# The organization of adherens junctions and desmosomes at the cardiac intercalated disc is independent of gap junctions

**David E. Gutstein*, Fang-yu Liu, Marian B. Meyers, Andrew Choo and Glenn I. Fishman**

Division of Cardiology, Department of Medicine, New York University School of Medicine, 550 First Avenue, New York, NY 10016, USA
*Author for correspondence (e-mail: david.gutstein@med.nyu.edu)

*Accepted 6 November 2002*
*Journal of Cell Science 116, 875-885 © 2003 The Company of Biologists Ltd*
*doi:10.1242/jcs.00258*

## Summary

**Adherens junctions and desmosomes are responsible for mechanically coupling myocytes in the heart and are found closely apposed to gap junction plaques at the intercalated discs of cardiomyocytes. It is not known whether loss of cardiac gap junctions, such as described in cardiac disease states, may influence the expression patterns of other intercalated disc-associated proteins. We investigated whether the major cardiac gap junction protein connexin43 (Cx43) may be responsible for regulating adherens junctions, desmosomes and their associated catenins, in terms of abundance and localization at the intercalated discs of cardiomyocytes. In order to study the effect of loss of cardiac gap junctions on the intercalated disc-associated proteins, we used a combination of immunoblotting, immunofluorescence with confocal microscopy and electron microscopy to evaluate heart tissue from mice with cardiac-specific conditional knockout of Cx43. We found that the cardiac adherens junctions, desmosomes and their associated catenins, as well as vinculin and ZO-1, maintain their normal abundance, structural appearance and localization in the absence of Cx43. We conclude from these data that Cx43 is not required for the organization of the cell adhesion junctions and their associated catenins at the intercalated disc in the adult cardiac myocyte.**

Key words: Connexin43, Gap junction, Adherens junction, Desmosome, Cadherin, Desmoplakin

## Introduction

Cardiac contractile function is dependent upon the highly coordinated electrical and mechanical activation of the heart's constituent myocytes. Thus, it is perhaps not surprising that the adherens junctions and desmosomes, which are responsible for mechanically coupling cardiac myocytes, are closely juxtaposed to the gap junctions at the intercalated disc (Angst et al., 1997; Peters et al., 1994). Despite significant progress in understanding the structural biology of each of these junctional components, a detailed characterization of the inter-relationships between electrical and mechanical junctions is lacking. Studies of connexin43 (Cx43) heterozygous knockout mice, in which expression of gap junction protein is reduced by about half, show no significant changes in adherens junction expression (Saffitz et al., 2000). By contrast, in experimentally induced myocardial infarction, loss of gap junctions in the peri-infarct region is accompanied by downregulation of cell adhesion junctions whereas, in transgenic murine models of cardiomyopathy, gap junction remodeling is associated with upregulation of adherens junction proteins and no changes in desmosomal protein expression (Ehler et al., 2001; Matsushita et al., 1999). Additionally, pulsatile stretch of cultured myocytes induces marked coordinate upregulation of cardiac gap junction and adherens junction proteins (Zhuang et al., 2000). These disparate observations leave unresolved the question of whether Cx43 abundance can directly influence the expression pattern of adherens junctions and desmosomes.

Adherens junctions, consisting of classical cadherins, link the intercalated disc to the actin cytoskeleton. Desmosomes, in which desmoplakin is a required component, attach to intermediate filaments (Gallicano et al., 1998). Knockout studies have revealed devastating consequences to heart development and fetal viability in the absence of adherens junctions and desmosomes, underscoring their importance to normal cardiac development and function (Gallicano et al., 1998; Ruiz et al., 1996). Adherens junctions and desmosomes both associate with catenin family proteins, which regulate cell-to-cell adhesion and the structure of the junctions at the intercalated disc (Linask et al., 1997; Mathur et al., 1994; Ruiz et al., 1996; Sacco et al., 1995; Zhurinsky et al., 2000a; reviewed by Zhurinsky et al., 2000b). In addition, β-catenin and p120 catenin are thought to function directly in signaling pathways (Anastasiadis et al., 2000; Daniel and Reynolds, 1999; Miller et al., 1999; Noren et al., 2000). Recent data have suggested that loss of Cx43 may be associated with alterations in the intracellular localization of p120 catenin and, thus, may affect p120 catenin-dependent signaling pathways (Xu et al., 2001). Furthermore, forced expression of the gap junction channel protein may influence β-catenin-dependent signaling (Ai et al., 2000).

In this study, using conditional knockout (CKO) mice in which Cx43 expression in cardiomyocytes is virtually absent (Gutstein et al., 2001a), we investigated whether cardiac gap junctions may be responsible, at least in part, for the structural

integrity of the intercalated disc. We also examined whether loss of cardiac gap junctions may lead to altered localization of the catenins associated with the intercalated disc, which would suggest the possibility of altered catenin-dependent signaling. Using a combination of immunoblotting, immunofluorescence with confocal microscopy and electron microscopy (EM) on CKO heart samples, we found that the adherens junctions and desmosomes appear structurally intact in the absence of Cx43. The distribution of other junction-associated proteins, such as the catenins, vinculin and ZO-1, is also unchanged in the Cx43 CKO hearts. We conclude from these data that Cx43 is not necessary for the organization of the cell adhesion junctions and their associated catenins at the intercalated disc of the postnatal cardiac myocyte.

## Materials and Methods

### Animal model

Cx43 CKOs (α-MHC-Cre:$Cx43^{flox/flox}$) were obtained by mating heterozygous CKO mice (α-MHC-Cre:$Cx43^{+/flox}$) with mice homozygous for the 'floxed' Cx43 allele ($Cx43^{flox/flox}$). $Cx43^{flox/flox}$ littermates were used as controls. One-month-old sex-matched CKO and control mice were used for all experiments.

### Antibodies

The Cx43 polyclonal antibody (custom manufactured by Research Genetics, Huntsville, AL) was directed against the same epitope used by Yamamoto et al. (Yamamoto et al., 1990). Other polyclonal primaries included: anti-α-catenin (Santa Cruz Biotechnology, Santa Cruz, CA); anti-desmoplakin (Serotec, Raleigh, NC); and anti-pan-cadherin (Sigma). Monoclonal primary antibodies included: anti-Cx43 and -ZO-1 (Zymed Laboratories, San Francisco, CA); anti-N-cadherin, -β-catenin and -p120 catenin (BD Transduction Laboratories, Lexington, KY); anti-pan-cadherin, -vinculin and -β-tubulin (Sigma-Aldrich, St Louis, MO); and anti-plakoglobin (Chemicon International, Temecula, CA).

Secondary antibodies included horseradish peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse IgG (Santa Cruz Biotechnology) for western blotting, and FITC- or Texas Red-conjugated goat anti-rabbit or anti-mouse antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) for immunofluorescence.

### Immunoblotting and densitometry

For the evaluation of total ventricular protein levels by immunoblotting, ventricular tissue was Dounce homogenized in the presence of Complete protease inhibitor cocktail (Roche, Mannheim, Germany). Equivalent amounts of protein per sample as determined by Bradford assay and confirmed with Coomassie staining were electrophoresed on SDS-PAGE gels and transferred onto nitrocellulose (Bio-Rad Laboratories, Hercules, CA). Blots were blocked followed by incubation with appropriate primary and secondary HRP-conjugated antibodies. Signal was detected with ECL chemiluminescent processing (Amersham Pharmacia Biotech, Little Chalfont, UK) and autoradiography. At least two separate experiments per protein were quantified by scanning the autoradiograms on a Bio-Rad Gel Doc 1000 and calculating band intensity with Quantity One software (Bio-Rad Laboratories).

### Fraction preparation and immunoprecipitation

To determine cytosolic and plasma membrane catenin concentrations, fractionation of samples was performed as described (Atkinson, 1973).

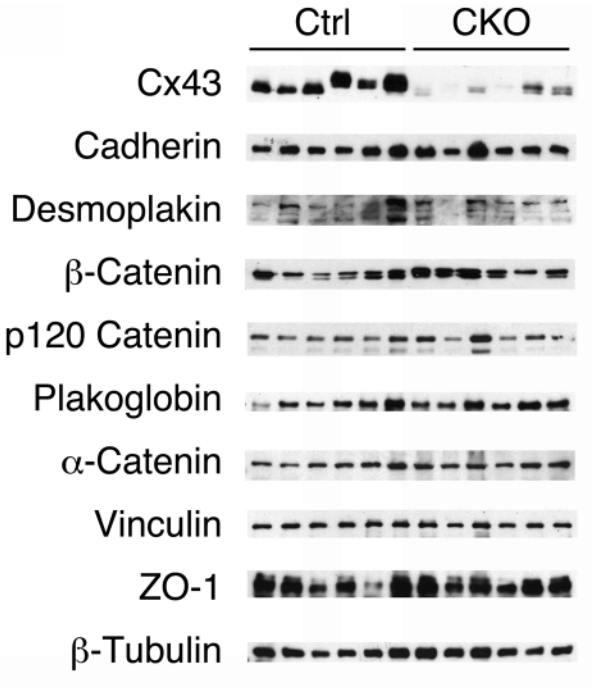



**Fig. 1.** Immunoblotting of intercalated disc-associated proteins in Cx43 CKO hearts. Immunoblots of total ventricular lysates using specific antibodies directed against components of the cardiac intercalated disc. Proteins detected are indicated to the left of each blot. Immunoblots represent either a re-probed or duplicate blot, with the same heart lysate loaded on the same lane for each of the different antibodies.

Briefly, heart samples were Dounce homogenized, centrifuged at 500 ***g*** for 10 minutes to remove insolubles and layered over a 45% sucrose cushion. After centrifuging at 7000 ***g*** for 20 minutes, the supernatant (cytosolic fraction) was separated from the cloudy layer immediately overlying the sucrose (plasma membrane fraction). The resulting cytosolic and plasma membrane fractions were analyzed by SDS-PAGE.

For immunoprecipitation, 150 μg of plasma membrane protein was incubated with polyclonal anti-pan-cadherin antibody. After addition of protein A-agarose-immobilized protein beads (Roche) to the samples, the protein A suspension was centrifuged at 5000 ***g*** and supernatant was removed. The protein A beads were washed in IP buffer and resuspended in Laemmli buffer (Laemmli, 1970) prior to incubation at 100°C and analysis by SDS-PAGE.

### Immunofluorescence and confocal microscopy

For immunofluorescence, hearts were frozen in Tissue Tek OCT compound (Sakura Finetek USA, Inc., Torrance, CA) upon sacrifice. Frozen sections (6 μm thick) were cut in an HM 560 cryostat (Microm, Walldorf, Germany) at –20°C, placed onto Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA) and fixed in acetone. The sections were blocked in PBS with 5% normal goat serum, 0.1% bovine serum albumin (BSA) and 0.1% sodium azide ($NaN_3$) at 37°C for 30 minutes and then incubated with primary antibodies in PBS with 1% BSA and 0.1% $NaN_3$ for 2 hours at 37°C. After washing in PBS, sections were incubated with secondary antibodies in PBS with 0.1% BSA and 0.1% $NaN_3$ at 37°C for 1 hour. Sections were washed again in PBS and mounted with Vectashield mounting medium (Vector Laboratories, Berlingame, CA).

Immunostained sections were examined with a TCS-SP confocal laser scanning microscope (Leica, Heidelberg, Germany). Double-stained sections were visualized after ensuring that the settings were

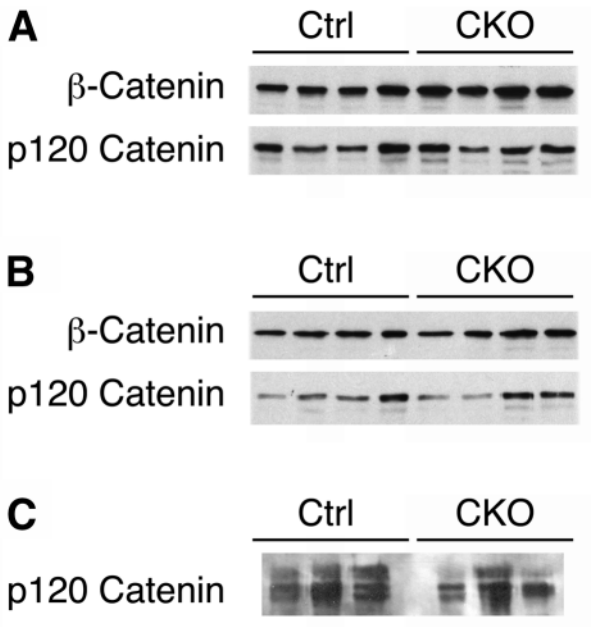



**Fig. 2.** Subcellular localization of β-catenin and p120 catenin in Cx43 CKO hearts. (A) Immunoblot analysis of cytosol fractions from control and CKO mouse hearts for the presence of β-catenin and p120 catenin. (B) Immunoblot analysis of neat plasma membrane fractions from control and CKO mouse hearts for the presence of β-catenin and p120 catenin. Coomassie staining was used to ensure equivalent loading of protein per lane in the cytosol and plasma membrane blots. (C) Immunoprecipitation of plasma membrane fractions with an anti-pan-cadherin antibody followed by blotting with an anti-p120 catenin antibody demonstrates an association of cadherin with p120 in control and CKO hearts.

such that there was no cross-over of signal between the FITC and Texas Red channels. Experiments were conducted in a minimum of four control and CKO hearts simultaneously, at least in duplicate.

For the relative quantification of adherens junction and desmosomal junction areas at the intercalated disc, images of intercalated discs in short-axis were collected in series (Gourdie et al., 1991; Kaprielian et al., 1998). Stacked images of intercalated discs were traced with IPLab software (Scanalytic, Fairfax, VA) for the calculation of area and mean fluorescence (after correcting for background) of each intercalated disc. For presentation only, stacked images of intercalated discs were deconvolved using Microtome deconvolution software (VayTek, Fairfield, IA).

### Electron microscopy

For EM, mice were anesthetized with pentobarbital prior to perfusion with 150 mM KCl and 5000 U/L heparin, followed by PBS. For

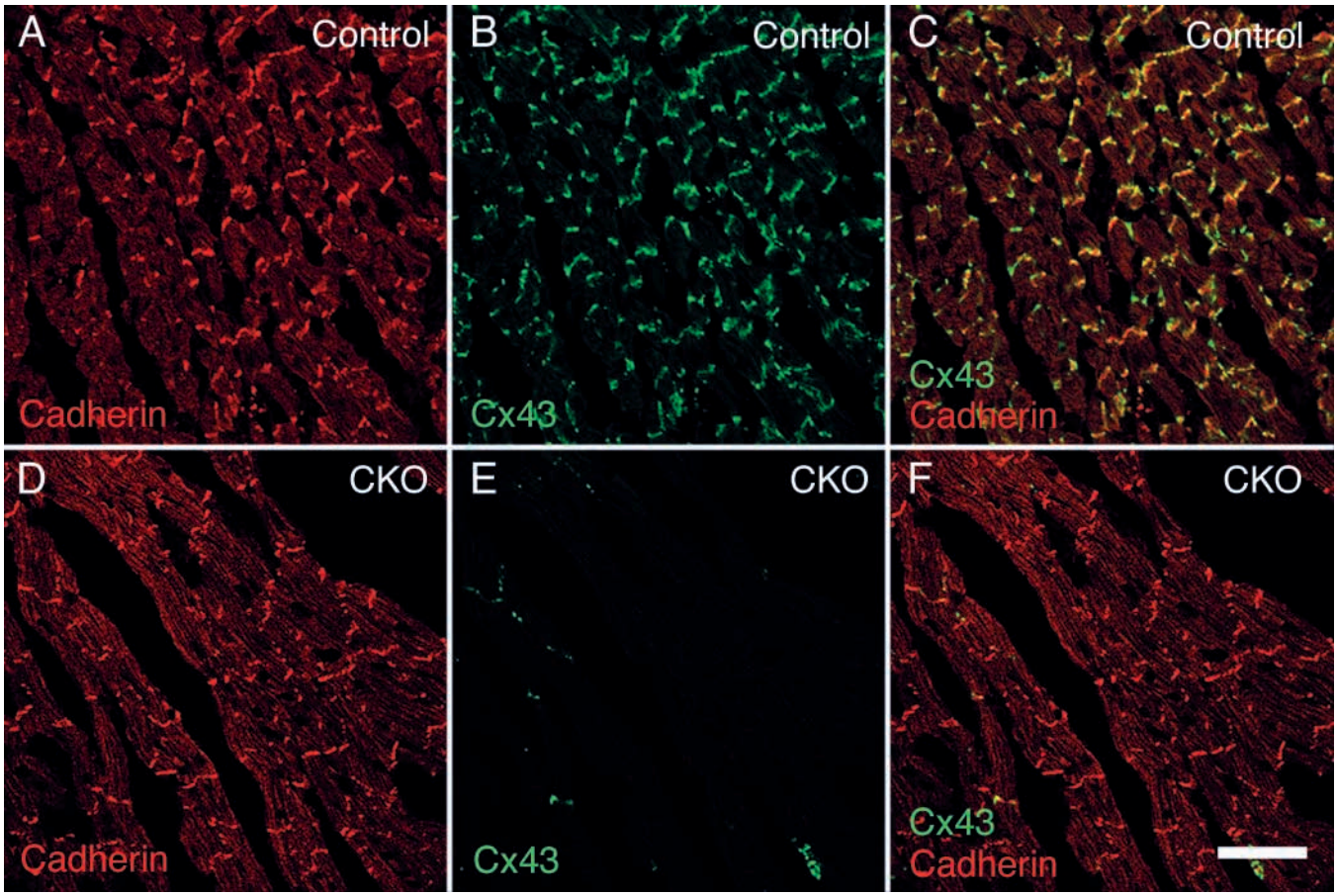



**Fig. 3.** Immunofluorescent staining for cadherin in control and Cx43 CKO mouse heart sections. Heart sections were double stained with a mouse anti-pan-cadherin antibody (A, control; D, CKO), and a rabbit anti-Cx43 antibody (B, control; E, CKO) and imaged with a confocal microscope. Merged images are shown in C (control) and F (CKO) and demonstrate that adherens junction localization to the intercalated disc, indicated by cadherin staining, is unchanged in the absence of Cx43. Magnification, 40×; bar, 50 μm.

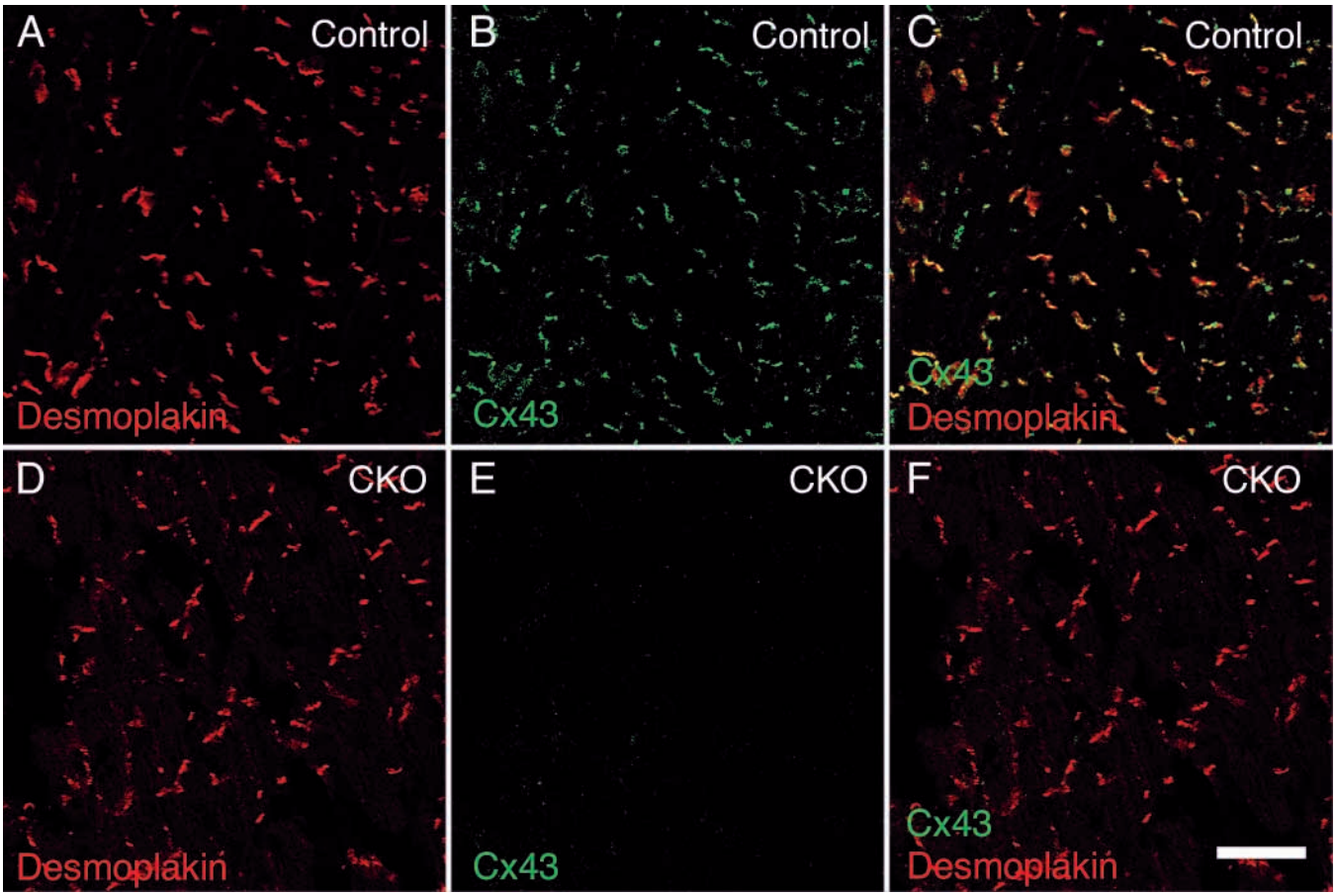



**Fig. 4.** Immunofluorescent staining for desmoplakin in control and Cx43 CKO mouse heart sections. Heart sections are double stained for desmoplakin (A, control; D, CKO) and Cx43 (B, control; E, CKO) and imaged with a confocal microscope. Merged images are shown in C (control) and F (CKO). No change in the pattern of desmoplakin staining in CKO hearts is evident in comparison with controls. Magnification, 40×; bar, 50 μm.

morphology, hearts were then perfusion fixed with 3% paraformaldehyde, 1% glutaraldehyde. The apical segment was then removed and immersed in fresh fixative, post-fixed in 1% $OsO_4$, stained with 1% uranyl acetate, dehydrated and embedded in Epon.

For immuno-EM, hearts were perfusion fixed with 3% paraformaldehyde in PBS. Apical segments were removed, washed in PBS and incubated in 0.05 M $NH_4Cl$, 0.05 M PBS, followed by dehydration and embedding in Lowicryl K4M (Electron Microscopy Sciences, Fort Washington, PA). For the immunoreaction, incubation with primary antibody was followed by application of protein A conjugated to 10 nm gold particles. Sections were viewed on a Hitachi 7000 electron microscope.

### Statistics

Data are expressed as mean±s.e. and comparisons between groups were performed with a two-tailed *t*-test using Microsoft Excel software. $P<0.05$ was considered statistically significant.

## Results

### Cadherin, desmoplakin and associated catenins are unchanged in overall abundance in the absence of Cx43

In order to study the regulation of adherens junction and desmosomal expression in the absence of gap junctions, we first evaluated the abundance of these proteins by immunoblotting for junctional and related proteins in total ventricular lysates from control and CKO hearts. As expected from our previous studies (Gutstein et al., 2001a), Cx43 levels were significantly reduced in the CKO hearts to 7.9±3.5% of the control levels ($P<0.05$). By contrast, expression of cadherins and desmoplakin were unchanged, indicating no difference in the overall abundance of these proteins in the absence of gap junctions. Furthermore, total ventricular levels of α- and β-catenin, plakoglobin and p120 catenin were unchanged in the absence of Cx43. Ventricular levels of the structural proteins vinculin and ZO-1 were also no different in the CKO hearts compared with controls. Interestingly, despite the reported interaction of β-tubulin and Cx43 (Giepmans et al., 2001), we found no change in β-tubulin expression in CKO hearts. Representative immunoblots from this series of experiments are shown in Fig. 1 and quantitative densitometry data are presented in Table 1.

### Deficiency of Cx43 does not affect subcellular localization of β- or p120 catenin

Recent data have shown that the subcellular localization of

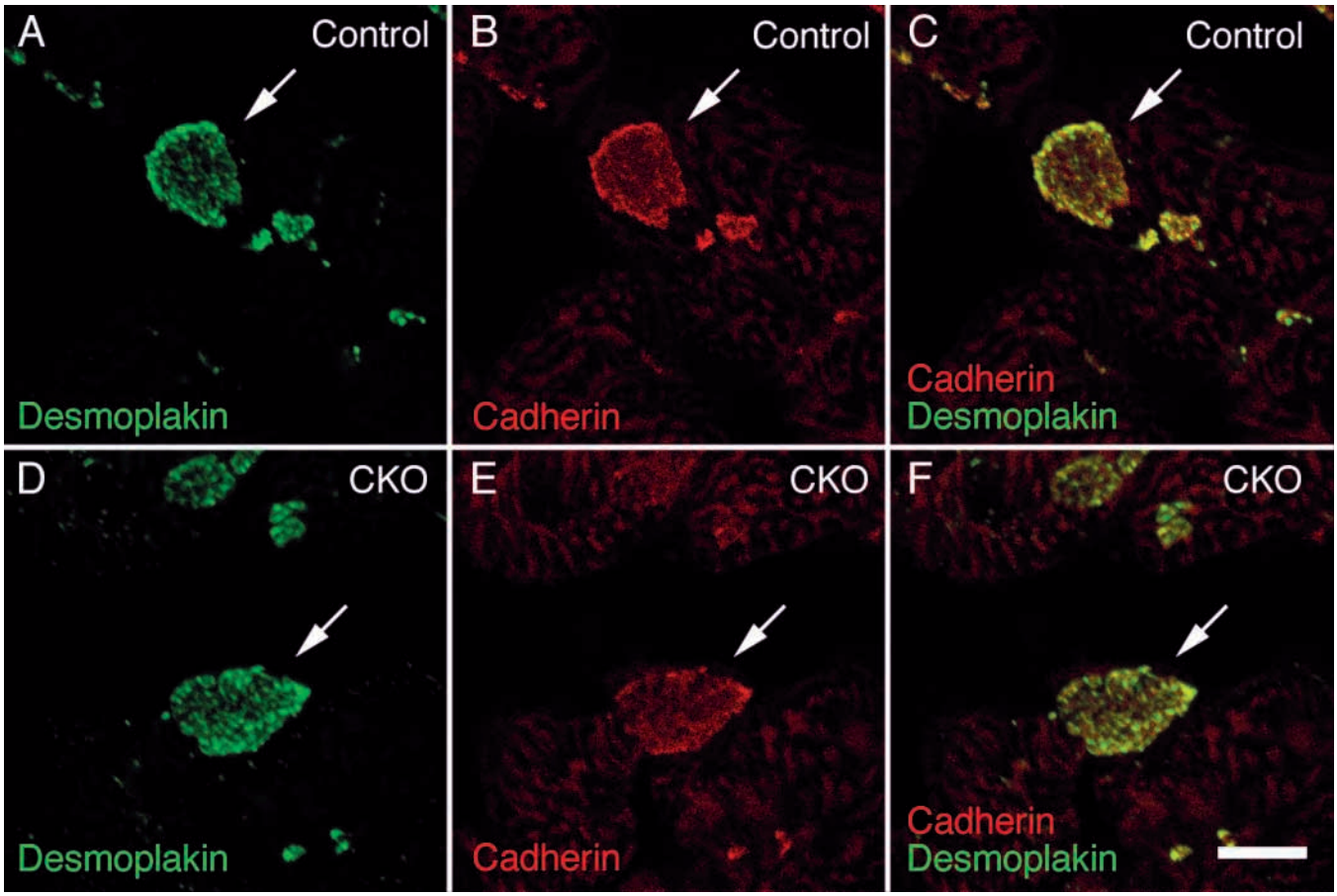



**Fig. 5.** Immunofluorescent images of desmoplakin and cadherin double-stained control and CKO hearts. Shown are stacked confocal images through the intercalated discs in en-face orientation (arrows) in mouse heart sections double stained for desmoplakin (A, control; D, CKO) and cadherin (B, control; E, CKO). Merged images (C, control; F, CKO) suggest that desmoplakin and cadherin are juxtaposed, but in general not co-localized, at the intercalated discs, in a similar pattern in both control and CKO hearts. Magnification, 63×; zoom, 2.96; bar, 10 μm.

p120 catenin is altered in the Cx43 germline knockout mouse, suggesting that p120 catenin-dependent intracellular signaling may also be altered, possibly accounting for some of the pathology associated with the loss of Cx43 (Xu et al., 2001). Since cytosolic β-catenin concentration mirrors its nuclear level (Giarre et al., 1998) and p120 catenin may complex with kaiso in the cytosol (Daniel and Reynolds, 1999), cytosolic levels of these catenins probably reflect their availability to influence signaling. In addition to their role in intracellular signaling, p120 catenin and β-catenin associate with cadherins at the plasma membrane. We studied both the cytosolic and plasma membrane fractions from four control and four CKO hearts. Levels of both β-catenin and p120 catenin were unaltered in the cytosolic fraction (Fig. 2A). Similarly, levels of both β-catenin and p120 catenin in neat plasma membrane fractions were not significantly changed in the CKO hearts compared with controls (Fig. 2B).

**Table 1. Expression of intercalated disc proteins**

| | Control | CKO | *P* value |
|---|---|---|---|
| Total ventricular lysate | | | |
| Cx43 | 1.00±0.32 | 0.079±0.035 | 0.017 |
| Cadherin | 1.00±0.11 | 1.01±0.11 | 0.97 |
| Desmoplakin | 1.00±0.23 | 0.68±0.16 | 0.29 |
| β-catenin | 1.00±0.15 | 1.34±0.13 | 0.12 |
| p120 catenin | 1.00±0.10 | 1.05±0.24 | 0.84 |
| α-catenin | 1.00±0.15 | 1.44±0.20 | 0.11 |
| Plakoglobin | 1.00±0.21 | 1.29±0.16 | 0.29 |
| Vinculin | 1.00±0.09 | 1.11±0.16 | 0.56 |
| ZO-1 | 1.00±0.25 | 1.12±0.14 | 0.50 |
| β-tubulin | 1.00±0.14 | 1.10±0.16 | 0.67 |
| Cytosolic fraction | | | |
| β-catenin | 1.00±0.05 | 0.91±0.05 | 0.24 |
| p120 catenin | 1.00±0.17 | 0.95±0.15 | 0.86 |
| Plasma membrane fraction | | | |
| β-catenin | 1.00±0.06 | 1.09±0.09 | 0.45 |
| p120 catenin | 1.00±0.28 | 1.08±0.27 | 0.85 |

Data are expressed as mean±s.e.m.

While p120 catenin concentration at the plasma membrane in the CKO hearts remained unchanged, we sought to determine whether p120 catenin remained associated with cadherin in the absence of Cx43 by using immunoprecipition. As demonstrated by immunoprecipitation of plasma membrane fractions with an anti-pan-cadherin antibody followed by blotting for p120 catenin, in the absence of Cx43 p120 catenin remained associated with cadherin at the plasma membrane (Fig. 2C).

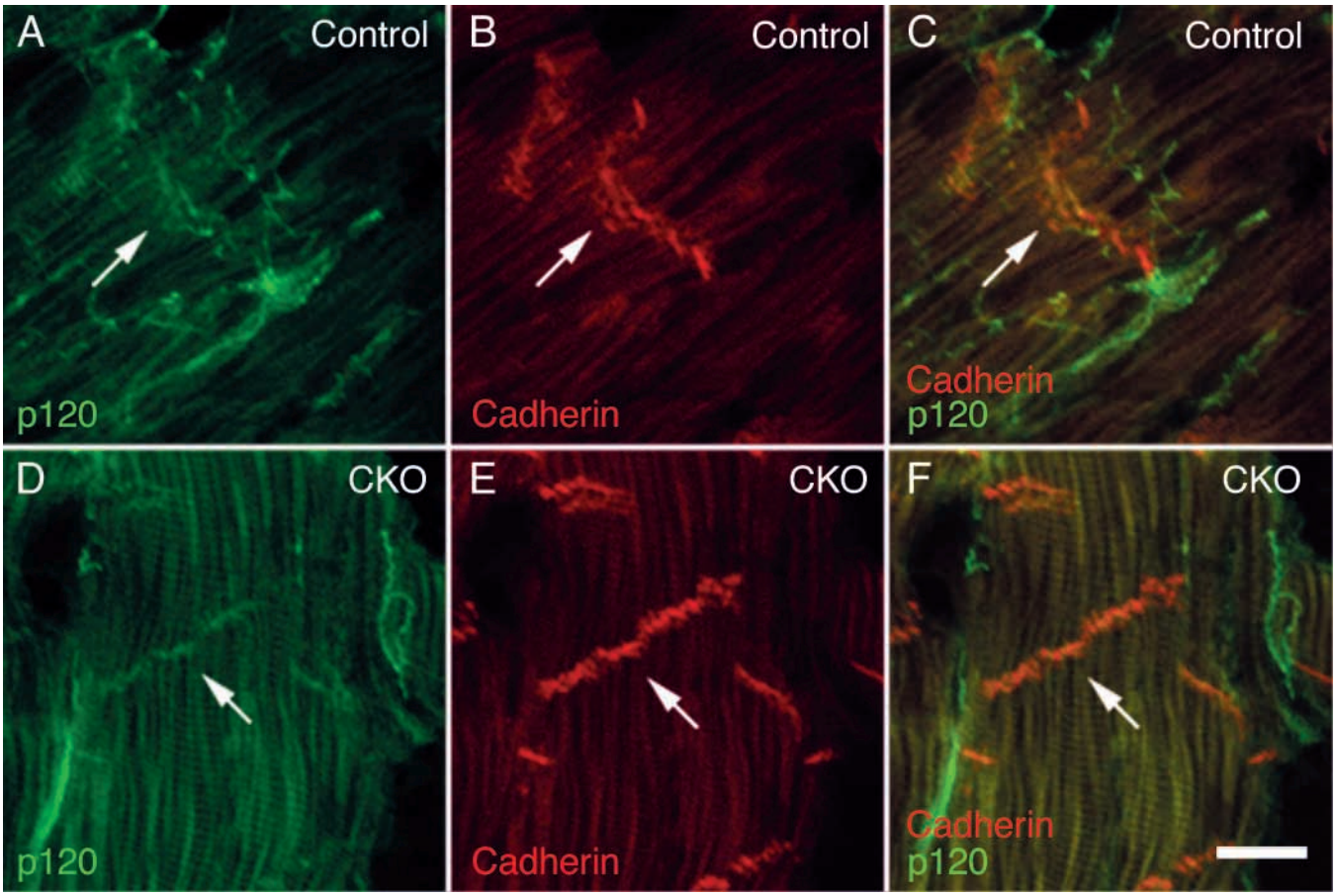



**Fig. 6.** p120 co-localizes with cadherin at the intercalated disc in control and CKO hearts. Stacked confocal images of heart sections double stained for p120 catenin (A, control; D, CKO) and cadherin (B, control; E, CKO) show co-localization (arrows) at the intercalated disc (merged images in C, control; F, CKO). Magnification, 63×; zoom, 2.96; bar, 10 μm.

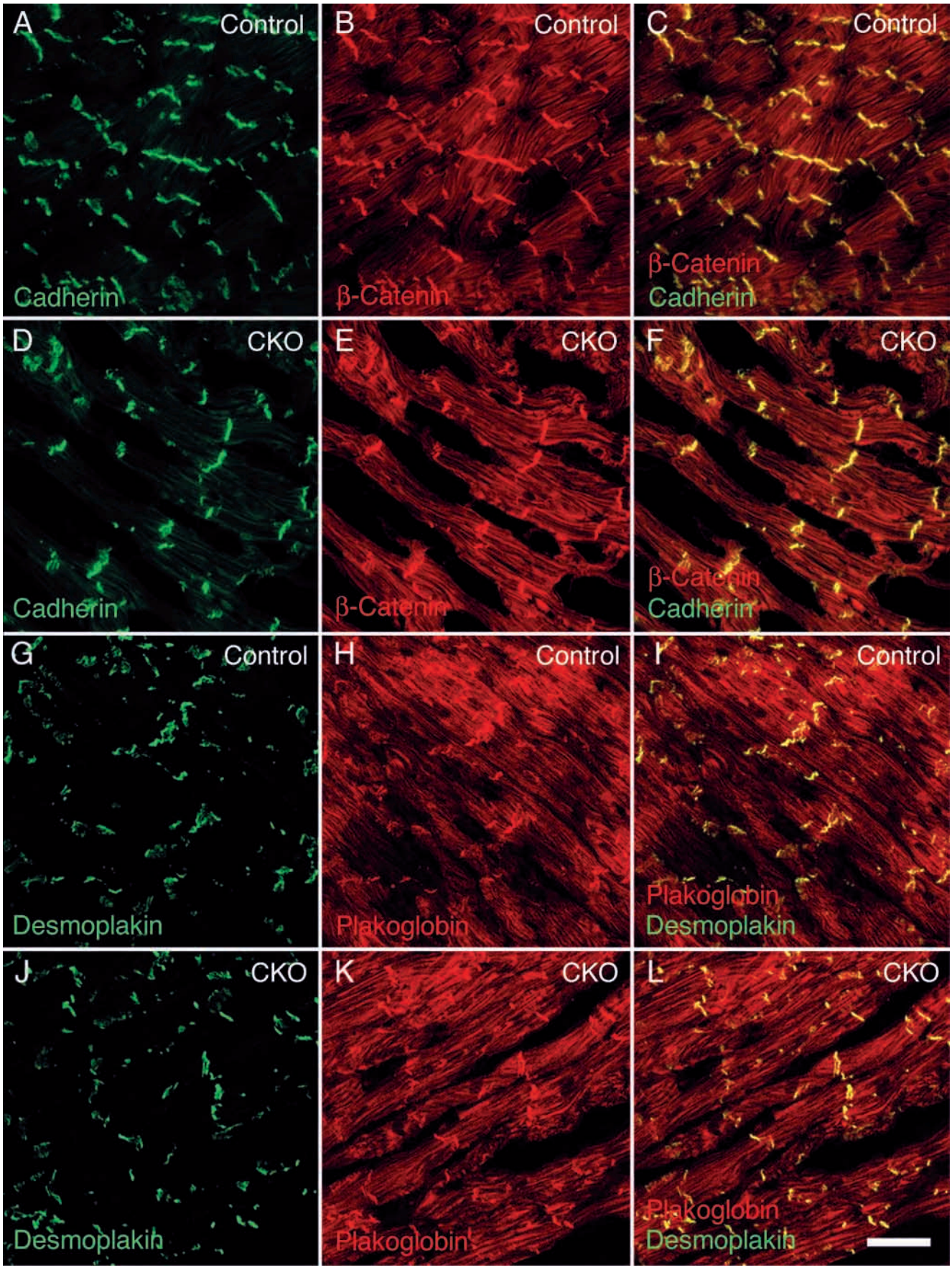
### Adherens junctions and desmosomes localize to the intercalated disc in a similar pattern in both control and CKO hearts

In order to assess the localization of adherens and desmosomal junction proteins at the intercalated disc in the absence of Cx43, we utilized immunofluorescence with confocal microscopy. Sections from control and CKO hearts double stained for Cx43 and cadherin showed that adherens-junction-associated cadherin staining was primarily localized to the intercalated discs in frozen sections from both control and CKO hearts (Fig. 3). While Cx43 staining was detected at the intercalated discs of control hearts, overlapping as well as non-overlapping but juxtaposed areas of Cx43 and cadherin staining were frequently seen (Fig. 3C), suggesting Cx43 and cadherin were closely associated at the intercalated disc. Only rare Cx43 staining was seen in CKO hearts, as demonstrated in Fig. 3E. Staining for the presence of N-cadherin, the major cardiac cadherin subtype, in the control and CKO hearts also showed no difference in localization (not shown).

In order to determine the localization of desmosomes in cardiac myocytes in the absence of Cx43, we double stained for Cx43 and desmoplakin, a necessary component of desmosomes (Fig. 4). As seen with adherens-junction-associated cadherin staining, gap junctions and desmosomes were often juxtaposed at the intercalated disc (Fig. 4E). However, despite their close proximity, double staining for desmoplakin and Cx43 revealed no changes in desmosomal localization in the absence of Cx43 compared with controls.

For the determination of relative localization of adherens junctions to desmosomes in the absence of gap junctions, we double stained for pan-cadherin and desmoplakin in control and CKO hearts and imaged with confocal microscopy in both the longitudinal and short-axis views. In order to minimize overlap, short-axis views of intercalated discs are presented in Fig. 5. In both control and CKO hearts, desmoplakin (green stain; A and D) and cadherin staining (red; B and E) had a similar distribution at the intercalated disc, although merged images (C

**Fig. 7.** Co-localization of β-catenin with cadherin and plakoglobin with desmoplakin at the intercalated disc in control and CKO mouse hearts. Frozen sections were double stained for cadherin (A, control; D, CKO) and β-catenin (B, control; E, CKO) or desmoplakin (G, control; J, CKO) and plakoglobin (H, control; K, CKO). Merged images of β-catenin and cadherin double staining are shown in C (control) and F (CKO) and those of plakoglobin and desmoplakin in I (control) and L (CKO). All images represent stacked confocal slices taken every 0.5 μm through the tissue section at a magnification of 63× and a zoom factor of 1.00. Bar, 30 μm.


A
Control
Cadherin
B
Control
β-Catenin
C
Control
β-Catenin
Cadherin
D
CKO
Cadherin
E
CKO
β-Catenin
F
CKO
β-Catenin
Cadherin
G
Control
Desmoplakin
H
Control
Plakoglobin
I
Control
Plakoglobin
Desmoplakin
J
CKO
Desmoplakin
K
CKO
Plakoglobin
L
CKO
Plakoglobin
Desmoplakin

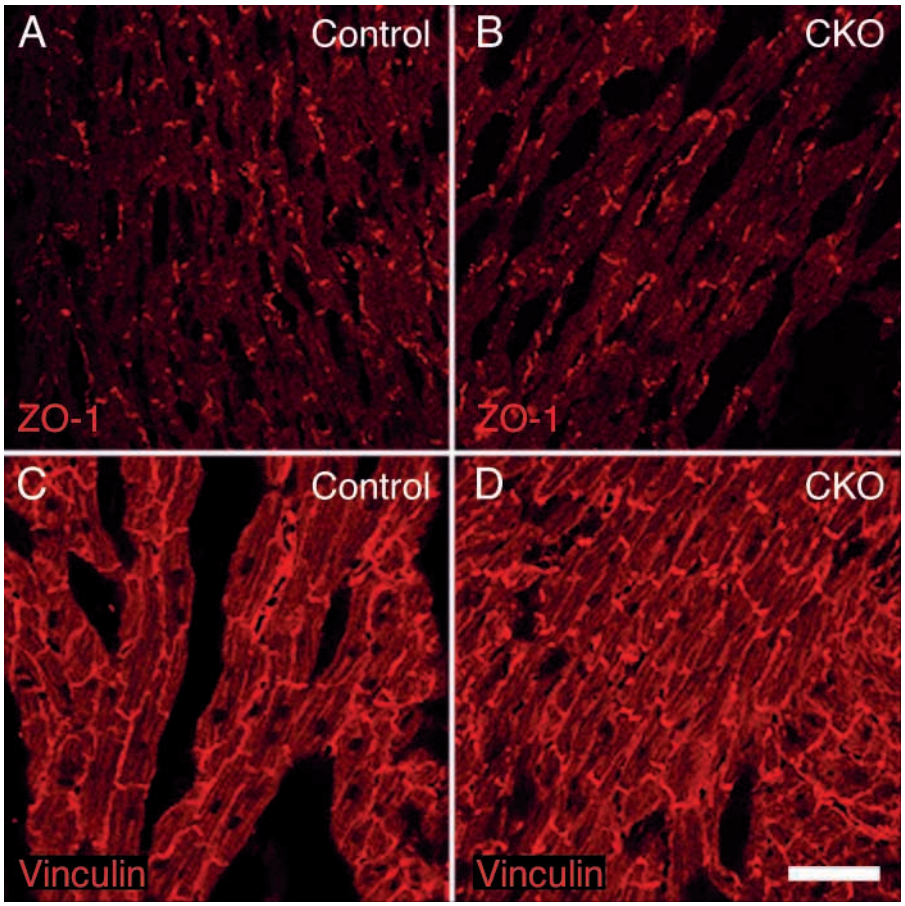



**Fig. 8.** Immunofluorescent staining for ZO-1 and vinculin in control and CKO mouse heart sections. ZO-1 (A, control; B, CKO) and vinculin (C, control; D, CKO) staining revealed similar patterns in control and CKO hearts. Magnification, 40×; bar, 50 μm.

and F) suggested that they are mainly juxtaposed (green and red stain in close proximity) rather than co-localized. We measured the area of the intercalated discs in control and CKO hearts stained with cadherin and desmoplakin. In hearts stained with cadherin, the intercalated disc area measured 93.8±10.7 $\mu m^2$ in controls ($n$=6) versus 106±9.1 $\mu m^2$ in the CKOs ($n$=5; $P$=0.42). Essentially identical values were obtained when the intercalated disc area was determined by visualizing desmoplakin expression (97.7±11.5 $\mu m^2$ in controls versus 109±7.4 $\mu m^2$ in CKOs; $n$=6 each; $P$=0.43).

Next, we used short-axis images to investigate whether adherens junctions and desmosomes were remodeled at the intercalated disc in the absence of gap junctions. Mean cadherin fluorescence in the CKO hearts was 87.7±12.8% of control values ($n$=7 controls and 8 CKO hearts; $P$=0.56), while desmoplakin fluorescence in the CKOs was 114±16.7% of control levels ($n$=7 control and CKO hearts; $P$=0.55). Thus, we found no significant differences in intercalated disc size or the abundance of adherens and desmosomal proteins at the intercalated disc in hearts devoid of Cx43 compared with littermate controls.

## Catenins co-localize at the intercalated disc with associated adhesion junctions in the presence and absence of Cx43

Given recent data suggesting altered p120 catenin intracellular localization in the absence of Cx43 in cultured explanted embryonic neural crest tissue (Xu et al., 2001), we investigated whether p120 and other catenins may be mis-localized in the CKO hearts. We double stained control and CKO heart sections for cadherin and one of its associated catenins, p120. We found that in both control and CKO hearts cadherin and p120 catenin staining co-localized at the intercalated disc (Fig. 6). While staining for cadherin was most abundant at the intercalated discs, p120 catenin was evident at the lateral borders of the myocytes in addition to the intercalated discs in both groups.

Next, we examined the localization of β-catenin and plakoglobin, catenins that associate with adherens junctions and desmosomes, respectively. Double staining for the presence of cadherin and β-catenin showed that these proteins co-localized at the intercalated disc in both control and CKO hearts (Fig. 7A-F). Plakoglobin and desmoplakin were similarly co-localized at the intercalated discs of both control and CKO hearts (Fig. 7G-L). While staining of β-catenin and plakoglobin was most intense at the intercalated disc, extensive cytoplasmic staining of both catenins was evident in control and CKO heart sections (β-catenin: Fig. 7B, control and Fig. 7E, CKO; plakoglobin: Fig. 7H, control and Fig. 7K, CKO).

## Localization of structural proteins ZO-1 and vinculin is unchanged in CKO hearts

We evaluated structural proteins related to the intercalated disc junctions, ZO-1 and vinculin, to determine their relative localization in the absence of Cx43. The cytoskeletal protein ZO-1 complexes with Cx43 in cardiac myocytes in a c-Src-mediated interaction and the association of ZO-1 and Cx43 is actually increased upon enzymatic disruption of intercellular contacts (Barker et al., 2002; Toyofuku et al., 2001; Toyofuku et al., 1998). ZO-1, which stained at the intercalated disc and along parts of the lateral cell borders, was similar in distribution in control and CKO sections (Fig. 8A,B). Vinculin, which tethers the adherens junction complex to the actin cytoskeleton via α-catenin, is downregulated in a guinea pig model of pressure overload hypertrophy (Wang and Gerdes, 1999). The distribution of vinculin, which extended along the entire sarcolemma in both controls and CKO myocytes, was not visibly different in the absence of Cx43 (Fig. 8C,D).

## Ultrastructure of CKO intercalated disc reveals absence of gap junctions without alterations in adherens junction or desmosome morphology

The morphology of the intercalated disc was evaluated in detail

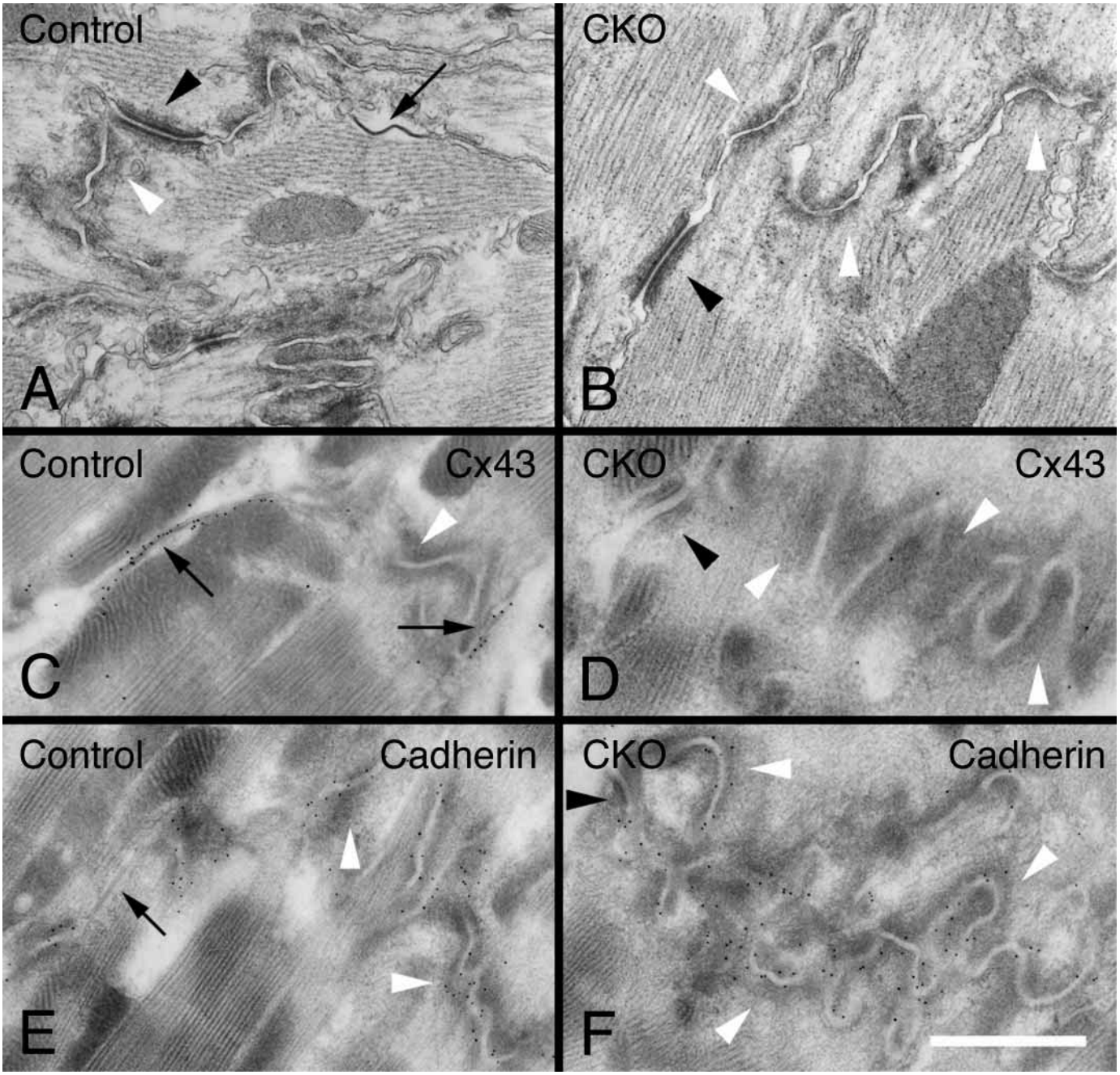



**Fig. 9.** Electron microscopy of the intercalated disc in control and CKO mice. Electron microscopy of the intercalated disc in a control mouse heart (A) shows a gap junction (arrow) in close proximity to adherens junctions (white arrowheads) and desmosomes (black arrowheads), while a representative intercalated disc in a CKO heart (B) shows no gap junctions but intact architecture of the remaining structures. Immuno-electron microscopy for Cx43 in a control heart (C) confirms the presence of Cx43 in the cardiac gap junction and demonstrates the absence of Cx43 label at the intercalated disc of a CKO mouse (D). Immuno-electron microscopy for cadherin in controls (E) and CKO hearts (F) demonstrates intact adherens junctions in both samples. Bars, 50 nm (A,B); 140 nm (C); 70 nm (D); 95 nm (E); 70 nm (F).

in the CKO hearts and compared with controls using EM. The morphology of the intercalated discs in the CKO hearts was no different than that of the controls, except for the near-complete absence of gap junctions. Structures that appeared to be adherens-type junctions and desmosomes in the CKO heart sections were unchanged in their localization at the intercalated disc, organization and general appearance when compared with the controls (Fig. 9A,B).

In order to confirm the absence of Cx43 in the CKO hearts, we performed immuno-labeling of the sections for Cx43, followed by EM. As expected, in addition to the morphological absence of gap junctions in the CKO hearts, there was also a paucity of Cx43 labeling (Fig. 9C,D). Rare gap junctions were present in the CKO hearts and these junctions demonstrated Cx43 labeling, but did not label for Cx45. Similarly, no specific Cx45 labeling of gap junctions was observed in control hearts.

Immuno-labeling for cadherin, followed by EM, however, revealed no difference in localization of the adherens junctions in the CKO hearts. Indeed, structures with the typical appearance of adherens junctions at the intercalated disc labeled for cadherin in both the CKO samples and in controls (Fig. 9E,F). The specificity of cadherin labeling is demonstrated by the lack of cadherin label on the gap junction in Fig. 9E (black arrow) and the desmosomal junction in Fig. 9F (black arrowhead).

## Discussion

In this study, we provide direct evidence that cell adhesion junctions in the heart, comprised of adherens junctions and desmosomes, as well as their associated catenins and the structural proteins ZO-1 and vinculin, are organized independently of the gap junctions. Indeed, despite the absence of Cx43 as detected by immunoblotting and immunofluorescence, and the consequent lack of gap junctions by EM, the localization, abundance and morphology of adherens junctions and desmosomes at the intercalated disc remained unchanged. This result adds important information to the understanding of the relationship between the various junctions at the intercalated disc and has significant implications for their regulation in heart disease.

Studies of intercalated-disc-related protein expression in animal models of heart disease have produced inconsistent results. For instance, cell adhesion junction proteins are downregulated along with Cx43 expression in the peri-infarct region of experimentally induced myocardial infarction (Matsushita et al., 1999), but are either upregulated or unchanged in genetic murine models of dilated cardiomyopathy (Ehler et al., 2001).

Observational investigations of the dynamics of adhesion junction and gap junction expression in cultured cells during postnatal mammalian heart development and in regenerating hepatocytes have suggested that gap junctions are not necessary for the establishment of the cell adhesion junctions at the intercalated disc (Angst et al., 1997; Fujimoto et al., 1997; Kostin et al., 1999). In addition, data from heterozygous Cx43-null mouse hearts indicate that intercalated disc structure, including N-cadherin abundance and distribution, was unchanged despite diminished (but not absent) Cx43 expression (Saffitz et al., 2000). Our studies presented here now provide the most direct data that Cx43 is not required for the organization of the adhesion junctions and their related proteins. Furthermore, we found no difference in cytosolic levels of p120 catenin or β-catenin in the CKO hearts by immunoblotting, suggesting that the availability of either catenin for complexing transcription factors and translocating to the nucleus was unchanged.

The CKO mouse provides an important model for the study of interactions between the gap junctions and the other junctions at the intercalated disc. The CKO mouse develops normally and has normal heart function by echocardiography, yet dies of sudden arrhythmic death starting at around two weeks of age. Thus, this model allows for the study of heart function and biochemical structure in the absence of Cx43, while avoiding the developmental abnormality and perinatal lethality of the Cx43 germline knockout. We chose to study one-month-old CKO mice because at the one-month point their survival curves have already started to diverge from littermate controls, yet sufficient numbers of mice could easily be generated for experimentation.

The results of this study have important potential implications for the nascent field of cardiomyocyte transplantation. It appears that it may be possible to introduce cultured cardiomyocytes into injured or infarcted segments of the heart in order to improve global function (Sakakibara et al., 2002). However, mechanical coupling in the absence of effective electrical coupling may negatively impact global ventricular function (Gutstein et al., 2001b). In addition, islands of poorly coupled cells may serve as arrhythmogenic foci. In this study, we have demonstrated normal cell adhesion junction distribution and morphology in the absence of gap junctions. In this light, for optimal effect, transplanted cardiomyocytes must be integrated electrically as well as mechanically into the recipient heart.

In summary, despite the absence of gap junctions in the CKO hearts, adherens junction and desmosmal distribution and morphology at the intercalated disc remain intact. Furthermore, the localization of catenins associated with the intercalated disc junctions is unchanged, suggesting that decreases in Cx43 expression may not directly influence catenin-dependent signaling. In addition, structural proteins associated with the intercalated disc junctions ZO-1 and vinculin are also unchanged in their distribution despite the loss of Cx43. The results of this study suggest that the gap junction is not necessary for the organization of the cell adhesion junctions and associated proteins in the cardiac intercalated disc.

Supported by HL04222 (D.E.G.), HL30557 and HL64757 (G.I.F.) from the NIH and a Burroughs-Wellcome Fund Clinical-Scientist Award in Translational Research (G.I.F.). Confocal laser scanning and EM was performed at the Mount Sinai School of Medicine microscopy core facility, supported with funding from a NIH shared instrumentation grant (1 SS10 RR0 9145-01) and NSF Major Research Instrumentation grant (DBI-9724504). We gratefully acknowledge the assistance of Scott Henderson and Vladimir Protopopov. The authors thank Manzoor Bhat, Edward A. Fisher and Sandra Mazur for their thoughtful review of this manuscript.

## References

**Ai, Z., Fischer, A., Spray, D. C., Brown, A. M. and Fishman, G. I.** (2000). Wnt-1 regulation of connexin43 in cardiac myocytes. *J. Clin. Invest.* **105**, 161-171.

**Anastasiadis, P. Z., Moon, S. Y., Thoreson, M. A., Mariner, D. J., Crawford, H. C., Zheng, Y. and Reynolds, A. B.** (2000). Inhibition of RhoA by p120 catenin. *Nat. Cell Biol.* **2**, 637-644.

**Angst, B. D., Khan, L. U., Severs, N. J., Whitely, K., Rothery, S., Thompson, R. P., Magee, A. I. and Gourdie, R. G.** (1997). Dissociated spatial patterning of gap junctions and cell adhesion junctions during postnatal differentiation of ventricular myocardium. *Circ. Res.* **80**, 88-94.

**Atkinson, P. H.** (1973). HeLa cell plasma membranes. *Methods Cell Biol.* **7**, 157-188.

**Barker, R. J., Price, R. L. and Gourdie, R. G.** (2002). Increased association of ZO-1 with connexin43 during remodeling of cardiac gap junctions. *Circ. Res.* **90**, 317-324.

**Daniel, J. M. and Reynolds, A. B.** (1999). The catenin p120(ctn) interacts with Kaiso, a novel BTB/POZ domain zinc finger transcription factor. *Mol. Cell. Biol.* **19**, 3614-3623.

**Ehler, E., Horowits, R., Zuppinger, C., Price, R. L., Perriard, E., Leu, M., Caroni, P., Sussman, M., Eppenberger, H. M. and Perriard, J. C.** (2001).

Alterations at the intercalated disk associated with the absence of muscle LIM protein. *J. Cell Biol.* **153**, 763-772.

**Fujimoto, K., Nagafuchi, A., Tsukita, S., Kuraoka, A., Ohokuma, A. and Shibata, Y.** (1997). Dynamics of connexins, E-cadherin and alpha-catenin on cell membranes during gap junction formation. *J. Cell Sci.* **110**, 311-322.

**Gallicano, G. I., Kouklis, P., Bauer, C., Yin, M., Vasioukhin, V., Degenstein, L. and Fuchs, E.** (1998). Desmoplakin is required early in development for assembly of desmosomes and cytoskeletal linkage. *J. Cell Biol.* **143**, 2009-2022.

**Giarre, M., Semenov, M. V. and Brown, A. M.** (1998). Wnt signaling stabilizes the dual-function protein beta-catenin in diverse cell types. *Ann. N. Y. Acad. Sci.* **857**, 43-55.

**Giepmans, B. N., Verlaan, I., Hengeveld, T., Janssen, H., Calafat, J., Falk, M. M. and Moolenaar, W. H.** (2001). Gap junction protein connexin-43 interacts directly with microtubules. *Curr. Biol.* **11**, 1364-1368.

**Gourdie, R. G., Green, C. R. and Severs, N. J.** (1991). Gap junction distribution in adult mammalian myocardium revealed by an anti-peptide antibody and laser scanning confocal microscopy. *J. Cell Sci.* **99**, 41-55.

**Gutstein, D. E., Morley, G. E., Tamaddon, H., Vaidya, D., Schneider, M. D., Chen, J., Chien, K. R., Stuhlmann, H. and Fishman, G. I.** (2001a). Conduction slowing and sudden arrhythmic death in mice with cardiac-restricted inactivation of connexin43. *Circ. Res.* **88**, 333-339.

**Gutstein, D. E., Morley, G. E., Vaidyu, D., Liu, F., Chen, F. L., Stuhlmann, H. and Fishman, G. I.** (2001b). Heterogeneous expression of gap junction channels in the heart leads to conduction defects and ventricular dysfunction. *Circulation* **104**, 1194-1199.

**Kaprielian, R. R., Gunning, M., Dupont, E., Sheppard, M. N., Rothery, S. M., Underwood, R., Pennell, D. J., Fox, K., Pepper, J., Poole-Wilson, P. A. et al.** (1998). Downregulation of immunodetectable connexin43 and decreased gap junction size in the pathogenesis of chronic hibernation in the human left ventricle. *Circulation* **97**, 651-660.

**Kostin, S., Hein, S., Bauer, E. P. and Schaper, J.** (1999). Spatiotemporal development and distribution of intercellular junctions in adult rat cardiomyocytes in culture. *Circ. Res.* **85**, 154-167.

**Laemmli, U. K.** (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

**Linask, K. K., Knudsen, K. A. and Gui, Y. H.** (1997). N-cadherin–catenin interaction: necessary component of cardiac cell compartmentalization during early vertebrate heart development. *Dev. Biol.* **185**, 148-164.

**Mathur, M., Goodwin, L. and Cowin, P.** (1994). Interactions of the cytoplasmic domain of the desmosomal cadherin Dsg1 with plakoglobin. *J. Biol. Chem.* **269**, 14075-14080.

**Matsushita, T., Oyamada, M., Fujimoto, K., Yasuda, Y., Masuda, S., Wada, Y., Oka, T. and Takamatsu, T.** (1999). Remodeling of cell–cell and cell–extracellular matrix interactions at the border zone of rat myocardial infarcts. *Circ. Res.* **85**, 1046-1055.

**Miller, J. R., Hocking, A. M., Brown, J. D. and Moon, R. T.** (1999). Mechanism and function of signal transduction by the Wnt/beta-catenin and Wnt/$Ca2^+$ pathways. *Oncogene* **18**, 7860-7872.

**Noren, N. K., Liu, B. P., Burridge, K. and Kreft, B.** (2000). p120 catenin regulates the actin cytoskeleton via Rho family GTPases. *J. Cell Biol.* **150**, 567-580.

**Peters, N. S., Severs, N. J., Rothery, S. M., Lincoln, C., Yacoub, M. H. and Green, C. R.** (1994). Spatiotemporal relation between gap junctions and fascia adherens junctions during postnatal development of human ventricular myocardium. *Circulation* **90**, 713-725.

**Ruiz, P., Brinkmann, V., Ledermann, B., Behrend, M., Grund, C., Thalhammer, C., Vogel, F., Birchmeier, C., Gunthert, U., Franke, W. W. et al.** (1996). Targeted mutation of plakoglobin in mice reveals essential functions of desmosomes in the embryonic heart. *J. Cell Biol.* **135**, 215-225.

**Sacco, P. A., McGranahan, T. M., Wheelock, M. J. and Johnson, K. R.** (1995). Identification of plakoglobin domains required for association with N-cadherin and alpha-catenin. *J. Biol. Chem.* **270**, 20201-20206.

**Saffitz, J. E., Green, K. G., Kraft, W. J., Schechtman, K. B. and Yamada, K. A.** (2000). Effects of diminished expression of connexin43 on gap junction number and size in ventricular myocardium. *Am. J. Physiol. Heart Circ. Physiol.* **278**, H1662-H1670.

**Sakakibara, Y., Tambara, K., Lu, F., Nishina, T., Sakaguchi, G., Nagaya, N., Nishimura, K., Li, R. K., Weisel, R. D. and Komeda, M.** (2002). Combined procedure of surgical repair and cell transplantation for left ventricular aneurysm: an experimental study. *Circulation* **106**, I193-I197.

**Toyofuku, T., Yabuki, M., Otsu, K., Kuzuya, T., Hori, M. and Tada, M.** (1998). Direct association of the gap junction protein connexin-43 with ZO-1 in cardiac myocytes. *J. Biol. Chem.* **273**, 12725-12731.

**Toyofuku, T., Akamatsu, Y., Zhang, H., Kuzuya, T., Tada, M. and Hori, M.** (2001). c-Src regulates the interaction between connexin-43 and ZO-1 in cardiac myocytes. *J. Biol. Chem.* **276**, 1780-1788.

**Wang, X. and Gerdes, A. M.** (1999). Chronic pressure overload cardiac hypertrophy and failure in guinea pigs: III. Intercalated disc remodeling. *J. Mol. Cell. Cardiol.* **31**, 333-343.

**Xu, X., Li, W. E., Huang, G. Y., Meyer, R., Chen, T., Luo, Y., Thomas, M. P., Radice, G. L. and Lo, C. W.** (2001). Modulation of mouse neural crest cell motility by N-cadherin and connexin 43 gap junctions. *J. Cell Biol.* **154**, 217-230.

**Yamamoto, T., Ochalski, A., Hertzberg, E. L. and Nagy, J. I.** (1990). LM and EM immunolocalization of the gap junctional protein connexin 43 in rat brain. *Brain Res.* **508**, 313-319.

**Zhuang, J., Yamada, K. A., Saffitz, J. E. and Kleber, A. G.** (2000). Pulsatile stretch remodels cell-to-cell communication in cultured myocytes. *Circ. Res.* **87**, 316-322.

**Zhurinsky, J., Shtutman, M. and Ben-Ze'ev, A.** (2000a). Differential mechanisms of LEF/TCF family-dependent transcriptional activation by beta-catenin and plakoglobin. *Mol. Cell. Biol.* **20**, 4238-4252.

**Zhurinsky, J., Shtutman, M. and Ben-Ze'ev, A.** (2000b). Plakoglobin and beta-catenin: protein interactions, regulation and biological roles. *J. Cell Sci.* **113**, 3127-3139.