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RESEARCH ARTICLE

CCN2/CTGF is required for matrix organization and to protect growth plate chondrocytes from cellular stress

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Abstract CCN2 (connective tissue growth factor (CTGF/CCN2)) is a matricellular protein that utilizes integrins to regulate cell proliferation, migration and survival. The loss of CCN2 leads to perinatal lethality resulting from a severe chondrodysplasia. Upon closer inspection of *Ccn2* mutant mice, we observed defects in extracellular matrix (ECM) organization and hypothesized that the severe chondrodysplasia caused by loss of CCN2 might be associated with defective chondrocyte survival. *Ccn2* mutant growth plate chondrocytes exhibited enlarged endoplasmic reticula (ER), suggesting cellular stress. Immunofluorescence analysis confirmed elevated stress in *Ccn2* mutants, with reduced stress observed in *Ccn2* overexpressing transgenic mice. In vitro studies revealed that *Ccn2* is a stress responsive gene in chondrocytes. The elevated stress observed in *Ccn2*−/− chondrocytes is direct and mediated in part through integrin α5. The expression of the survival marker NFκB and components of the autophagy pathway were decreased in *Ccn2* mutant growth plates, suggesting that CCN2 may be involved in mediating chondrocyte survival. These data demonstrate that absence of a matricellular protein can result in increased cellular stress and highlight a novel protective role for CCN2 in chondrocyte survival. The severe chondrodysplasia caused by the loss of CCN2 may be due to increased chondrocyte stress and defective activation of autophagy pathways, leading to decreased cellular survival. These effects may be mediated through nuclear factor κB (NFκB) as part of a CCN2/integrin/NFκB signaling cascade.

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Introduction

Chondrocytes secrete large amounts of extracellular matrix (ECM) as they differentiate. Disruptions in this process often result in chondrodysplasias, a diverse group of congenital birth defects characterized by short stature and skeletal deformity. The most common causes of chondrodysplasias are mutations that lead to structural alterations in genes that encode ECM proteins. These alterations prevent proper protein folding and maturation, leading to their retention in the endoplasmic reticulum (ER), and/or the incorporation of the mutated product into the ECM, interfering with matrix structure and function (Bateman et al. 2009).

ER enlargement is a hallmark of defective protein folding and cellular stress. ER and other forms of cellular stress activate the Unfolded Protein Response (UPR), an adaptive mechanism to restore cell homeostasis and viability (Rasheva and Domingos 2009). The UPR ameliorates cellular stress by attenuating protein synthesis and increasing chaperone protein levels to facilitate correct protein folding and clearing of misfolded protein aggregates. The glucose-regulated protein 78 (GRP78) also known as BiP, is a resident ER chaperone and the main initiator of the UPR (Bertolotti et al. 2000). BiP activates UPR effector molecules that function to restore cellular homeostasis. ER and other forms of cellular stress also activate anti-oxidative stress genes, which promote cell survival; however, prolonged stress can lead to activation of CCAAT/enhancer-binding protein-homologous protein (CHOP) and caspases to induce cell death (Rasheva and Domingos 2009). Although ER sensing of unfolded proteins is the most common inducer of ER stress, the UPR is activated by multiple stress pathways, including hypoxia and ECM detachment (Avivar-Valderas et al. 2011; Walter and Ron 2011). Stress pathways are major players in multiple pathologies including osteoarthritis (Rasheva and Domingos 2009) and identifying proteins involved in cellular stress pathways may provide new therapeutic targets.

Nuclear factor κB (NFκB/p65/relA) is a transcription factor that plays pivotal roles in cell proliferation, differentiation and survival (Marcu et al. 2010). ER stress activates NFκB via tumor necrosis factor-α (TNF-α) receptor associated factor 2 (TRAF2) and inositol requiring enzyme 1 (IRE1) in vitro (Kaneko et al. 2003). The role of NFκB activation in various forms of cellular stress is unclear, but several studies show that NFκB exerts pro-survival functions (Nozaki et al. 2001; Sirabella et al. 2009). In the growth plate, NFκB is essential for chondrocyte viability (Park et al. 2007; Wu et al. 2007). However, the mechanisms that control NFκB activity in the growth plate are unknown.

Autophagy is a lysosomal degradation pathway used to recycle cellular components and activate cell survival under conditions of starvation and stress (Mehrpour et al. 2010). Autophagy is essential for maintaining ER homeostasis, and evidence indicates that during prolonged ER stress, autophagy is activated to assist in the removal of misfolded proteins (Li et al. 2008). Crosstalk between cellular stress pathways, autophagy and NFκB-mediated cellular survival has been confirmed previously (Copetti et al. 2009; Sirabella et al. 2009), but whether such crosstalk occurs in chondrocytes is unknown.

The term "matricellular" was coined to encompass a structurally diverse group of proteins that reside in the ECM, but serve no structural functions (Bornstein 2009). Members of the CCN (Cyr61/CTGF/Nov) family of matricellular proteins are critical regulators of cell-ECM interactions. The six members of the CCN family contain four conserved domains that mediate interactions with integrins, growth factors, and ECM components including heparin sulfate proteoglycans, fibronectin and aggrecan (Jun and Lau 2011). Connective tissue growth factor (CTGF/CCN2) is best known for its role in fibrosis, where its overexpression exacerbates excess collagen deposition in multiple organs (Brigstock 2010). CCN2 overexpression is involved in many other pathological processes, such as atherosclerosis, osteoarthritis and cancer (Chen and Lau 2009).

Although most studies have focused on the mechanisms by which CCN2 overexpression leads to pathological changes, CCN2 is indispensible for endochondral bone formation. Global loss of CCN2 in mice results in severe chondrodysplasia and lethality at birth (Ivkovic et al. 2003). This phenotype is accompanied by a significant decrease in chondrocyte proliferation, delayed chondrocyte differentiation, impaired ECM production and insufficient vascular invasion (Hall-Glenn et al. 2012; Ivkovic et al. 2003).

CCN2 induces NFκB activity in ATDC5 chondrocytic cells through integrin αvβ3-mediated mechanisms to enhance migration (Tan et al. 2009). Reciprocally, the CCN2 promoter contains an NFκB responsive element that activates CCN2 expression during the mechanical stretch response in smooth muscle cells (Chaqour et al. 2006). These studies raise the possibility that CCN2 may regulate NFκB in the growth plate. Given the essential role of NFκB in chondrocyte survival, and evidence that CCN2 induces its expression and activity in multiple cell types, we investigated whether CCN2 plays a role in survival of growth plate chondrocytes. We show that CCN2 is essential for chondrocyte survival, protecting growth plate chondrocytes from pro-apoptotic pathways activated by cellular stress, and promoting chondrocyte survival. We provide evidence

consistent with the possibility that CCN2 enhances chondrocyte survival through activation of NFκB and autophagy. Additionally, we provide evidence that these effects are mediated in part through the ability of CCN2 to engage integrins.

Materials and methods

Mouse strains and cell lines *Ccn2* knockout mice were generated, genotyped and maintained as described (Ivkovic et al. 2003). *Ccn2*−/− embryos were obtained from timed heterozygote matings, with embryonic day (E) 0.5 representing the detection of a post-copulatory plug.

CCN2 floxed inducible transgenic mice (*Ccn2fxTg*) were generated by pronuclear injection as described (Araki et al. 1995; Voncken 2011). These mice were crossed to *Col2a1Cre* mice (Ovchinnikov et al. 2000) to induce overexpression of CCN2 in chondrocytes. Genotyping was performed on DNA isolated from tail biopsies with the following primers: Forward: 5′-TCTTCTGCGATTTCGGCTCC-3′; Reverse: 5′-AATGTGTCTTCCAGTCGGTAG-3.′ Mouse embryonic fibroblasts (MEFs) from *Ccn2fxTg* embryos were isolated and cultured as described (Lengner et al. 2004). MEFs were infected for 24 hours with adenoviral Cre-recombinase (Ad-CRE) and empty vector controls (Ad-CNT) (University of Iowa Gene Transfer Vector Core) at a multiplicity of infection of 300. RNA was isolated using Qiagen RNeasy Kit, and quantitative RT-PCR (qRT-PCR) was used to quantify relative CCN2 expression normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described (Kawaki et al. 2008).

Experiments on mice were performed with four sets of WT and *Ccn2*−/− littermates at E16.5, E18.5 and postnatal day (P) 0 (N=8 per genotype). Animals were treated in accordance with the National Institutes of Health guidelines for care and use of animals, and approved by the UCLA Institutional Animal Care and Use Committee.

Transmission electron microscopy WT and *Ccn2*−/− E18.5 hindlimbs (N=3 per genotype) were prepared for electron microscopy as previously described (Szafranski et al. 2004). The ultrastructural analysis was performed at the Center of Regenerative Medicine for Skeletal Tissues (Department of Clinical Research, University of Bern), in Bern, Switzerland.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays TUNEL assays (Roche) were performed on WT and *Ccn2*−/− growth plates according to the manufacturer's protocol. Cellular counts of chondrocytes were quantitated and normalized to total DAPI counts.

Immunofluorescent staining Embryos were fixed and embedded as described (Ivkovic et al. 2003). 7 μm sections were generated using a Lecia GM40 Microtome. For fluorescent staining, Tyramide Signal Amplification was performed according to the manufacturer's protocol (Invitrogen). The following antibodies were used: CCN2 L-20 (1:1,000; Santa Cruz Biotechnology), BiP (1:500; Cell Signaling), CHOP (1:400; Cell Signaling), Calnexin (1:300; Chemicon), NFκB/RelA/p65 (1:400; Cell Signaling), Apg/Atg12 (1:400; Chemicon) and Apg8b/LC3 (1:200; Abgent). Secondary antibodies were conjugated with AlexaFluor-594 and 488 (Invitrogen). Sections were counterstained with DAPI (Sigma) and mounted with Prolong Gold reagent (Sigma). Immunofluorescence was visualized on an Olympus Bx60 Microscope.

Sternal chondrocyte isolation E16.5 sterna were isolated and pooled by genotype into conical tubes. Garnet bead mix (Invitrogen) was added to 15 ml of HEPES (Sigma) buffered DMEM (HDMEM) (GIBCO). The connective tissue from the sterna was removed by mechanical shaking for 30 min. The media was aspirated, and 0.03 % bacterial collagenase (Chlostridium; Sigma) in HDMEM was added. Additional shaking was performed for 10 min. Cleared sterna were further digested in 0.01 % collagenase overnight. The following day, chondrocytes were filtered (70 μm Fisher) and plated.

Alginate chondron cultures Isolated sternal chondrocytes were suspended in 1.1 % sodium alginate in 1X Phosphate Buffered Saline (PBS) (Sigma) (Lee et al. 1997). The cell/alginate suspension was extruded drop-wise into 0.1 M Calcium Chloride (Sigma) and beads were polymerized at room temperature for 10–15 min. The beads were then washed with 1X PBS, transferred to T25 flasks and incubated in differentiation media containing 50 ng/ml ascorbic acid (Sigma). After 7–10 days in culture, the chondrons were released using 10 mM sodium citrate (Sigma). Chondron clusters were plated using Cell-tak adhesive (BD Biosciences) and cultured for 1–3 days for immunofluorescence (Hamamura et al. 2009).

Thapsigargin-induced ER stress in ATDC5 cells ATDC5 chondrosacrcoma cells (RCB0565-Riken Cell Bank) were cultured as described (Altaf et al. 2006). ER stress was induced by a 24 h treatment with thapsigargin (THG, Sigma) diluted in DMSO at the following concentrations: 0, 40, 80 and 160nM (Hamamura et al. 2009). All experiments were preformed in triplicate and repeated twice.

Integrin blocking and recombinant CCN2 assays Sternal chondrocytes were isolated as described above. Integrin blocking assays were performed as previously described

(Nishida et al. 2007). Briefly, sternal chondrocytes were plated and serum starved for 6–8 h. Chondrocytes were then treated for 24 h with an anti-rat integrin α5 blocking antibody (1:100; CD49e BD Pharmingen) or 5 % rat control serum (Sigma). In a separate set of experiments, ATDC5 cells were serum starved followed by a 1 h treatment with recombinant protein containing only the C terminal (CT) domain of CCN2 (150 ng/ml; Peprotech) or BSA added to the media, and then by a 1 h treatment with integrin α5 blocking antibody and/or 80nM THG or DMSO control. Cells were lysed and RNA extracted using a Qiagen RNA purification kit following the manufacturer's instructions. cDNA was generated using Superscript III (Invitrogen). The following primer sequences were used: Integrin alpha 5 Forward: 5′-AGCGCATCTCTCACCATCTT-3′ and Reverse: 3′-TCAGGTTCAGTGCGTTCTTGT-5′, and normalized to GAPDH as described (Nishida et al. 2007).

CCN2 overexpression in ATDC5 cells ATDC5 cells were infected with a bicistronic adenovirus expressing CCN2 and green florescent protein (AdCCN2-GFP) adenovirus or an adenoviral control (Ad-CNT) vector for 24 h at a multiplicity of infection of 200 (University of Iowa) (Hall-Glenn et al. 2012). After 24 h, cells were analyzed by immunofluorescence. Experiments were performed in triplicate and repeated twice.

Western blot analysis Proteins were isolated from *Ccn2fxTg* and WT sterna through lysis in RIPA buffer with 1X protease (Roche) and 1X phosphatase inhibitors (Sigma). 30 μg of protein lysates were separated by gel electrophoresis and transferred to 0.45 μm nitrocellulose membranes (Biorad). Membranes were blocked in milk and incubated at a 1:2,000 dilution of the following primary antibodies overnight at 4 °C: CCN2 (L-20; Santa Cruz Biotech) and β-actin (Sigma). The blots were incubated with the following secondary antibodies: Donkey anti-goat horseradish peroxidase (HRP) and Goat anti-rabbit HRP (1:5,000; Biorad). Membranes were developed using Pierce ECL HRP chemoluminescent reagent (ThermoScientific). The blots were repeated twice.

Quantitative reverse transcriptase PCR (qRT-PCR) All qRT-PCR reactions were performed with a SYBR Green Real-time PCR Master Mix (Fermentas) with a Mx3005P QPCR System (Stratagene). Relative expression of *Ccn2*, *Bip* and *Chop* were quantitated and normalized to *Gapdh* as described and performed in triplicate (Hamamura et al. 2009; Kawaki et al. 2008). *Atg12* and *Lc3* levels were quantitated and normalized to *Gapdh* as previously described (Kouroku et al. 2007; Marino et al. 2010).

Statistical analysis

Immunofluorescent quantitation of the levels of CCN2, BiP and CHOP expression was performed through ImageJ analysis and calculated as a percentage of DAPI positive total cell counts. Three images were taken per independent experiment, followed by quantitation and averaging. At least three independent WT and mutant littermate growth plates were examined with each marker. All in vitro experiments were performed in triplicate and repeated twice. All graphs are represented as fold induction over normalized untreated controls. A normal distribution of the data was assumed, and statistical analysis was performed using Student's *t*-test (95 % confidence interval).

Results

Loss of CCN2 results in cellular stress in the growth plate

Ccn2 mutant growth plates and cultured chondrocytes exhibit decreased ECM production (Ivkovic et al. 2003; Nishida et al. 2007). However, the consequences on the overall organization of the cartilage ECM were not previously investigated. Therefore, transmission electron microscopy was performed on E18.5 WT and *Ccn2* mutant growth plates. Unexpectedly, ultrastructural examination revealed enlarged and distended ERs in proliferating and hypertrophic chondrocytes in *Ccn2* mutants (Fig. 1a–d). WT proliferating (Fig. 1a) and hypertrophic (Fig. 1c) chondrocytes contained an organized rough ER (rER) with a limited amount of protein evenly distributed throughout the cisternae. However, in *Ccn2* mutants, rER cisternae were dilated (Fig. 1b, d). Large vacuoles filled with an electron-lucid granular substance were also observed in mutants, indicative of accumulated intracellular proteins (Fig. 1b, d). Moreover, the nuclear chromatin in mutant chondrocytes was condensed (Fig. 1d), indicating most chondrocytes were undergoing cell death. The mechanism by which chondrocytes undergo physiological cell death is a matter of debate, but condensed chromatin and TUNEL labeling are reliable markers of chondrocyte death (Ahmed et al. 2007). TUNEL staining showed that *Ccn2*−/− growth plates exhibited an increase in the percent of chondrocytes undergoing cell death in both the proliferative and hypertrophic zones compared to WT growth plates (Fig. 1e–g). Ultrastructural analysis also revealed major defects in ECM assembly in mutants. Fewer collagen fibrils were observed throughout the growth plate in *Ccn2* mutants compared to WT littermates (Fig. S1).

Because we noted distended ER in proliferative as well as hypertrophic chondrocytes, we examined CCN2 expression and confirmed that CCN2 protein is expressed both in the

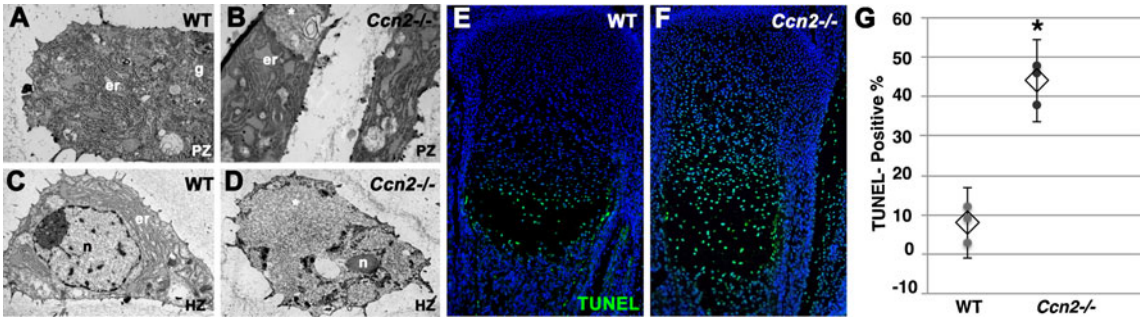

Fig. 1 The Loss of CCN2 results in chondrocyte stress and death. Electron microscopy was performed on WT and *Ccn2* mutant chondrocytes in E18.5 growth plates. **a**, **b** WT and *Ccn2*−/− proliferative chondrocytes, respectively. **c**, **d** WT and *Ccn2*−/− hypertrophic chondrocytes, respectively. The *Ccn2* mutant ER appears devoid of ribosomes and is engorged with an electron lucid substance, which is presumably composed of misfolded protein aggregates. Large vacuoles are also present and filled with granulated material (*asterisk*). **e**, **f** WT and *Ccn2* mutant growth plates analyzed by TUNEL staining showing increased cell death in the proliferative zone in mutants. **g** Quantitation of TUNEL-positive cells. *Open triangles* represent the average of the data points. *g* golgi, *n* nucleus; *r* endoplasmic reticulum. Representative images are shown

proliferative and hypertrophic zones (Ivkovic et al. 2003) (Fig. 2a), and that CCN2 protein is absent in mutant growth plates (Fig. 2b) (Ivkovic et al. 2003). Next, we tested whether the distended ER in *Ccn2* mutant chondrocytes is associated with activation of ER stress pathways by examining expression of the UPR activator, BiP, and the apoptosis inducing protein, CHOP. Consistent with previous studies (Tsang et al. 2007), low levels of BiP were observed in the hypertrophic zone (Fig. 2c), and CHOP (Fig. 2e) was undetectable in E16.5 WT growth plates. Increased expression of BiP (Fig. 2d) and CHOP (Fig. 2f) was observed in the hypertrophic zones of *Ccn2* mutant growth plates, but not in the proliferative zone. Expression of calnexin, a calcium dependent ER chaperone, was elevated throughout the proliferative and prehypertrophic zones in *Ccn2* mutants (Fig. 2g, h). Although calnexin is upregulated by ER stress in chondrocytes (Vranka et al. 2001), the observation that BiP and CHOP were not elevated in the proliferative zone in *Ccn2* mutants indicates that these chondrocytes are not undergoing a classical UPR. Nonetheless, these results show that the absence of CCN2 in the growth plate results in defective ECM assembly, increased cellular stress, and chondrocyte cell death in both the proliferative and hypertrophic zones.

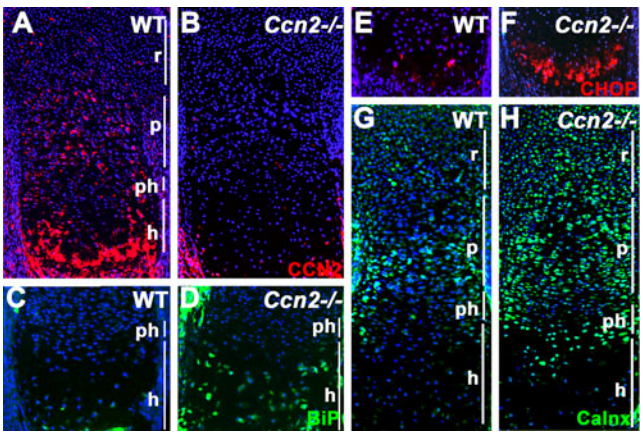

Fig. 2 Depletion of CCN2 results in increased cellular stress and defective chaperone expression in vivo. All images are E16.5 tibial growth plates. **a**, **b** Immunofluorescence staining for CCN2 in WT and *Ccn2*−/− growth plates, respectively, showing expression of CCN2 in proliferative chondrocytes in addition to hypertrophic chondrocytes. **b** The *Ccn2* mutant allele is transcriptionally null, and in accordance, no CCN2 expression is observed in *Ccn2*−/− growth plates. **c**, **d** Immunofluorescence staining for BiP reveals very low levels of expression in WT growth plates (**c**), and elevated expression in the hypertrophic zones of *Ccn2* mutant littermates (**d**). **e**, **f** The pro-apoptotic marker CHOP is seen at very low levels, restricted to the hypertrophic zone, in WT growth plates (**e**), but is strongly upregulated in the hypertrophic zone in *Ccn2* mutant littermates (**f**). **g**, **h** Expression of the resident ER chaperone calnexin is restricted to lower proliferative zone chondrocytes in WT growth plates (**g**), but is upregulated throughout the growth plates of *Ccn2* mutants (**h**). Representative images are shown

CCN2 Is a stress responsive gene in vitro

The finding that loss of CCN2 was associated with increased chondrocyte stress raised the possibility that CCN2 may be responsive to cellular stress in chondrocytes. Therefore, we tested whether CCN2 is upregulated in chondrocytes during chemically induced ER stress. Thapsigargin (THG) induces cellular stress by inhibiting sarco/endoplasmic reticulum calcium ATPases, thus blocking calcium release from ER stores (Yang et al. 2005). Prior to cell death, the transcription of ECM components is downregulated and protective ER stress response genes such as BiP are activated by THG in chondrocytes (Yang et al. 2005). ATDC5 chondrocytic cells were treated with increasing concentrations of THG. *Bip* and *Chop* levels were examined using qRT-PCR. Consistent with previous studies, THG induced dose-dependent increases in *BiP* (Fig. 3a) and *Chop* mRNA (Fig. 3b) (Hamamura et al. 2009). In contrast to the expression of ECM components, which are

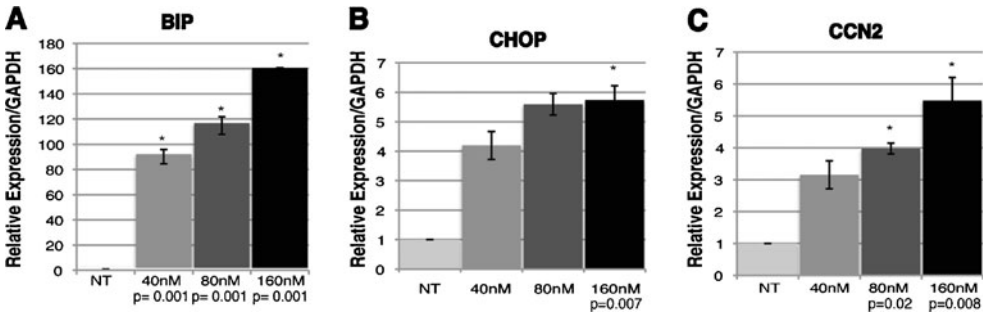

Fig. 3 CCN2 is a stress responsive gene in chemically induced cellular stress. ATDC5 chondrocytes were cultured for 24 h in the presence of Thapsigargin (THG) at the indicated concentrations. mRNA expression levels of *Ccn2* (A), *BiP* (B) and *Chop* (**c**) were assayed by Quantitative Reverse Transcriptase PCR (qRT-PCR). Assays were performed in triplicate and a p value of >0.05 was considered significant compared to no THG (*). *p* values shown are for 160nM THG

downregulated by THG (Nugent et al. 2011), *Ccn2* mRNA levels were increased by THG with the same kinetics as *BiP* and *Chop* (Fig. 3c), suggesting that CCN2 is a stress-responsive gene in chondrocytes in vitro.

CCN2 depletion induces cellular stress in chondron cultures

The above experiments demonstrate that CCN2 expression is induced by stress in vitro. However, they do not demonstrate that the cellular stress observed in *Ccn2*−/− growth plates is due to a direct role for CCN2 in chondrocytes. Recent studies have shown that the presence of ECM protects chondrocytes from ER stress (Nugent et al. 2011). Therefore, a 3-dimensional culture system was developed in which chondrocytes were maintained in alginate, yielding chondroctye clusters (chondrons) (Fig. S2) (Lee et al. 1997). The accumulation of ECM components can be observed (Fig. S2A, B). THG treatment induced cellular stress in chondrons, with BiP localization transitioning from the ER lumen in control cells (Fig. S2C), to the nucleus/perinuclear region in THG treated chondrons (Fig. S2D). The re-localization of BiP to the nucleus has not been reported previously in chondrocytes, but is consistent with previous reports in other cell types (Ni et al. 2011). CHOP levels were also increased in THG treated chondrons (Fig. S2F), compared to untreated controls (Fig. S2E). Therefore, chondrons in alginate are sensitive to THG-induced stress. Next, the impact of loss of *Ccn2* on THG-induced stress was tested. First, we confirmed that CCN2 is expressed in WT chondrons (Fig. 4a) and is absent in *Ccn2*−/− chondrons (Fig. 4b, c). WT chondrons expressed low levels of BiP (Fig. 4d). *Ccn2*−/− chondrons displayed upregulated BiP expression and a higher proportion of nuclear BiP-positive cells (Fig. 4e, f). CCN2 mutant chondrons also exhibit increased expression of CHOP (Fig. 4g–i). These results suggest that CCN2 plays a direct role in the protection of chondrocytes against THG-induced stress in vitro.

The protective effect of CCN2 against chondrocyte stress may be mediated through integrin α5

CCN2 is thought to mediate its functions by acting as a ligand for integrins (Jun and Lau 2011). CCN2 induces expression of integrin α5, and binds to integrin α5β1 to promote chondrocyte proliferation and ECM secretion (Nishida et al. 2007). Integrin α5β1 is also required for chondrocyte survival (Pulai et al. 2002). Recent data demonstrate that multiple stress pathways are activated as a survival mechanism during cell detachment from the ECM (Avivar-Valderas et al. 2011). Taken together, these findings suggest that one of the pro-survival functions of integrin α5β1 in chondrocytes may be the suppression of cellular stress. To test this possibility, sternal chondrocytes were treated with or without an integrin α5-blocking antibody for 24 h (Fig. 5a). *BiP* and *Chop* mRNA expression levels were upregulated in response to treatment with the integrin α5-blocking antibody (Fig. 5a). Consistent with our findings that loss of *Ccn2* leads to increased cellular stress in chondron cultures (Fig. 4), recombinant C-terminal CCN2 (rCCN2)-treated ATDC5 cells exhibited a decrease in *Chop* mRNA levels compared to BSA treated controls in the presence of THG (Fig. 5b). The C-terminal domain of rCCN2 contains the integrin α5β1-binding domain (Gao and Brigstock 2006). Next, ATDC5 cells were pre-treated for 1 h with recombinant C-terminal rCCN2 added to the medium prior to stress induction for 1 h in the presence of the α5-blocking antibody. As in Fig. 5a, treatment with integrin α5-blocking antibody led to increased *Chop* mRNA expression (Fig. 5c, columns 1 and 2), whereas treatment of ATDC5 cells with THG leads to an approximately 2-fold induction in *Chop* mRNA (Fig. 5b, columns 1 and 2). Also, treatment with THG in the presence of the integrin α5-blocking antibody leads to an approximately 10-fold induction of *Chop* (Fig. 5c, columns 1 and 3). Moreover, while rCCN2 prevented THG-induced *Chop* expression (Fig. 5b, columns 2 and 4), treatment with integrin α5-blocking antibody prevented the protective effect of rCCN2 (Fig. 5c,

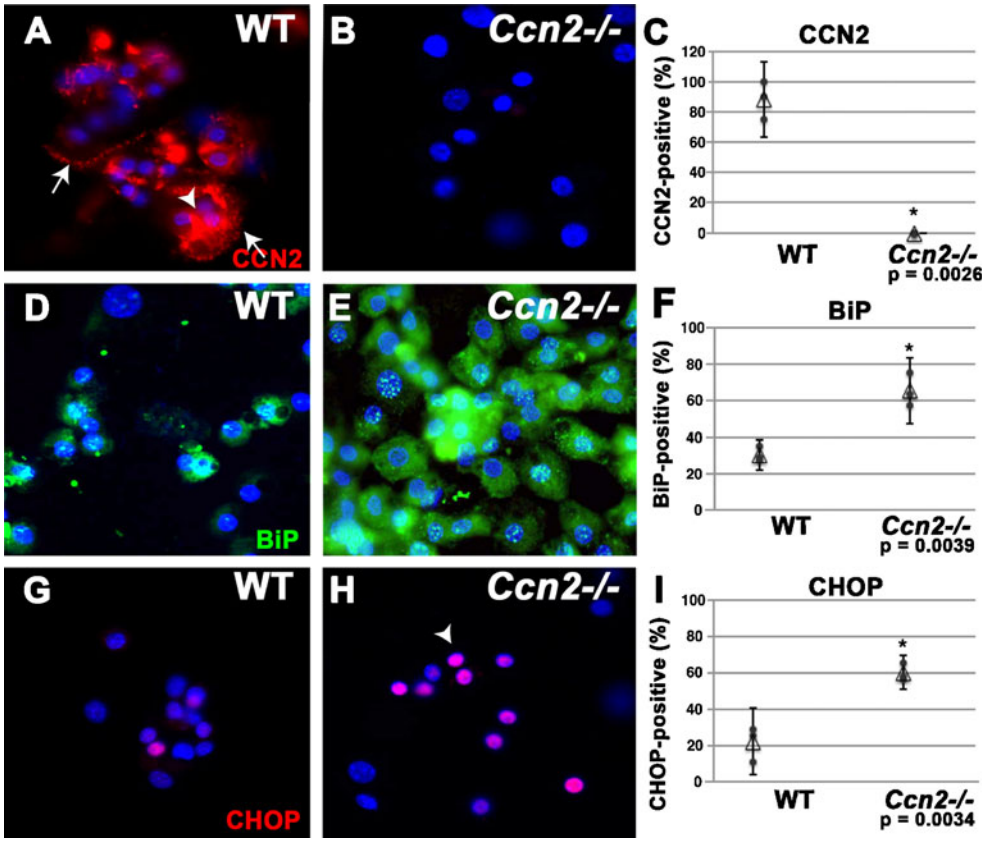

Fig. 4 CCN2 depleted chondrons exhibit elevated cellular stress. Primary sternal chondrocytes were isolated from E16.5 WT and *Ccn2* −/− sterna, and cultured in alginate for 10 days to yield chondrons. CCN2, BiP and CHOP protein expression were analyzed through immunofluorescence. **a**–**c** CCN2 expression. CCN2 protein expression was abundant in WT chondrons (**a**), where punctate CCN2 protein expression was observed intracellularly (*arrowhead*) and pericellularly (*arrows*). CCN2 protein was not detected in *Ccn2* mutant chondrons. **b**, **c** Quantitation of number of CCN2-expressing cells. **d**–**f** BiP expression in WT and *Ccn2*−/− chondrons. **d** BiP is detected in the ER in some cells in WT chondron clusters. **e** Nearly all *Ccn2*−/− chondrocytes express BiP, and many nuclei are positive. **f** Quantitation of the percentage of nuclear BiP-expressing cells. **g**–**i** CHOP expression. **g** Few WT chondrocytes express CHOP. In contrast, most *Ccn2*−/− chondrocytes express CHOP (H). **i** Quantitation of the percentage of CHOP-expressing chondrocytes. **c**, **f**, **i** CCN2, BiP, and CHOP expression was analyzed in triplicate using ImageJ analysis and calculated as a percentage of DAPI positive total cell counts. *Asterisks* indicate statistical significance with a *p*-value of 0.003. *Open triangles* in (**c**), (**f**), and (**i**) represent the averages of the data points. Representative images are shown

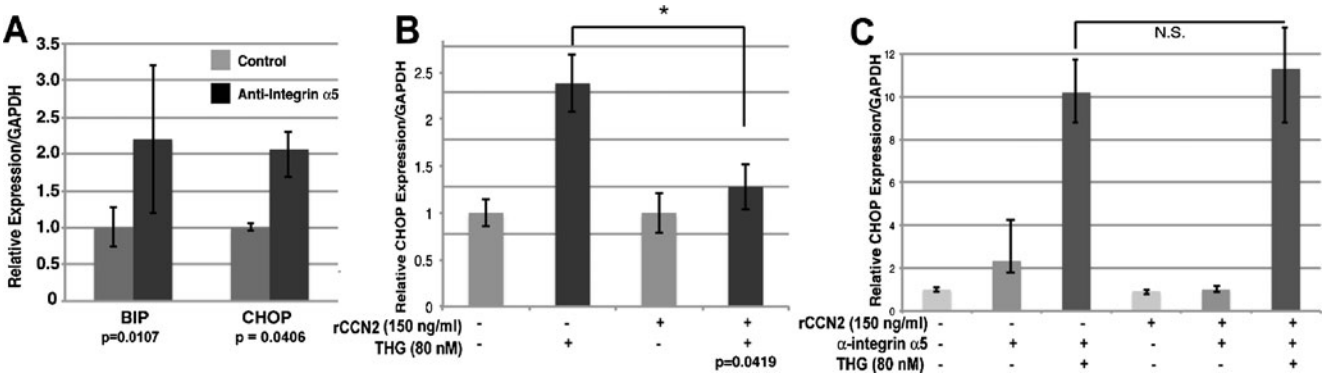

Fig. 5 Blocking Integrin α5 results in chondrocyte stress. Primary sternal chondrocytes were serum starved and treated with anti-integrin α5 blocking antibody or goat serum (control) for 24 h, after which BiP and CHOP mRNA levels were quantitated by qRT-PCR and normalized to GAPDH. BiP and CHOP expression were increased by treatment with the blocking antibody. **b** rCCN2 protects against THG-induced stress, resulting in *Chop* mRNA induction. **c** This affect is attenuated upon treatment with α5 blocking antibody and THG compared to DMSO and rat serum controls

columns 3 and 6). These results, although preliminary, suggest that integrin α5 may be required for the ability of CCN2 to protect against cellular stress in chondrocytes in vitro.

Overexpression of CCN2 attenuates chondrocyte stress

Since loss of CCN2 induces cellular stress in cartilage, we generated transgenic mice that overexpress CCN2 in chondrocytes to determine whether increased CCN2 could lead to reduced stress. An inducible transgenic construct was generated, consisting of a floxed transcriptional STOP signal placed upstream of the *Ccn2* coding sequence (Fig 6a). Transgenic lines were established and mated to *Col2Cre* mice (Ovchinnikov et al. 2000) to induce chondrocyte-specific overexpression of CCN2 (Fig 6a). A 3–5 fold induction of CCN2 protein was observed in *Ccnfx2Tg*;*Col2Cre* mice (Fig. 6b). The specificity of induction was confirmed in isolated mouse embryonic fibroblasts (MEFs) from *Ccn2fxTg* embryos infected with adenoviral Cre (Ad-CRE) (Fig. 6c). *Ccn2fxTg*;*Col2Cre* mice were viable with no obvious morphological differences at birth (Fig. 6d), but exhibited progressive overgrowth of cartilage elements (data not shown). A complete characterization of these transgenics will be presented elsewhere. The growth plates of P0 *Ccn2fxTg* tibiae had normal chondrocyte organization, although both the hypertrophic zone and chondrocytes within it were slightly smaller than in WT littermates (Fig. 6e, f). Consistent with a protective function for CCN2, levels of BiP were reduced in *Ccn2fxTg*;*Col2Cre* mice compared to WT controls (Fig. 6g, h).

CCN2 regulates NFκB expression and autophagy

NFκB is required for chondrocyte survival (Park et al. 2007; Wu et al. 2007). CCN2 induces nuclear localization of NFκB and activates pro-survival mechanisms, while inhibiting stress-mediated apoptosis in a number of cell types (Gao and Brigstock 2005). NFκB is activated by integrin α5β1 in breast cancer epithelial cells (Sen et al. 2010) and α5β1 is a major receptor for CCN2 in chondrocytes (Sen et al. 2010). Our earlier results suggested that CCN2 protects against chondrocyte stress through

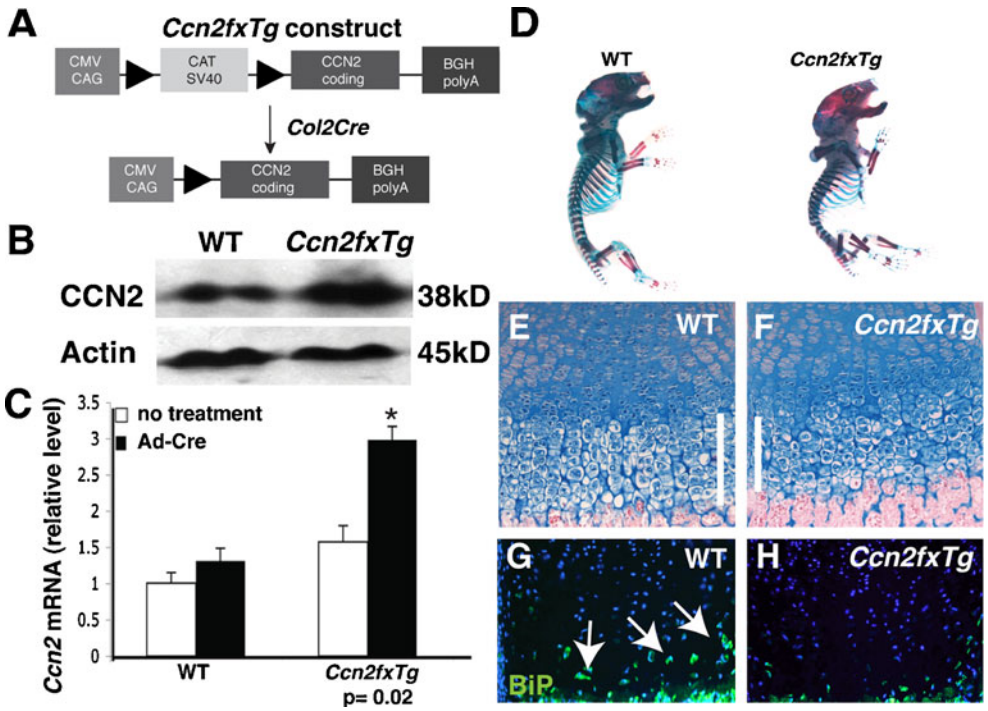

Fig. 6 CCN2 overexpression attenuates ER stress in vivo. **a** Schematic of the CCN2 floxed transgenic construct. A floxed cassette consisting of a STOP cassette and a chloroacetyltransferase (CAT) gene is flanked by loxP sites and inserted between,the CMV/chicken β actin (CAG) promoter and the murine CCN2 coding sequence, followed by a bovine growth hormone polyA signal sequence. Upon crossing to a *Col2Cre* mouse, the silencer is excised, leading to transcription of CCN2 from the transgene in cartilage. **b** Western blot of protein isolated from sterna of WT and *Ccn2fxTg*;*Col2Cre* P0 pups with actin controls. **c** *Ccn2* mRNA levels in mouse embryonic fibroblasts (MEFs) from E11.5 *CCN2fxtg* E11.5 embryos infected with AdCre or Adcontrol. *Ccn2* mRNA levels are not significantly different in WT vs. *Ccn2fxTg* MEFs in the absence of Cre, but are increased approximately 2.5-fold in *Ccn2fxTg* mice in the presence of Cre. (D) Skeletal preparations of postnatal day 0 (P0) *Ccn2fxTg*;*Col2Cre* and WT littermates, showing no apparent morphological differences. **e**, **f** Growth plate histology of P0 WT (**e**) and *Ccn2fxTg*;*Col2Cre* (**f**) littermate. The hypertrophic zone is slightly shorter and hypertrophic chondrocytes are smaller in transgenics. **g**, **h** Overexpression of *Ccn2* results in decreased BiP expression (**h**) compared to WT littermate (**g**) in P0 growth plates

integrin α5 in vitro (Fig. 5). We therefore investigated whether CCN2 might exert pro-survival functions in chondrocytes at least in part through regulation of NFκB. NFκB localization was examined in E16.5 WT and mutant growth plates. In WT proliferative zones, NFκB was predominantly localized to the nucleus (Fig. 7a), similar to previous reports (Park et al. 2007; Wu et al. 2007). In *Ccn2*−/− growth plates, NFκB was present in the cytoplasm, but the number of cells exhibiting nuclear localization was decreased (Fig. 7b,c). These data are thus consistent with previous in vitro studies showing that CCN2 induces NFκB activation (Gao and Brigstock 2005), and raise the possibility that CCN2 exerts pro-survival effects in chondrocytes at least in part through NFκB.

Autophagy is an important survival mechanism in normal chondrocytes and in response to stress (Lotz and Carames 2011). We examined the expression of autophagosome markers Atg12 and LC3 (Atg8), which conjugate with one another to stabilize autophagosome formation (Mehrpour et al. 2010). In E16.5 WT growth plates, Atg12 and LC3 were expressed throughout the growth plate, with the highest levels in the hypertrophic zone (Fig. 7d, f), consistent with previous literature showing that autophagy is required for terminal chondrocyte differentiation (Srinivas et al. 2009). However, in *Ccn2*−/− growth plates Atg12 and LC3 protein (Fig. 7e,g) and mRNA (Fig. 7h, i) levels were decreased.

Because NFκB and autophagy have pro-survival functions in chondrocytes, we investigated whether CCN2 can induce NFκB and the expression of autophagy genes. ATDC5 cells infected with a CCN2 overexpressing adenovirus (Ad-CCN2-GFP) exhibited increased CCN2 expression within 24 h after infection (Fig. 8a, b). Consistent with in vivo studies (Fig. 7), CCN2 expression induced nuclear NFκB expression (Fig. 8c, d). Furthermore, CCN2 stimulated the expression of LC3 (Fig. 8e, f). These data suggest that CCN2 may protect against chondrocyte stress by upregulating pro-survival NFκB and autophagy pathways.

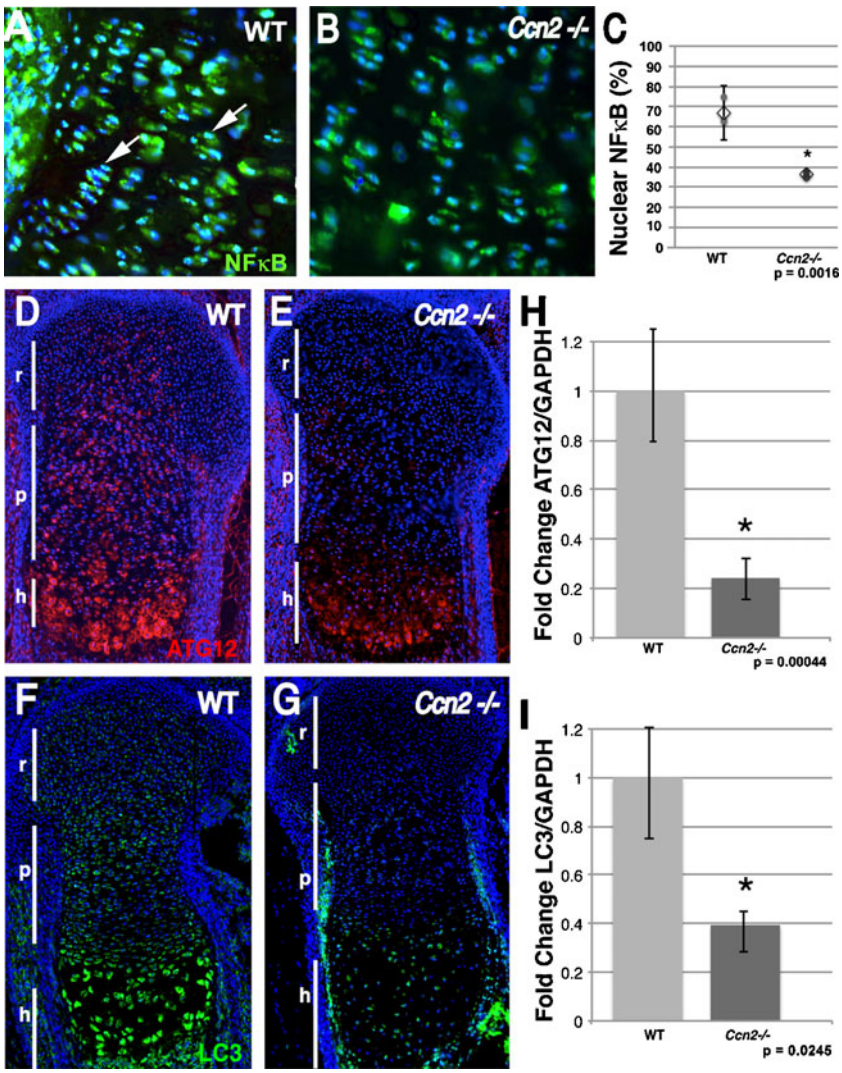

Fig. 7 Decreased NFκB and autophagy-mediated cell survival in *Ccn2* mutants. NFκB expression was visualized by immunofluorescence in E16.5 WT and *Ccn2* mutant growth plates. **a** In WT growth plates, NFκB was primarily localized to the nucleus throughout the proliferative zone. **b** In *Ccn2*−/− growth plates, NFκB expression in the proliferative zone was decreased and localized mainly to the cytoplasm. **c** NFκB nuclear expression throughout the growth plate was quantitated using Image J analysis as the percentage of DAPI positive nuclei. Growth plate immunostaining was performed in triplicate; *asterisk* denotes a *p*-value of 0.003, indicating statistical significance. *Open triangle* indicates the average of the data points. **d**, **e** E16.5 WT (D) and *Ccn2*−/− (**e**) growth plates immunostained for Atg12. **f**, **g** E16.5 WT (F) and *Ccn2*−/− (**g**) growth plates immunostained for Atg8 (LC3). All sections were counterstained with DAPI. Stains were performed at least twice and representative images are shown. **h**, **i** *Atg12* and *LC3* mRNA levels were quantitated by qRT-PCR in WT and *Ccn2*−/− sternal chondrocytes

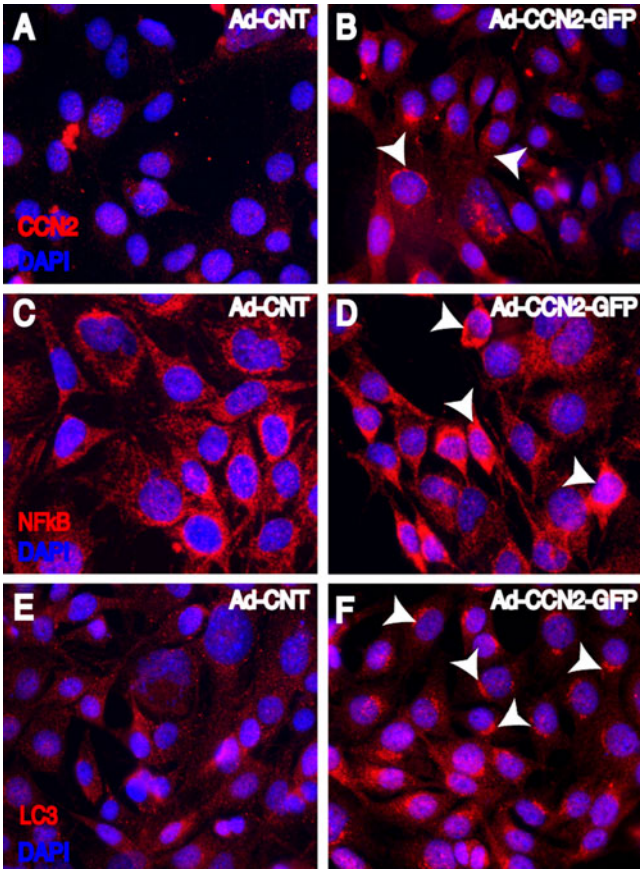

Fig. 8 Ectopic CCN2 expression results in increased NFκB and expression of autophagy genes in ATDC5 cells. NFκB and autophagy marker LC3 were examined in ATDC5 cells infected with Ad-CCN2-green florescent protein (Ad-CCN2-GFP) and Ad-control (Ad-CNT) constructs. **a**, **b** CCN2 expression is upregulated in Ad-CCN2-GFP ATDC5 cells (**b**) compared to Ad-CNT treated cells (**a**). **c**, **d** Nuclear NFκB is increased in Ad-CCN2-GFP cells (**d**) compared to control cells (**c**). **e**, **f** Expression of LC3/Atg8 is increased in Ad-CCN2-GFP cells (**f**) compared to Ad-CNT treated cells (**e**). (*Red*- CCN2, NFκB and LC3); (*Blue* = DAPI counterstain). Representative images are shown

Discussion

The data reported here provide evidence that CCN2 is important for ECM assembly and chondrocyte survival in the growth plate. Ultrastructural analysis revealed that loss of CCN2 leads to defective ECM organization and chondrocyte death, accompanied by increased cellular stress. The activation of stress pathways in vivo was confirmed by elevated levels of BiP and CHOP in *Ccn2*−/− growth plates. These results were recapitulated in vitro, where loss of *Ccn2* in chondron cultures also resulted in cellular stress. This finding suggests that the role of CCN2 in protecting chondrocytes from stress is direct, rather than an indirect consequence of defective growth plate angiogenesis. The finding that *Ccn2* expression is induced by stress, unlike structural ECM proteins, whose expression is downregulated, suggests that an important function of CCN2 may be to mediate chondrocyte survival.

The precise mechanisms by which CCN2 protects against cellular stress remain unclear. Integrin α5β1 is essential for chondrocyte survival in vitro (Pulai et al. 2002). Integrin α5 expression was decreased in *Ccn2*−/− chondrocytes, leading to defective chondrocyte adhesion and downstream integrin signaling (Nishida et al. 2007). Evidence that CCN2 may exert a pro-survival effect through modulation of cell-ECM attachment comes from the finding that treatment of chondrocytes with an integrin α5-blocking antibody leads to increased cellular stress, while treatment of ATDC5 cells with C-terminal rCCN2 (which contains the α5β1 binding site) (Gao and Brigstock 2006) mediates a modest protective effect during chemically induced stress, which is attenuated in the presence of an integrin α5-blocking antibody. Currently, no in vivo studies have been conducted to assess the role of α5 containing integrins in cartilage. However, the loss of β1 integrin leads to reduced ECM production, growth plate disorganization and impaired survival (Aszodi et al. 2003), consistent with a potential role for α5β1 in ECM organization and chondrocyte survival.

In addition to induction of pro-apoptotic stress pathways, the loss of *Ccn2* also results in decreased expression of nuclear NFκB and autophagy genes in vivo. In accordance, overexpression of *Ccn2* in chondrocytes resulted in induction of nuclear NFκB and autophagy gene expression. A limitation of these experiments is that the ability of overexpression of CCN2 to rescue NFκB and autophagy gene expression in *Ccn2*−/− chondrocytes was not tested in vitro. Thus, we cannot determine whether the effect of CCN2 is direct or indirect. However, both NFκB and autophagy exert pro-survival functions in growth plate chondrocytes (Srinivas et al. 2009; Wu et al. 2007). The mechanism by which CCN2 induces the expression of NFκB in chondrocytes is unknown but probably occurs through integrin signaling, as previously observed in other cell types (Gao and Brigstock 2005; Zahir et al. 2003).

CCN2 expression is correlated with the onset of osteoarthritis (OA) and synovial damage (Blaney Davidson et al. 2006; Omoto et al. 2004). Paradoxically, recombinant CCN2 expedites repair of articular cartilage during chemically induced knee OA (Nishida et al. 2004). These opposing effects of CCN2 may be due to its ability to modulate NFκB. While NFκB has pro-survival functions in chondrocytes (Park et al. 2007; Wu et al. 2007), it also promotes the expression of pro-inflammatory genes in OA chondrocytes (Marcu et al. 2010). Whether NFκB has a pro-survival function in OA chondrocytes is unknown. Further investigations of the mechanism by which CCN2 promotes

survival in growth plate chondrocytes, and its role in the maintenance of articular cartilage may provide new avenues to treat and prevent OA.

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Author contributions KML, PB, EBH and FHG were involved in the conception and design of the experiments. FHG, AD, AA, LA, RRB, JRO and EBH performed the experiments and analyzed the data. FFS, FvN and RG generated crucial reagents. FHG and KML wrote the article.

References

Ahmed YA, Tatarczuch L, Pagel CN, Davies HM, Mirams M, Mackie EJ (2007) Physiological death of hypertrophic chondrocytes. Osteoarthr Cartil OARS Osteoarthr Res Soc 15(5):575–586

Altaf FM, Hering TM, Kazmi NH, Yoo JU, Johnstone B (2006) Ascorbate-enhanced chondrogenesis of ATDC5 cells. Eur Cell Mater 12:64–69, discussion 69–70

Araki K, Araki M, Miyazaki J, Vassalli P (1995) Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase. Proc Natl Acad Sci U S A 92(1):160–164

Aszodi A, Hunziker EB, Brakebusch C, Fassler R (2003) Beta1 integrins regulate chondrocyte rotation, G1 progression, and cytokinesis. Genes Dev 17(19):2465–2479

Avivar-Valderas A, Salas E, Bobrovnikova-Marjon E, Diehl JA, Nagi C, Debnath J, Aguirre-Ghiso JA (2011) PERK integrates autophagy and oxidative stress responses to promote survival during extracellular matrix detachment. Mol Cell Biol 31(17):3616–3629

Barbieri O, Astigiano S, Morini M, Tavella S, Schito A, Corsi A, Di Martino D, Bianco P, Cancedda R, Garofalo S (2003) Depletion of cartilage collagen fibrils in mice carrying a dominant negative Col2a1 transgene affects chondrocyte differentiation. Am J Physiol Cell Physiol 285(6):C1504–C1512

Bateman JF, Boot-Handford RP, Lamande SR (2009) Genetic diseases of connective tissues: cellular and extracellular effects of ECM mutations. Nature reviews. Genetics 10(3):173–183

Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D (2000) Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nat Cell Biol 2(6):326–332

Blaney Davidson EN, Vitters EL, Mooren FM, Oliver N, Berg WB, van der Kraan PM (2006) Connective tissue growth factor/CCN2 overexpression in mouse synovial lining results in transient fibrosis and cartilage damage. Arthritis Rheum 54(5):1653–1661

Bornstein P (2009) Matricellular proteins: an overview. J Cell Commun Signal 3(3–4):163–165

Brigstock DR (2010) Connective tissue growth factor (CCN2, CTGF) and organ fibrosis: lessons from transgenic animals. J Cell Commun Signal 4(1):1–4

Chaqour B, Yang R, Sha Q (2006) Mechanical stretch modulates the promoter activity of the profibrotic factor CCN2 through increased actin polymerization and NF-kappaB activation. J Biol Chem 281(29):20608–20622

Chen CC, Lau LF (2009) Functions and mechanisms of action of CCN matricellular proteins. Int J Biochem Cell Biol 41(4):771–783

Copetti T, Bertoli C, Dalla E, Demarchi F, Schneider C (2009) p65/RelA modulates BECN1 transcription and autophagy. Mol Cell Biol 29(10):2594–2608

Gao R, Brigstock DR (2005) Activation of nuclear factor kappa B (NF-kappaB) by connective tissue growth factor (CCN2) is involved in sustaining the survival of primary rat hepatic stellate cells. Cell Commun Signal 3:14

Gao R, Brigstock DR (2006) A novel integrin alpha5beta1 binding domain in module 4 of connective tissue growth factor (CCN2/CTGF) promotes adhesion and migration of activated pancreatic stellate cells. Gut 55(6):856–862

Hall-Glenn F, De Young RA, Huang BL, van Handel B, Hofmann JJ, Chen TT, Choi A, Ong JR, Benya PD, Mikkola H, Iruela-Arispe ML, Lyons KM (2012) CCN2/connective tissue growth factor is essential for pericyte adhesion and endothelial basement membrane formation during angiogenesis. PLoS One 7(2):e30562

Hamamura K, Goldring MB, Yokota H (2009) Involvement of p38 MAPK in regulation of MMP13 mRNA in chondrocytes in response to surviving stress to endoplasmic reticulum. Arch Oral Biol 54(3):279–286

Ivkovic S, Yoon BS, Popoff SN, Safadi FF, Libuda DE, Stephenson RC, Daluiski A, Lyons KM (2003) Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. Development 130(12):2779–2791

Jun JI, Lau LF (2011) Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets. Nat Rev Drug Discov 10(12):945–963

Kaneko M, Niinuma Y, Nomura Y (2003) Activation signal of nuclear factor-kappa B in response to endoplasmic reticulum stress is transduced via IRE1 and tumor necrosis factor receptor-associated factor 2. Biol Pharm Bull 26(7):931–935

Kawaki H, Kubota S, Suzuki A, Lazar N, Yamada T, Matsumura T, Ohgawara T, Maeda T, Perbal B, Lyons KM, Takigawa M (2008) Cooperative regulation of chondrocyte differentiation by CCN2 and CCN3 shown by a comprehensive analysis of the CCN family proteins in cartilage. J Bone Miner Res 23(11):1751–1764

Kouroku Y, Fujita E, Tanida I, Ueno T, Isoai A, Kumagai H, Ogawa S, Kaufman RJ, Kominami E, Momoi T (2007) ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. Cell Death Differ 14(2):230–239

Lee GM, Poole CA, Kelley SS, Chang J, Caterson B (1997) Isolated chondrons: a viable alternative for studies of chondrocyte metabolism in vitro. Osteoarthr Cartil 5(4):261–274

Lengner CJ, Lepper C, van Wijnen AJ, Stein JL, Stein GS, Lian JB (2004) Primary mouse embryonic fibroblasts: a model of mesenchymal cartilage formation. J Cell Physiol 200(3):327–333

Li J, Ni M, Lee B, Barron E, Hinton DR, Lee AS (2008) The unfolded protein response regulator GRP78/BiP is required for endoplasmic reticulum integrity and stress-induced autophagy in mammalian cells. Cell Death Differ 15(9):1460–1471

Lotz MK, Carames B (2011) Autophagy and cartilage homeostasis mechanisms in joint health, aging and OA. Nat Rev Rheumatol 7(10):579–587

Marcu KB, Otero M, Olivotto E, Borzi RM, Goldring MB (2010) NF-kappaB signaling: multiple angles to target OA. Current Drug Targets 11(5):599–613

Marino G, Fernandez AF, Cabrera S, Lundberg YW, Cabanillas R, Rodriguez F, Salvador-Montoliu N, Vega JA, Germana A, Fueyo A, Freije JM, Lopez-Otin C (2010) Autophagy is essential for mouse sense of balance. J Clin Investig 120(7):2331–2344

Mehrpour M, Esclatine A, Beau I, Codogno P (2010) Overview of macroautophagy regulation in mammalian cells. Cell Res 20(7):748–762

Ni M, Zhang Y, Lee AS (2011) Beyond the endoplasmic reticulum: atypical GRP78 in cell viability, signalling and therapeutic targeting. Biochem J 434(2):181–188

Nishida T, Kubota S, Kojima S, Kuboki T, Nakao K, Kushibiki T, Tabata Y, Takigawa M (2004) Regeneration of defects in articular cartilage in rat knee joints by CCN2 (connective tissue growth factor). J Bone Miner Res 19(8):1308–1319

Nishida T, Kawaki H, Baxter RM, Deyoung RA, Takigawa M, Lyons KM (2007) CCN2 (Connective Tissue Growth Factor) is essential for extracellular matrix production and integrin signaling in chondrocytes. J Cell Commun Signal 1(1):45–58

Nozaki S, Sledge GW Jr, Nakshatri H (2001) Repression of GADD153/CHOP by NF-kappaB: a possible cellular defense against endoplasmic reticulum stress-induced cell death. Oncogene 20(17):2178–2185

Nugent AE, McBurney DL, Horton WE Jr (2011) The presence of extracellular matrix alters the chondrocyte response to endoplasmic reticulum stress. J Cell Biochem 112(4):1118–1129

Omoto S, Nishida K, Yamaai Y, Shibahara M, Nishida T, Doi T, Asahara H, Nakanishi T, Inoue H, Takigawa M (2004) Expression and localization of connective tissue growth factor (CTGF/Hcs24/CCN2) in osteoarthritic cartilage. Osteoarthr Cartil 12(10):771–778

Ovchinnikov DA, Deng JM, Ogunrinu G, Behringer RR (2000) Col2a1-directed expression of Cre recombinase in differentiating chondrocytes in transgenic mice. Genesis 26(2):145–146

Park M, Yong Y, Choi SW, Kim JH, Lee JE, Kim DW (2007) Constitutive RelA activation mediated by Nkx3.2 controls chondrocyte viability. Nat Cell Biol 9(3):287–298

Pulai JI, Del Carlo M Jr, Loeser RF (2002) The alpha5beta1 integrin provides matrix survival signals for normal and osteoarthritic human articular chondrocytes in vitro. Arthritis Rheum 46(6):1528–1535

Rasheva VI, Domingos PM (2009) Cellular responses to endoplasmic reticulum stress and apoptosis. Apoptosis 14(8):996–1007

Sen T, Dutta A, Chatterjee A (2010) Epigallocatechin-3-gallate (EGCG) regulates gelatinase-B (MMP-9) by involvement of FAK/ERK/NFkappaB and AP-1 in the human breast cancer cell line MDA-MB-231. Anti-cancer drugs 21(6):632–644

Sirabella R, Secondo A, Pannaccione A, Scorziello A, Valsecchi V, Adornetto A, Bilo L, Di Renzo G, Annunziato L (2009) Anoxia-induced NF-kappaB-dependent upregulation of NCX1 contributes to Ca2+ refilling into endoplasmic reticulum in cortical neurons. Stroke J Cereb Circ 40(3):922–929

Srinivas V, Bohensky J, Shapiro IM (2009) Autophagy: a new phase in the maturation of growth plate chondrocytes is regulated by HIF, mTOR and AMP kinase. Cells Tissues Organs 189(1–4):88–92

Szafranski JD, Grodzinsky AJ, Burger E, Gaschen V, Hung HH, Hunziker EB (2004) Chondrocyte mechanotransduction: effects of compression on deformation of intracellular organelles and relevance to cellular biosynthesis. Osteoarthr Cartil 12(12):937–946

Tan TW, Lai CH, Huang CY, Yang WH, Chen HT, Hsu HC, Fong YC, Tang CH (2009) CTGF enhances migration and MMP-13 up-regulation via alphavbeta3 integrin, FAK, ERK, and NF-kappaB-dependent pathway in human chondrosarcoma cells. J Cell Biochem 107(2):345–356

Tsang KY, Chan D, Cheslett D, Chan WC, So CL, Melhado IG, Chan TW, Kwan KM, Hunziker EB, Yamada Y, Bateman JF, Cheung KM, Cheah KS (2007) Surviving endoplasmic reticulum stress is coupled to altered chondrocyte differentiation and function. PLoS Biol 5(3):e44

Voncken JW (2011) Genetic modification of the mouse: general technology—pronuclear and blastocyst injection. Methods Mol Biol 693:11–36

Vranka J, Mokashi A, Keene DR, Tufa S, Corson G, Sussman M, Horton WA, Maddox K, Sakai L, Bachinger HP (2001) Selective intracellular retention of extracellular matrix proteins and chaperones associated with pseudoachondroplasia. Matrix Biol 20(7):439–450

Walter P, Ron D (2011) The unfolded protein response: from stress pathway to homeostatic regulation. Science 334(6059):1081–1086

Wu S, Flint JK, Rezvani G, De Luca F (2007) Nuclear factor-kappaB p65 facilitates longitudinal bone growth by inducing growth plate chondrocyte proliferation and differentiation and by preventing apoptosis. J Biol Chem 282(46):33698–33706

Yang L, Carlson SG, McBurney D, Horton WE Jr (2005) Multiple signals induce endoplasmic reticulum stress in both primary and immortalized chondrocytes resulting in loss of differentiation, impaired cell growth, and apoptosis. J Biol Chem 280(35):31156–31165

Zahir N, Lakins JN, Russell A, Ming W, Chatterjee C, Rozenberg GI, Marinkovich MP, Weaver VM (2003) Autocrine laminin-5 ligates alpha6beta4 integrin and activates RAC and NFkappaB to mediate anchorage-independent survival of mammary tumors. J Cell Biol 163(6):1397–1407