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## The Matricellular Protein CCN1 (CYR61) Is Essential For Cardiac Development

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### Abstract

The matricellular protein CCN1 (CYR61) regulates cell adhesion, migration, proliferation, survival, and differentiation through binding to integrin receptors and heparan sulfate proteoglycans. Here we show that *Ccn1*-null mice are impaired in cardiac valvuloseptal morphogenesis, resulting in severe atrioventricular septal defects (AVSD). Remarkably, haploinsufficiency for *Ccn1* also results in delayed formation of the ventricular septum in the embryo and persistent ostium primum atrial septal defects (ASD) in ~20% of adults. Mechanistically, *Ccn1* is not required for epithelial-to-mesenchymal transformation or cell proliferation and differentiation in the endocardial cushion tissue. However, *Ccn1* deficiency leads to precocious apoptosis in the atrial junction of the cushion tissue and impaired gelatinase activities in the muscular component of the interventricular septum at E12.5, when fusion between the endocardial cushion tissue and the atrial and ventricular septa occurs, indicating that these defects may underlie the observed AVSD. Moreover, human *CCN1* maps to 1p21-p31, the chromosomal location of an AVSD susceptibility gene. Together, these results provide evidence that deficiency in matrix signaling can lead to autosomal dominant AVSD, identify *Ccn1*<sup>+/-</sup> mice as a genetic model for ostium primum ASD, and implicate *CCN1* as a candidate gene for AVSD in humans.

### INTRODUCTION

Atrioventricular septal defect (AVSD) is a common family of genetic disorders, accounting for ~7.5% of the recognized congenital heart disease (CHD) in humans<sup>1</sup>. During cardiac development, the endocardial cushion tissue expands through formation of the cardiac jelly, a specialized extracellular matrix (ECM) between the endocardium and myocardium, into which endocardial cells that have undergone epithelial-to-mesenchymal transformation (EMT) invade. Subsequent growth and remodeling of the cushion tissue, coupled with its fusion with the developing atrial septum and muscular interventricular septum, lead to formation of the definitive atrioventricular (AV) septa and valves<sup>2, 3</sup>. Defects in this process are manifested in a spectrum of abnormalities with varying severities, ranging from partial forms of AVSD with ostium primum atrial septal defects (ASD) to complete forms with absence of the AV septa and lack of partitioning of the AV valve into separate mitral (left) and tricuspid (right) valves. While AVSD is frequently associated with Down's syndrome (trisomy 21), non-syndromic AVSD also occur. Although non-syndromic AVSD may be a sporadic trait or the result of

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#### DISCLOSURE

None

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multifactorial inheritance, strong evidence also support the presence of susceptibility genes for AVSD that are autosomal dominant and incompletely penetrant<sup>4-6</sup>.

CCN1 (CYR61, cysteine-rich angiogenic inducer 61; NM\_001554) is a secreted, cysteine-rich protein associated with the ECM. A dynamically expressed protein, CCN1 serves regulatory rather than structural roles and can be considered a matricellular protein<sup>7</sup>. CCN1 promotes cell adhesion, migration, proliferation, differentiation, and survival or death in a cell type-dependent manner<sup>8</sup>. CCN1 also induces angiogenesis *in vivo* and *Ccn1*-null mice suffer embryonic lethality, in part due to placental vascular insufficiency and compromised embryonic vessel integrity<sup>9, 10</sup>. Mechanistically, CCN1 binds and functions through specific integrin Whereas CCN1 promotes cell adhesion, migration, and proliferation in fibroblasts through integrins  $\alpha_6\beta_1$ ,  $\alpha_v\beta_5$ , and  $\alpha_v\beta_3$ , respectively, the pro-angiogenic activities of CCN1 in endothelial cells are mediated through  $\alpha_v\beta_3$ . Recently, the specific binding sites of CCN1 for these integrins and HSPGs have been identified<sup>11, 12</sup>.

Human *CCN1* has been mapped to chromosome 1p22-p31<sup>13</sup>, the same region (1p21-p31) as an AVSD susceptibility gene identified in a large kindred<sup>4</sup>. In this study, we show that *Ccn1*-null mice exhibit AVSD with complete penetrance. Remarkably, haploinsufficiency for *Ccn1* also results in ostium primum ASD in 20% of adults, resembling a common form of partial AVSD recognized clinically in humans. These results implicate *CCN1* as a candidate gene for AVSD. *Ccn1* deficiency does not affect endocardial cushion tissue formation or EMT, but results in accelerated apoptosis in the cushion tissue and reduced gelatinase activity in the developing ventricular septum and AV valves. These results show that the matricellular protein CCN1 is essential for heart septation, and underscore the importance of matrix signaling in valvuloseptal morphogenesis.

## MATERIALS AND METHODS

### Animals, histology and immunohistochemistry

Generation of *Ccn1*-deficient mice, knockin of a *lacZ* reporter gene, and detection of  $\beta$ -galactosidase activity were described previously<sup>10</sup>. For histology, formalin-fixed embryos or post-natal hearts were embedded in paraffin, and 7  $\mu$ m sections were stained with hematoxylin and eosin. For immunohistochemistry, embryos fixed in 4% paraformaldehyde were sectioned and stained with antibodies against  $\alpha$ -smooth-muscle actin antibody (clone 1A4; Sigma) or activated caspase-3 (5A1, Cell signaling), and detected with alkaline phosphatase (AP) chromogen (Zymed). For proliferation assay, sections were stained with polyclonal anti-Ki67 antibodies (Novocastra Lab) and Alexa Fluor 594-conjugated secondary antibody (Molecular Probes), and counterstained with 4',6-diamidino-2-phenylindole dilactate (DAPI; Sigma). Images were acquired through a fluorescence microscope using a digital camera, and superimposition of Alexa Fluor 594 (red) and DAPI (blue) staining was done using the AxioVision (Zeiss) software.

### *In situ* hybridization

Embryos were fixed in 4% paraformaldehyde and paraffin embedded; 20  $\mu$ m sections were hybridized to digoxigenin-labeled *Mox-1* anti-sense riboprobe (generous gift of Christopher Wright, Vanderbilt) and visualized with AP-conjugated anti-digoxigenin antibody (Roche) and AP-chromogen. Sense riboprobe of *Mox-1* was used as control (data not shown).

### AV cushion explant culture

AV cushion explants were cultured on collagen gels as described<sup>14</sup>. E9.5 (somite #21~26) AV cushion tissues were microdissected and cultured on type I collagen (BD Biosciences) gels,

then fixed and stained with fluorescein phalloidin (Invitrogen) and counterstained with DAPI after 48 hrs.

### TUNEL assay and zymography

Paraffin sections (10  $\mu$ m) of 4% paraformaldehyde-fixed embryos were subjected to TUNEL assay using ApopTag Red *In Situ* Apoptosis Detection Kit (Chemicon) per vendor's instructions. *In situ* zymography was performed as described<sup>15</sup>. H9c2 cardiomyocytes (ATCC) were treated with 4  $\mu$ g/ml recombinant CCN1 protein<sup>16</sup> in DMEM containing 0.1% BSA. Conditioned medium was collected after 1–3 days and subjected to gelatin gel zymography following standard protocol with precast 10% zymogram gel containing gelatin (Bio-Rad).

### Quantitative real-time reverse transcriptase PCR (qRT-PCR)

cDNA reverse transcribed from total RNA isolated from E12.5 embryonic hearts was quantified using a MyiQ single-color real-time PCR detection system (Bio-Rad). PCR reaction in triplicate contains iQ SYBR Green Supermix (Bio-Rad), cDNA template and 200 nM primer pairs for either *Ccn1* or *cyclophilin* (internal control). Sense (S) and antisense (AS) primers are: *Ccn1*-S, 5'-GCAGCAAGACCAAGAAATCC-3'; *Ccn1*-AS, 5'-TTCTGGTCTGCAGAGGTGTG-3'. *Cyclophilin*-S, 5'-GGCAAATGCTGGACCAAACAC-3'; *cyclophilin*-AS, 5'-TTCTGGACCCAAAACGCTC-3'.

### Statistical analysis

Values are expressed as mean  $\pm$  standard derivations. Comparisons were made by Student's *t* test; statistical significance was set at  $P < 0.05$ .

## RESULTS

### Expression of *Ccn1* during heart development

We have previously placed the *lacZ* gene under the control of the endogenous *Ccn1* promoter, thereby allowing *Ccn1* expression to be monitored by following  $\beta$ -galactosidase activity<sup>10</sup>. As judged by X-gal staining, *Ccn1* is expressed in the E8.5 myocardium and endocardium of the bulbus cordis region in the primitive heart (Fig. 1A). Prominent cardiac expression of *Ccn1* was seen at E10.5 (Fig. 1B), especially in the truncus arteriosus (arrowhead, Fig. 1C), which later divides to form the aorta and pulmonary trunk. *Ccn1* expression was also seen around the AV canal at later stages and in the AV cushion tissue and septum (Fig. 1D,E). At E12.5, *Ccn1* was expressed at the junctions between the muscular component of the ventricular septum and the membranous mesenchyme of the cushion tissue, and between the mesenchymal cap of the atrial septum and the AV cushion tissue (Fig. 1F). By E13.5, expression around the atrial junction had subsided, but was prominent in the developing AV valvular leaflets (Fig. 1G). Expression was high in the vessel wall of the aorta, although little or no expression was seen in the pulmonary and aortic valve leaflets (Fig. 1H). Thus, *Ccn1* expression coincides with the temporal and spatial pattern of key events in atrioventricular valvuloseptal morphogenesis. The levels of *Ccn1* mRNA expressed in E12.5 embryonic hearts were examined by quantitative real-time RT-PCR, confirming a true null phenotype in *Ccn1*<sup>-/-</sup> embryos and haploinsufficiency in *Ccn1*<sup>+/-</sup> embryos (Fig. 1I)<sup>10</sup>.

### *Ccn1*-null mice exhibit AVSD

Given the cardiac expression of *Ccn1* described above, we examined the effects of *Ccn1* deficiency in the heart. The atrial and ventricular septa were formed by E14.5 in WT embryos, as were the AV valves (Fig. 2A,B). *Ccn1*<sup>-/-</sup> embryos, however, exhibited AVSD with ~100% penetrance, with 55% (10/18) showing complete AVSD and 45% (8/18) showing VSD (Fig.

2E-G). Severe malformation of the atrial and ventricular septa and defects in AV valve morphogenesis were evident. During AV septation and valve formation, the common AV canal is partitioned by the fusion of the inferior and superior endocardial cushion tissues into separate left and right components, which undergo further morphogenesis to form the mitral and tricuspid valves<sup>3</sup>. Fusion of the inferior and superior cushion tissue was impaired in affected *Ccn1*<sup>-/-</sup> embryos as evidenced by gaps between the two endocardial cushions, resulting in a common AV valve orifice that is a hallmark of complete AVSD (Fig. 2F,G). Nearly all *Ccn1*-null embryos showed defects in maturation of the interventricular septum (IVS), which forms by the fusion of a muscular component that extends as an outgrowth of the ventricular wall, and a membranous component that forms from the AV cushion tissue. This fusion is defective in *Ccn1*-null embryos (Fig. 2E), consistent with the expression of *Ccn1* in the junction between the muscular component of the septum and the membranous mesenchyme (black arrow, Fig. 1F).

### Haploinsufficiency in *Ccn1* leads to atrial septal defects

Surprisingly, *Ccn1*<sup>+/-</sup> embryos also display AVSD. This finding is significant because autosomal dominant inheritance has been observed in non-syndromic AVSD in humans<sup>4, 6</sup>. Histological analysis showed that ~67% of *Ccn1*<sup>+/-</sup> embryos (18/27) exhibited cardiac defects of varying severity at E14.5, including complete AVSD (4/27), VSD (11/27), or dysplastic mitral valves (3/27)(Fig. 2C,D). Although VSD phenotypes in *Ccn1*<sup>+/-</sup> hearts were conspicuous at E13.5 and E14.5, they were not observed after E15.5 (n=35), indicating that IVS formation is delayed in *Ccn1*<sup>+/-</sup> embryos but not persistently impaired. However, ~20% of adult *Ccn1*<sup>+/-</sup> mice (5/25) show persistent ASD (Fig. 3). Although cardiac septal defects might form as secondary effects of placental abnormalities<sup>17</sup>, these cardiac defects are not likely related to the placenta since the *Ccn1*<sup>+/-</sup> placenta develop normally<sup>10</sup>.

Septation of the common atrial chamber begins at E10.5 with the formation of the septum primum, which approaches the AV cushion tissue from the atrial chamber roof. The opening between the two atrial chambers, the ostium primum, is obliterated upon fusion of the septum primum with the AV cushion and is replaced by the ostium secundum, which forms by apoptosis in the septum primum. The septum secundum then forms, with the foramen ovale serving as the opening between the atria<sup>3</sup>. Postnatal fusion of the septum primum and septum secundum closes the openings of both septa and ensures complete separation of the left and right atria after birth when pulmonary circulation is established (Fig. 3A,B). In affected *Ccn1*<sup>+/-</sup> neonates, the septum primum failed to fuse completely with the cushion tissue and the ostium primum remained (Fig. 3C,D). Consistent with a role in this fusion event, *Ccn1* was expressed in the AV cushion where fusion with the mesenchymal cap of the septum primum occurs (Fig. 1F). Although formation of the septum secundum was successful, postnatal fusion of the two septa was precluded by the defective septum primum. Consequently, blood cells were trapped between the atria as the hemodynamic flow was in disarray (Fig. 3C,D), and the foramen ovale remained patent in affected adult *Ccn1*<sup>+/-</sup> mice (Fig. 3E,F).

### EMT and differentiation of cushion mesenchyme

Central to valvuloseptal morphogenesis is the AV cushion tissue, which forms when endocardial cells undergo EMT and invade into the cardiac jelly<sup>2, 18</sup>. We therefore examined whether EMT occurs in AV cushion tissue explants isolated from E9.5 *Ccn1*<sup>-/-</sup> embryos. Endocardial cells that successfully undergo EMT are capable of migrating into collagen gel on which the explant is cultured (Fig. 4A-D). The percentages of cells migrating into collagen gel in wild-type and *Ccn1*<sup>-/-</sup> cushion explants were indistinguishable (Fig. 4E), indicating that *Ccn1* is not essential for EMT. We next investigated the differentiation of the mesenchymal cells within the cushion tissue using molecular markers. Smooth muscle  $\alpha$ -actin is expressed as mesenchymal cells gain characteristics of smooth muscle-like myofibroblasts<sup>19</sup>, and

*Mox-1* is a marker of more differentiated mesenchymal cells in the cushion<sup>20</sup>. Expression of both smooth muscle  $\alpha$ -actin and *Mox-1* was similar in wild-type and *Ccn1*<sup>-/-</sup> embryonic hearts (Fig. 4F), indicating that EMT and further differentiation of cushion tissue mesenchymal cells were successful in *Ccn1*<sup>-/-</sup> embryos.

### Cell proliferation

Valvuloseptal morphogenesis begins with the expansion of the cushion tissue and fusion with the AV septa, followed by extensive remodeling and shrinkage to form the mature AV septa and valves. Coordinated with these morphogenetic events are highly regulated cell proliferation and programmed cell death in the cushion tissue<sup>21</sup>, and BMP-4 deficiency causes septal defects due to reduced cushion mesenchymal cell proliferation<sup>22</sup>. As judged by Ki67 staining, the percentages of proliferating cells in WT and *Ccn1*-null AV cushion were similar as the cushion tissue actively expands at E11.5 and E12.5 (Fig. 5). Thus, cell proliferation in the AV cushion is not affected by *Ccn1* deficiency.

### Precocious apoptosis in the *Ccn1*-deficient cushion tissue

CCN1 can induce apoptosis in fibroblasts but promote endothelial cell survival *in vitro*<sup>16, 23</sup>. Consistent with its pro-survival function in endothelial cells, vascular cells of large vessels in *Ccn1*-null mice suffer apoptosis<sup>10</sup>. As previously reported, very few apoptotic cells were detected in the WT AV cushion tissue as it began to fuse with the AV septa at E12.5 (Fig. 6A,B)<sup>24</sup>. By contrast, a large number of TUNEL- or activated caspase-3-positive apoptotic cells were detected in the *Ccn1*<sup>-/-</sup> cushion tissue proximal to the atrial septum (Fig. 6A-E, K-N) where *Ccn1* is expressed (Fig. 1F). These results suggest that CCN1 is required for cushion tissue cell survival at this embryonic stage, and its absence results in apoptosis. This early onset of apoptosis in the E12.5 cushion tissue may be detrimental to atrial septum fusion, leading to the ASD phenotype. By E13.5, the atrial septum primum fusion has occurred, and expression of *Ccn1* was diminished in the atrial fusion site of the AV cushion (Fig. 1G), correlating with a dramatic increase in apoptosis in the WT cushion tissue (Fig. 6F,G), consistent with previous reports<sup>24</sup>. Thus, while programmed cell death occurs in the AV cushion upon fusion with the atrial septum<sup>21</sup>, precocious onset of apoptosis in *Ccn1*-null mice may prevent the fusion event from occurring successfully. Although *Ccn1* deficiency also leads to VSD, no abnormal cell death was detected at the fusion site between the ventricular septum and the cushion tissue (Fig. 6).

### Gelatinase deficiency in the *Ccn1*-null heart

Matrix metalloproteinases (MMPs) and their regulators are thought to be involved in aspects of cardiac development as regulators of growth factors activity and ECM composition<sup>25, 26</sup>. Several MMPs have been identified as key targets of CCN1-regulated signaling pathways in fibroblasts<sup>27</sup>, prompting us to examine MMP activity in embryos. Gelatinases (MMP2 and MMP9) degrade type IV collagen in basement membrane and are of particular importance in tissue remodeling. Gelatinase activity was detected by *in situ* gelatin zymography in the E12.5 WT heart (Fig. 7A,D). This activity was blocked by the inhibitor 1, 10-phenanthroline (PNT), confirming that it is due to a metalloprotease (Fig. 7B,E). By contrast, gelatinase activity was greatly diminished in the *Ccn1*<sup>-/-</sup> heart, particularly in the muscular component of the IVS and the valvular leaflets where defects are observed (Fig. 7C,F). To test whether CCN1 is able to directly regulate the expression of gelatinases, which are synthesized as zymogens and are proteolytically cleaved to the active forms, we treated H9c2 cardiomyocytes with purified CCN1 protein. Indeed, CCN1 treatment dramatically increased both secreted pro- and activated-MMP-2 (Fig. 7G). Thus, CCN1 is an inducer of MMP2 in cardiomyocytes, and a deficiency in gelatinase activities in *Ccn1*-null mice may compromise the matrix remodeling process that is crucial for fusion of the IVS and AV cushion tissue.

## DISCUSSION

ECM signaling plays a critical role in developmental processes. As demonstrated in this study, the matricellular protein CCN1 is indispensable for cardiovascular development, and nullizygoty in *Ccn1* results in severe AVSD and embryonic death. Although heterozygous *Ccn1* mice are largely viable, 20% of them display ostium primum ASD. Furthermore, *CCN1* maps to the same human chromosomal location (1p21-p31) as that of a susceptibility gene for non-syndromic AVSD, *AVSD1*, mutations in which also leads to predominantly ostium primum ASD<sup>4, 13</sup>. These results provide the first evidence that haploinsufficiency in an ECM protein can lead to autosomal dominant ASD, and implicate *CCN1* as a candidate gene for AVSD in humans.

CHDs are the most common group of birth defects in humans, encountered in ~1% of live births and 10% of stillborns<sup>1</sup>. Consistent with the complex nature of cardiac development, nullizygoty of a multitude of genes in mice result in heart defects<sup>28</sup>. Whereas human CHDs exhibit predominantly autosomal dominance rather than homozygous recessive modes of transmission, only a very few genes, encoding cardiac-specific transcription factors, have been identified to cause autosomal dominant CHD due to haploinsufficiency in mice. Heterozygous mutations in these transcription factor genes, including *Tbx1* (DiGeorge syndrome)<sup>29</sup>, *Tbx5* (Holt-Oram syndrome)<sup>30</sup>, and *Nkx2-5* (conduction defects, tetralogy of Fallot)<sup>31</sup>, also cause CHDs in humans. Since *CCN1* is a matricellular protein that acts through binding to integrins and HSPGs, *Ccn1* mutant mice provide a unique animal model for addressing the role of matrix signaling in heart development. Aside from *Ccn1* and *AVSD1*, another genetic locus known to be associated with non-syndromic AVSD is *CRELD1*, which encodes a predicted matricellular cell adhesion molecule based on its sequence<sup>6, 32</sup>. Although the activities of *CRELD1* have not yet been examined, these findings are consistent with the notion that matrix signaling is crucial for AV septation and valvular formation.

The AV cushion tissue is central to valvuloseptal morphogenesis, and malformations in the cushion tissue, such as those caused by deficiency in versican, hyaluronan synthase 2 or the TGF- $\beta$  type III receptor, result in AVSD<sup>28, 33</sup>. Although *Ccn1* is not required for the formation of the AV cushion, EMT of endocardial cells, or proliferation or further differentiation of cushion mesenchymal cells, *Ccn1* deficiency leads to precocious apoptosis in the AV cushion (Fig. 4-6). Programmed cell death plays a key role in cardiac development and is often found at causative factor in the AVSD phenotype of transforming growth factor- $\beta$ 2 (TGF- $\beta$ 2)-deficient mice<sup>34</sup>. The early onset of apoptosis in the AV cushion in *Ccn1*-deficient mice is likely an impediment to successful fusion between the cushion tissue and the atrial septum, and may lead to defects in tissue remodeling. This finding also indicates that *CCN1* is required for cushion tissue mesenchymal cell survival. *CCN1* is known to promote the survival of endothelial cells in culture through the engagement of integrin  $\alpha_v\beta_3$ <sup>16, 23</sup>, consistent with aberrant vascular cell apoptosis in *Ccn1*-null embryos<sup>10</sup>. *CCN1* may serve a similar pro-survival function for the endocardium-derived cushion mesenchymal cells, and thus *Ccn1* deficiency results in apoptosis.

MMPs and their inhibitors have been shown to mediate various aspects of cardiac development, including heart tube formation, cardiac neural crest migration, and EMT of the endocardial cushions<sup>25</sup>. The *Ccn1*-null hearts displayed a significant reduction in gelatinase activities in the interventricular septum, left ventricular wall, and the AV valves, whereas residual activity was detected in the right ventricular wall (Fig. 7). *CCN1* regulates the expression of genes involved in matrix remodeling in fibroblasts<sup>27</sup>, and upregulates MMP-2 levels and enhances its activation in cardiomyocytes (Fig. 7G). Although deficiency in gelatinase activities may contribute to cardiac defects, it is by itself insufficient to cause cardiac abnormalities. No heart defects have been reported in mouse models with null mutations in MMP-2 (gelatinase A),

MMP-9 (gelatinase B), or both<sup>35–37</sup>. However, loss of MMP-2 and/or -9 protects against the tissue fibrosis seen following myocardial infarction in adults, demonstrating that these gelatinases do have significant effects on myocardiocytes *in vivo*<sup>38</sup>.

*Ccn2* (CTGF, connective tissue growth factor) is a closely related homologue of *Ccn1*, and both genes are highly expressed in the myocardium and AV cushion during heart development, and play a role in adaptation of the heart to cardiovascular stress<sup>39, 40</sup>. Although *Ccn2*-null mice show apparently normal heart development through birth<sup>41</sup>, there may be significant functional redundancies between these genes that allow *Ccn1* to compensate for the loss of *Ccn2* in the cardiovascular system, since CCN1 and CCN2 have similar activities *in vitro*<sup>42</sup>. Hence, while specific functions of CCN1 are clearly indispensable, the potential roles of CCN2 and possible overlapping functions between CCN1 and CCN2 in cardiac development remain to be established.

How *Ccn1* is integrated into the regulatory circuits that control heart development is currently unknown, although consensus binding sites for the cardiac transcription factors Nkx2.5 and GATA-4 are found on its promoter<sup>43</sup>. Mechanistically, CCN1 can function to regulate cell survival/death and the expression of genes involved in matrix remodeling. In addition, CCN1 may potentially modulate the activities of other regulatory pathways through interactions with growth or morphogenic factors or their receptors<sup>44, 45</sup>. Since haploinsufficiency in *Ccn1* leads to ostium primum ASD resembling that observed in man, the possibility that mutations in *CCN1* may lead to clinical AVSD clearly warrants further investigation.

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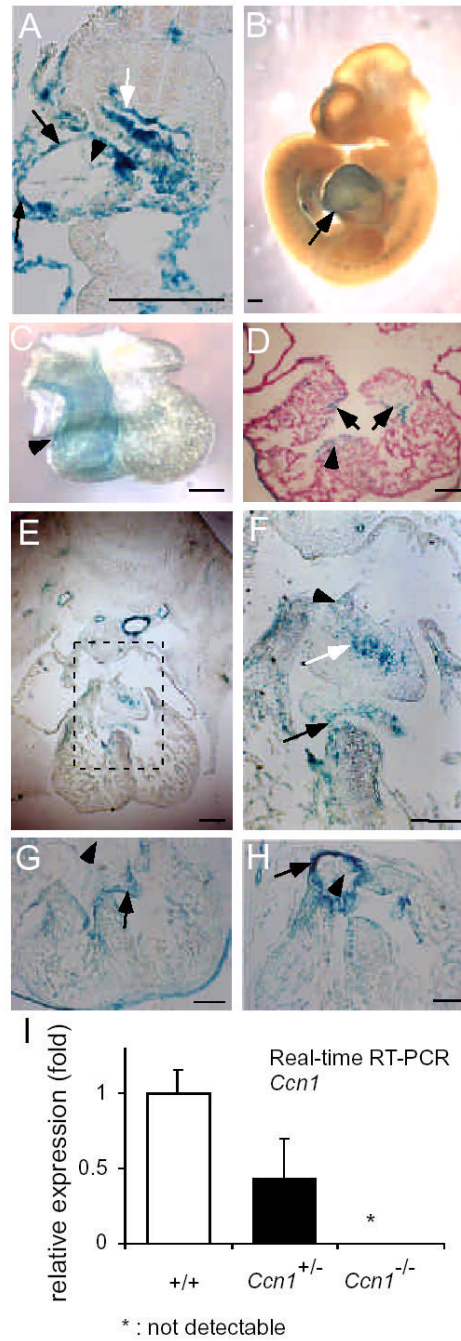
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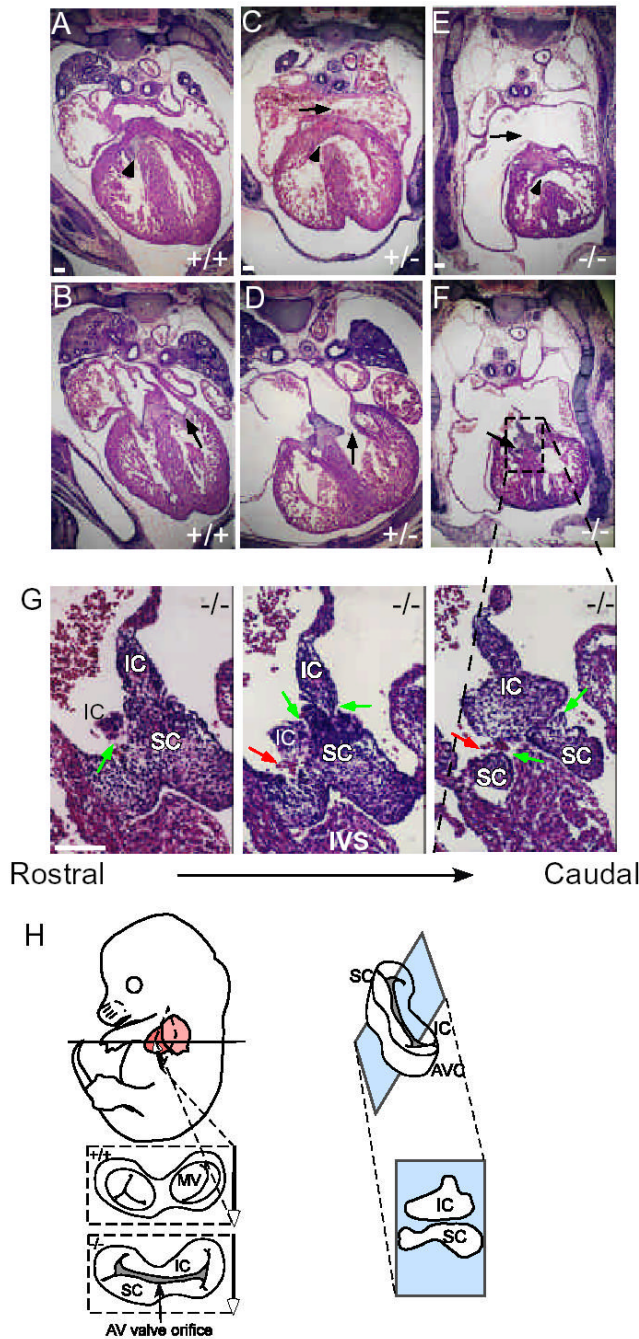
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**Figure 1.**

Expression of  $\beta$ -galactosidase driven by the *Ccn1* promoter. (A) Intense X-gal staining in the myocardium (black arrow) of the bulbus cordis of the primitive heart of E8.5 *Ccn1*<sup>+/-</sup> embryos; less staining was seen at endocardium (black arrowhead). White arrow points to the foregut. (B) Whole-mount E10.5 embryo shows prominent cardiac staining (arrow). (C) X-gal staining was detected in the truncus arteriosus (arrowhead) at E10.5. (D) Expression in the developing atrioventricular cushion tissue (arrows) and ventricular septum (arrowhead) at E11.5. (E) X-gal staining in the E12.5 embryonic heart. (F) Higher magnification view of dashed box in E, showing staining in the mesenchymal cap of the atrial septum primum (black arrowhead), the cushion mesenchyme (white arrow), and at the junction between the ventricular septum and

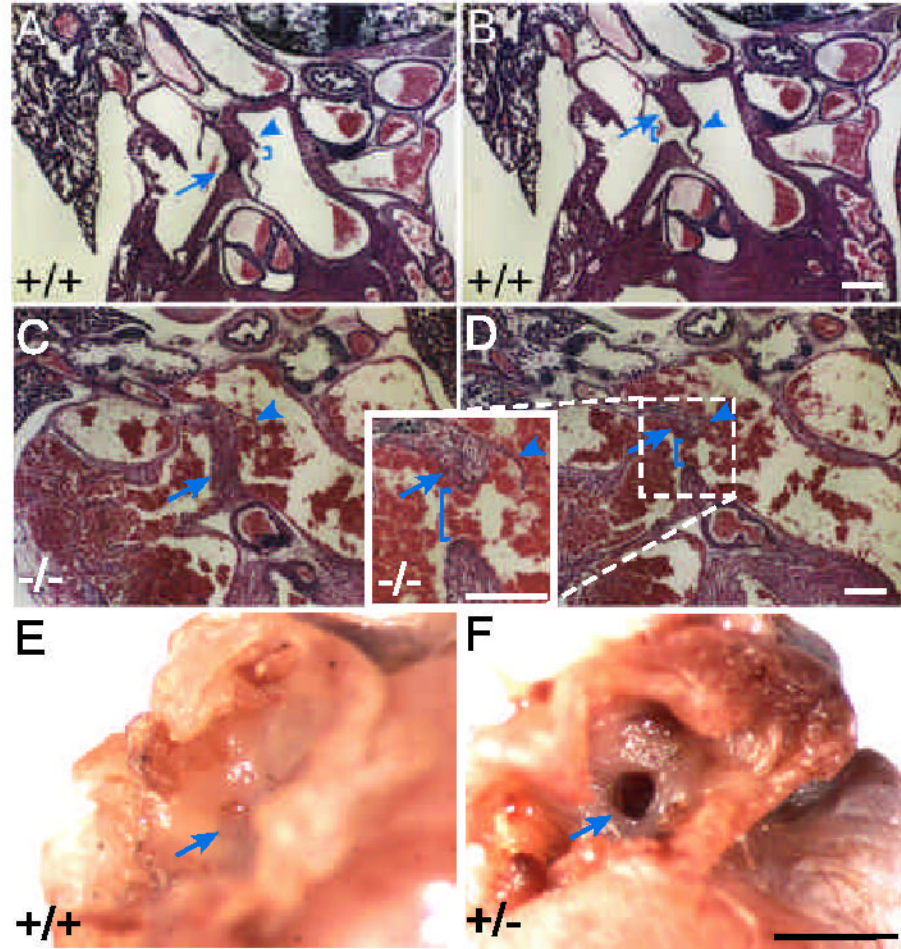
the cushion tissue (black arrow). (G) At E13.5, expression was observed at the valvular leaflets (arrow) and the ventricular septum junction, while expression around the atrial fusion sites had diminished (arrowhead). (H) Expression was weak in aortic leaflets (arrowhead), but remained intense in aortic wall at E13.5 (arrow in H). (I) The levels of *Ccn1* mRNA in E12.5 hearts were quantified by qRT-PCR, normalized to cyclophilin mRNA as internal control. No detectable signal was observed in *Ccn1*<sup>-/-</sup> (n=3 per genotype,  $P < 0.03$  between +/+ and +/-). Bars: 200  $\mu$ m.



**Figure 2.**

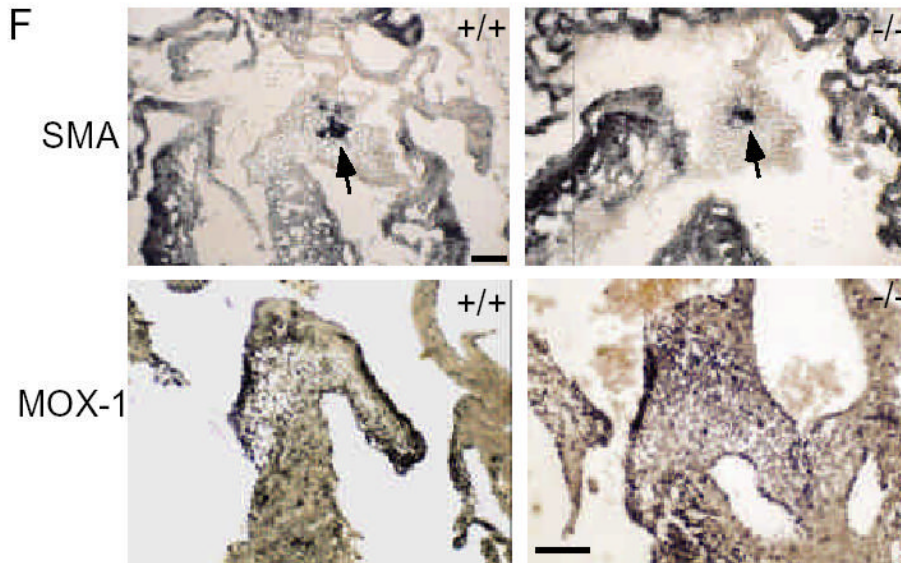
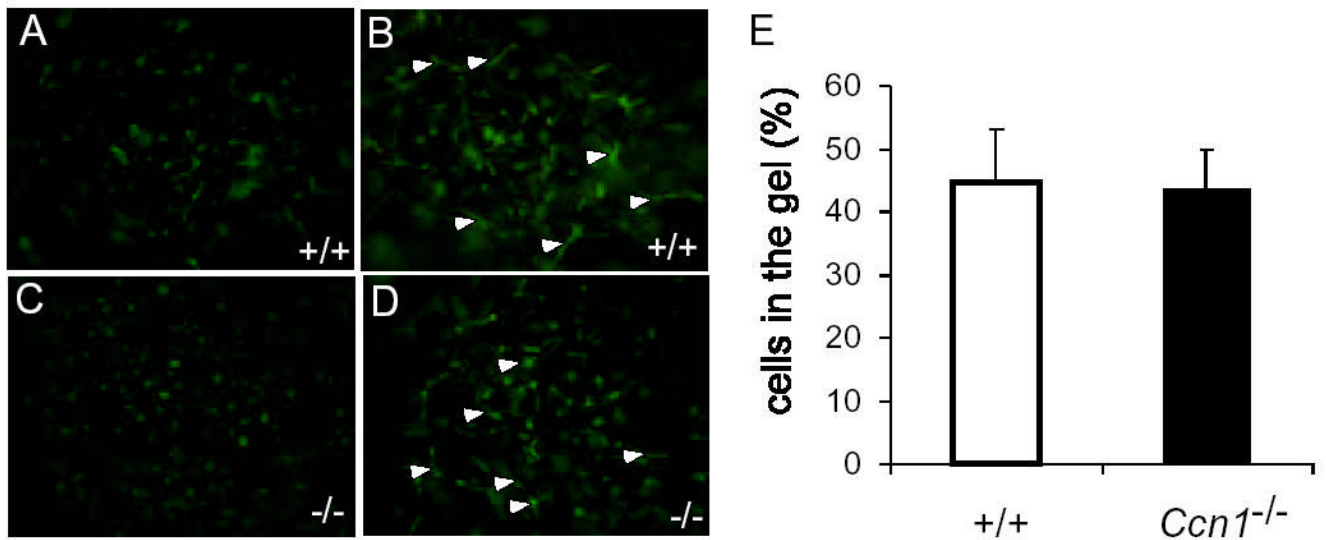
Atrioventricular septal defects in *Ccn1*-deficient embryos. Transverse sections of E14.5 embryonic hearts were H & E stained. (A,B) WT embryos showed well developed four-chambered hearts, with complete closure of the ventricular septum (arrowhead, A) and extended AV valvular structure (arrow, B). (C,D) *Ccn1*<sup>+/-</sup> embryos displayed VSD (arrowheads in C), ASD (arrow, C), and dysplastic atrioventricular valves (arrow, D). (E,F) *Ccn1*<sup>-/-</sup> hearts exhibited VSD (arrowhead, E), ASD (arrow, E), and AV valve orifice (arrow, F). (G) High magnification views of dashed box in F and its adjacent sections reveal evidence of AV valve orifice, including gaps between superior (SC) and inferior (IC) endocardial cushions of AV canal (green arrows) with blood cells in between the two AV cushions (red

arrows). IVS: interventricular septum. (H) Left, diagram illustrates the partitioning of the AV canal (AVC) into tricuspid (TV) and mitral (MV) valves, viewed from the atrial perspective. A hallmark of complete AVSD is the presence of a common AV valve orifice resulting from impaired fusion between the inferior (IC) and superior (SC) cushion tissue. Right, the plane of section in sections shown in (G), showing how the IC and SC are viewed. Bars: 100  $\mu\text{m}$ .



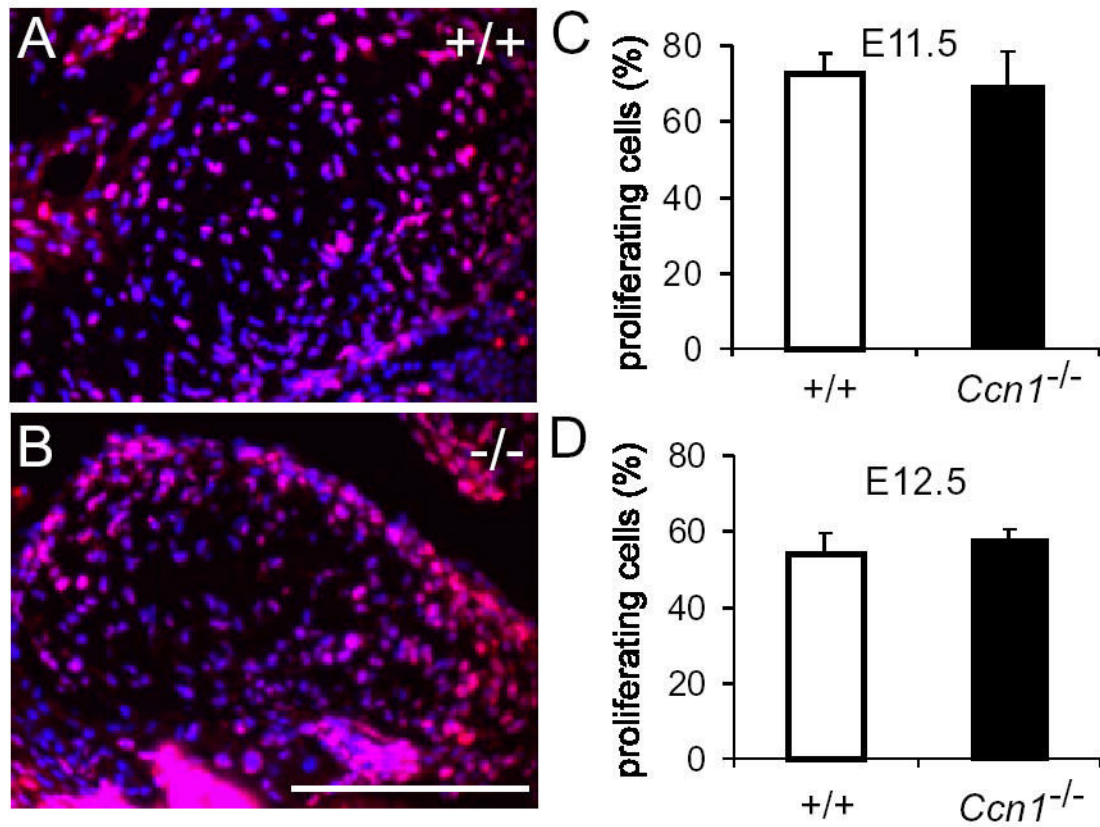
**Figure 3.**

Haploinsufficiency in *Ccn1* results in ASD. (A,B) WT neonatal hearts were sectioned and H & E stained. The septum primum (arrowheads) and septum secundum (arrows) were properly formed, separating the two atrial chambers. The ostium secundum (bracket in A) and fossa ovalis (bracket in B) are seen. (C,D) In *Ccn1*<sup>+/-</sup> pups, fusion of the septum primum (arrowheads) with the cushion tissue was unable to occur, leaving a detached septum primum and foramen ovale (brackets in D). Formation of the septum secundum (arrows) appeared normal. (E,F) In hearts of 10 month old mice, the fossa ovalis (arrow, E) was closed in the WT but the embryonic foramen ovale remained patent (arrow, F) in the *Ccn1*<sup>+/-</sup> mouse. White bars: 100 μm, black bar: 1 mm.



**Figure 4.**

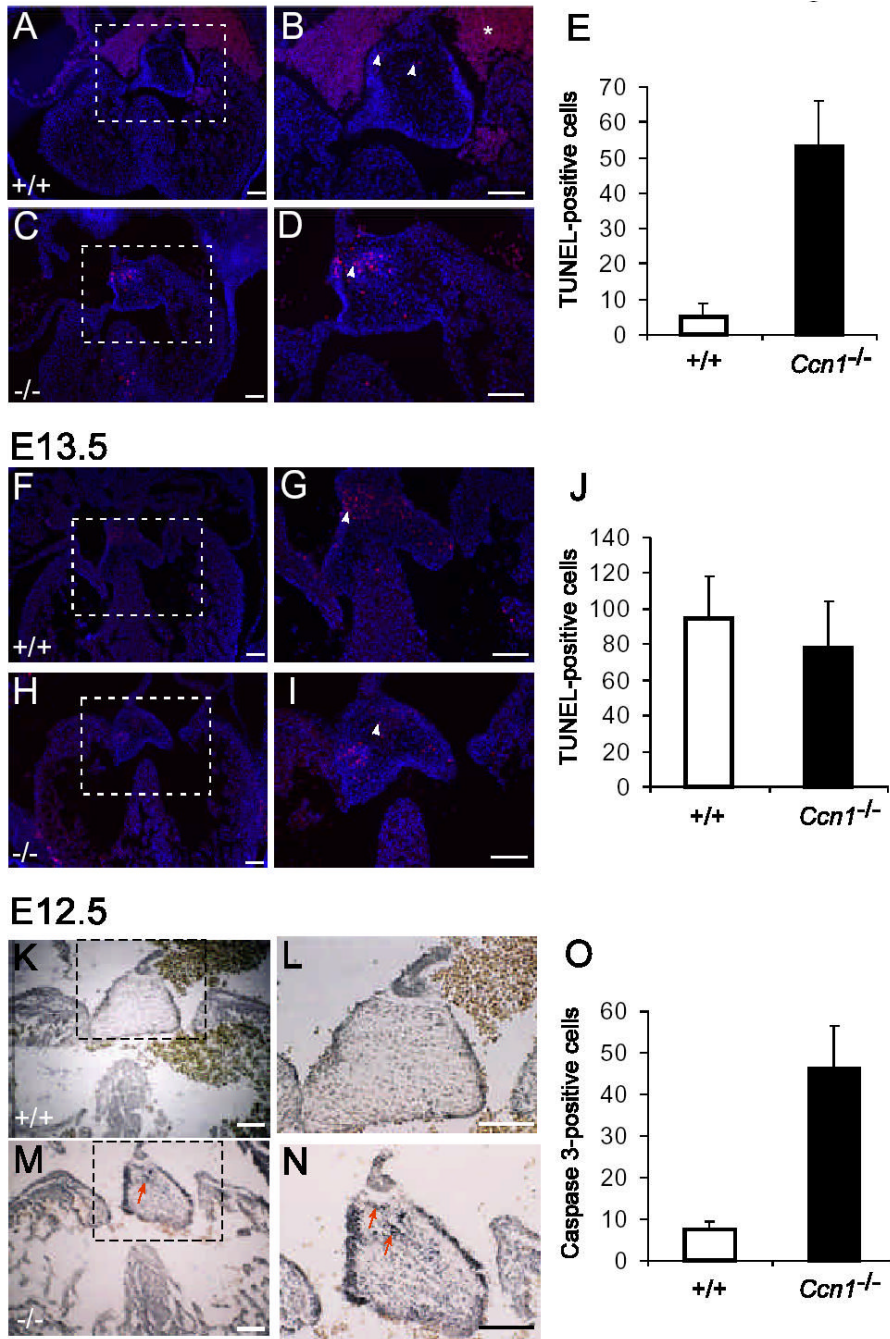
Epithelial-mesenchymal-transformation in AV cushion explant culture. E9.5 AV cushions were isolated from *Ccn1*<sup>+/+</sup> (A,B) and *Ccn1*<sup>-/-</sup> (C,D) embryos and cultured on collagen gel for 48 hrs. (A,C) Undifferentiated cells remaining on the gel surface. (B,D) Differentiated cells (arrowheads) migrated into the collagen gel. (E) Percentages of cells migrated into the collagen gel over the total number of cells in AV explants from *Ccn1*<sup>+/+</sup> (n=6) and *Ccn1*<sup>-/-</sup> (n=5) embryos ( $P>0.80$ ). (F) Immunostaining of smooth muscle  $\alpha$ -actin (SMA, arrows) in E13.5 cushions showed similar patterns in *Ccn1*<sup>+/+</sup> and *Ccn1*<sup>-/-</sup> embryonic hearts. *Mox-1* expression was detected in E14.5 cushions and developing valves in both wild-type and *Ccn1*<sup>-/-</sup> embryos by *in situ* hybridization. Bars: 100  $\mu$ m.



**Figure 5.**

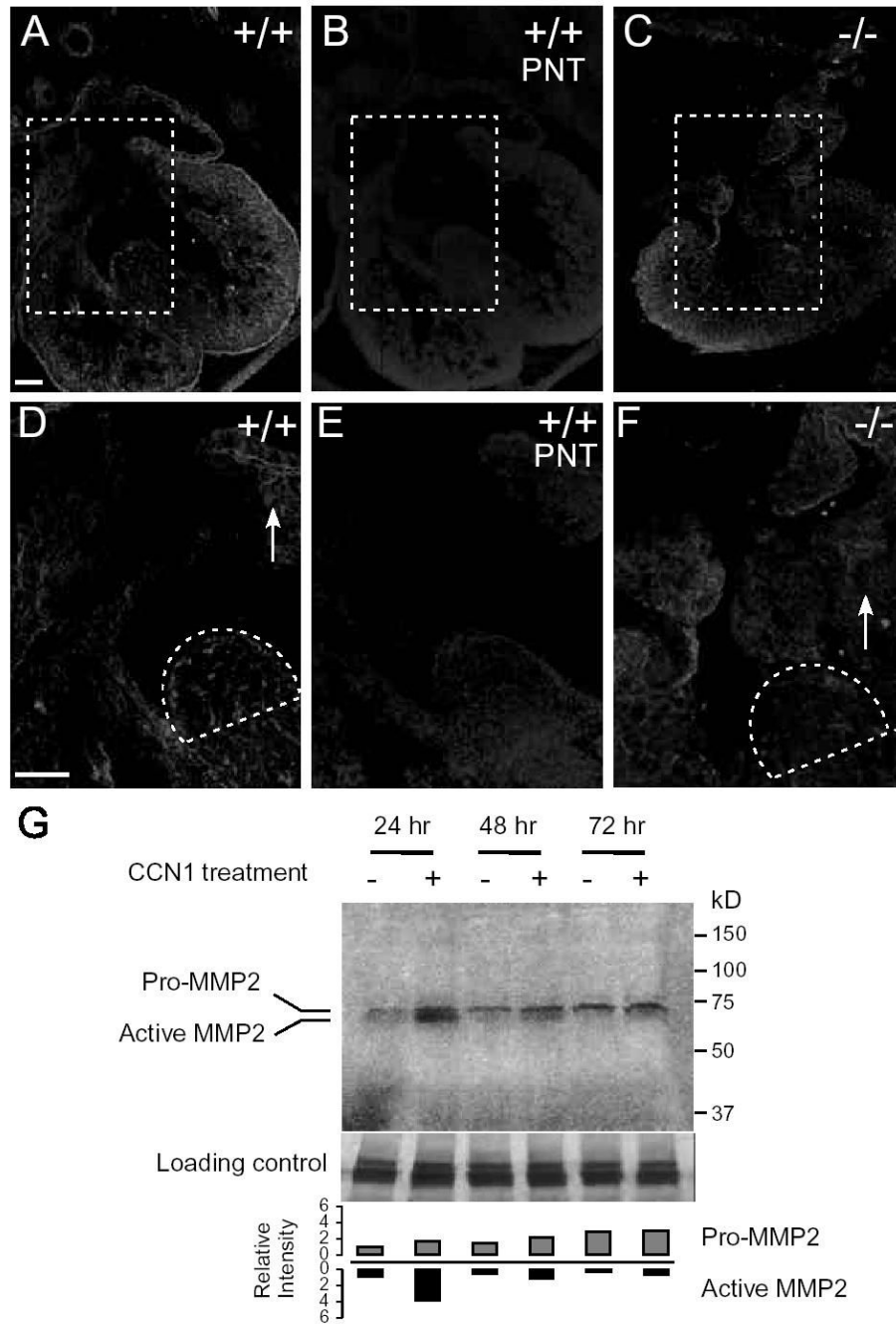
Cell proliferation in the AV cushion tissue. E11.5 (shown in A, B) and E12.5 AV cushion tissues of wild-type (+/+) and *Ccn1*<sup>-/-</sup> embryos were immunostained for Ki67 (red) and counterstained with DAPI (blue). Results were quantified, showing equivalent proliferation rates in wild-type and *Ccn1*<sup>-/-</sup> embryos in E11.5 (C, n=6 each;  $P>0.59$ ) and E12.5 embryos (D, n=5 each,  $P>0.34$ ).





**Figure 6.** Apoptosis in the AV cushion. Apoptotic cells were identified in sections of embryonic hearts using TUNEL assay and counterstained with DAPI (A-J, arrowheads), or by immunohistochemical staining for activated caspase-3 (K-O, arrows). Higher magnifications of dashed boxes in A, C, F, H, K, and M are shown in B, D, G, I, L, and N, respectively. Precocious apoptosis was observed in E12.5 *Ccn1*<sup>-/-</sup> AV cushion (C,D), compared to the *Ccn1*<sup>+/+</sup> cushion (A,B) by either TUNEL assay (E;  $P < 0.001$ ) or immunohistochemical staining of activated caspase-3 (K-O;  $P < 0.001$ ). At E13.5, similar numbers of TUNEL-positive cells were observed in AV cushions and valvular leaflets of both WT (F, G) and *Ccn1*<sup>-/-</sup> (H, I) hearts (J;  $P > 0.30$ ). Apoptotic cells were counted from transverse sections of AV cushions of

*Ccn1*<sup>+/+</sup> and *Ccn1*<sup>-/-</sup> hearts (each group n=5), and the average numbers of TUNEL-positive cells in 10  $\mu\text{m}$  sections are shown in E, J, and O. Error bars are standard deviations. The star in B indicates background staining from red blood cells. Bars = 100  $\mu\text{m}$ .



**Figure 7.** Gelatinase zymography. Gelatinase activity was visualized by *in situ* zymography as bright fluorescence in E12.5 hearts (A-F). D, E and F are the higher magnification views of dashed boxes in A, B and C, respectively. Enzyme activities can be seen in the hearts of wild-type E12.5 embryos (A,D), including the tip of the ventricular septum (dashed area in D) and the valvular leaflets (arrow). (B,E) The MMP inhibitor 1,10-phenanthroline (PNT) blocked this activity. (C,F) *Ccn1*<sup>-/-</sup> hearts show deficiency in gelatinase activity at the ventricular septum (dashed) and the valvular leaflets (arrow). (G) Conditioned media of H9c2 cardiomyocytes cultured with or without purified CCN1 were collected 24, 48 or 72 hrs after treatment and analyzed by gelatin zymogram, showing induction of active MMP-2 by CCN1 at 24 hrs. The

intensity of bands corresponding to either the pro- or active MMP-2 were quantified with ImageJ (NIH). The same samples were electrophoresed in acrylamide gel and Coomassie blue-stained, showing equal protein in each sample (lower panel). Four independent experiments were performed with similar results.