The Cx43-like Connexin Protein Cx40.8 Is Differentially Localized during Fin Ontogeny and Fin Regeneration

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Abstract

Connexins (Cx) are the subunits of gap junctions, membranous protein channels that permit the exchange of small molecules between adjacent cells. Cx43 is required for cell proliferation in the zebrafish caudal fin. Previously, we found that a Cx43-like connexin, cx40.8, is co-expressed with cx43 in the population of proliferating cells during fin regeneration. Here we demonstrate that Cx40.8 exhibits novel differential subcellular localization in vivo, depending on the growth status of the fin. During fin regeneration, Cx40.8 is found at the plasma membrane, but Cx40.8 is retained in the Golgi apparatus during regeneration. We next identified a 30 amino acid domain of Cx40.8 responsible for its dynamic localization. One possible explanation for the differential localization is that Cx40.8 contributes to the regulation of Cx43 in vivo, perhaps modifying channel activity during ontogenetic growth. However, we find that the voltage-gating properties of Cx40.8 are similar to Cx43. Together our findings reveal that Cx40.8 exhibits differential subcellular localization in vivo, dependent on a discrete domain in its carboxy terminus. We suggest that the dynamic localization of Cx40.8 differentially influences Cx43-dependent cell proliferation during ontogeny and regeneration.

Introduction

Connexins, the subunits of gap junction channels, are part of a large multigene family that includes about 20 genes in mammals [1]. Connexins are integral membrane proteins that consist of four transmembrane domains, two extracellular loops, one cytoplasmic loop, and cytoplasmic amino- and carboxy-termini. Six connexin proteins oligomerize to form one connexon (he,ring channel), and the docking of two connexons at the plasma membranes of neighboring cells forms a single gap junction channel. Channels associate together at the plasma membrane to form gap junction plaques, permitting the passage of ions and small molecules (<1200 Da) between adjacent cells. Interestingly, most tissues express a unique complement of 2–7 connexin genes, suggesting that distinct homomeric and heteromeric gap junction channels contribute to cell-cell coupling and functional diversity (reviewed in [2]). Channel composition can determine metabolic selectivity and specificity [3,4,5]. One possible role of this diversity in connexin expression is that regulated hetero-oligomerization of different isotypes may in turn regulate tissue function.

Gap junctional intercellular communication (GJIC) is necessary for normal tissue development, evidenced by the identification of mutations in connexins that cause human disease [2,6,7]. In particular, missense mutations in human CX43 cause the craniofacial and limb skeletal malformations associated with oculodentodigital dysplasia (ODDD) [8]. Importantly, the function of Cx43 appears to be conserved during skeletal morphogenesis. Mutations in zebrafish cx43 cause short bony fin ray segments associated with the short fin (sof) phenotype [9,10]. In addition to the sof j7e1 allele that has reduced mRNA and protein levels [11,12], three non-complementing ENU-induced alleles were shown to cause missense mutations (sof j7e2 codes Cx43-F30V, sof j7e3 codes Cx43-P191S, and sof j7e4 codes Cx43-F209I). Each missense allele can form gap junction plaques, but channels exhibit aberrant ionic coupling properties [10]. Recent studies revealed a positive correlation between segment length, level of cell proliferation, and level of GJIC [10,12]. Therefore, the zebrafish fin represents a valuable system to understand how Cx43 function contributes to the development of the vertebrate skeleton, including cell proliferation and bone growth.

The zebrafish caudal fin consists of 16–18 segmented bony rays, where fin length depends on the number and size of bony segments [13]. In addition to ontogenetic growth, fins also have the capacity for regenerative growth, where lost tissue is replaced rapidly due to an accelerated growth rate. Amputation is immediately followed by wound healing and establishment of the regeneration blastema (reviewed in [14,15]). The blastema is a specialized structure composed primarily of proliferating cells, and is required for outgrowth. Interestingly, expression of cx43 is up-regulated in the proliferating cells of the blastema [9], and all four sof alleles exhibit reduced levels of cell proliferation during regeneration [12]. Moreover, cx43-gene knockdown also causes reduced levels of cell proliferation [12]. Thus, Cx43 is required to promote cell proliferation during the rapid growth of fin

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regeneration. It remains unclear how Cx43 activity is regulated in this tissue. One possibility is that other connexins accomplish this role.

The zebrafish genome includes a large connexin gene family (n = 37) [16], almost twice the size of human (n = 21) and mouse (n = 20) families. Previously, we identified and characterized cx40.8, a cx43-like gene in the zebrafish genome [17]. The cx40.8 gene shares 80% nucleotide identity with cx43, and is co-expressed with cx43 in the population of dividing cells during fin regeneration. Therefore Cx40.8 is a reasonable candidate for influencing Cx43 function. However, Cx40.8-EGFP localized to intracellular vesicles and not to gap junction plaques when expressed alone in HeLa cells [17], in contrast to typical connexins such as zebrafish Cx43-EGFP [10]. Although, co-transfection with Cx43-mApple permits Cx40.8-EGFP to localize to the plasma membrane in gap junction plaques. Since cx43 and cx40.8 are both expressed in the proliferating cells of the regenerating fin, and since Cx43 and Cx40.8 can co-localize to common gap junction plaques in HeLa cells, one possibility is that Cx40.8 influences Cx43 function in dividing cells. Here, we test this hypothesis during zebrafish fin regeneration. Strikingly, we find that Cx40.8 exhibits differential subcellular localization either to the plasma membrane (during ontogeny) or to the Golgi apparatus (during regeneration). This dynamic localization is dependent on a 30 amino acid sequence immediately following the fourth transmembrane-spanning domain (TM4) of Cx40.8. We also show that the channel properties of Cx40.8 are similar to Cx43, suggesting that Cx40.8 does not directly influence Cx43-based GJIC. Together, these findings reveal that Cx40.8-dependent subcellular localization is correlated with the growth rate of fins, perhaps by regulating a non-channel function of Cx43.

Results

Cx40.8 is differentially localized during ontogeny and regeneration

Previous studies have shown that Cx40.8 is restricted to intracellular vesicles when expressed in HeLa cells [17]. To evaluate Cx40.8 localization in vivo, an antibody was generated against an internal peptide located in the carboxy terminus (see Materials and Methods). First we evaluated antibody-specificity using lysates prepared from regenerating fins. A single band was identified at the expected size (Figure 1A), demonstrating that the antibody recognizes Cx40.8 in fins. Peptide competition experiments confirmed the specificity of the antibody. Antibody recognition for its epitope was challenged by prior incubation with the peptide used to generate the antibody (i.e. “competed”). We prepared lysates from bacteria expressing a GST-Cx40.8CT fusion protein, and loaded increasing amounts of lysate on two identical SDS gels, followed by immunoblotting. One blot was probed with the anti-Cx40.8 antibody and the other was probed with the “competed” antibody. As expected, the immunoblot treated with competed antibody showed reduced antibody binding to the GST-Cx40.8CT fusion protein compared to the non-competed antibody (Figure 1B,C). Together, these results demonstrate that the anti-Cx40.8 antibody is specific and recognizes endogenous Cx40.8 protein by immunoblotting.

Next, Cx40.8 localization in fins was evaluated during ontogenic and regenerative growth by confocal microscopy (Figure 2). During fin regeneration, Cx40.8 is found intracellularly in crescent-like structures adjacent to the nuclei, consistent with localization in the Golgi apparatus (Figure 2B). These results were consistent with previous findings that Cx40.8-EGFP remains in intracellular vesicles in HeLa cells, and were therefore not unexpected. In contrast, during ontogeny, Cx40.8 is observed at the plasma membrane (Figure 2A), suggesting a dynamic localization of Cx40.8 that depends on growth status of the fin. Importantly, Cx43 was not found to exhibit differential localization, but is located at the plasma membrane during both ontogeny and regeneration [12]. Thus, given that the Cx43 and Cx40.8 antibodies exhibit distinct immunolocalization patterns in regenerating fins, the newly developed Cx40.8 antibody does not cross-react with endogenous Cx43. Our findings described above reveal that during the relatively slow growth of ontogeny, Cx40.8 is located at the plasma membrane. During the relatively rapid growth of regeneration, Cx40.8 is located intracellularly. Together with our previous findings that cx43 and cx40.8 are co-expressed in...
the population of dividing cells [17], and that Cx43 is required for cell proliferation [12], it is possible that Cx40.8 localization to the plasma membrane attenuates Cx43-dependent cell proliferation during ontogeny.

We next directly assessed whether Cx40.8 is localized to the Golgi apparatus during fin regeneration. To accomplish this, we utilized a novel zebrafish transgenic line that tags the amino-terminal domain of the Golgi-retained enzyme galactotransferase (GalT) with GFP (i.e. Tg(bact:galT-gfp)). In the transgenic line, GalT-GFP is expressed under the control of the zebrafish β-actin promoter (AV and KCS, in preparation), resulting in GFP-labeled Golgi apparatus in most cells of the fin. We found that Cx40.8 immunostaining co-localizes with GalT-GFP (Figure 3 A–C). To further establish that the two labels co-reside in the Golgi apparatus, we treated regenerating fins with a drug that specifically disrupts the Golgi apparatus, Brefeldin A (BFA, [18]). BFA should cause dispersal of both Cx40.8 and GalT-GFP if they are in fact Golgi residents. In fin rays injected with the carrier DMSO, GalT-GFP and Cx40.8 co-localized as expected (Figure 3 D–F). In contrast, fin rays injected with 10 μg/ml BFA exhibited disruption of both the Cx40.8 signal and the GalT-GFP (Figure 3 G–I). Together, these data provide compelling evidence that newly synthesized Cx40.8 localizes to the Golgi apparatus during fin regeneration. We speculate that it is eventually mobilized to the plasma membrane following the transition from regenerative to ontogenetic fin growth. In order to reveal how Cx40.8 may be retained in the Golgi, we next attempted to define the region of Cx40.8 responsible for its intracellular localization.

A carboxy-terminal domain downstream of TM4 is responsible for the intracellular localization of Cx40.8

Since Cx40.8 is found in intracellular vesicles in HeLa cells but at the plasma membrane when co-transfected with Cx43 [17], we reasoned that Cx40.8 may contain an intrinsic signal regulating its subcellular localization. To test this hypothesis, multiple chimeric forms of Cx43 and Cx40.8 were generated to identify the domain responsible for the intracellular localization of Cx40.8. These chimeras were generated as EGFP fusion proteins in order to directly visualize subcellular localization. Cx43 and Cx40.8 are about 80% identical at the amino acid levels, where the sequences of the carboxy-termini are the least conserved [17]. Therefore, we subdivided the carboxy termini of Cx43 and Cx40.8 into three similarly sized regions, BCD for Cx43 and bcd for the analogous regions in Cx40.8 (Figure 4).

To test whether the carboxy terminus is indeed responsible for subcellular location, we first generated chimeras in which the carboxy terminus of Cx43 and Cx40.8 were swapped (i.e. Cx43-bcd-EGFP contains the N-terminus of Cx43 but the C-terminus of Cx40.8 and Cx40.8-BCD-EGFP contains the N-terminus of Cx40.8 but the C-terminus of Cx43). Results show that Cx40.8-BCD-EGFP forms gap junction plaques at the plasma membrane, resembling the behavior of Cx43, whereas Cx43-bcd-EGFP remains intracellular, resembling the behavior of Cx40.8 (Figure 5C,D). Thus, the putative signal responsible for connexin localization appears to reside in the C-termini of these connexins. To determine more precisely the Cx40.8 localization domain, chimeras were generated between the three different sub-domains. Importantly, swapping the B/b domains had the same effect on

Figure 3. Cx40.8 co-localizes with the GalT-GFP transgene found in the Golgi during regeneration. (A–C) Co-localization in untreated regenerating fins. (D–F). Co-localization in regenerating fins treated with 0.1% DMSO carrier. (G–I) Fins injected with 10 μg/ml BFA/0.1% BFA to disrupt the Golgi show dispersal of both Cx40.8 and GalT-GFP signals. In D-I, nuclei (blue) are stained with TO-PRO3 detected in the far red channel. Arrows point to areas of overlap in F (intact Golgi) and I (remnants of intact Golgi). Scale bars in C and F, 10 μm.
connexin location as swapping the entire carboxy-termini suggesting that this domain is responsible for subcellular localization. Indeed, the effect was reciprocal, such that Cx43-bCD-EGFP was located in intracellular vesicles, while Cx40.8-Bcd-EGFP was found at the plasma membrane in gap junction plaques (Figure 5E,F). In contrast, swapping the C/c or D/d domains did not coincidently reverse the location of the connexins (Figure 5G–J). Together, these findings demonstrate that the carboxy-terminal domain closest to TM4 (i.e. juxta-TM4) determines the subcellular localization of both Cx43 and Cx40.8. A comparison of the sequences of these domains is included in Figure 4.

To establish conclusively that the Cx40.8 "b" domain is responsible for the intracellular localization of Cx40.8 in HeLa cells, two additional constructs were tested. The first construct deletes the "b" region from Cx40.8 (i.e. Cx40.8-cd-EGFP) and results in localization of Cx40.8-cd-EGFP into gap junction plaques at the plasma membrane (Figure 5L). Thus, "b" is necessary for the restriction of Cx40.8 to intracellular vesicles. The second construct, Cx43-bBCD-EGFP, inserts the "b" region of Cx40.8 ahead of the juxta-TM4 region of Cx43. As anticipated, this construct is retained in intracellular vesicles (Figure 5K). However, an alternate "b"-containing construct, Cx43-BbCD-EGFP, allows Cx43 to travel to the plasma membrane (not shown). We conclude from these experiments that the Cx40.8 "b" domain is required for intracellular localization of Cx40.8, and that the location of the "b" sequence in the carboxy terminus contributes to its effects on connexin localization. Indeed, the juxta-TM4 domains of both Cx43 and Cx40.8 appear to directly influence the subcellular localization of each protein (i.e. provided that the domains are located immediately downstream of TM4).

Although EGFP fusions to connexins are widely utilized for following subcellular localization, it remained possible that such fusions could lead to aberrant localization. For example, Cx43-EGFP fusions fail to bind to the recycling factor ZO-1, leading to reduced turnover and expanded gap junctions [19,20]. Other fluorescent proteins have been found to alter the typical trafficking of connexins [i.e. DsRed forms an obligate tetramer, causing connexins to be improperly retained, [21]]. In order to rule out the possibility that the EGFP tag is confounding our results, we next evaluated untagged versions of selected constructs using connexin-specific antibodies. First, we find that untagged Cx43 and untagged Cx40.8 behave as their EGFP-tagged counterparts (Figure 6 A,B). More importantly, we find that the untagged Cx43-bBCD is retained intracellularly, confirming that "b" can impart the intracellular retention of Cx43 when inserted adjacent to TM4.

**Figure 5.** The Cx40.8-b domain is responsible for the intracellular localization of Cx40.8. HeLa cells were singly transfected with EGFP fusions of each construct. (A) Cx43-BCD-EGFP, (B) Cx40.8-bcd-EGFP, (C) Cx43-bcd-EGFP, (D) Cx40.8-bcd-EGFP, (E) Cx43-bcD-EGFP, (F) Cx40.8-bcD-EGFP, (G) Cx43-bCd-EGFP, (H) Cx40.8-bCd-EGFP, (I) Cx43-BCd-EGFP, (J) Cx40.8-bCd-EGFP, (K) Cx43-bBCD-EGFP, (L) Cx40.8-cd-EGFP. Asterisks denote constructs in which the localization depends on a component of the carboxy terminus. Arrows identify gap junction plaques.

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Further, we find that the untagged Cx40.8-cd is able to travel to the plasma membrane and establish gap junction plaques (Figure 6D). These data indicate that the EGFP tag does not influence the trafficking of these proteins. More importantly, these data further confirm that the Cx40.8 "b" directs the intracellular retention of Cx40.8.

Cx40.8 co-assembles with Cx43 in gap junction channels

We previously found that Cx40.8 is capable of co-localizing to gap junction plaques in HeLa cells when co-transfected with Cx43 [17], suggesting that Cx43 and Cx40.8 could associate in common gap junction channels. Increasing the primary magnification during fluorescence microscopy can be used to further investigate this hypothesis [22]. For example, co-transfection of HeLa cells with human Cx26-EGFP and human Cx43-mApple demonstrates that these connexins occupy a common gap junction plaque (Figure 7C,D and [22]). However, discrete domains of green and red are observed, revealing that Cx26-EGFP and Cx43-mApple do not hetero-oligomerize in common connexons, nor do they establish homomeric heterotypic gap junction channels (i.e. homomeric heterotypic channels would be comprised of one Cx26-EGFP connexon and one Cx43-mApple connexon). In contrast, co-transfection of human Cx43-EGFP and human Cx43-mApple results in plaques that are uniformly yellow, suggesting that the two connexins are located in common gap junction channels (Figure 7A,B). Similarly, when zebrafish Cx43-mApple was co-expressed with zebrafish Cx40.8-EGFP, the resulting gap junction plaques were yellow (Figure 7E,F). This finding suggests that Cx43 and Cx40.8 co-assemble into common gap junction channels when co-expressed in HeLa cells (see Figure S1 for single channel images).

Co-association of Cx43 and Cx40.8 in common gap junction channels may cause novel functional properties, such as modulation of channel permeability or electrical-gating properties. We addressed this possibility by assessing the channel properties of Cx40.8-cd-EGFP alone, which is capable of trafficking to the plasma membrane and establishing gap junction plaques (compared to full length Cx40.8 which does not). We performed dual whole cell voltage clamp in N2a cells expressing either Cx43-

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**Figure 6. Untagged Cx43 and Cx40.8 constructs behave similarly as GFP tagged constructs in transiently transfected HeLa cells.** (A) Cx43 localizes to the plasma membrane. (B) Cx40.8 is retained intracellularly. (C) Cx43-bBCD is retained intracellularly. (D) Cx40.8-cd localizes to the plasma membrane. The Cx43 antibody [12] was used to detect untagged Cx43 and untagged Cx43-bBCD. The Cx40.8 antibody was used to detect untagged Cx40.8 and untagged Cx40.8-cd. Flared arrows identify gap junction plaques located at the plasma membrane; Plain arrows identify the plasma membrane in the absence of gap junction plaques; n, nucleus. doi:10.1371/journal.pone.0031364.g006

**Figure 7. Cx43-mApple and Cx40.8-EGFP co-assemble in common gap junction channels.** High resolution fluorescence microscopy was used to provide evidence for co-association of Cx43-mApple and Cx40.8-EGFP in common gap junction channels. Constructs that were co-transfected in HeLa cells are indicated to the left of the panels (A, B) Homo sapiens (Hs) Cx43-mApple + Hs Cx43-EGFP show uniformly yellow plaques, suggesting co-association. (C, D) Hs Cx43-mApple + Hs Cx26-EGFP show discrete green and red domains, revealing a lack of co-association. Arrows indicate green Hs Cx26-EGFP localization and arrowheads indicate red Hs Cx43-mApple localization. (E, F) Danio rerio (Dr) Cx43-mApple + Dr Cx40.8-EGFP show uniform yellow distribution. doi:10.1371/journal.pone.0031364.g007
EGFP or Cx40.8-cd-EGFP. Transjunctival current ($I_j$) was measured while transjunctival voltage ($V_j$) was varied up to ±180 mV in 30 mV steps. Voltage was stepped in one cell of the pair while the other cell remained clamped at 0 mV. Step duration was either 400 ms or 4 s. Representative current families from the 4 s protocol for cell pairs expressing Cx43-EGFP and Cx40.8-cd-EGFP constructs are shown (Figure 8 A,B). Cx43-EGFP-expressing pairs showed voltage dependent inactivation over the course of the 4 s voltage step, with transjunctival conductance ($G_j$) declining to 50.4 ± 21.5% at $V_j$ of +180 mV ($n = 4$), confirming results obtained previously from paired oocyte recordings [10]. Similarly, Cx40.8-cd-EGFP containing plaques inactivated to 44.6 ± 5.6% ($n = 6$). Data for the 400 ms protocols followed this trend (Cx43: 74.1 ± 14.0%, $n = 7$; Cx40.8-cd: 78.5 ± 17.2%, $n = 9$). Steady state $G_j$ from the 4 s protocol was plotted as a function of $V_j$, and normalized to the conductance at ±30 mV (Figure 8C). We find that the steady state $G_j$ was similar over most of the voltage range for both constructs. These results, taken together with the high sequence homology of the two connexins [17], reveal that voltage-dependent conductance properties of Cx43 and Cx40.8 are comparable. We cannot rule out the possibility that removal of the “b” domain influences the voltage-dependent conductance of Cx40.8. However, given that the domains predicted to regulate voltage-dependent channel activity (reviewed in [23]) are highly similar between Cx40.8 and Cx43, the finding that their voltage properties are also similar is not unexpected. We therefore conclude that it is unlikely that Cx40.8 dramatically modulates the electrical properties of Cx43 channels. Other possibilities for how Cx40.8 may regulate Cx43 function will be evaluated in future experiments.

**Discussion**

The four major findings of this study elucidate a novel mechanism that may regulate gap junction assembly and function. First, Cx40.8 exhibits dynamic subcellular localization in zebrafish fins, where it is retained in the Golgi apparatus during regeneration and is later mobilized to the plasma membrane during ontogeny. Thus, Cx40.8 retention is associated with the rapid growth during regeneration, and Cx40.8 at the plasma...
Subcellular Localization of Cx40.8

Materials and Methods

Statement on the ethical treatment of animals

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols used for this manuscript were approved by Lehigh's Institutional Animal Care and Use Committee (IACUC) [identification #488, approved 6-3-2011]. Lehigh University’s Animal Welfare Assurance Number is A-3077-01.

Fish maintenance

The fish used in this study were derived from the C32 strain and were raised in a 14 light:10 dark photoperiod at 25°C [30]. Tg(bact:galt-gfp) zebrafish were generated using Invitrogen Gateway technology to fuse amino acids 1–6 from human BAG1L with GFP under the control of the beta-actin promoter and inserted into the zebrafish genome using sites derived from the tol2 transposon. Lines were maintained on the TAB14 background.

Immunoblots and Cx40.8 antibody

Amino acids 269–285 (CSAPVPNLGYNLDTVDK) of zebrafish Cx40.8 were chosen as the antigen in conjunction with Quality Controlled Biochemicals (www.qcb.com). QCB completed peptide synthesis, immunization, rabbit maintenance, and affinity purifications of bleeds. Escherichia coli lysates expressing GST-Cx40.8CTI fusion protein were grown to confluency before adding 0.3 mM IPTG to induce protein expression. Lysates were prepared using 50 µg/ml lysozyme in lysis buffer (100 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM EDTA pH 8.0, complete protease inhibitor cocktail, Roche). In addition, 2.2 N NaOH and 8% BME were added to the mixture. Total protein was precipitated using TCA and pellets were resuspended in SDS buffer. Samples were diluted in SDS buffer and decreasing volumes of sample were loaded in a total volume of 20 µL for the antibody competition, identical gels were prepared. The anti-Cx40.8 antibody was either used directly (1:2000) or following pre-incubation with the peptide made as the Cx40.8 antigen. Wildtype
fins (5 dpa regenerates) were homogenized in homogenization buffer (5 mM Tris- HCl, 5 mM EDTA, 5 mM EGTA pH 8.0, 0.1 mM PEFABLOC, 1 mM Na3VO4, 1 mM Na2VO4 protease inhibitor cocktail) using a 5 mm generator (Pro 200 homogenizer, ProScientific, Rockford, IL). Protein samples were separated using 12% SDS-PAGE before transfer to nitrocellulose membranes. Following transfer, blots were rinsed in 40% isopropanol, rinsed in distilled water, and then blocked in 5% milk in Super TBST for 30 min at room temperature. Blots were then incubated with anti-Cx40.8 (1:2000) for 1 hr at room temperature, rinsed for 40 min in TBST, and then incubated with peroxidase-conjugated goat anti-rabbit IgG (1:250,000, pre-absorbed with fin tissue, Thermo Scientific, Rockford, IL) for 1 hr at room temperature. Following incubation, blots were rinsed in TBST for 40 min at room temperature. Using the Amersham ECL Plus Western Blotting Detection System (GE Healthcare, UK), blots were developed and exposed to X-ray film (CL-XPosure film, Thermo Scientific, Rockford, IL).

Immunofluorescence and confocal microscopy

Wildtype fins (9–12 months for ontogenetic fins, or 5 dpa regenerates) were harvested and fixed in 2% PFA in 0.1 mM phosphate buffer (PB) for 30 min at room temperature. Fins were washed (3×10 min) in 25 mM PB and incubated in Trypsin/EDTA (Gibco) for 10 min on ice. Fins were next blocked (50 mM Tris-HCl; pH 7.4, 250 mM NaCl, 0.3% Triton-X 100, 6% goat serum) for 30 min at room temperature and incubated with Cx40.8 (1:200) antibody overnight at 4°C. After incubation, fins were washed in 25 mM phosphate buffer (3×5 min) and incubated in goat anti-rabbit Alexa 488 (1:200, Molecular probes) antibody and propidium iodide for 2 hr at room temperature followed by 1× PBS washes (3×10 min). For Tg(bact:galT-gfp), 5 dpa regenerates were harvested and processed identically except for the antibodies. For primary antibodies, the rabbit Cx40.8 antibody (1:200) and the mouse EGFP (Clontech, 1:100) antibodies were used together. The secondary antibodies were the goat anti-rabbit Alexa 546 and the goat anti-mouse Alexa 488. In the experiments with Brefeldin A (BioLegend, San Diego, CA), the goat anti-rabbit Alexa 546 and the goat anti-mouse Alexa 488. Antibodies were used together. The secondary antibodies were the goat anti-rabbit Alexa 546 and the goat anti-mouse Alexa 488. When paired with a 5 μg of plasmid DNA and imaged 21–24 hours later. Transfections were evaluated by standard immunofluorescence on a Nikon Eclipse E80 microscope. For double transfections, 1 μg of each plasmid was transfected. Analyses were completed on a Nikon Eclipse TE2000-U using the 100× lens in combination with a 1.5× optivar to increase the primary magnification.

Dual whole cell patch clamping

For dual whole cell voltage clamp recording, the poly-L-lysine cover glass was placed into a chamber at room temperature perfused with oxygenated extracellular solution (containing in mM: 135 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 1 BaCl2, 5 dextrose, 5 HEPES, and 2 pyruvate, pH 7.2–7.4). N2a cells were imaged on a Nikon (EF-4) Phystoiated equipped with epifluorescence and infra red differential interference contrast optics. GFP positive cell pairs expressing connexin constructs were located by fluorescence and imaged using a Hamamatsus C5700 video camera (Hamamatsu City, Japan). Cells contacting multiple GFP positive neighbors were excluded. Patch pipettes were pulled from thick walled borosilicate glass capillary tubes (WPI 1B120F-4) using a two-stage puller (Narishige PC-10, Tokyo, Japan) to a resistance of 4–6 MΩ when back-filled with internal solution (containing in mM: 125 CsCl, 0.5 CaCl2, 10 EGTA, and 10 HEPES, pH 7.2). Patch pipettes were then brought into contact with cells using motorized micromanipulators (Model 7500, Siskiyou Instruments) and gigahm seals were established. Whole cell configuration was achieved and cells were initially voltage clamped to 0 mV using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA). Series resistance was compensated at 60–80%. The signal was filtered at 1–2 kHz and digitized at 10 kHz with a Digidata 1440 data acquisition board. Recording protocols were generated using Clampex 10.2 software (Molecular Devices, Sunnyvale, CA).

Voltage clamp methods followed [31]. Trans junctional potential (Vj) was varied up to ±180 mV in steps of 30 mV. Step duration was either 400 ms or 4 s. Recordings were analyzed using Clampex 10.2 software. Trans junctional conductance values were
calculated from mean transjunctonal current (Ij) amplitudes from a 15 ms window measured at 5 ms after pulse onset. Steady state Gj was computed from Ij measured at 5 ms before the termination of the pulse. Gj was maximal and generally linear at voltages <60 mV so steady state Gj was normalized to the conductance value calculated at ±30 mV. Data in the results text are expressed as percent of maximum Gj ± SD. Error bars in figures represent SE.

Supporting Information

Figure S1 Cx43-mApple and Cx40.8-EGFP co-assemble in common gap junction channels. High resolution fluorescence microscopy was used to provide evidence for co-association of Cx43-mApple and Cx40.8-EGFP in common gap junction channels. Plane views are shown. Top: In HeLa cells co-transfected with Hs-Cx43-EGFP and Hs-Cx43-mApple, both green and red plaques completely overlap. Middle: In HeLa cells co-transfected with Hs-Cx26-EGFP and Hs-Cx43-mApple, discrete domains of green and red plaques are observed. Bottom: In HeLa cells co-transfected with Dr-Cx40.8-EGFP and Dr-Cx43-mApple, both green and red plaques completely overlap. (TIF)

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Author Contributions

Conceived and designed the experiments: SVG MKI RMB KCS. Performed the experiments: SVG DME RMB SNO AV RJ MKI. Analyzed the data: SVG DME RMB SNO AV KCS RJ MKI. Contributed reagents/materials/analysis tools: RMB AV KCS. Wrote the paper: SVG DME RMB SNO KCS MKI.

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