Requirement of plakophilin 2 for heart morphogenesis and cardiac junction formation

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P lakophilins are proteins of the armadillo family that function in embryonic development and in the adult, and when mutated can cause disease. We have ablated the plakophilin 2 gene in mice. The resulting mutant mice exhibit lethal alterations in heart morphogenesis and stability at mid-gestation (E10.5–E11), characterized by reduced trabeculation, disarrayed cytoskeleton, ruptures of cardiac walls, and blood leakage into the pericardiac cavity. In the absence of plakophilin 2, the cytoskeletal linker protein desmoplakin dissociates from the plaques of the adhering junctions that connect the cardiomyocytes and forms granular aggregates in the cytoplasm. By contrast, embryonic epithelia show normal junctions. Thus, we conclude that plakophilin 2 is important for the assembly of junctional proteins and represents an essential morphogenetic factor and architectural component of the heart.

Introduction

Proteins of the armadillo family are characterized by repeats of the arm motif, and include molecules such as β-catenin, plakoglobin, p120ctn, and the plakophilins (Cowin et al., 1986; Nagafuchi and Takeichi, 1989; Ozawa et al., 1989; Peifer and Wieschaus, 1990; McCrea et al., 1991; Hülsken et al., 1994; Hatzfeld, 1999). Arm repeat proteins bind to the cytoplasmic part of glycoproteins of the cadherin family of cell adhesion molecules, thus forming plaques to which bundles of cytoskeletal filaments are tethered (for reviews see Takeichi, 1995; Smith and Fuchs, 1998; Green and Gaudry, 2000). β-Catenin also plays a crucial role in the canonical Wnt pathway, which transduces developmental and oncogenic signals from the cell surface to the nucleus (Behrens et al., 1996; Eastman and Grosschedl, 1999; Hecht et al., 1999; Bienz and Clevers, 2000). Mutations of plakoglobin in humans (Naxos disease) and in mice have been reported to result in heart, skin, and hair follicle dysfunctions, a complex phenotype that might involve both adhesion and signaling disorders (Bierkamp et al., 1996; Ruiz et al., 1996; McKoy et al., 2000). A remarkably similar triad of cardiomyopathy, woolly hair, and keratoderma has been noted in patients suffering from mutations in the gene encoding desmplakin, another junctional plaque protein (Ng et al., 2000; Rampazzo et al., 2002; Alcalai et al., 2003).

Plakoglobin 1 mutations in humans result in skin fragility-ectodermal dysplasia syndrome, consistent with the restriction of plakoglobin expression to certain stratified epithelia and its function in keratinocyte adhesion (Magrath et al., 1997; Whittock et al., 2000; Hamada et al., 2002; Sprecher et al., 2004).

Two types of “classical” cell–cell junctions are found in vertebrates, adherens junctions and desmosomes, which have one plaque protein in common, plakoglobin (Cowin et al., 1986). In adherens junctions that comprise morphologically diverse forms such as puncta adhaerentia, fasciae adhaerentes, and zonulae adhaerentes, β-catenin links adherens to α-catenin, thus mediating interaction with the actin cytoskeleton (Bolier et al., 1985; Nagafuchi and Takeichi, 1989; Ozawa et al., 1989; Hülsken et al., 1994; Aberle et al., 1996). Typical desmosomes (maculae adhaerentes), which are characterized by the plaque protein desmoplakin (Franke et al., 1982), are found in epithelial cells, cardiomyocytes, meningothelial cells, and dendritic reticulum cells of lymph node follicles, where they anchor bundles of intermediate-sized filaments (IFs) of the cytokeratin, the desmin, or the vimentin type, respectively (Kartenbeck et al., 1983, 1984; Franke and Moll, 1987; Kuruc and Franke, 1988; van der Loop et al., 1995). In addition, desmoplakin has also been identified in the complexus adhaerens, an adhering junction characteristic of some endothelia, where it is essential for vascular development, as demonstrated by gene ablation in mice (Schmelz and Franke, 1993; Schmelz et al., 1994; Valiron et al., 1996; Kowalczyk et al., 1998; Gallicano et al., 2001; Zhou et al., 2004).
The plakophilin subfamily of arm repeat proteins comprises three members, plakophilins 1–3 (Kapprell et al., 1988; Hatzfeld et al., 1994, 2000; Heid et al., 1994; Schmidt et al., 1994, 1997, 1999; Mertens et al., 1996, 1999; Bonné et al., 1999). The arm protein p0071, occasionally also called plakophilin 4, is more closely related to another armadillo subfamily comprising proteins p120ctn, ARVCF, and neurojungin (Schmidt et al., 1999). Plakophilins 1–3 are juxtamembranous constituents of plaques of desmosomes and certain related junctions where they are tightly associated with other arm proteins, cadherins and desmoplakin, and are involved in the anchorage of IFs (Hatzfeld and Nachtsheim, 1996; Mertens et al., 1996, 1999; Schmidt et al., 1997, 1999; Bonné et al., 1999; Kowalczyk et al., 1999; North et al., 1999; Hatzfeld et
al., 2000; Bornslaeger et al., 2001; Chen et al., 2002; Koeser et al., 2003). Plakophilins are also detected in the nucleus; for example, plakophilin 2 has been found in association with nucleoplasmic RNA polymerase III complexes (Mertens et al., 1996, 2001; Schmidt et al., 1997, 1999; Bonné et al., 1999). Although the function of $\beta$-catenin in the nucleus is well known (Behrens et al., 1996; Eastman and Grosschedl, 1999; Hecht et al., 1999; Bienz and Clevers, 2000), similar roles for plakophilins have not yet been established. Plakophilin 2 occurs in all proliferative epithelial tissues and tumors as well as in the cardiomyocytes and Purkinje fiber cells of the heart (Mertens et al., 1996, 1999).

Striking similarities exist between the phenotypes generated by human and mouse mutations of desmosomal proteins (Ruiz et al., 1996; Bierkamp et al., 1996; Gallicano et al., 1998, 2001; Armstrong et al., 1999; McKoy et al., 2000; Norgett et al., 2000; Vasioukhin et al., 2001; Rampazzo et al., 2002; Alcalai et al., 2003). No mutations for plakophilin 2, which is the only plakophilin gene expressed in the heart, had been reported. Here, we describe the phenotype of plakophilin 2-deficient mouse embryos, showing distortions of heart morphogenesis and stability, followed by cardiac rupture, blood leakage, and embryonic death. The analysis of the molecular organization of the affected tissue has allowed us to propose a molecular mechanism for these alterations as well as a general role of plakophilin 2 in junctional plaque organization.

### Results

#### Generation of plakophilin 2 mutant mice

We generated a null mutation of the plakophilin 2 gene by homologous recombination in embryonic stem (ES) cells. In the targeting vector, a neo cassette inserted in opposite transcriptional orientation replaced a 7.8-kb genomic fragment from the NotI site in exon 1 to the BamHI site in intron 1 (Fig. 1 a). Homologous recombination yielded a chimeric line, which transmitted the mutation to several generations of transgenic mice. The targeting event was confirmed by Southern blot analysis using a probe within the intron flanking the insertion site. The targeted allele was used to generate a null mutation in ES cells and to establish a null mutation line. The insertion allele was used to generate a conditional mutation in ES cells and to establish a conditional mutation line.

The phenotype of plakophilin 2-deficient mouse embryos is characterized by severe heart defects, including atria wall thinning, ventricular trabeculation loss, and blood leakage in the pericardial cavity. These defects lead to embryonic death at various stages of development. The analysis of the molecular organization of the affected tissue has allowed us to propose a molecular mechanism for these alterations as well as a general role of plakophilin 2 in junctional plaque organization.

#### Table I. Viability of embryos resulting from heterozygous intercrosses

| Stage   | +/+ | +/− | −/− |
|---------|-----|-----|-----|
| E9.75   | 32% (15) | 40% (19) | 28% (13) |
| E10.75  | 26% (25) | 48% (46) | 26% (25) |
| E11.5   | 29% (21) | 49% (35) | 22% (16) |
| E13.75  | 30% (4) | 69% (9) | 0% |

*The number of dead homozygous mutant embryos; the number of tested embryos is in parentheses.
mologous recombination events were identified by Southern blot analyses (Fig. 1 b); an external probe (ext) yielded a novel Xba fragment of 10 kb in the mutant, Δpckp2 (the wild-type [wt] fragment is 16 kb), and the neo probe produced a 4.5-kb HindIII fragment. We generated a null mutation in the plakophilin 2 gene after homologous recombination because insertion of neo prevents splicing between exon 1 and 2 and leads to an early stop of plakophilin 2 translation after 43 aa. Using two lines of mutant ES cells, we produced plakophilin 2 mutant chimeric and heterozygous mice that were healthy and fertile. However, matings between heterozygous mice produced no live plakophilin 2–deficient offspring, implying that the mutant embryos died during embryogenesis. To determine the time of death, embryos from different developmental stages were genotyped by PCR (Fig. 1 c) and inspected visually. Up to day 10.75 of embryogenesis (E10.75), the expected Mendelian ratio of homozygous mutant embryos was observed (Table I); however the mutant embryos showed blood accumulation in the pericardial and peritoneal cavities. At E11.5, the number of viable plakophilin 2 −/− embryos declined, as judged by PCR genotyping and lack of heart beating. Western blot analyses using an antibody against the COOH terminus of plakophilin 2 indicated absence of full-length or truncated protein in E10.75 plakophilin 2 −/− embryos (Fig. 1 d). In wt embryos at E10.75, plakophilin 2 was prominent in cardiomyocytes of the atrium and the ventricle of the heart (Fig. 1 e), and expression was lost in the null mutation embryos (see also below, Fig. 4 B, and Fig. 8). At E13.75, plakophilin 2 was intensely synthesized in the wt cardiomyocytes, but less in the surrounding epicard (Fig. 1 f).

**Defects in heart morphogenesis of plakophilin 2 −/− embryos**

To determine the cause of lethality in plakophilin 2–deficient mice, we examined embryos at E9.5 and later stages of development. Homozygous embryos at E10.75 were pale in the head and the dorsal trunk region, i.e., blood was not distributed homogenously but had accumulated in the pericardial cavity and also in the peritoneal cavity (Fig. 2, a and b). Similarly, blood was not found in the blood vessels of the yolk sacs of homozygous plakophilin 2 −/− embryos (Fig. 2, c and d; see section through blood vessels in Fig. 2, e and f). The vasculature appeared to be largely intact, as determined by PECAM staining, although some minor alterations in the overall pattern of the blood vessel network could be recognized (Fig. 2, g and h). Transverse sections through the forming heart of the plakophilin 2 mutants at E10.75 showed reduced trabeculation in the heart ventricles (Fig. 2, i and j) and thinner walls of the atria, as in some places two cell layers instead of three to four in the wt embryos were observed (Fig. 2, k and l; see also Fig. 3 and Sedmera et al., 2000). No alterations in cell proliferation and apoptosis were seen in mutant hearts, as determined by anti-phosphohistone 3 and TUNEL staining (unpublished data). Overt rupture of heart walls, as previously found in plakoglobin-deficient mutant embryos (Ruiz et al., 1996), could not be detected, indicating that blood leakage occurred through small perforations of the beating heart (the position of a possible small leakage site is denoted by an arrowhead in Fig. 2 l). Homozygous embryos of earlier stages (E9.5–E10) did not show blood leakage into the pericard, whereas embryos of E11.5–E12 displayed, in addition to blood leakage, swollen pericardial and peritoneal cavities, before they became necrotic (unpublished data).

**Changes of cytoskeletal architecture and composition of adhering junctions in hearts of plakophilin 2 −/− embryos**

Desmoplakin represents a major and functionally essential component of adhering junctions in the intercalated disks that connect cardiomyocytes (Franke et al., 1982; Gallicano et al., 2001). In the wt embryonic heart at E10.75, desmoplakin was distributed throughout the trabeculae in a complex punctate pattern at cell–cell borders of both proliferating and differentiating cardiomyocytes (Fig. 3; see also Moorman and Lamers, 1999).

Confocal laser-scanning, double-label immunofluorescence microscopy was used to examine molecular localization in the intercalated disks of wt and mutant embryonic hearts (Fig. 4, A and B). In wt hearts at E10.75, desmoplakin (green fluorescence) showed far-reaching colocalization with the junctional proteins plakoglobin, N-cadherin, β-catenin, plakophilin 2, desmoglein Dsg2 (red fluorescence), α-catenin, and p120catenin, as demonstrated by the merged yellow fluorescence (Fig. 4 A, right column; unpublished data). However, in the plakophilin 2–deficient mutants desmoplakin did not colocalize with any of these junction proteins (Fig. 4 B, right column), but was rather dispersed over the cytoplasm, mostly away from the intercalated disks. Our immunolocalization analyses also confirmed that plakophilin 2 was absent in these mutants (Fig. 4 Bd’). Surprisingly, the desmosomal cadherin desmoglein 2 could not be localized at significant levels in the plakophilin 2–devoid hearts (Fig. 4 Be’).
Electron microscopy showed that throughout mid-gestation, the cardiomyocytes of the wt embryos were connected by well-organized intercalated disks that were rich in both types of adhering junctions, the *fascia adhaerens*-like and the desmosome-like subforms (Fig. 5 a). In the hearts of *plakophilin 2*–/– mutants, these two morphotypes of adhering junctions were difficult to distinguish (Fig. 5 b). Electron microscopy and immunogold labeling of wt hearts demonstrated that desmoplakin was located in the plaques of both the desmosome-like and *fascia adhaerens*-type junctions (Fig. 5 c, e, and g), confirming previous observations (Borrmann, 2000), although a somewhat higher intensity of labeling was generally seen in the more desmosome-like junctions (Fig. 5 e, compare right-hand with left-hand junction). “Fused” type junctions with continuous desmoplakin or plakophilin 2 labeling were also consistently observed in wt hearts (Fig. 5, g and h). Both morphotypes of adhering junctions were also strongly positive for the major transmembrane glycoprotein, N-cadherin (Fig. 5 k).

By contrast, in the hearts of the *plakophilin 2*–/– embryos the location of desmoplakin was drastically altered: immunogold labeling demonstrated that desmoplakin was virtually absent from all junctions (Fig. 5 d). Instead, desmoplakin immunolabel was found in sparse distribution over the cytoplasm, often appearing in granular aggregates (green dots). (d’): Complete absence of plakophilin 2 immunostaining in the mutant embryos is shown. (e’) Note that Dsg2 is not detectably enriched in the intercalated disks of the mutants. Bars, 50 μm.

**Figure 4.** Major architectural and compositional changes of the adhering junctions in the intercalated disks of hearts in *plakophilin 2*-deficient E10.75 mouse embryos. Laser-scanning, double-label immunofluorescence microscopy of cryostat sections through cardiac tissue, performed with wt and *plakophilin 2*-deficient (*pkp2*–/–) embryos. Specific combinations of antibodies were used: rabbit antibodies to desmoplakin (DP, marked by green fluorescence) and murine mAbs to diverse other cardiac adhering junction components (red fluorescence): (a and a’) plakoglobin (PG); (b and b’) N-cadherin (*N-Cad*); (c and c’) β-catenin (*β-Cat*); (d and d’) plakophilin 2 (*PKP2*); (e and e’) desmoglein 2 (*Dsg2*). The merged fluorescence images are shown in the right-hand column. (A) In the wt embryos, the special adhering junctions of the intercalated disks show far-reaching colocalization (yellow) of desmoplakin with (a) plakoglobin, (b) N-cadherin, (c) β-catenin, (d) plakophilin 2, and (e) desmoglein 2. Other known plaque proteins of cardiac adhering junctions show identical localization, including p120<sup>ctn</sup> and α-catenin (see also Janssens et al., 2001). (B) By contrast, in the *pkp2*–/– embryos none of the plaque proteins colocalize with desmoplakin (a’–e’). In the mutants, desmoplakin is dispersed over the cytoplasm, often appearing in granular aggregates (green dots). (d’) Complete absence of plakophilin 2 immunostaining in the mutant embryos is shown. (e’) Note that Dsg2 is not detectably enriched in the intercalated disks of the mutants. Bars, 50 μm.
(e.g., α- and β-catenin) was not significantly altered in the hearts of the wt and the plakophilin 2–deficient embryos (Fig. 5, k and l; unpublished data). In many places, IF arrays appeared locally displaced by the aggregates of desmoplakin granules (Fig. 5, i and j). The extent of changes of cytoskeletal organization in the absence of plakophilin 2 is presented at higher magnification in Fig. 6: we frequently observed conspicuous IF arrays, which appeared as extensive swirls of disordered filaments around the dense desmoplakin aggregates (Fig. 6, arrow). In addition, some desmoplakin immunolabel was always identified in association with the extensive IF tangles (Fig. 6).

Remarkably, the ultrastructure of adherens junctions and desmosomes in epithelial tissues, such as in the forming epidermis and in stomach mucosa, was not altered in the plakophilin 2–deficient mutant mice (Fig. 7). Similarly, no significant morphological changes were noted in the aorta as well as in endothelia of blood vessels (unpublished data).

**Biochemical differences of molecular complexes in adhering junctions of embryonic hearts**

It has previously been demonstrated that in tissues as well as in monolayer cell cultures of epithelial cells and of cardiomyocytes, junctional proteins such as plakophilins are to a large extent associated with the cytoskeleton and thus not readily extractable by nondenaturing detergents such as Triton X-100 or NP-40 at moderate ionic strength (Mertens et al., 1996, 2001; Bornslaeger et al., 2001; Koeser et al., 2003). Similarly, we found that in the forming hearts of wt embryos large proportions of desmoplakin, plakoglobin, β-catenin, and plakophilin 2 (but also of the transmembrane glycoproteins N-cadherin and desmoglein 2) were detergent insoluble (Fig. 8, left column). In contrast, in plakophilin 2–deficient embryonic hearts, desmoplakin and desmoglein 2, and in part also plakoglobin were largely Triton X-100 soluble (i.e., less stably associated with the cytoskeleton), whereas the cytoskeletal associations of N-cadherin and β-catenin were unchanged (Fig. 8, compare left and right column). In addition, low amounts of desmoglein 2 were recovered from mutant embryonic hearts (Fig. 8, compare left and right columns). We conclude from these biochemical data that the association of desmoplakin and plakoglobin, but also of desmosomal cadherins with their partners in the cardiac junctional plaques, is strongly reduced in the plakophilin 2–deficient embryonic hearts, consistent with reduced architectural stability of the intercalated disks.

*Figure 4 (continued from previous page)*
Discussion

Ablation in mice of the gene encoding plakophilin 2, a widespread protein with dual location in intercellular junctions and in nuclei, results in lethal cardiac damage at mid-gestation. Defective cell–cell adhesion at the intercalated disks and leakage of blood into the pericardial cavity is observed, and electron microscopic as well as biochemical data demonstrate a markedly reduced association of plaque proteins such as desmoplakin and plakoglobin with the junctional structures. These alterations occur at similar time periods (E10.5–E12) with similar pathological alterations as those previously shown for the ablations of another arm protein plaque constituent, plakoglobin (Bierkamp et al., 1996; Ruiz et al., 1996), and in part for the cell–cell adhesion molecule N-cadherin (Radice et al., 1997). Ablations of the genes encoding the cytoskeletal linker protein desmoplakin (Gallicano et al., 1998) and the desmosomal cadherin Dsg2 (Eshkind et al., 2002), two other major components of cardiac adhering junctions, disrupt earlier stages of embryogenesis, but specific damage in embryonic hearts has been noted upon partial rescue of desmplakin (Gallicano et al., 2001; Zhou et al., 2004). We conclude from these results that (1) the developing embryonic heart is particularly vulnerable to diverse forms of weakening of cardiomyocyte adhesion, and (2) plakophilin 2 is a key organizer of cardiac architecture during embryogenesis. The latter conclusion is in line with several observations on plakophilin 2–binding proteins in other cultured cells (Chen et al., 2002; Koeser et al.,...
The fact that plakophilin 2 is the only plakophilin present in cardiac adhering junctions may explain this extraordinary sensitivity in mutant hearts. The analysis of the molecular changes in the cytoskeletal architecture of the plakophilin 2 mutant hearts has, however, revealed striking differences to ablations of plakoglobin and N-cadherin: while in plakoglobin-deficient embryonic hearts desmoplakin remains firmly associated with the plaques of the adhering junctions (Ruiz et al., 1996), this protein is no longer associated with the junctions in the absence of plakophilin 2. Rather, in the plakophilin 2–deficient embryonic hearts desmoplakin is dispersed over the cytoplasm, where it frequently forms sizable aggregates located between the myofibril and IF arrays, away from the intercalated disks. We conclude therefore that it is plakophilin 2, possibly together with plakoglobin, which is essential for fixing desmoplakin to the junctional plaques of cardiomyocytes. Our work also shows that both plakophilin 2 and desmoplakin are not required for the anchorage of myofibrils to adhering junctions of cardiomyocytes (see Fig. 5, a and b), as this has also been shown for plakoglobin (Isac et al., 1999). By contrast, the IF arrays normally interspersed between the myofibrils and enriched at the intercalated disks (e.g., Kartenbeck et al., 1983; Milner et al., 1996) are often displaced in the mutant, but still display associations with desmoplakin, including formations of swirls around the desmoplakin aggregates. These associations between IFs and desmoplakin away from the plasma membrane reflect the intimate binding of these proteins, compatible with results in cultured epithelial cells (Stappenbeck and Green, 1992; Stappenbeck et al., 1993; Kouklis et al., 1994; Bornslaeger et al., 1996, 2001; Smith and Fuchs, 1998; Kowalczyk et al., 1999; Vasioukhin et al., 2001; Chen et al., 2002). Our immunolocalization and biochemical data also demonstrate that in the plakophilin 2–deficient mouse embryos, several proteins of the armadillo family such as β-catenin, p120ctn, and also part of plakoglobin remain at the adhering junctions of the cardiac intercalated disks, despite the absence of plakophilin 2 and desmoplakin. This indicates that their binding to other plaque components, including the cytoplasmic portion of N-cadherin, is sufficient to secure their junctional integration. Plakophilin 2 binding, as reported from cell transfection and yeast two-hybrid experiments (Chen et al., 2002), is therefore not needed for association of β-catenin, p120ctn, and part of plakoglobin with the cardiac junctions. However, the desmosomal cadherins (e.g., Dsg2) were largely absent from desmosomal junctions and were detergent extractable from mutant hearts. This higher detergent solubility of Dsg2 may be due to its exclusion from the cardiac junctions, similar to the detergent-extractable forms of nascent desmoglein from cultured cells (e.g., Pasdar and Nelson, 1989; Pasdar et al., 1991). Thus, our observations are compatible with reports on cultured cells that plakophilin 2...
congestive heart failure (Gerull et al., 2002). After our report, cardiac muscles, can result in dilated cardiomyopathy with mutation of titin, a large cytoskeletal and signaling protein of genes coding for components of the contractile apparatus have impaired myocardial contractility and ventricular dilatation, and frequently also affect myofibril function. Mutations in plakophilin 2 give rise to a wide spectrum of interferences with plakophilin 2 function, might impair heart function and play a role in human heart disease.

### Materials and methods

#### Generation of mutant mice
The mouse plakophilin 2 gene was isolated from a λ FIX II 129/SVJ library (Stratagene). Restriction analysis and PCR amplification were used to map and subclone fragments that encompass the exon containing the translation initiation site and downstream exons. The targeting vector was created using standard molecular biology techniques, and ES cells were electroporated and selected as described previously (Huelsken et al., 2000). Recombined loci were analyzed by Southern blotting, and Western blotting was used to demonstrate that the modified plakophilin 2 locus produced a null allele. Two independent heterozygous ES cell clones of the mutated allele were used to generate chimeric mice by blastocyst injection (Huelsken et al., 2000), and mutant animals were bred on a C57Bl6 background. PCR genotyping was performed using oligonucleotides 5′-GATCCGGACGCCAGCACAGTC-3′ and 5′-AGGGTCTGACCGGCAGG-3′ for the wt allele, and 5′-GATCCGGACGCCAGCACAGTC-3′ and 5′-CTTCCAGAGGCGATGGCAATTA-3′ for the mutant allele.

#### Histology and immunohistochemistry
Embryos and yolk sacs were fixed in PBS containing 4% formaldehyde and then embedded in paraffin. For whole-mount immunohistochemistry, embryos were fixed in 4% paraformaldehyde containing 0.1% sodium azide and 0.2% Triton X-100, then incubated with the primary antibodies for 1 h, followed by incubation with secondary POD-conjugated donkey anti–rat antibody. DAB was used for color detection.

#### Immunofluorescence microscopy
Cryostat sections of wt and plakophilin 2–deficient mutant embryos were fixed in PBS containing 4% formaldehyde and then embedded in paraffin. For whole-mount immunohistochemistry, embryos were fixed in 4% paraformaldehyde containing 0.1% sodium azide and 0.2% Triton X-100, then incubated with the primary antibodies for 1 h, followed by incubation with secondary POD-conjugated donkey anti–rat antibody. DAB was used for color detection.

#### Electron and immunoelectron microscopy
Embryos were dissected and fixed in 8% formaldehyde/0.1% glutaraldehyde in HEPES, osmicated, and embedded in Epon (Polysciences) using standard procedures. For immunoelectron microscopy, 50-μm-thick cryostat sections were mounted on coverslips, fixed for 10 min with 2% formaldehyde in PBS, permeabilized with 0.1% saponin in PBS for 5 min, and incubated for 1 h with the primary antibodies. After three washes with PBS, specimens were incubated with secondary, Nanogold-
conjugated antibodies (BioTrend) for 2–4 h. Further treatment, including silver enhancement of the gold particles, was as described previously (Langbein et al., 2002). Samples were dehydrated and flat-embedded in Epon. Ultrathin sections were examined with an electron microscope (model EM 910; Carl Zeiss Microlmaging, Inc.).

Detergent extraction, gel electrophoreses, and Western blots
For Triton X-100 extraction, embryonic hearts were snap-frozen in liquid nitrogen and genotyped. Hearts were pooled, sonicated in lysis buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 0.5 mM CaCl2, and 1% Triton X-100), and centrifuged at 20,000 g for 15 min. Protein concentration was measured, and equal amounts of the Triton X-100 insoluble and soluble fractions were subjected to SDS-PAGE and transferred to nitrocellulose. And blots were probed several antibodies (see above) including 

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