulation fusion activity increased in cardiomyocytes, and (iii) ryanodine receptor-2-mediated calcium oscillations increased fusion activity in HEK293 cells and complementing changes occurred in Mfn1. Weakened cardiac contractility *in vivo* in alcoholic animals is also associated with depressed mitochondrial fusion. Thus, attenuated mitochondrial fusion might contribute to the pathogenesis of cardiomyopathy.

mitochondria | fusion | calcium | alcohol | cardiomyopathy

Cardiac contractions require a constant energy supply, which is provided by mitochondrial metabolism. ATP is needed for excitation-contraction coupling (ECC) for both contraction and relaxation in each cycle (1, 2). ECC-associated cytoplasmic  $Ca^{2+}$  transients ( $[Ca^{2+}]_c$ ) are propagated to the mitochondrial matrix (3) to regulate  $Ca^{2+}$ -dependent mitochondrial dehydrogenases, ATP synthesis, and intracellular  $Ca^{2+}$  homeostasis (4). Thus, cardiac mitochondria are forced to work permanently and likely require quality control mechanisms to keep them in a functioning state.

In many tissues, mitochondria are permanently rebuilt through evolutionarily conserved cyclic processes of fusion and fission. Fusion involves content exchange, allowing complementation of mitochondrial solutes, proteins, and DNA. Fission allows segregation of damaged components (5, 6). Both fusion and fission are promoted by mitochondrial movements that can bring distant mitochondria close to one another and can also separate interacting structures (7). However, the spatial arrangements and mitochondrial morphology are determined by sarcomers in ventricular myocytes, the contractile units of the adult mammalian heart. The gaps among densely packed parallel myofibrils are inhabited by bullet-like mitochondria that run longitudinally, interacting intimately with the junctional sarcoplasmic reticulum (SR), in a conformation that facilitates  $Ca^{2+}$  exchange and supports ECC (4, 8). Despite the spatial restrictions intrinsic to adult cardiomyocyte mitochondria, interactions among these

organelles have been proposed based on functional observations. Reactive oxygen species (ROS)-induced ROS release (9, 10) and so-called “energy-transmitting cables” (11) suggest the presence of intermitochondrial signaling. Recent data describing intermitochondrial junctions and spatially organized cristae arrays indicate that physical structures support the communication among cardiac mitochondria (12). Reports on genetic targeting of mitochondrial fusion or fission in cardiac-derived cell lines (13) and neonatal cardiomyocytes (14), as well as regulation of mitochondrial fission by  $[Ca^{2+}]_c$  transients in neonatal cardiomyocytes (15), suggest that mitochondrial dynamics might be an active process in the heart; however, there are no published studies like these in fully differentiated adult ventricular myocytes (AVCMs).

In cultured AVCMs, low-frequency mitochondrial matrix content exchange has been shown, largely mediated by stable connecting structures known as nanotunnels (16). In adult skeletal muscle fibers, the other form of striated muscle, we (17) and others (18–20) have recently demonstrated that mitochondria are dynamically connected and undergo frequent fusion events dependent on an outer mitochondrial membrane (OMM) fusion protein, mitofusin 1 (Mfn1), and the inner mitochondrial membrane

## Significance

Mitochondrial function is supported by dynamic quality control processes, such as mitochondrial fusion. Cardiac contractility depends on mitochondrial metabolism, yet in cardiomyocytes, mitochondria are confined among myofibrils, raising questions about the possibility of mitochondrial physical communication. Here we demonstrate that mitochondrial continuity is robust and fusion is frequent in freshly isolated rat ventricular myocytes, manifesting both as rapid content mixing events between adjacent organelles and slower, often long-distance events. We show that mitochondrial fusion decreases dramatically in culture because of the decay in contractile activity and, more specifically, the underlying calcium oscillations, which involve mitofusin 1 (Mfn1) abundance. In addition, we show that attenuation of cardiac contractility *in vivo* in alcoholic animals is also associated with depressed mitochondrial fusion.

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(IMM) fusion protein, Opa1 (17). In addition, recent studies have suggested that skeletal muscle reticular mitochondria support membrane potential propagation (19). In the heart, interference with such fusion proteins causes massive damage. Cardiac-specific ablation of Mfn1 and Mfn2 leads to mitochondrial fragmentation, impaired oxidative metabolism, and dilated cardiomyopathy (21) and compromises heart development (22). Moreover, targeting of Opa1 is detrimental to cardiac myocyte differentiation (22) and contractile function (23). In addition, imbalanced processing of Opa1 in the heart leads to mitochondrial fission and heart failure (24). Considering that mitochondrial fusion proteins in the heart have also been implicated in other functions, including cristae remodeling (25), SR-mitochondria communication (26), mitophagy (27), and apoptosis (28), the specific contribution of fusion to these cardiac impairments remains elusive.

Cardiac contractile activity and the corresponding energy needs fluctuate continually. In skeletal muscle, scanning electron microscopy has shown that acute exercise results in increased structural interactions among mitochondria (29). In addition, elevated Mfn1 and Mfn2 mRNA levels (30), as well as increased mitochondrial continuity (20), have been observed after exercise. Finally, slow-twitch/oxidative fibers that are permanently stimulated by action potentials exhibit a higher frequency of fusion events than fast-twitch/glycolytic fibers that exhibit isolated acute trains of stimulation (20, 31). Thus, it is plausible that cardiac contractile activity might exert some control over cardiac mitochondrial fusion. We speculate that the low frequency of mitochondrial fusion events previously documented in adult cardiomyocytes kept in culture for 48–72 h (16) might be a consequence of the decay in contractile and/or metabolic activity.

Contractile activity becomes depressed in dilated cardiomyopathy, a common cause of which is chronic alcohol consumption (32). Mitochondria are a primary target of alcoholic tissue injury (33), and altered mitochondrial ultrastructure has been reported in alcoholic cardiac cells (34). In addition, our previous studies documented attenuated mitochondrial fusion dynamics in both skeletal muscle fibers (17) and hepatocytes (35) isolated from ethanol (EtOH)-fed rats. To investigate whether suppressed mitochondrial quality control might be a factor in the progression of alcoholic cardiac muscle dysfunction, in the present work we evaluated cardiac mitochondrial dynamics in chronically EtOH-fed rats.

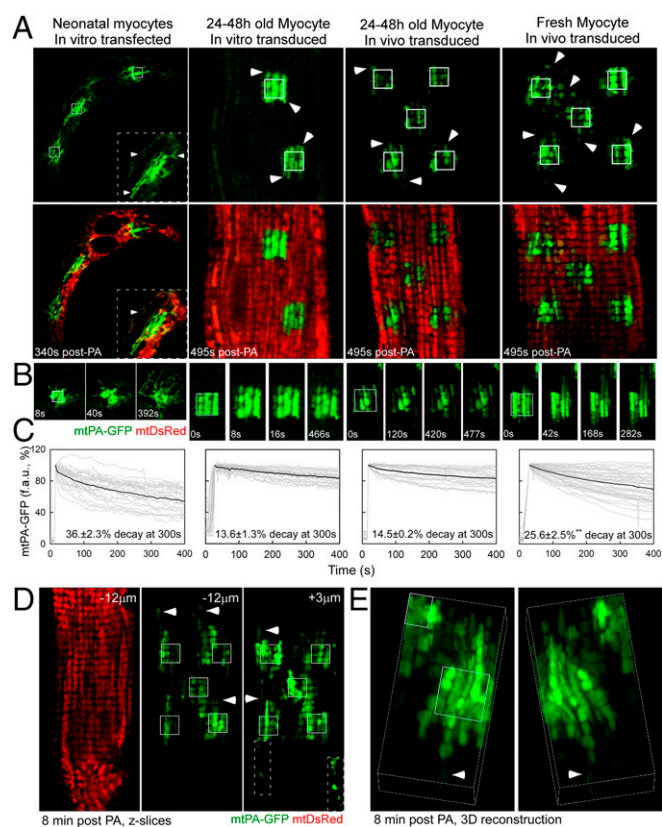
The technical challenges of measuring mitochondrial dynamics and the scanty information available on mitochondrial content exchange in contracting cardiomyocytes have precluded answering fundamental questions about mitochondrial quality control in the heart, including the specific mechanisms of content exchange, the dependence of content exchange events on ECC activity and the possible involvement of content exchange processes in cardiac disease. Here we applied photoactivatable fluorescent protein techniques to evaluate mitochondrial dynamics in cardiomyocytes and in intact heart.

## Results

**Matrix Continuity Among Mitochondria in Rat Cardiac Myocytes: Effect of Maturation State and Cell Culture.** To study the interactions among mitochondria in cardiac myocytes, we expressed two mitochondrial matrix-targeted soluble fluorescent proteins, mtDsRed and mtPA-GFP, and evaluated their distribution by confocal microscopy. We selected between three and five 25- $\mu\text{m}^2$  square areas in each cell and illuminated them to achieve two-photon (2P) photoactivation (PA) of PA-GFP, and followed the spreading of mtPA-GFP out of the illuminated regions. The fluorescence decay within the PA areas represents the sum of diffusion within the mitochondrial network, mitochondrial fusion, and mitochondrial movement (17). Following the

strategy described in a recent study (16), we first evaluated 24- to 48-h-cultured neonatal ventricular cardiomyocytes (NVCs). NVCs showed robust spreading of mtPA-GFP (Fig. 1A and B, column 1 and Movie S1), reaching distant organelles within 5 min (quantified in Fig. 1C).

In contrast to mitochondria in NVCs, mitochondria in AVCMs rearranged into longitudinal columns of apparently unconnected organelles and subgroups of less well-organized perinuclear organelles (Fig. 1A and B, columns 2–4 and Fig. S1). AVCMs were first transformed in vitro by adenoviral mtDsRed and mtPA-GFP. Cells were infected promptly after plating and imaged 24–36 h later, once the fluorescent protein expression reached detectable levels. We focused on the interactions among intermyofibrillar mitochondria, given their role in regulating contractile function and their strategic orientation toward the SR (4). Spreading of mtPA-GFP beyond the edges of the PA area (Fig. 1A–C, column 2 and Movie S2) demonstrated some continuity



**Fig. 1.** Mitochondrial matrix continuity in cardiac myocytes. (A) Representative images of myocytes after 2P PA of mtPA-GFP (white outlined squares). Arrowheads indicate mtPA-GFP beyond the PA regions. mtDsRed reveals the whole mitochondrial population of in vitro-transfected NVCs (column 1) and 24–48 h in vitro-cultured (column 2) or in vivo-transduced (column 3) and freshly isolated in vivo-transduced (column 4) AVCMs. (B) Time series of one PA region for each condition. (C) mtPA-GFP fluorescence decay kinetics in a PA region in in vitro-transfected NVCs ( $n = 20$ ) and AVCMs ( $n = 30$ ) and in vivo-transduced and cultured ( $n = 20$ ) and freshly isolated AVCMs ( $n = 47$ ). Gray curves denote individual regions; black curves represent the mean.  $P < 0.01$ . (D) Mitochondrial distribution in a freshly isolated AVCM (Left), and the diffusion of mtPA-GFP at  $-12 \mu\text{m}$  below (Center) and  $+3 \mu\text{m}$  above (Right) the 2P illuminated focal plane. (Inset) The spread of mtPA-GFP along a continuous chain of connected mitochondria. Arrows highlight organelles located distant from the PA regions. (E) A 3D reconstruction of a freshly isolated myocyte. The arrowhead indicates a distant organelle containing mtPA-GFP.

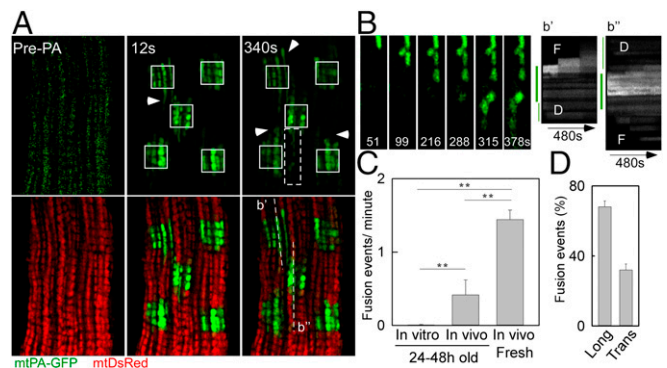


among mitochondria in AVCMs; however, this diffusion was limited and only over short distances.

We reasoned that because culture conditions negatively affect contractile function (36), they also might alter mitochondrial dynamics. Thus, our next step was to perform *in vivo* adenoviral infection of the ventricular walls of adult rat hearts. Then, 7–10 d later, we isolated AVCMs and plated them for microscopic evaluation at 24–48 h postharvest or promptly after plating. Fig. 1 *A–C*, column 3 and *Movie S3* show diffusion of mtPA-GFP comparable to that observed in *in vitro*-transformed AVCMs (column 2). Freshly isolated *in vivo*-transduced AVCMs exhibited significant mitochondrial continuity, characterized by both longitudinal and transversal diffusion of mtPA-GFP (Fig. 1*A*, column 4 and *Movies S4* and *S5*), extending >5  $\mu\text{m}$  away from the PA region (Fig. 1*B*). We also found a significant decrease in mtPA-GFP fluorescence within the PA regions, with 25.6% decay at 300 s, as opposed to 14.5% in cultured AVCMs ( $P < 0.01$ ) (Fig. 1*C*).

We further evaluated the diffusion of mtPA-GFP above and below the PA focal plane in fresh AVCMs by means of Z-stack imaging at 8 min after PA. Mitochondria containing photoactivated mtPA-GFP were found even 12  $\mu\text{m}$  below (–12  $\mu\text{m}$ ) the PA focal plane (Fig. 1*D*). In addition, at the +3  $\mu\text{m}$  focal plane, a discrete row of mitochondria projected toward the periphery, extending 12  $\mu\text{m}$  away from the PA region. Finally, high-magnification 3D projections from a different cell (Fig. 1*E*) showed mitochondrial matrix continuity in both longitudinal and transversal orientations in a freshly isolated AVCM. We performed a comparable 3D analysis in *in vitro*- and *in vivo*-transduced, AVCMs cultured for 24–48 h and found a drastic decrease in mitochondrial continuity compared with freshly isolated myocytes (Fig. *S1B*). We conclude that mitochondria in AVCMs form complex networks with matrix content exchange, and that culture conditions cause suppression of mitochondrial communication. Moreover, in NVCMs, mitochondria are less ordered and communicate actively with their neighbors.

**Active Mitochondrial Fusion Dynamics in Freshly Isolated AVCMs.** We next evaluated the mitochondrial fusion activity in freshly isolated AVCMs transduced with the reporters *in vivo*. When mtPA-GFP is photoactivated, mtDsRed becomes photobleached in the same area, producing green-only mitochondria surrounded by red-only mitochondria. During the merging of green-only and red-only mitochondria, abrupt complementary changes in green and red fluorescence validate fusion pore opening and matrix content mixing driven by the concentration gradient. We observed fusion events among intermyofibrillar mitochondria, showing diverse orientations and kinetics (Fig. 2 *A* and *B*). Images of an AVCM before and 12 s after PA highlight the early spreading of PA-GFP (Fig. 2*A*), which indicates preexisting matrix continuity among individual mitochondria. Further examination of the distribution of mtPA-GFP in discrete mitochondria located in the proximity of the PA areas revealed abrupt increases in the area of the fluorescent protein, corresponding to mitochondrial fusion events; as an example, a series of organelles underwent sequential fusion events in longitudinal orientation (Fig. 2*B*). Another example shows a subregion of a PA area and its surroundings, presenting four sequential fusion events in both longitudinal and transversal orientations, characterized by a decrease in PA-GFP fluorescence in the donor mitochondrion and a parallel increase in a nearby acceptor mitochondrion (Fig. *S2A* and *Movie S6*). Noticeably, NVCMs also exhibited sequential fusion events among neighboring mitochondria (Fig. *S2B*). In quantitative analyses of the fusion events in AVCMs maintained under different culture conditions (Fig. *S2C*), freshly isolated AVCMs exhibited  $1.4 \pm 0.1$  events/min, whereas cultured *in vivo*-transduced AVCMs showed four-fold fewer events, and cultured *in vitro*-transformed AVCMs showed

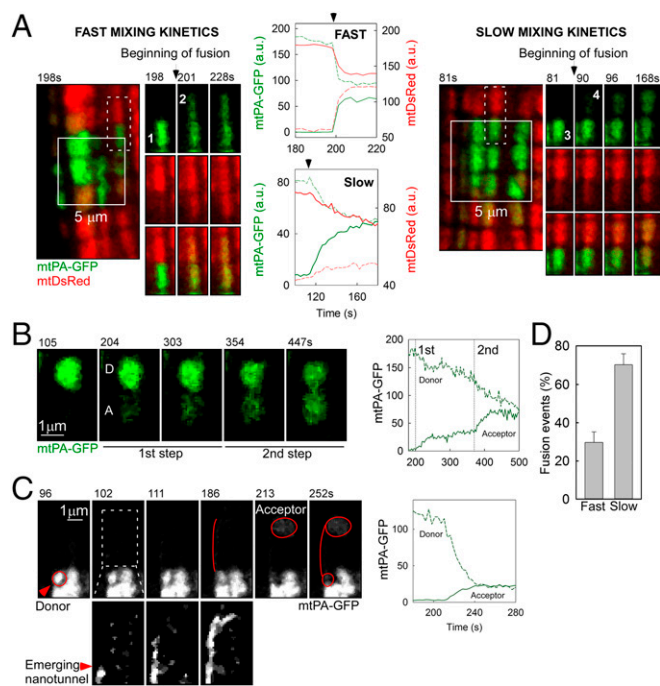


**Fig. 2.** Mitochondrial fusion in freshly isolated AVCMs. (*A*) A representative AVCM before and after 2P PA of mtPA-GFP (white squares). At 12 s, the spreading of mtPA-GFP fluorescence indicates matrix continuity among mitochondria (arrowhead). At 340 s, newly formed communication (arrowheads) is observed. (*B, Left*) mtPA-GFP time series for the area marked by the white-dashed box in *A*, denoting serial fusion events in a longitudinal orientation. (*B, Right*) Here *b'* and *b''* represent a line-scan analysis of the indicated dashed lines in *A*. The thick green line indicates the PA area. *D*, initial diffusion of mtPA-GFP toward the vicinity of the PA region, also denoted by the thin green line on the left. *F*, step-like mtPA-GFP fluorescence transfer corresponding to fusion event. (*C*) Fusion frequency in cultured and freshly isolated AVCMs (in *in vitro*-transformed AVCMs cultured for 24–48 h: 26 cells, seven experiments; in *in vivo*-transformed AVCMs cultured for 24 h: nine cells, two experiments; in *in vivo*-transformed freshly isolated AVCMs: 34 cells, four experiments).  $**P < 0.01$ . (*D*) Frequency of longitudinal (Long) and transverse (Trans) fusion events.

almost no events (a rare event is shown in Fig. *S2C*). Thus, AVCM mitochondria regularly undergo fusion, and this activity decays in culture.

Transmission electron microscopy (TEM) in fixed heart muscles has demonstrated that intermyofibrillar mitochondria run parallel to myofibrils, mostly forming columns of individual mitochondria, accompanied by some transversally oriented organelles. Such a distribution in live AVCMs is also supported by our confocal microscopy data. Moreover, the fusion events occurred predominantly among longitudinally oriented organelles (Fig. 2*D*), although 32% of the interactions occurred transversally.

**Slow Matrix Content Mixing Events in AVCMs.** Different from other cells that we have studied previously, including mouse embryonic fibroblasts (MEFs), H9c2 cells, hepatocytes, and skeletal muscle fiber cells (7, 17, 35), AVCMs exhibited diverse mixing kinetics of the matrix-targeted fluorescent proteins (Fig. 3). Two representative examples are shown in Fig. 3*A* and *Movie S7*. In the figure, the left panel shows an event with fast mixing kinetics between two longitudinally oriented mitochondria. This mixing event was completed within 12 s, as validated by the time course of both PA-GFP and DsRed in the donor and acceptor mitochondria. The complementary equilibration of both fluorescent proteins confirms that a fusion event occurred. The right panel shows a comparable fusion event example in terms of location relative to the PA region and size of both the PA-GFP donor and the acceptor. The transfer of the fluorescent proteins was slower, however, taking 70 s to reach equilibrium. The slow mixing kinetics applied to both fluorescent proteins, arguing against the possibility of an artifact associated with the conformation of either protein. Thus, based on the mixing kinetics, we discriminated between fast and slow fusion events, which are completed in <12 s and >12 s of mitochondrial matrix mixing, respectively. Overall, slow mixing kinetics fusion events are prevalent in AVCMs (Fig. 3*D*) and are likely to represent a tissue-specific feature of cardiac mitochondrial fusion.

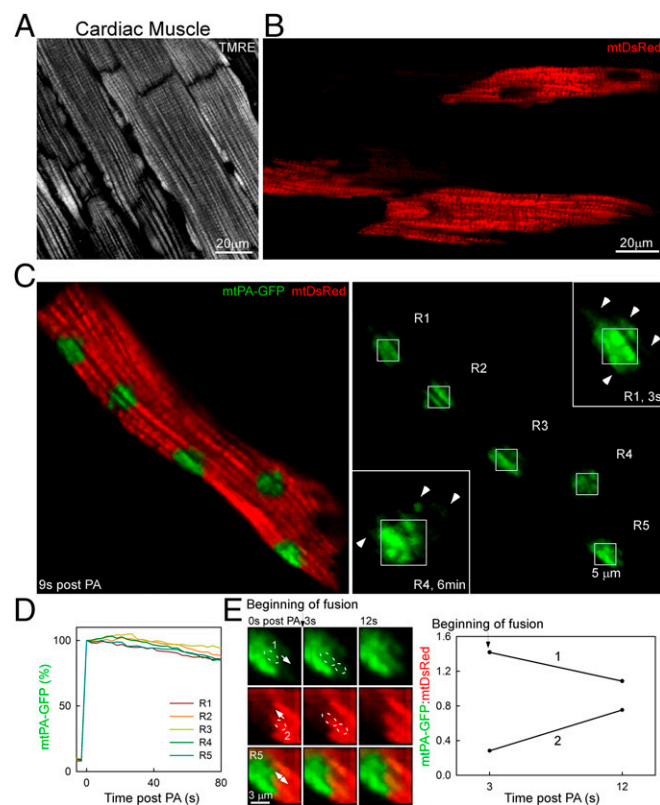


**Fig. 3.** Kinetics of fusion events in AVCMs. (A) Examples of fusion with fast (Left; mitochondria 1 and 2) and slow (Right; mitochondria 3 and 4) mixing kinetics. The locations of the involved organelles are indicated by white dashes. The time series shows the transfer of mtPA-GFP (photoactivated within the PA area) and mtDsRed (photobleached at the same time) in opposite directions. Central plots represent the quantification of mtPA-GFP and mtDsRed exchange. (B) A slow mixing kinetics fusion event displaying a two-step mixing kinetics, between donor (D) and acceptor (A) mitochondria. (Left) Representative image series. (Right) Quantitative plot. (C) A slow mixing kinetics fusion event between two distant mitochondria (D and A) connected by a narrow mitochondrial structure, from D to A. (Bottom Left) The dashed rectangle with enhanced brightness highlighting an emerging nanotunnel-like structure. (Right) Slow mixing kinetics leading to equilibration of fluorescence. (D) Frequency of fast and slow events ( $n = 51$  cells).

We next searched for possible explanations for the slow matrix mixing kinetics. We found some evidence suggesting the IMM fusion pore might open in an intermittent fashion (Fig. 3B shows a two-step fusion event), similar to the fusion between exocytotic vesicles and the plasma membrane (37). Overall, 11% of the slow mixing kinetics events displayed detectable multistep mixing kinetics (Fig. S3A and Movie S8). Furthermore, the previously described narrow intermitochondrial nanotunnels (16) also might support slow content mixing. Indeed, we observed a nanotunnel-like structure growing out from a small PA-GFP-carrying mitochondrion and reaching a distant mitochondrion, which was associated with slow mixing kinetics (Fig. 3C). We also detected slow mixing kinetics between two non-neighbor mitochondria (Fig. S3A). In addition, we found narrow tubules apparently emerging from larger mitochondria (Fig. S3B and Movie S9). These examples demonstrate a fluorescence distribution consistent with the presence of nanotunnels, and demonstrate that these structures make mitochondrial soluble content exchange possible, accompanied by slow mixing kinetics.

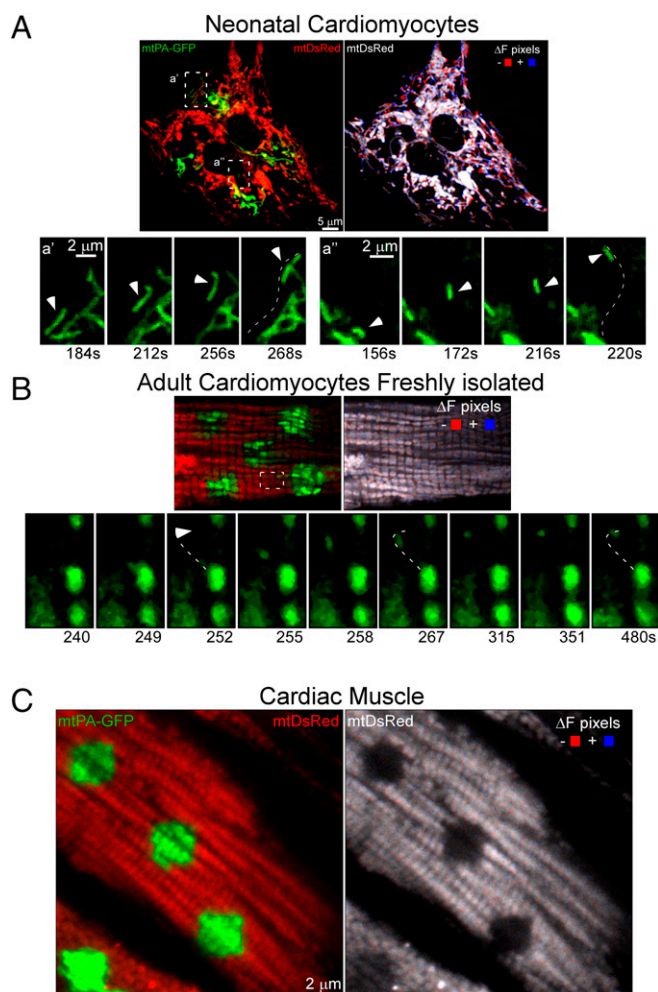
**Mitochondrial Fusion Dynamics in Whole Heart.** To image mitochondria in intact tissue, in vivo-infected whole hearts were perfused and examined by confocal microscopy. The mitochondrial structure in the outer layer of myocytes in the ventricular wall was first visualized using tetramethylrhodamine, ethyl ester (TMRE), which accumulates in the mitochondria in a membrane

potential-dependent manner (Fig. 4A). Cardiomyocytes of the ventricular wall of in vivo-transformed hearts exhibited mtDsRed fluorescence distribution comparable to TMRE localization (Fig. 4B). Approximately 40% of the myocytes expressed mtDsRed. Once we found a myocyte positioned parallel to the coverslip and showing well-defined organelles, we applied 2P illumination in discrete 25- $\mu\text{m}^2$  square regions and followed the time course after the PA of mtPA-GFP. Immediately after the end of PA, mtPA-GFP diffused into the surrounding mitochondria around the PA regions, mostly in longinudinal orientations (Fig. 4C, Inset, R1). Interestingly, at 6 min after PA, region 4 showed a new mitochondrion located 1  $\mu\text{m}$  away from the corner of R4 and transversally oriented to the PA-GFP-containing mitochondria inside it, possibly indicating a fusion event. Owing to contraction, recording the kinetics of PA-GFP spreading was difficult; nonetheless, some decay in PA-GFP fluorescence was apparent even in the first 80 s after PA (Fig. 4D). Despite the complexity of the recording, we could identify fusion events. One example shows the mixing of mtPA-GFP and mtDsRed between two neighbor mitochondria (Fig. 4E). Therefore, these data confirm that the observed mitochondrial fusion activity found in isolated myocytes also occurs within cardiac muscle.



**Fig. 4.** Mitochondrial continuity and fusion events in the whole heart. (A) Ventricular myocytes in the outer layer of the ventricular wall of a TMRE-perfused whole heart. (B) Ventricular myocytes from an in vivo-transformed whole heart showing mtDsRed fluorescence. (C) A ventricular myocyte in the whole heart. Right side mtPA-GFP image denotes five PA regions (R1–5). R1 and R4 were zoomed in at 3 s and 6 min, respectively. (D) Plot showing the mtPA-GFP decay kinetics in the PA area. (E) Images of a time series of a fusion event between two adjacent mitochondria, 1 and 2. The plot shows the opposite fluorescence transfer as the mtPA-GFP/mtDsRed ratio for each organelle.



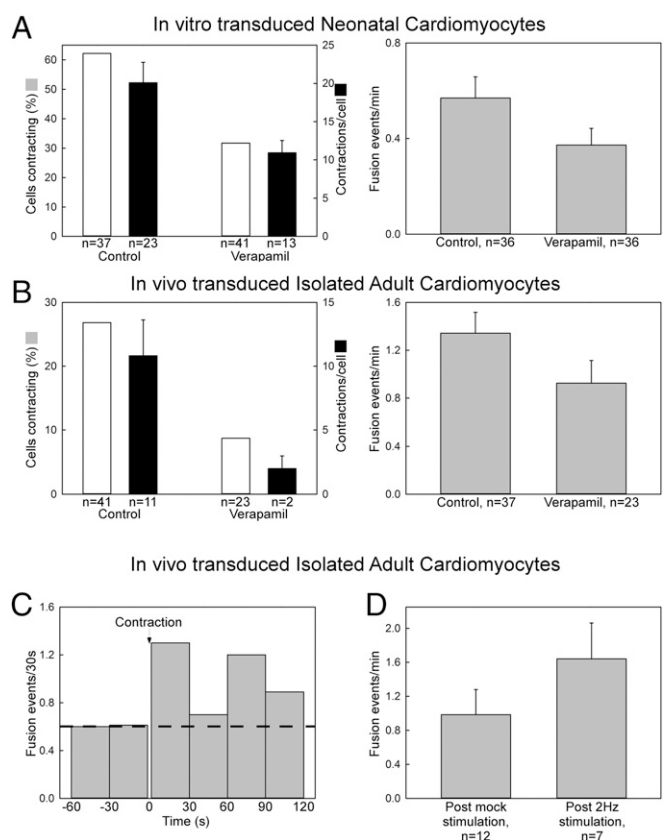


**Fig. 5.** Mitochondrial motility in heart cells. (A) NVCs. (Left) In the green and red image acquired at 184 s post-PA of mtPA-GFP, the white boxes labeled 'a' and 'a'' show regions in which isolated mitochondrial displacements are apparent. (Bottom) Time series illustrating the movement of a single mitochondrion in the 'a' and 'a'' regions. The trajectory of each organelle is indicated by a white-dashed line. (Right) Processed image shows both mtDsRed fluorescence (grayscale) and the sites of mitochondrial movement calculated by subtraction of F(mtDsRed) at 4 s from F(mtDsRed) at 16 s. Red indicates negative changes; blue, positive changes. (B, Upper Left) In vivo-transformed AVCMs at 180 s post-PA of mtPA-GFP shows minimal displacement of individual mitochondria. (B, Right) There were few moving mitochondria, as indicated by the low number of  $\Delta F$  pixels. The dashed lines show one mitochondrion moving toward the upper side of the cell. (B, Bottom) A detailed view of the trajectory of this directional movement. (C) Mitochondrial movement in an AVCM in the whole heart at 20 s after PA.

**Motility of Mitochondria in Cardiac Myocytes.** Given the relevance of mitochondrial movements as a facilitator of mitochondrial encounters and fusion events (7, 38), we studied motility in ventricular myocytes. NVCs exhibited highly active mitochondrial movements throughout the cells (Fig. 5A). In contrast, cultured AVCMs showed minimal motility (data not shown). Moreover, mitochondria in the whole heart did not exhibit motility (Fig. 5C); however, some motility activity was present in freshly isolated AVCMs (Fig. 5B). In addition to Brownian-like movements (Fig. 5B), we found an isolated displacement motility event restricted to a small region (Fig. 5B, Lower). The limited motility observed in the AVCMs indicates that mitochondrial movements do not have a major role in supporting mitochondrial fusion in cardiac muscle.

### Mitochondrial Fusion Depends on Excitation-Contraction Activity.

The forgoing results indicate a negative effect of cell culture conditions on mitochondrial communication in AVCMs. The regular ECC activity decays in AVCMs after isolation and culture. This raises the possibility that ECC function is required to support mitochondrial fusion, which we tested more directly using the following approach. We first treated the cells with verapamil, an inhibitor of  $\text{Ca}^{2+}$  entry via L-type  $\text{Ca}^{2+}$  channels to suppress contractile activity in neonatal and freshly isolated ventricular myocytes. This treatment caused fewer cells to contract and contracting cells to show fewer contractions (Fig. 6A and B, Left), and partially decreased the mitochondrial fusion event rate in both NVCs and freshly isolated AVCMs (Fig. 6A and B, Right). We also analyzed the rate of fusion events before and after the occurrence of a spontaneous contraction in AVCMs, and detected an increase in mitochondrial fusion frequency in the first 2 min after a spontaneous contraction (Fig. 6C). Finally, we evaluated the short-term effect of ECC activity



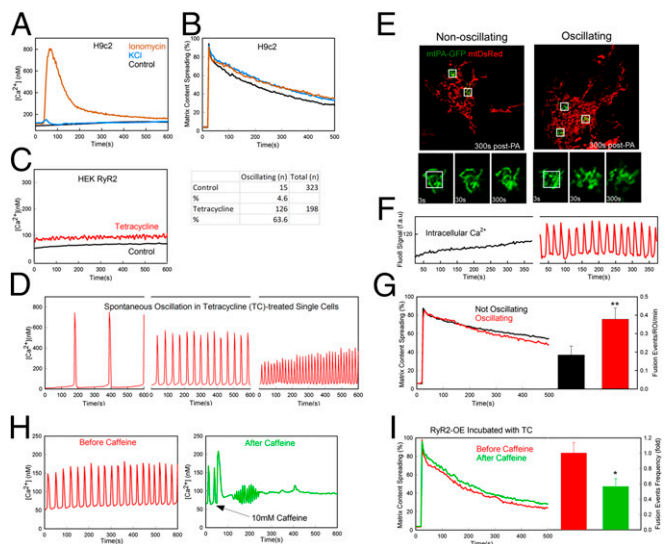
**Fig. 6.** Effect of contractile activity on mitochondrial fusion dynamics. (A) NVCs transfected with mtDsRed and mtPA-GFP were treated with verapamil 10  $\mu\text{M}$  for 48 h, followed by confocal microscopy evaluation. (Left) Bar chart showing the percentage of contracting cells (white) and the number of contractions per cell (black). (Right) Mitochondrial fusion event frequency. (B) Freshly isolated in vivo-transduced AVCMs were treated with verapamil 10  $\mu\text{M}$  for at least 4 h and no longer than 6 h. (Left) Bar chart showing the percentage of contracting cells (white) and the number of contractions per cell (black). (Right) Fusion event frequency. (C) Temporal distribution of fusion events relative to a spontaneous contraction in non-stimulated in vivo-transduced freshly isolated AVCMs. The cells did not show spontaneous contraction in the minutes before the contraction evaluated ( $n = 10$  cells). (D) Freshly isolated in vivo-transduced AVCMs expressing mtDsRed that responded by contraction to isolated electric pulses were field-stimulated (5 min) without 2,3-butanedione monoxime (BDM) during confocal microscopy and then evaluated for fusion events in 10 mM BDM-supplemented 0.25% BSA extracellular medium (ECM).

in freshly isolated AVCMs by field-stimulating the cells for 5 min. (Fig. S4 shows a schematic presentation of the stimulation protocol.) After stimulation, we found a 60% increase in mitochondrial fusion compared with control cells (Fig. 6D). Thus, we conclude that regular ECC facilitates the mitochondrial fusion activity of AVCMs.

**RyR2-Mediated  $[Ca^{2+}]_c$  Oscillations Are Sufficient to Promote Mitochondrial Fusion.** Each ECC event in AVCMs is triggered by plasma membrane depolarization and an ensuing  $[Ca^{2+}]_c$  transient. To isolate the potential role of the depolarization and  $[Ca^{2+}]_c$  increase from contraction, we first used noncontracting H9c2 cardiac myoblasts. Depolarization evoked by increased KCl and  $[Ca^{2+}]_c$  elicited by a  $Ca^{2+}$  ionophore, ionomycin, failed to induce an increase in mitochondrial connectivity (Fig. 7A and B). However, ionomycin causes a single and prolonged  $[Ca^{2+}]_c$  rise, whereas AVCMs display RyR2-mediated  $[Ca^{2+}]_c$  oscillations. A surrogate for these  $[Ca^{2+}]_c$  oscillations without contractile activity is provided by HEK293 cells expressing RyR2 (HEK-RyR2) in a tetracycline-inducible manner (39). Indeed, tetracycline-pretreated HEK-RyR2 cells displayed a moderately elevated average  $[Ca^{2+}]_c$  compared with nontreated cells (Fig.

7C), owing to an increase in the fraction of cells with  $[Ca^{2+}]_c$  spiking from 4.6% to 63.6%, manifesting in the form of baseline-spike  $[Ca^{2+}]_c$  oscillations (Fig. 7D). Simultaneous imaging of  $[Ca^{2+}]_c$  and mitochondrial dynamics (with mtDsRed and mtPA-GFP) revealed no difference in mitochondrial morphology between nonoscillating and oscillating HEK-RyR2 cells (Fig. 7E and F); however, the spread of the matrix-targeted fluorescent proteins, and specifically the number of mitochondrial fusion events, was enhanced in the oscillating cells (Fig. 7G). Interestingly, when caffeine (10 mM), a stimulator of RyR2, was added, a single and large  $[Ca^{2+}]_c$  transient occurred, and the oscillations stopped (Fig. 7H). This was followed by decreases in mitochondrial matrix continuity and fusion activity (Fig. 7I). Taken together, these results suggest that the effect of cardiac contractile activity on mitochondrial fusion could be mediated by the  $[Ca^{2+}]_c$  oscillations.

To identify the potential target of ECC activity/ $[Ca^{2+}]_c$  oscillations in the control of mitochondrial fusion, we quantified the abundance of OMM and IMM fusion proteins in freshly isolated and overnight-cultured AVCMs. Immunoblotting indicated significantly decreased Mfn1 in the overnight-cultured AVCMs, but no change in Mfn2 and Opa1 (Fig. S5A). To test for a cause-and-effect relationship between Mfn1 decrease and decay in mitochondrial fusion activity, we used an adenovirus carrying a mouse Mfn1 expression construct (which shares 99% homology with rat Mfn1) to in vitro-transform freshly isolated AVCMs in vivo-infected with AdmtDsRed and AdmtPA-GFP. The cells were kept in culture for 24–48 h before mitochondrial fusion was tested. We found that exogenous expression of Mfn1 in the cultured AVCMs rescued mitochondrial continuity and fusion frequency compared with cells transformed with a control adenovirus, AdLacZ (Fig. S5C). The efficiency of AdMfn1 for actually rescuing the expression of Mfn1 was validated in MEF Mfn1<sup>-/-</sup> cells in terms of both Mfn1 abundance and mitochondrial morphology (Fig. S5B). Complementing the results in AVCMs, HEK-RyR2 cells displayed higher Mfn1 levels compared with plain HEK cells (Fig. S5D). Thus, these results indicate that ECC activity/RyR2-mediated  $[Ca^{2+}]_c$  oscillations help maintain Mfn1 abundance as a mechanism to support mitochondrial fusion activity. A decrease in Mfn1 might account for the long-term effects of ECC activity, but is unlikely to explain the short-term changes in fusion activity observed on pacing in cultured AVCMs (Fig. 6D) and cessation of  $[Ca^{2+}]_c$  oscillations in HEK-RyR2 cells (Fig. 7I and Discussion).

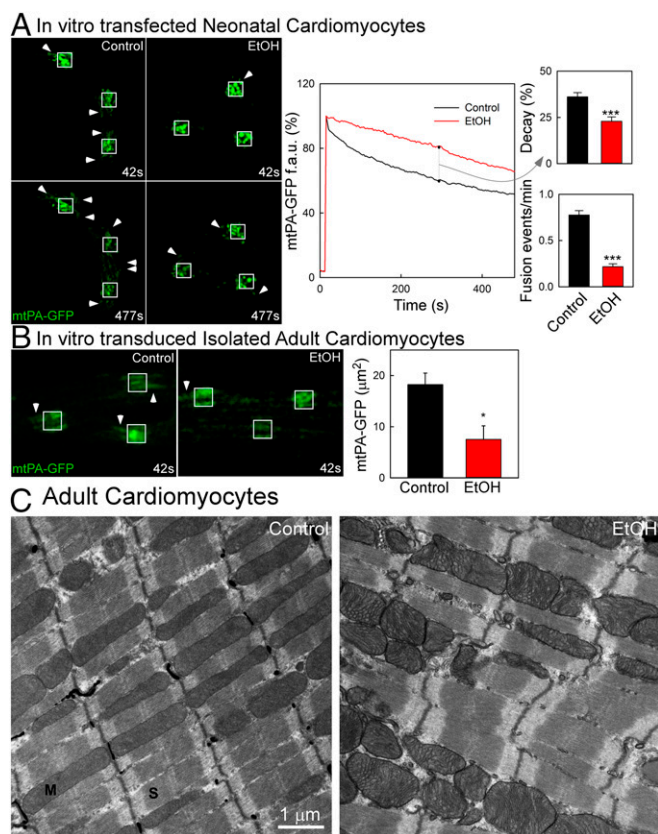


**Fig. 7.** Effect of calcium oscillations and plasma membrane depolarization on mitochondrial dynamics in H9c2 and HEK cells. (A) Fura2-AM-loaded H9c2 cells were treated with 60 mM KCl ( $n = 179$ ), treated with 5  $\mu$ M ionomycin ( $n = 159$ ), or left untreated (control;  $n = 348$ ) at 30 s.  $[Ca^{2+}]_c$  was recorded every 3 s for 10 min. (B) The change in mtPA-GFP/mtDsRed is shown for H9c2 cells treated with 60 mM KCl ( $n = 31$ ), treated with 5  $\mu$ M ionomycin ( $n = 27$ ), or untreated from 5 min before imaging ( $n = 36$  cells). (C, Left) HEK-RyR2 cells induced with tetracycline show increased mean basal  $[Ca^{2+}]_c$  compared with WT because of spontaneous oscillations. (C, Right) Percentage of cells in each group showing spontaneous oscillation. (D) Samples of HEK-RyR2 cells showing spontaneous calcium oscillation measured with Fluo8-AM show greater amplitude when the oscillations occur at a lower frequency. (E) Spreading of mtPA-GFP and recovery of mtDsRed in nonoscillating and oscillating HEK-RyR2 cells (Upper) and time series of one PA region from the cells in the upper panel (Lower). (F)  $[Ca^{2+}]_c$  recorded with Fluo8 (fluorescence arbitrary units) in the cells shown in E. (G) Comparison of mtPA-GFP spreading between oscillating and nonoscillating HEK-RyR2 cells. Mean spreading rates show little difference (Left); however, the oscillating cells ( $n = 30$ ) show roughly a twofold increase in fusion events per PA region compared with the nonoscillating cells ( $n = 34$ ) (Right).  $**P < 0.01$ . (H)  $[Ca^{2+}]_c$  in a single oscillating HEK-RyR2 cell before and after treatment with 10 mM caffeine ( $n = 314$  and 297, respectively). (I) Comparison of GFP diffusion and relative rates of fusion frequency in HEK-RyR2 cells before and after caffeine treatment ( $n = 12$  and 13, respectively, for PA-GFP diffusion;  $n = 6$  and 7, respectively, for mitochondrial fusion;  $P < 0.05$ ).

**Suppression of Cardiac Mitochondrial Fusion by Chronic EtOH Exposure.** Environmental and dietary stressors target the heart's contractile performance and represent a leading cause of death worldwide. Among these stressors, chronic EtOH consumption leads to dilated cardiomyopathy (32). Given that mitochondria are well-known targets of EtOH (33), and that our previous data have shown suppression of mitochondrial fusion in skeletal muscle by prolonged EtOH exposure (17), we performed experiments to evaluate the effect of chronic EtOH on mitochondrial fusion in cardiomyocytes. We first treated NVCMS in vitro with 50 mM EtOH for 48 h, which represents an in vitro model for chronic alcohol exposure. The EtOH-treated NVCMS exhibited a 40% decrease in mitochondrial continuity and a 75% decrease in mitochondrial fusion activity (Fig. 8A).

As an in vivo model, we used EtOH-fed (a 32% EtOH-containing diet for 6–9 mo) and pair-fed rats in which EtOH-induced contractile dysfunction was documented as decreased ejection fraction and fractional shortening (Materials and Methods). AVCMs isolated from the EtOH-fed rats showed unaltered resting mitochondrial membrane potential, as assessed by the potentiometric dye TMRE (EtOH:  $23 \pm 4\%$  uptake, pair-fed:  $21 \pm 1\%$  uptake;  $n = 5$ ), though previous studies reported an increased sensitivity to  $Ca^{2+}$ -induced depolarization and permeability





**Fig. 8.** Effect of prolonged EtOH exposure on cardiac mitochondrial fusion dynamics. (A, Left) Diffusion of mtPA-GFP in control and EtOH-treated (50 mM for 48 h) NVCMs. Arrowheads indicate diffusion of mtPA-GFP due to pre-established connections (42 s) or newly formed connections due to fusion events (477 s). (A, Center) mtPA-GFP fluorescence decay in the PA area. (A, Right) Bar charts showing a greater percentage of decay at 300 s post-PA in control cells ( $n = 57$  ROIs) compared with EtOH-treated cells ( $n = 59$ ) (Upper Right) and a higher fusion event frequency in control cells ( $n = 63$  cells) compared with EtOH-treated cells ( $n = 33$ ) (Lower). (B) In vitro-transduced AVCMs from control and EtOH-fed rats (age 6–9 mo). Images of control and EtOH-treated myocytes show limited mtPA-GFP diffusion (arrowheads). Bar charts denote diffusion kinetics for control ( $n = 12$ ) and EtOH ( $n = 10$ ), expressed as the area covered by the PA-GFP PA areas. (C) Ultrastructure of normal and alcoholic AVCMs. Shown are representative images of longitudinal arrangements of sarcomeres (S) and mitochondria (M).

transition (33, 40). Here we infected in vitro the freshly harvested AVCMs with mtPA-GFP and mtDsRed, and found significantly reduced mitochondrial continuity in the cells derived from the EtOH-fed rats (Fig. 8B). We next measured the levels of mitochondrial fusion proteins Mfn1, Mfn2, and Opa1 in mitochondrial fractions of control and EtOH-fed rats. No significant change was detected (Fig. S6), but in three of the five pairs Mfn1 was apparently somewhat decreased in the EtOH-fed rat, suggesting that EtOH can target mitochondrial fusion in the heart without altering the abundance of fusion proteins though a moderate decrease in Mfn1 might be involved.

To further evaluate the dysregulation induced by chronic EtOH exposure, we studied the ultrastructure of mitochondria in the ventricular muscles of hearts harvested from control and EtOH-fed rats (Fig. 8C). Mitochondrial cross-sections accounted for 34% and 37% in hearts from control and EtOH-fed animals, respectively (no significant difference); however, the individual mitochondrial cross-section areas in the hearts from EtOH-fed rats showed a tendency for enlargement (control:  $0.74 \pm 0.14$  and EtOH:  $0.81 \pm 0.15 \mu\text{m}^2$ ,  $n = 3$ ,  $P = 0.056$ ). In addition,

mitochondria that showed signs of swelling, distortion, and/or resorption of cristae were more prevalent in the cardiac muscles of the EtOH-fed rats (Fig. S7). Using a scoring system for cristae abundance and form ranging from 0 (worst) to 4 (best), the mean score for control was significantly higher in control rats compared with the EtOH-fed rats ( $2.45 \pm 0.23$  vs.  $1.92 \pm 0.2$ ;  $P < 0.05$ , paired  $t$  test) (Fig. S7B). Comparing the prevalence of individual score grades, grade 0 (no well-defined crista) and grade 1 (no crista in  $>50\%$  of the mitochondrial area) were significantly more prevalent in the EtOH group (Fig. S7C). Thus, our results suggest that chronic EtOH consumption causes suppression of mitochondrial fusion activity in cardiac muscle and leads to distortion of the ultrastructure.

## Discussion

We have demonstrated that in the adult heart, mitochondria exhibit active fusion dynamics to support matrix content exchange among individual organelles. Distinctively, content exchange among cardiac mitochondria often occurs with slower kinetics compared with that in neonatal heart or in any previously studied cell types. A systematic comparison of freshly isolated and cultured ventricular myocytes revealed that mitochondrial fusion activity decays rapidly when cardiomyocytes are transferred to cell culture. This effect might result from a decrease in contractile activity, as demonstrated by the ability to down-regulate and up-regulate mitochondrial fusion by suppressing and restoring cardiomyocyte contractions, respectively. More specifically, we have shown that oscillatory, but not sustained,  $\text{Ca}^{2+}$  transients significantly increase mitochondrial fusion frequency in RyR2-expressing HEK cells, suggesting that the  $\text{Ca}^{2+}$  component of the ECC alone can regulate cardiac mitochondrial communication, and that this mechanism seems to involve Mfn1. Finally, we also have shown that chronic alcohol-fed rats, which exhibit a cardiomyopathy phenotype, show impaired mitochondrial fusion and ultrastructural impairments.

Although mitochondrial fusion has been visualized in diverse differentiated cells, such as neurons (41), hepatocytes (35), and skeletal muscle fibers (17, 18), previous studies of cardiac mitochondrial fusion have been largely confined to evaluating cardiac function after genetic manipulation of the expression of fusion and fission proteins (13, 14, 21, 22). Mitochondrial “kissing” was recently described (16) in cultured AVCMs studied at 48–72 h postisolation. However, our study illuminates the active communication between intermyofibrillar mitochondria in noncultured adult cardiomyocytes, as well as in situ within cardiac muscle. This is a major step forward, because the fusion activity under these conditions is much higher than that in cultured primary cardiomyocytes, indicating that cardiac mitochondrial fusion activity has been underestimated until now.

The lesser mitochondrial continuity and motility observed in cultured AVCMs compared with NVCM is in accordance with our previous observations in differentiated and nondifferentiated cardiac-derived H9c2 cells and in skeletal myoblasts, and indicates decreased mitochondrial continuity and fusion in differentiated striated muscle (17, 42). Mitochondrial fusion is supported by mitochondrial motility, which brings mitochondria into contact to create the opportunity for fusion (7, 38). Multi-directional orientation patterns of microtubules and abundant mitochondrial movements occur in NVCMs (43), H9c2 cells (44), and skeletal myoblasts and myotubes (17). In contrast, in AVCMs and skeletal muscle fibers, mitochondria are spatially confined to the space among the myofilaments and have no opportunity to move toward distant mitochondria. This might account for the lower frequency of fusion events seen in cultured AVCMs and other differentiated muscle paradigms (17, 45). In addition to the structural confinement of mitochondria, in vivo and cell culture conditions seem to affect mitochondrial motility differently, with

mitochondria showing little movement in freshly isolated striated and vascular smooth muscle but vigorous displacement in proliferating cultured striated and smooth muscle cells (17, 46).

Despite the similar fiber organization in adult skeletal muscle fibers and AVCMs, the former exhibit 0.5 fusion event/min, compared with 1.4 fusion events/min in the latter. This difference may be linked to tissue-specific abundance of mitochondrial fusion and fission proteins; however, compared with skeletal muscle, cardiac muscle contains more abundant and larger mitochondria. Unexpectedly, we also found that the fusion-mediated exchange of soluble matrix content shows slower kinetics than that in skeletal muscle and other previously characterized paradigms. Based on our observations and the literature, several factors might explain the unusual complementation kinetics of cardiac mitochondria. First, our evidence for discrete and partial repetitive steps of mtPA-GFP transfer shows a similarity to neurotransmitter release by exocytosis, where fusion of neurotransmitter-filled vesicles with the plasma membrane occurs through partial opening of a fusion pore, leading to a so-called “kiss-and-run” between the membranes, which remain connected via a nanotubule that opens up to a larger pore and then recloses to a nanotube (37). Thus, it might be speculated that the IMM undergoes fast intermittent fusion pore openings as well. Second, the observation of narrow connectors that resemble those previously described in heart mitochondria (16, 34), along with the results of Lavorato et al. (47) showing that in a model of catecholaminergic polymorphic ventricular tachycardia (RyR2-A4860G), nanotunnel structures coupled with slow matrix mixing events become more frequent, support the idea of nanotunnels mediating slow mitochondrial fusion in AVCMs. Third, ample ultrastructural evidence shows that the IMM is highly folded and the numerous cristae define narrow compartments of the matrix specifically in cardiac mitochondria (12, 48, 49), which might have more restricted communication compared with the matrix areas of mitochondria in other tissues. Thus, multiple nonexclusive mechanisms might contribute to the slow content mixing during fusion of heart mitochondria.

A striking observation of the present study is the time-dependent decay in mitochondrial fusion activity when AVCMs are placed into cell culture. The decreases in mitochondrial continuity and fusion activity are accompanied by a previously reported loss of transversal tubules and, consequently, dysregulation of the ECC-associated  $Ca^{2+}$  transients (36) and suppressed contractile activity. Therefore, we reasoned that the decay in mitochondrial fusion activity might result from the loss of regular contractile activity or  $Ca^{2+}$  transients. Indeed, we found that attenuation of the  $Ca^{2+}$  transients and contraction leads to depressed fusion activity, and that stimulation of contraction in cultured AVCMs positively affects mitochondrial fusion activity. Until now, it was thought that in contrast to vesicular fusion, mitochondrial fusion does not depend on  $Ca^{2+}$  (50); however, a number of posttranslational modifications regulate mitochondrial dynamics at different levels, including phosphoregulation of Drp1 (51), redox modification of Mfn2 (52), Opa1 proteolytic processing (24), and others. In the heart, mechanical forces and stretch activate various signaling pathways, including such as NADPH-derived ROS, leading to hypertrophy through the activation of diverse signaling programs (53). Whether or not these pathways extended to mitochondrial fusion regulatory proteins was unknown. Our present data show activation of mitochondrial fusion by contraction at both short-term and long-term levels.

An adaptation of mitochondrial communication to persistent contraction is in accordance with previous studies in skeletal muscle showing that exercise enhances the apparent communication among mitochondria (29) and induces expression of mitochondrial fusion proteins (30). Our data show that the amount

of the mitochondrial fusion protein Mfn1 decreases in response to a lack of contractile activity in cultured AVCMs, and that the rescue of Mfn1 by exogenous expression in cultured AVCMs leads to a recovery of mitochondrial fusion, highlighting this protein as the key player in striated muscle mitochondrial fusion regulation, in agreement with our previous data in skeletal muscle (17). In contrast to Mfn2, Mfn1 has an exclusive mitochondrial fusion-related function in muscle, whereas Mfn2 is also involved in cardiac mitochondria-SR interactions (26).

We also show that RyR2-dependent oscillatory  $[Ca^{2+}]_c$  transients improve mitochondrial connectivity and fusion frequency and also support Mfn1 up-regulation, which may reflect a  $Ca^{2+}$ -induced transcriptional regulation. Extensive studies have shown regulation of gene expression by  $Ca^{2+}$  in striated muscle and neurons, also known as excitation-transcription coupling (54–56). RyR1- and IP3R-dependent calcium transients differentially control gene expression in skeletal muscle (54). In the heart, IP3R-induced  $Ca^{2+}$  transients lead to activation of nuclear factor of activated T cells and hypertrophy (55). Interestingly, repetitive oscillatory  $Ca^{2+}$  signals are more efficient than sustained transients for inducing  $Ca^{2+}$ -dependent transcriptional factors (57). Along the line of these findings, ECC-evoked  $Ca^{2+}$  transients might regulate mitochondrial fusion at a transcriptional level in the heart; however, we also observed a rapidly reversible effect of  $Ca^{2+}$  oscillations on the maintenance of mitochondrial fusion activity in HEK-RyR2-expressing cells. This observation, together with the rapid enhancement of mitochondrial fusion activity observed on pacing or spontaneous contractions in AVCMs, suggest a fairly direct and acute positive effect of  $Ca^{2+}$  oscillations on mitochondrial fusion.

Given that Mfn1 abundance is unlikely to increase and decrease within minutes, an alternative mechanism must be involved in the acute effect of  $Ca^{2+}$  oscillations, such as  $Ca^{2+}$ -dependent regulation of mitochondrial metabolism, which is central to fusion activity (20, 58, 59), or  $Ca^{2+}$  binding to some components of the fusion machinery or to one of its posttranslational modifiers. Importantly, stopping and restarting of ECC activity does not occur under physiological conditions; thus, we can only claim that the maintenance of mitochondrial fusion activity involves a  $Ca^{2+}$  oscillation/contraction-dependent factor in the heart. The potential pathophysiological significance of this factor is highlighted by the work of Lavorato et al. (47), who report that a mutation in RyR2 (A4860G), which alters the normal  $Ca^{2+}$  oscillation phenotype, causes a striking change in mitochondrial fusion dynamics.

Several lines of circumstantial evidence implicate mitochondria in either the initiation or progression of cardiomyopathy (60). Mitochondrial involvement in alcoholic cardiomyopathy is intriguing, because mitochondria represent a primary cellular target of alcohol (33). Alcoholic cardiomyopathy is characterized by reduced contractility, along with arrhythmias and fibrosis (61, 62). Alterations in hearts from alcoholic patients at the level of mitochondrial communication were suggested based on early ultrastructural studies (34), and recent studies have reported impairment of mitochondrial DNA integrity by EtOH (63). Given the relevance of mitochondrial fusion to mtDNA stability (5), our results provide a potential structural explanation of these latter findings. A loss of mtDNA integrity was also found in skeletal muscle-conditional knockout of Mfn1 and Mfn2 (64). Moreover, our recent findings in skeletal muscle from alcohol-fed rats show that chronic EtOH consumption leads to inhibition of mitochondrial fusion and mitochondrial calcium uptake efficiency, accompanied by dysregulation of ECC-derived calcium transients, leading to fatigue (17). Our present results indicate that in the heart, alcohol targets mitochondrial dynamic communication via a mechanism that might be linked to the alcohol-induced reduced contractility. Given the apparent bidirectional dependence between contractile and mitochondrial fusion



activities, the pathogenesis of cardiomyopathy might include contractile and mitochondrial fusion impairments that facilitate one another. Based on our findings, it might be speculated that targeting the fusion-fission process may help improve cardiac function in alcoholics.

## Materials and Methods

**cdNA Constructs and Adenovirus.** mtDsRed and mtPA-GFP plasmid DNA have been described previously (7). Type V adenoviruses carrying mtDsRed, mtPA-GFP, LacZ, and mouse Mfn1 were produced by Vector Biolabs.

**Cardiac Myocyte Isolation, Cell Lines, and Cultures.** NVCMs and AVCMs (14, 65), H9c2 cells, WT cells, and RyR2-overexpressing HEK293T cells (39) were cultured as described in *SI Materials and Methods*.

**Animal Treatment.** The EtOH-consuming adult male Sprague–Dawley rat model is described elsewhere (17). All procedures were done in accordance with the National Institutes of Health's Guidelines on the Use of Laboratory Animals and were approved by Thomas Jefferson University's Committee on Animal Care. Cardiac function was monitored by echocardiography as described earlier (66). These studies validated significant EtOH-dependent decreases in ejection fraction (EF) and fractional shortening (FF) (EF: control,  $78 \pm 2\%$ ; EtOH,  $72 \pm 2\%$ ;  $P < 0.02$ ; FF: control,  $48 \pm 2\%$ ; EtOH,  $43 \pm 2\%$ ;  $P < 0.02$ ). Thus, in the chronic EtOH-fed rats, contractile dysfunction develops in the heart.

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