Myosin VI facilitates connexin 43 gap junction accretion

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ABSTRACT

In this study, we demonstrate myosin VI enrichment at Cx43 (also known as GJA1)-containing gap junctions (GJs) in heart tissue, primary cardiomyocytes and cell culture models. In primary cardiac tissue and in fibroblasts from the myosin VI-null mouse as well as in tissue culture cells transfected with siRNA against myosin VI, we observe reduced GJ plaque size with a concomitant reduction in intercellular communication, as shown by fluorescence recovery after photobleaching (FRAP) and a new method of selective calcein administration. Analysis of the molecular role of myosin VI in Cx43 trafficking indicates that myosin VI is dispensable for the delivery of Cx43 to the cell surface and connexon movement in the plasma membrane. Furthermore, we cannot corroborate clathrin or Dab2 localization at gap junctions and we do not observe a function for the myosin-VI–Dab2 complex in clathrin-dependent endocytosis of annular gap junctions. Instead, we find that myosin VI was localized at the edge of Cx43 plaques by using total internal reflection fluorescence (TIRF) microscopy and use FRAP to identify a plaque accretion defect as the primary manifestation of myosin VI loss in Cx43 homeostasis. A fuller understanding of this derangement may explain the cardiomyopathy or gliosis associated with the loss of myosin VI.

KEY WORDS: Connexin, Endocytosis, Gap junction, Myosin

INTRODUCTION

Cardiac function depends on the precise conduction of electrical activity throughout the myocardium, and disturbances to this highly coordinated process are the hallmark of acquired heart disease. Gap junctions (GJs) are semi-crystalline arrays of double-membrane-spanning intercellular channels that facilitate action potential propagation by electrochemically coupling neighboring cardiomyocytes. The basic subunit of the gap junction is the connexin, of which there are over 21 types expressed to variable levels in cardiac tissue (Goodenough, 1981; Laird et al., 1991). Ultimately, understanding the continuous remodeling, it also leaves connexins particularly susceptible to trafficking defects (Berthoud, 2004; Fallon and Goodenough, 1981; Laird et al., 1991). Ultimately, understanding of molecular mechanisms that govern the delicate balance of gap junction formation and internalization is paramount to the maintenance of intercellular communication.

Gap junction regulation is largely controlled by protein–protein interactions and post-translational modifications involving the Cx43 C-terminal tail. Plaque formation requires phosphorylation by casein kinase 1 and stabilization depends on the interaction of the Cx43 C-terminus with the actin-binding scaffolding protein ZO-1 (also known as TJP1) (thereby linking tight and GJs) (Cooper, 2002; Giepmans and Moolenaar, 2007). GJ plaque removal and decreased gap junction intercellular communication (GJIC) are associated with many processes, including phosphorylation by both Src and PKC, ubiquitylation by the protein ligase Nedd4, binding by the endocytic protein Eps15 and interaction with 14-3-3 proteins (Catarino et al., 2011; Girao et al., 2009; Smyth et al., 2014; Solan and Lampe, 2007; Toyofuku et al., 2001). Ultimately, internalized AGJs are degraded by autophagy in a ubiquitin-dependent manner (Bejarano et al., 2012; Fong et al., 2012). Knowledge of this unidirectional pathway has long been accompanied by the fact that Cx43 exhibits a surprisingly short half-life for a junctional protein (ranging from 1.5–5 h), and while rapid turnover facilitates continuous remodeling, it also leaves connexins particularly susceptible to trafficking defects (Berthoud, 2004; Fallon and Goodenough, 1981; Laird et al., 1991). Ultimately, understanding of molecular mechanisms that govern the delicate balance of gap junction formation and internalization is paramount to the maintenance of intercellular communication.
addition, the Dab2-binding partner myosin VI was also found to localize to Cx43 GJs and it was suggested that myosin VI plays a role in the clathrin-mediated endocytosis of GJ plaques. However, these studies used the no insert (NI) isoform of myosin VI, which has no reported role in clathrin-mediated endocytosis. Furthermore, studies have questioned the role of clathrin in the internalization of double membrane GJs, suggesting that clathrin instead targets plasma membrane-localized connexons that have yet to form GJs (Johnson et al., 2013).

Myosin VI is the only unconventional myosin motor protein that moves toward the minus end of actin filaments, and it is expressed in four different splice variants in a tissue-specific manner. The large insert isoform is selectively expressed in polarized epithelial cells, where it is targeted to the apical domain to facilitate clathrin-mediated uptake of cell surface receptors (Ameen and Apodaca, 2007; Buss et al., 2001). In contrast, the no insert splice variant is widely expressed in most cell types and tissues, functioning in cargo sorting in the endocytic pathway, exocytosis, autophagy and the regulation of actin filament dynamics (Bond et al., 2011; Chibalina et al., 2007; Rogat and Miller, 2002; Tumbarello et al., 2012). At the plasma membrane, myosin VI functions at the interface between cell surface proteins and the cortical cytoskeleton, stabilizing cell–cell adhesion and anchoring apical hair cell membrane to the cortical actin meshwork (Geisbrecht and Montell, 2002; Heintzelman et al., 1994; Millo et al., 2004; Self et al., 1999). To mediate this variety of cellular functions, myosin VI interacts with a broad range of cargo adaptor proteins that bind to regions in the C-terminal cargo-binding tail with either an RRL sequence [binding partners GIPC1, TAX1BP1, NDP52 (also known as CALCOCO2) and optineurin] or with a WWY motif [binding partners TOM1, LMTK2 and Dab2] (Spudich et al., 2006; Tumbarello et al., 2013).

Interestingly, the loss of myosin VI in mice and humans manifests as two pathologies affiliated with connexin derangements: sensorineural deafness and hypertrophic cardiomyopathy (Avraham et al., 1995; Hegan et al., 2015; Mohiddin et al., 2004; Williams et al., 2013). In this study, we further investigate the association of myosin VI with Cx43 GJs in heart tissue and describe a new role for myosin VI in GJ homeostasis. Previous studies have separately reported myosin VI localization to GJ plaques in cell culture models and intercalated discs in heart sections (Karolczak et al., 2014; Piehl et al., 2007). We show that: (1) myosin VI recruitment to Cx43 GJs observed in native heart tissue extends to isolated cardiomyocytes; (2) myosin VI is not required for delivery of Cx43 to, or its movement within, the plasma membrane; and (3) loss of myosin VI instead impairs GJ plaque formation and downregulates intracellular communication.

RESULTS

Myosin VI localizes to GJs in heart tissue and primary cardiomyocytes

Given previous observations of myosin VI colocalization with its binding partner Dab2 on GJs in HeLa cells expressing GFP–Cx43 (Piehl et al., 2007), we first examined the subcellular localization of endogenous myosin VI and Cx43 in the adult mouse heart and neonatal rat ventricular myocytes (NRVMs). Whole frozen heart sections were fixed and analyzed by immunohistochemistry. We detected myosin VI throughout cardiac tissue, and it was enriched on Cx43 structures at the intercalated disc (Fig. 1A, arrowheads). We then isolated primary NRVMs and, by immunofluorescence, we confirmed that myosin VI strongly decorates Cx43 gap junction plaques (Fig. 1B, arrowheads). Unlike myosin VI, neither Dab2 nor clathrin localized to Cx43 GJs in our primary cardiomyocyte experiments using NRVMs (Fig. S1B,C). Western blotting confirmed that myosin VI is detected in whole-heart homogenates (Fig. S3A) and purified cardiomyocytes (Fig. S1A).

In addition to primary heart tissue, we also used normal rat kidney (NRK) cells, which endogenously express Cx43 and myosin VI, to confirm the colocalization of these proteins. Using retroviral transduction, we created cell lines stably expressing Cx43–TagRFP-T, in which we transiently expressed HaloTag (HT)-conjugated myosin VI. HT–myosin-VI strongly colocalized with Cx43–TagRFP-T-labeled GJs (Fig. 1C, arrowheads), thus supporting the localization of myosin VI at GJs observed in whole-heart sections and primary NRVMs.

Myosin VI recruitment to Cx43 GJs requires the RRL binding motif

Myosin VI is composed of a conserved catalytic domain and a C-terminal cargo-binding domain that contains ubiquitin- and lipid-binding domains and mediates binding to adaptor proteins via RRL and WWY motifs (Fig. 2A) (Chibalina et al., 2007; Morriswood et al., 2007; Sahlender et al., 2005; Tumbarello et al., 2012). To determine which binding domain and potential adaptor protein are required for myosin VI localization to Cx43 GJs, we transfected NRK cells stably expressing Cx43–TagRFP-T with GFP–myosin-VI full-length constructs (Fig. 2B) harboring amino acid substitutions known to disrupt these binding domains. Fixed cell fluorescence microscopy demonstrated that the ΔWWY mutant mimics wild type localization of myosin VI to Cx43 (arrowheads), while the loss of the RRL binding motif prevented myosin VI targeting to Cx43 (arrows).

To confirm this observation, line plots drawn through GJ plaques were quantified as depicted (Fig. 2C). Line plots were oriented with the GFP–myosin-VI-expressing cell to the right. While not included in Fig. 2B, motor domain (A600V and K157R) and ubiquitin-binding (A1013G) mutants were also tested for GJ recruitment. Myosin VI fluorescence intensity reached a local maximum at the Cx43 GJ plaque center (0 µm) in all plots except RRL mutants (Fig. 2D). These results clearly establish that myosin VI localization to Cx43 GJs does not depend on motor activity, the A1013 ubiquitin-binding site or the WWY-binding partners TOM1, LMTK2 or Dab2.

We then asked if the RRL-binding partners OPTN, TAX1BP1 or GIPC1 also localize to Cx43 GJs. NDP52 was omitted because rodents express a truncated isoform that lacks the myosin VI-binding zinc finger domain (Tumbarello et al., 2015). In fixed primary NRVMs, antibodies raised against OPTN, TAX1BP1 and GIPC1 failed to decorate Cx43 GJ plaques (Fig. S2A, arrowheads). We obtained the same result using NRK cells stably expressing Cx43–TagRFP-T and HT–myosin-VI transfected with GFP–OPTN, GFP–TAX1BP1 or GFP–GIPC1 (Fig. S2B, arrowheads). These results suggest that myosin VI targets to Cx43 either directly, via an unknown binding partner or ubiquitin binding through the MyUb domain – a recently described region that overlaps with the RRL binding region (He et al., 2016).

Myosin VI depletion leads to a reduction in GJ plaque size

To investigate the ramifications of myosin VI loss on GJ maintenance and morphology, we isolated heart tissue from the Snell’s waltzer (sv) myosin VI-null mouse. While blotting whole-heart homogenates showed only a subtle decrease in total Cx43 (Fig. S3A,B), a selective loss of Cx43 from the plasma membrane is observed by immunohistochemistry in fixed sv hearts. In the myosin VI-null heart, Cx43 still localized to the intercalated disc, but individual Cx43 structures were smaller (Fig. 3A). Quantification of
all Cx43 structures in sv hearts (between 26,000 and 47,000 per heart) verified this reduction in average Cx43 area compared to wild-type hearts (Fig. 3B).

We also depleted myosin VI by using siRNA in RPE cells and HeLa cells stably expressing Cx43. As we observed for whole-heart homogenates, the knockdown of myosin VI in HeLa cells (Fig. S3C) did not lead to a marked change in total Cx43; however, fixed cell fluorescence showed a reduction in the Cx43–GFP-labeled GJ plaque size when myosin VI was silenced in HeLa stable cell lines (Fig. 3C). Quantification of all Cx43 GJ plaques from these images (Fig. 3D) confirmed that the average plaque size is reduced when myosin VI is silenced.

To further corroborate these observations, we isolated and immortalized mouse embryonic fibroblasts (MEFs) from wild-type and sv mice. Here, western blotting for Cx43 (Fig. 3E) showed a sizeable reduction in the amount of total Cx43 in sv MEFs (Fig. 3F). By immunofluorescence, we again observed a decrease in Cx43 GJ plaque size when myosin VI was lost (Fig. 3G). Quantification of these microscopy experiments verified this observation (Fig. 3H). These results indicate that the primary effect of myosin VI loss is a reduction in Cx43 GJ plaque size in heart tissue, HeLa cells and immortalized sv MEFs.

**Myosin VI depletion impairs GJ intercellular communication**

We then asked whether the reduction in GJ plaque size is associated with a concomitant reduction in GJIC in myosin VI-null cells. For these experiments, we used the BioPen microfluidic pipette (Ainla et al., 2010) to generate a hydrodynamically confined fluid sphere to selectively load multiple cells with calcein AM (Fig. 4A), which converts into a GJ-permeable dye within cells. To demarcate the zone of calcein AM administration, 1 μg/ml wheat germ agglutinin conjugated to Alexa Fluor 647 (WGA–647) was added to a solution of 20 μM calcein AM and cells were loaded for 10 min (Fig. 4B). After 60 min, a tiled image of the administration site was obtained,
showing that sv MEFs transfer substantially less calcein AM across a monolayer compared to wild-type cells (Fig. 4C). To quantify this difference, line plots were drawn across the calcein administration zone and the intensities were averaged for each channel (Fig. 4D). Line plots from multiple experiments were normalized, averaged and plotted for WGA–647 (Fig. 4E) to confirm equivalent administration of calcein AM. In contrast, calcein AM spread (Fig. 4F) was much lower in sv MEFs compared to wild-type cells.

To confirm these findings and to verify this new method, we also conducted fluorescence recovery after photobleaching.
FRAP experiments (Abbaci et al., 2007; Bastide et al., 1995) with wild-type and sv MEFs. In accordance with our micropipette loading assay, calcein AM fluorescence recovery as calculated by linear regression analysis (Santiquet et al., 2012) was reduced when myosin VI was lost (Fig. 5A). In this analysis, total fluorescence recovery (Fig. 5B) was decreased in the absence of myosin VI, but the more striking effect was a decrease in the rate of fluorescence recovery (Fig. 5C). These results indicate that the reduction in GJ size due to myosin VI loss corresponds with defective GJIC.
Plaques accumulate more slowly without myosin VI, but not due to defective Cx43 forward trafficking

Considering known roles for myosin VI in protein secretion, we next investigated whether the forward trafficking of Cx43 from the ER to the plasma membrane was delayed in myosin VI-depleted cells (Bond et al., 2011; Chibalina et al., 2010). We first analyzed the formation of endogenous GJ plaques in sv MEFs using a brefeldin A (BFA) forward trafficking assay. Treatment with BFA induces a
Fig. 5. Loss of myosin VI compromises GJ intercellular transport.
(A) Wild-type (WT) and sv (SV) MEFs were loaded with calcein AM and cells were photobleached for FRAP analysis. Fluorescence recovery as a function of time was plotted (mean, s.e.m. is indicated by the region between the dashed lines, n=3). (B) Quantification of fluorescence intensity at t, F∞ as calculated by linear regression analysis (mean±s.d., n=3). *P<0.05. (C) Quantification of recovery rate. (mean±s.d., n=3). ***P<0.0001.

Myosin VI contributes to Cx43 accretion at GJ plaques
To further assess whether loss of myosin VI causes a defect in Cx43 trafficking to the cell surface, we quantified the plasma membrane-localized population of Cx43 connexons using a surface biotinylation assay in wild-type and sv MEFs. While total Cx43 was markedly reduced in sv MEFs, the amount of Cx43 connexons in the plasma membrane was not significantly different compared to wild-type MEFs (Fig. 7A,B). Since less Cx43 is incorporated into GJ plaques in sv MEFs compared to wild-type cells (see Fig. 3G,H), it follows that these cells contain a larger fraction of free Cx43 in the plasma membrane as compared to wild-type MEFs.

We next performed single-particle-tracking photoactivation localization microscopy (sptPALM) in wild-type and sv MEFs expressing a monomeric (m)EOS3–Cx43 fusion protein to test whether there were differences in the mobility of the free molecules in the plasma membrane of these cells. As shown in Fig. 7C, no difference in mEOS3–Cx43 mobility outside of plaques was seen in the two cell types, indicating myosin VI depletion does not affect free Cx43 movement in the plasma membrane (Fig. 7C).

We then addressed whether myosin VI serves a role in GJ plaque stability or biogenesis, since our localization studies show a strong enrichment of myosin VI on Cx43 GJs. We noticed that on rare occasions, Cx43 GJ plaques extend close to the coverslip. This allowed us to take advantage of the reductions in the out-of-focus light that total internal reflection fluorescence (TIRF) microscopy affords. Using this technique, we imaged NRK cells transfected with Cx43–GFP and HT–myosin VI, observing that HT–myosin VI localizes to the edge of GJ plaques (arrowhead, Fig. 7D; Movie 1). To investigate Cx43 GJ accretion (i.e. assembly into a GJ plaque), we used a photobleaching assay in wild-type or sv MEFs transfected with Cx43–GFP and HT–myosin VI, suggesting a defect in the ability of Cx43 to be recruited free Cx43 movement in the plasma membrane (Fig. 7C).

DISCUSSION
In this study, we used new and traditional assays of GJ trafficking and function to redefine numerous aspects of the role of myosin VI in Cx43 GJ homeostasis. First, we confirmed a previous report that endogenous myosin VI localizes to GJs in heart tissue...
Karolczak et al., 2014) and we extended this finding to primary mouse cardiomyocytes and identified the RRL or MyUB binding motif as the basis of this localization. Second, we showed that the loss of myosin VI leads to a reduction in GJ plaque size not only in myosin VI siRNA HeLa knockdown cells but also in cardiac tissue and fibroblasts from the Snell's waltzer mouse. Third, we observed a concomitant reduction in GJ intercellular communication using a new method of selective calcein administration. Finally, we found that the observed reduction in GJ size was not due to the defective delivery of Cx43 to the cell surface via the biosynthetic pathway but instead was caused by impaired plaque accretion (Fig. 8).

Prior work has suggested that localization of myosin VI at GJ plaques is linked to its role in GJ internalization by a clathrin- and Dab2-mediated process (Piehl et al., 2007). While our work confirms myosin VI localization at GJs in primary cardiac tissues and isolated cardiomyocytes, it also suggests that this localization is not involved in GJ internalization. None of our cells showed clathrin or Dab2 localized at GJ plaques. Moreover, we found that WWY motif of myosin VI (which interacts with Dab2) is dispensable to GJ localization of myosin VI. Therefore, myosin VI function at GJs is unlikely to be related to clathrin-mediated endocytosis.

This conclusion is consistent with the fact that the cells used in the Piehl et al. study did not express the LI isoform of myosin VI that
is involved in clathrin-mediated endocytosis (Aschenbrenner et al., 2003; Buss et al., 2001). The putative Dab2-binding motif (xPxY) that exists in the Cx43 tail, therefore, may be more important for Cx43 hemichannel rather than plaque endocytosis.

In our studies examining myosin VI recruitment to GJs, we identified a requirement of the RRL binding motif on myosin VI, but known RRL-binding partners – OPTN, TAX1BP1 and GIPC1 – failed to localize to GJ plaques. This raises the possibility that

Fig. 7. Plasma membrane-localized Cx43 exhibits an accretion defect in myosin VI-null MEFs. (A) The surface of wt and sv (SV) MEFs was biotinylated at 4°C and biotinylated proteins were isolated using NeutrAvidin beads. Whole-cell lysates and biotinylation fractions were immunoblotted with an antibody against Cx43. MVI, myosin VI. (B) Quantification of western blot Cx43 intensity normalized by Bio-Rad Stain-Free tryptophan labeling (total protein) (mean±s.d., n=3). ****P<0.0001; ns, not significant. (C) Wild-type and sv MEFs were transfected with mEOS3–Cx43 and imaged on a Nikon TIRF microscope for single-particle-tracking photoactivation localization microscopy analysis. The diffusion coefficient of mobile mEOS3–Cx43 (Cx43 D250ms; mean±s.d.) was quantified for at least seven cells for each phenotype. (D) NRK cells stably expressing Cx43–GFP (green) and HT–myosin-VI were incubated with the JF646-HaloTag ligand (red) before imaging using TIRF microscopy to identify peripheral recruitment of myosin VI to Cx43 GJ plaques (arrowheads). (E) WT and sv MEFs were transfected with Cx43–GFP (Fire LUT) and imaged using a 3i Spinning Disk microscope for FRAP experiments. Square regions of Cx43 plaques were photobleached (dotted box) and imaged over 40 min to measure Cx43 accretion. WT MEF accretion (arrowhead) was greater than that observed in sv MEFs (arrows). (F) Cx43 accretion was quantified using ImageJ (mean±s.d., n=4). *P<0.05. Scale bars: 10 µm.
myosin VI targets to GJs by some other binding partner. Alternatively, since recent studies suggest that myosin VI harbors a second ubiquitin-binding motif, which overlaps with the RRL binding site and is disrupted by the ΔRRL mutant (He et al., 2016), it is possible that interactions with ubiquitylated substrates (including ubiquitylation sites in the Cx43 C-terminal tail) determine myosin VI localization to GJs.

Regardless of the recruitment mechanism, we observed a striking reduction in GJ plaque size when myosin VI was lost. These findings suggest an alternative to the prevailing endocytosis model of myosin VI function in GJ homeostasis, with myosin VI instead functioning to deliver Cx43 molecules into plaques, facilitating the established role of actin in this process (see Fig. 8).

Actin-containing microfilaments and GJs have been long observed in close proximity to each other (Larsen et al., 1979). To date, Cx43 linkages to actin include the tight junction protein ZO-1 and the actin-binding protein drebrin. Studies have shown that both dominant-negative ZO-1 and drebrin silencing by siRNA cause Cx43 loss from the intercellular junction and reduced cell communication (Butkevich et al., 2004; Toyofuku et al., 1998). Furthermore, actin polymerization inhibition by cytochalasin D has been shown to reduce dye transmission between cells and impede recovery of photobleached Cx43 GJ plaques (Martin et al., 2001; Thomas et al., 2001). Other studies have claimed that actin depolymerization also reduces plaque formation (Shaw et al., 2007).

To date, an association of hypertrophic cardiomyopathy with myosin VI loss has been described for a number of patients and two different mouse models (Avraham et al., 1995; Mohiddin et al., 2004; Williams et al., 2013). Numerous studies have investigated the expression and localization of myosin VI in skeletal muscle throughout myotube differentiation and in response to denervation, but investigations in cardiac muscle have been less robust (Karolczak et al., 2013, 2014). One study confirmed an increase in the heart-weight-to-body-weight ratio in sv mice, and found that the absence of myosin VI caused an increase in interstitial fibrosis that was unrelated to mouse age; however, this investigation focused on myosin VI expression in vascular endothelial cells of the heart and lungs and did not comment on myosin VI localization to the intercalated disc as previously described (Hegan et al., 2015; Karolczak et al., 2014).

In summary, this work identifies a new role for myosin VI in the construction and maintenance of GJ plaques between cells, setting the stage for further investigations into other connexin derangements. Mice lacking myosin VI had a modest reduction in the GJ intercalated disc size in their hearts, so it will be interesting to assess the maintenance of Cx43 homeostasis, including Cx43...
accretion rates into GJs, in response to pathological insults such as ischemia and chronic elevations in afterload. Apart from the heart, Cx43 is also the major connexin expressed in other cell types – namely, astrocytes. The sv mice have been reported to display a profound reactive astrogliosis (Osterweil et al., 2005), so it will be interesting to see whether this is linked to reduced GJ function in reactive astrocytes in the brain.

MATERIALS AND METHODS

Reagents and antibodies

Affinity-purified rabbit antibodies against myosin VI, TAX1BP1 and optineurin were generated as previously described (Buss et al., 1998; Morriswood et al., 2007; Sahlender et al., 2005). Commercial antibodies against the following proteins were used: Cx43 [C6219; 1:200 immunofluorescence (IF), 1:2000 western blotting (WB)] and actin (A2066; 1:5000 WB) polyclonal antibodies (Sigma), Cx43 (O5-763; 1:200 IF) monoclonal antibody (Millipore), GAPDH (6C5; 1:1000 WB) and clathrin (X22; 1:100 IF) monoclonal antibodies (Abcam), Dab2 (H-110; 1:100 IF) and GIPC1 (N-19; 1:100 IF) polyclonal antibodies (Santa Cruz Biotechnology) and pan-cadherin (4068; 1:1000 WB) polyclonal antibody (Cell Signaling). Phalloidin–Alexa-Fluor-647, Calcein AM, WGA–647 and Hoechst 33342 were purchased from ThermoFisher. Bafilomycin A1, brefeldin A and birin were purchased from Sigma-Aldrich. JF646-HaloTag and Hoechst 33342 fluorescence microscopy.

HaloTag ligand conjugation, the far-red JF646-HaloTag ligand (HTL) was diluted 1:200 and then 1:5 in complete medium. Cells were incubated in DMEM and transfected into cells using Oligofectamine (ThermoFisher) previously described in Tumbarello et al., 2012) were purchased from Dharmacon and handled was identical to that for proteins tagged with fluorescent proteins on day five. Efficiency of depletion was assessed by western blotting.

Western blotting

Whole hearts were fixed in 4% formaldehyde and cryoprotected with 0.2% Triton X-100 (Sigma) in PBS. Fixed cells were blocked with 1% bovine serum albumin (BSA) before incubation with primary antibodies and detection using Alexa Fluor 488- or 568-conjugated secondary antibodies and 647-conjugated phalloidin (Invitrogen). Coverslips were mounted on slides using ProLong Anti-fade mounting medium (ThermoFisher) and Lab-Tek coverglasses were covered with PBS. Images were acquired on a Nikon Ti3 spinning disk system operated by Nikon Elements software.

Immunofluorescence

Cells were grown on glass coverslips (Carl Zeiss) or in Nunc Lab-Tek coverglass chambers (ThermoFisher). Cells were washed with PBS, fixed with 4% formaldehyde ( Electron Microscopy Services) and permeabilized with 0.2% Triton X-100 (Sigma) in PBS. Fixed cells were blocked with 1% bovine serum albumin (BSA) before incubation with primary antibodies and detection using Alexa Fluor 488- or 568-conjugated secondary antibodies and 647-conjugated phalloidin (Invitrogen). Coverslips were mounted on slides using ProLong Anti-fade mounting medium (ThermoFisher) and Lab-Tek coverglasses were covered with PBS. Images were acquired on a Nikon Ti3 spinning disk system operated by Nikon Elements software.

Live-cell imaging

Cells were grown on Lab-Tek coverglass chambers, glass-bottom dishes (MatTek) or 15-mm round coverslips under normal conditions. Cells were imaged on a Nikon Ti3 spinning disk or a 3i spinning disk system. All
systems were equipped with temperature regulation, while the Nikon system was also equipped with CO₂ control. For 3i imaging, phenol red-free DMEM was supplemented with 2 mM L-glutamine, 10% FBS and 20 mM HEPES was used. TIRF microscopy was conducted on a Nikon Ti3 TIRF system with an Andor EMCCD camera. For this imaging modality, #1.5 coverslips were coated with 20 μg/ml fibronectin (Millipore).

FRAP studies
FRAP studies were conducted using a 3i spinning disk microscope and 3i SlideBook software. For GJC studies, single cells were photobleached with 405 nm wavelength light and imaged for 10 min to measure fluorescence recovery. Calcein recovery was quantified using ImageJ. Specifically, three regions of interest (ROIs) were measured over time: the photobleached ROI, ROI(t); the whole-cell ROI, Tot(t); and an ROI for background fluorescence, BG(t). For each time point, the background intensity was removed, photobleaching was accounted for by dividing the photobleached ROI by the whole-cell ROI and each time point was normalized by the initial intensity (Eqn 1). Normalized values were plotted over time.

\[
\frac{\text{ROI}(t) - \text{BG}(t)}{\text{Tot}(t) - \text{BG}(t)} \times \frac{\text{Tot}(0) - \text{BG}(0)}{\text{ROI}(0) - \text{BG}(0)}
\]

For GJ plaque FRAP experiments, a square region of a GJ plaque was photobleached with 405 nm light and plaques were imaged every 5 min for 40 min. Cx43 plaque accretion was quantified manually using ImageJ to calculate the mean pixel intensity at the growing plaque edge.

Micropipette-assisted calcein loading
A microfluidic pipette (BioPen, Fluicell) controlled by a micromanipulation apparatus (Eppendorf) was used to selectively load a region of cells. Using an administration pressure of 200 mBar, a solution containing Alexa Fluor 647 (ThermoFisher) was first used to demarcate the fluid sphere. The micropipette was lowered until the area of dye fit inside the 60× field of view and matched that administered for other samples. The micropipette was then switched to medium containing calcein AM and WGA conjugated to Alexa Fluor 647 (ThermoFisher) and loading was imaged on a Nikon spinning disk microscope. After 10 min, the micropipette was removed and cells were incubated for 60 min in the microscope environmental chamber before a 4×4 field image was captured and stitched together using Nikon Elements Software.

Single-particle-tracking photoactivation localization microscopy
stPALM experiments were conducted as described previously (Manley et al., 2008). Briefly, MEFs were transfected with mEOS3–Cx43 and seeded on fibronectin-coated #1.5 coverslips for TIRF microscopy. Cells were imaged using a Nikon Ti3 TIRF system equipped with an Andor EMCCD camera. mEOS3 molecules were photoconverted using a laser intensity, pulse length and duration chosen to maintain a sparse population for localization and tracking. Measurements were taken for seven MEFs each transfected with mEOS3–Cx43. Molecules were localized using the QuickPALM ImageJ plugin and tracks were assembled using MATLAB (The Mathworks, Inc., Natick, MA). A linear fit of mean squared displacement for 250 ms trajectories with at least 15 steps was used to calculate the diffusion coefficient D for each condition.

Image processing
All images were processed using the ImageJ (NIH) FIJI build and compiled in Adobe Illustrator. Live-cell images were converted into .avi movies using ImageJ and converted into .mov files using Quicktime (Apple). Custom ImageJ macro scripts were created to quantify GJ size, intensity and number using threshold-defined regions of interest (ROIs). For myosin VI GJ line profiles, 10 μm lines were drawn across all junctional structures (pointing toward the center of the cell). Channel intensity was then calculated along this line, saved and averaged with all other GJ plaques for the specified cell. For micropipette calcein-loading experiments, lines were manually drawn across the administration region and line plots were generated.

Presentation of data and statistics
All graphs were produced using GraphPad Prism software. Bar graphs represent the mean plus the s.d. or s.e.m. as specified. Statistics were calculated using the unpaired Student’s t-test.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
B.W. and F.B. designed the research and wrote the manuscript. B.W. performed the research and analyzed data for all figures. Experiments were conducted in both the J.L.-S. and F.B laboratories, and F.B and J.L.-S. are joint senior authors. P.S. assisted with microscopy and analysis for Fig. 7C. G.G.H. was responsible for advice and reagents for GJ experiments and critical reading of the manuscript.

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References


Catarino, S., Ramalho, J. S., Pereira, P. and Gira (2012). Internalized gap junctions are degraded by autophagy. Autophagy 9, 244-250.


