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Regulation of the Catabolic Cascade in Osteoarthritis by the Zinc-ZIP8-MTF1 Axis

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SUMMARY

Osteoarthritis (OA), primarily characterized by cartilage degeneration, is caused by an imbalance between anabolic and catabolic factors. Here, we investigated the role of zinc (Zn^{2+}) homeostasis, Zn^{2+} transporters, and Zn^{2+}-dependent transcription factors in OA pathogenesis. Among Zn^{2+} transporters, the Zn^{2+} importer ZIP8 was specifically upregulated in OA cartilage of humans and mice, resulting in increased levels of intracellular Zn^{2+} in chondrocytes. ZIP8-mediated Zn^{2+} influx upregulated the expression of matrix-degrading enzymes (MMP3, MMP9, MMP12, MMP13, and ADAMTS5) in chondrocytes. Ectopic expression of ZIP8 in mouse cartilage tissue caused OA cartilage destruction, whereas *Zip8* knockout suppressed surgically induced OA pathogenesis, with concomitant modulation of Zn^{2+} influx and matrix-degrading enzymes. Furthermore, MTF1 was identified as an essential transcription factor in mediating Zn^{2+}/ZIP8-induced catabolic factor expression, and genetic modulation of *Mtf1* in mice altered OA pathogenesis. We propose that the zinc-ZIP8-MTF1 axis is an essential catabolic regulator of OA pathogenesis.

INTRODUCTION

Osteoarthritis (OA) is the most common of all arthropathies and is a leading cause of disability with a large socioeconomic cost. To date, however, no effective disease-modifying therapies for OA have been developed. OA is primarily characterized by cartilage destruction but also involves other pathological changes, including synovial inflammation, osteophyte formation, and subchondral bone sclerosis, in all tissues of joints (Bian et al., 2012; Loeser et al., 2012; Little and Hunter, 2013). OA pathogenesis is caused by an imbalance between anabolic and catabolic factors. A variety of etiologic risk factors and pathophysiological processes contribute to the progressive nature of the disease. Important among potential OA-causing mechanisms are mechanical stresses, including joint instability and injury, and factors that predispose toward OA, such as aging. These factors lead to the activation of biochemical pathways in chondrocytes that result in degradation of the extracellular matrix by matrix metalloproteinases (MMPs) and aggrecanases (ADAMTSs). Among matrix-degrading enzymes, MMP3, MMP13, and ADAMTS5 are known to play crucial roles in OA cartilage destruction (Blom et al., 2007; Glasson et al., 2005; Little et al., 2009).

Matrix-degrading enzymes require zinc (Zn^{2+}) as a structural component (Page-McCaw et al., 2007). Indeed, Zn^{2+} acts as an activator or coactivator of a variety of proteins by providing a structural scaffold, for example in the form of zinc fingers and zinc clusters (Prasad, 1995). Zn^{2+} is also found in the cytoplasm and intracellular compartments in a labile form, and changes in these Zn^{2+} levels affect various cellular functions. In fact, an imbalance in Zn^{2+} homeostasis is linked to abnormal embryonic development, immune dysfunction, neurological problems, and increased mortality and disease (Fukada et al., 2011). Zn^{2+} may also be overtly toxic when accumulated in excess in cells. Therefore, normal cell functioning requires tight regulation of Zn^{2+} homeostasis. Zn^{2+} homeostasis is primarily regulated by membrane Zn^{2+} transporters of the Slc30a family (ZNT) of exporters and Slc39a family (ZIP) of importers (Cousins et al., 2006). The ZNT family, consisting of ten members in mammals (ZNT1–ZNT10), mediates Zn^{2+} efflux from cells or influx into intracellular vesicles from the cytosol. There are 14 members of the ZIP family of Zn^{2+} importers in mammals (ZIP1–ZIP14) that promote Zn^{2+} influx from the extracellular fluid or intracellular vesicles into the cytoplasm. Zn^{2+} transporters exhibit tissue-specific functions (Liuzzi et al., 2005; Kitamura et al., 2006), and abnormalities in the function of certain Zn^{2+} transporters are associated with human diseases, such as acrodermatitis enteropathica (Küry et al., 2002). Zn^{2+} influx modulates a number of transcription factors in various cell types. Among them, metal-regulatory transcription factor-1 (MTF1) regulates expression of a variety of target genes and thereby regulates cellular

adaptation to various stress conditions, primarily exposure to heavy metals, but also hypoxia and oxidative stress (Laity and Andrews, 2007; Günther et al., 2012). Zn^{2+} homeostasis is additionally regulated by metal-dependent transcriptional control of storage proteins. For instance, metallothioneins (MTs), whose genes are well-known targets of MTF1, act as Zn^{2+}-storage proteins and thereby regulate cellular Zn^{2+} homeostasis. MTs also act as antioxidants and protect cells from oxidative stress (Laity and Andrews, 2007; Colvin et al., 2010; Günther et al., 2012).

There has been a growing interest in the potential role of Zn^{2+} in the pathogenesis of OA. Clinical studies indicate highly elevated serum Zn^{2+} levels in OA patients (Ovesen et al., 2009) and specific accumulation of Zn^{2+} in the tidemark region of articular cartilage in aged populations (Roschger et al., 2013). The association of Zn^{2+} with OA pathogenesis is broadly appreciated in the context of its role as a structural component of matrix-degrading enzymes required for the maturation and activation of these enzymes. However, how Zn^{2+} homeostasis is regulated during the onset and progression of OA and how it contributes to the pathological transition of articular chondrocytes remain unknown. Here, we investigated the roles of Zn^{2+} homeostasis, homeostasis-regulating Zn^{2+} transporters, and downstream transcription factors and their target genes in OA pathogenesis. We report here that the zinc-ZIP8-MTF1 axis regulates OA pathogenesis.

RESULTS

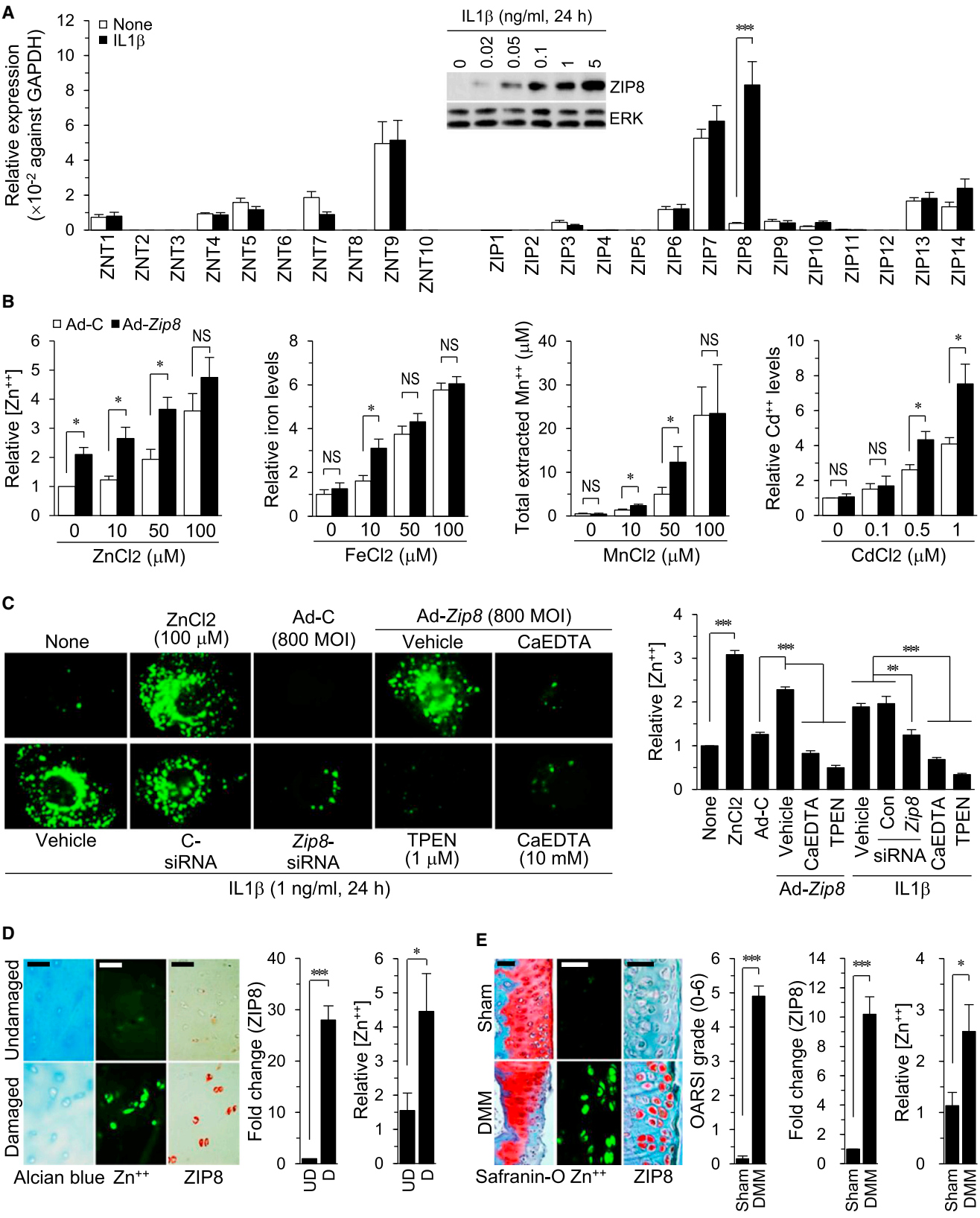
The Zn^{2+} Influx Mediator ZIP8 Is Upregulated in Chondrocytes under Pathological Conditions and in OA Cartilage

To elucidate the role of Zn^{2+} homeostasis and associated regulatory genes in OA pathogenesis, we initially examined expression levels of metal ion transporters—the Slc30a family (ZNT) of exporters and Slc39a family (ZIP) of importers—in primary cultures of mouse articular chondrocytes. Among the examined transporters, ZNT9 and ZIP7 mRNAs were expressed at relatively high levels in chondrocytes, whereas many of the remaining transporters were barely detectable (Figure 1A). However, following treatment with interleukin (IL)1β, a proinflammatory cytokine that promotes catabolism in arthritic cartilage (Kapoor et al., 2011), ZIP8 expression levels were markedly increased, making it the most dominantly expressed transporter among the ZNT and ZIP family members (Figure 1A). This led us to investigate the role of ZIP8 in OA pathogenesis. ZIP8 is known to transport Zn^{2+}, nontransferrin-bound Fe^{2+}, Mn^{2+}, and Cd^{2+} (Dalton et al., 2005; Wang et al., 2012). We therefore examined whether ZIP8 transports these metal ions in mouse articular chondrocytes. Overexpression of ZIP8 in chondrocytes caused significant Zn^{2+} influx (Figures 1B and 1C), whereas cellular levels of Fe^{2+}, Mn^{2+}, and Cd^{2+} were not affected by ZIP8 overexpression alone (Figure 1B). However, in a certain range of each metal ion supplementation, ZIP8 effectively drove influx of these metal ions (Figure 1B), suggesting that the transport efficiency of each metal ion by ZIP8 is determined by the cellular microenvironmental context, with Zn^{2+} evidently being the most efficiently transported by ZIP8 under our experiment conditions. Thus, we further characterized the regulation of Zn^{2+} influx by ZIP8. The ZIP8-mediated increase in Zn^{2+} influx was abolished by the cell-impermeable metal ion chelator, calcium-saturated EDTA (CaEDTA), or the cell-permeable chelator, N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) (Figure 1C). IL1β, which increased both transcript and protein levels of ZIP8 (Figure 1A, inset), caused an influx of Zn^{2+} in chondrocytes that was blocked by downregulating *Zip8* with small interfering RNA (siRNA) or the metal ion chelators CaEDTA or TPEN (Figure 1C).

The upregulation of ZIP8 and Zn^{2+} influx in chondrocytes under pathological conditions (i.e., exposure to IL1β) suggests the possible association of ZIP8-mediated Zn^{2+} influx with OA pathogenesis. This was evaluated by examining expression of ZIP8 and Zn^{2+} levels in OA cartilage of human and mouse models. Damage of OA-affected human cartilage was established based on Alcian blue staining (Figure 1D). Zn^{2+} and ZIP8 protein and mRNA levels were markedly elevated in OA-affected human cartilage but were barely detectable in undamaged regions of arthritic cartilage (Figure 1D). We also used destabilization of the medial meniscus (DMM) surgery as a mouse model of OA and compared it with normal cartilage in a sham-operated joint (Glasson et al., 2007). Similar to human OA cartilage, mouse OA cartilage showed a marked increase in Zn^{2+} and ZIP8 protein and mRNA levels (Figure 1E).

ZIP8-Mediated Zn^{2+} Influx Induces Upregulation of Matrix-Degrading Enzymes in Chondrocytes

The increased expression of ZIP8 and elevated Zn^{2+} levels in OA cartilage suggests their possible involvement in OA pathogenesis. A hallmark of OA chondrocytes is their increased production of Zn^{2+}-bound matrix-degrading enzymes such as MMPs and ADAMTSs. We therefore asked whether cellular Zn^{2+} influx driven by ZIP8 is sufficient to cause expression of matrix-degrading enzymes. Among the examined enzymes, MMP3, MMP9, MMP12, MMP13, and ADAMTS5 were significantly upregulated by infection of chondrocytes with an adenoviral *Zip8* expression construct (Ad-*Zip8*); this effect was blocked by metal ion chelation with CaEDTA or TPEN (Figure 2A). Consistent with this, treatment of chondrocytes with $ZnCl_2$ upregulated MMP3, MMP9, MMP12, and MMP13, whereas treatment with $CdCl_2$ or $MnCl_2$ did not cause a marked expression of matrix-degrading enzymes (Figure 2B). $FeCl_2$ treatment caused moderate expression of matrix-degrading enzymes. However, chelation of ferric or ferrous with Zn/DFO or 2,2-bipyridyl, respectively, did not affect Ad-*Zip8*-induced expression of matrix-degrading enzymes (Figure 2C), consistent with the fact that ZIP8 did not cause Fe^{2+} influx without exogenous $FeCl_2$ supplementation. Furthermore, preincubation of TPEN with $ZnCl_2$ effectively abolished the inhibitory effects of TPEN on the ZIP8-mediated expression of matrix-degrading enzymes, whereas $FeCl_2$, $CdCl_2$, and $MnCl_2$ showed no significant effects on TPEN inhibition of the ZIP8-mediated upregulation of matrix-degrading enzymes (Figure 2D). These results collectively suggest that Zn^{2+}, among the metal ions transported by ZIP8, plays a major role in ZIP8 regulation of the expression of matrix-degrading enzymes. Among the ZIP8-regulated matrix-degrading enzymes, MMP3, MMP13, and ADAMTS5 are crucial effectors of OA cartilage destruction (Blom et al., 2007; Glasson et al., 2005; Little



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et al., 2009). Indeed, upregulation of these enzymes by IL1β was abolished by knockdown of *Zip8* with specific siRNA or by metal ion chelation with CaEDTA or TPEN (Figures 2E and 2F). Collectively, these results suggest ZIP8-mediated Zn^{2+} influx exerts a catabolic function in chondrocytes by virtue of its role in upregulating matrix-degrading enzymes.

ZIP8 Overexpression in Cartilage Tissue Causes OA Pathogenesis in Mice

The role of ZIP8-mediated Zn^{2+} influx in OA pathogenesis was directly examined by intra-articular (IA) injection of Ad-*Zip8* into mouse knee joints. We have previously shown that the adenoviral system effectively delivers genes into mouse joint tissues (Yang et al., 2010; Ryu et al., 2011, 2012; Oh et al., 2012) and further confirmed gene delivery via this system by injecting Ad-*eGFP* (Figure S1B available online). Ad-*Zip8* injection caused ZIP8 overexpression in cartilage, meniscus, ligament, and synovium (Figures 3A and S1A). Three weeks after Ad-*Zip8* injection, chondrocytes of cartilage tissue exhibited markedly increased levels of Zn^{2+}, MMP3, and MMP13 (Figure 3A) with concomitant cartilage destruction (Figures 3B and 3C). IA injection of Ad-*Zip8* also caused synovitis (Figure S1C). Consistent with this, overexpression of ZIP8 in primary cultured mouse fibroblast-like synoviocytes caused upregulation of various catabolic factors, including matrix-degrading enzymes, cytokines, and chemokines (Figure S1D). Additionally, Ad-*Zip8*-induced MMP expression, cartilage destruction, and synovitis were significantly blocked by coinjection of TPEN (Figures 3A, 3B, and S1C). Other OA manifestations, such as osteophyte development and subchondral bone sclerosis, were not observed at 3 weeks after Ad-*Zip8* injection. However, 8 weeks after IA injection, joint tissues exhibited osteophyte development and subchondral bone sclerosis with more severe cartilage destruction (Figure S1E). Collectively, these results demonstrate that ZIP8-mediated Zn^{2+} influx acts as a catabolic regulator of OA pathogenesis.

The in vivo role of ZIP8-mediated Zn^{2+} influx in OA pathogenesis was further validated using chondrocyte-specific *Zip8*-overexpressing transgenic (TG) mice (*Col2a1-Zip8*), in which *Zip8* expression is driven by the promoter and enhancer region of mouse *Col2a1* (Yang et al., 2010; Oh et al., 2012). *Col2a1-Zip8* TG mice expressed markedly enhanced levels of ZIP8 in cartilage and meniscus, but not in the synovium or ligaments, of joint tissues (Figures 3D and S2A). *Zip8* TG mice also showed increased levels of Zn^{2+}, MMP3, and MMP13 in cartilage tissue (Figure 3D). Consistent with this, aged (12-month-old) *Zip8* TG mice exhibited spontaneous cartilage destruction (Figure 3E), with Osteoarthritis Research Society International (OARSI) grades ranging from 1 to 6 (Figure S2D) and subchondral bone sclerosis (Figure 3F) compared with age-matched wild-type (WT) mice; synovitis was not observed in *Zip8* TG mice (Figure 3G). Additionally, DMM surgery in young (10–12 weeks old) *Zip8* TG mice showed significantly enhanced OA cartilage destruction (Figure 3H) and subchondral bone sclerosis, whereas synovitis and osteophyte development were not affected (Figures S2E and S2F). These features of *Zip8* TG mice clearly support the notion that ZIP overexpression in cartilage tissue is sufficient to cause OA pathogenesis.

Zip8 Knockout in Mice Inhibits Experimental OA Pathogenesis

We next confirmed the catabolic role of ZIP8 in OA pathogenesis using *Zip8*-knockout (KO) mice. Because homozygous deletion of *Zip8* in mice ($Zip8^{-/-}$) is embryonic lethal (Gálvez-Peralta et al., 2012), we evaluated the effect of *Zip8* knockout on OA pathogenesis using chondrocyte-specific conditional KO (CKO) mice ($Zip8^{fl/fl}$;*Col2a1-Cre*). *Zip8*-CKO mice showed a significant reduction in OA cartilage destruction following DMM surgery (Figure 4A), with concomitant inhibition of ZIP8 expression, Zn^{2+} influx, and expression of MMP3 and MMP13 in cartilage tissue (Figure 4B). Consistent with this, IL1β-induced Zn^{2+} influx and expression of MMP3, MMP13, and ADAMTS5 were reduced in primary cultured chondrocytes isolated from *Zip8*-CKO mice (Figures 4C and 4D). In addition to cartilage destruction, DMM-operated *Zip8*-CKO mice exhibited significant inhibition of subchondral bone sclerosis, whereas synovitis and osteophyte formation were not affected (Figures 4E and 4F). The results collectively suggest that genetic deletion of *Zip8* inhibits experimental OA pathogenesis in mice. Similar to *Zip8* CKO mice, $Zip8^{+/-}$ mice also exhibited reduced OA cartilage destruction and subchondral bone sclerosis following DMM surgery (data not shown).

ZIP8-Mediated Zn^{2+} Influx Upregulates Matrix-Degrading Enzymes by Enhancing MTF1 Transcriptional Activity

Next, we elucidated the regulatory mechanisms by which ZIP8-mediated Zn^{2+} influx mediates catabolic factor expression and OA pathogenesis. To do this, we first identified transcription factors activated by ZIP8-mediated Zn^{2+} influx in chondrocytes using a transcription factor array kit. Among the 37 transcription factors examined, MTF1, NRF1 and NRF2, NF-κB, SP1, p53, C/EBP, and AP1 showed more than a 3-fold increase in transcriptional activity following Ad-*Zip8* infection (Figure S3A). Pharmacological inhibition of p53, AP1, or NF-κB or siRNA-mediated knockdown of *Nrf* or *Cebpb* had no significant effect

Figure 1. The Zn^{2+} Influx Mediator ZIP8 Is Upregulated in Chondrocytes under Pathological Conditions and in OA Cartilage

(A) mRNA levels of metal ion transporters determined by qRT-PCR in articular chondrocytes treated with IL1β (n = 10). Inset is western blotting of ZIP8 in chondrocytes treated with IL1β.

(B) Cellular levels of Zn^{2+}, Fe^{2+}/Fe^{3+}, Mn^{2+}, and Cd^{2+} were measured in chondrocytes infected with Ad-C or Ad-*Zip8* following treatment with indicated concentrations of $ZnCl_2$, $FeCl_2$, $MnCl_2$, or $CdCl_2$.

(C) Cellular Zn^{2+} levels were imaged and quantified in chondrocytes treated with $ZnCl_2$ or IL1β, with or without control (C) or *Zip8* siRNA, or in chondrocytes infected with Ad-*Zip8*, with or without the metal chelator, CaEDTA or TPEN (n = 5–12).

(D and E) Staining of cartilage with Alcian blue or safranin-O, imaging and quantification of Zn^{2+} levels with fluorophore, detection of ZIP8 by immunostaining, and quantification of ZIP8 mRNA levels by qRT-PCR in human OA cartilage (D) or mouse OA cartilage induced by DMM surgery (E) ($n \geq 8$).

Scale bar, 50 μm. Values are presented as means ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). NS, not significant. See also Table S1.

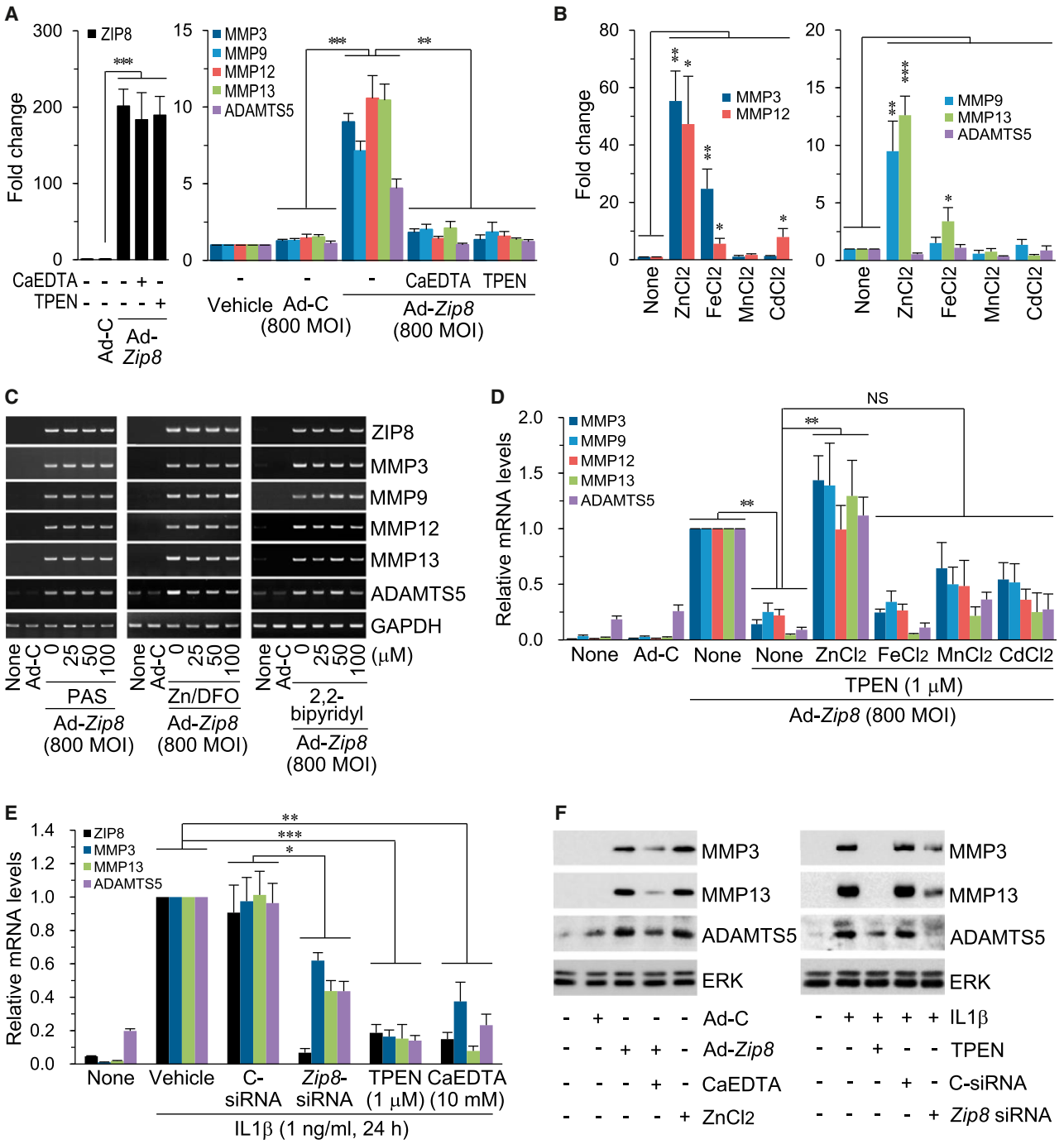

Figure 2. ZIP8-Mediated Zn^{2+} Influx Induces Upregulation of Matrix-Degrading Enzymes in Chondrocytes

(A and B) mRNA levels were quantified by qRT-PCR in chondrocytes infected with Ad-C or Ad-*Zip8* with or without CaEDTA or TPEN ($n \geq 6$) (A) or treated with 100 μM of $ZnCl_2$, $FeCl_2$, or $MnCl_2$ and 1 μM of $CdCl_2$ ($n = 6$) (B).

(C) The indicated mRNAs were detected in chondrocytes infected with Ad-C or Ad-*Zip8* in the absence or presence of the indicated concentrations of iron chelators, Zn/DFO, 2,2-bipyridyl, or Mn^{2+} chelator para-aminosalicylic acid (PAS) ($n = 4$).

(D) mRNA levels of matrix-degrading enzymes in chondrocytes infected with Ad-C or Ad-*Zip8* with or without TPEN (1 μM) or TPEN preincubated with 1 μM of the indicated metal ion ($n = 5$).

(E) qRT-PCR analysis ($n \geq 6$) of mRNA levels of ZIP8 and matrix-degrading enzymes in chondrocytes treated with IL1β, with or without control or *Zip8* siRNA, TPEN, or CaEDTA.

(F) Expression of matrix-degrading enzymes, determined by western blotting.

Values are presented as means ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). See also Table S1.

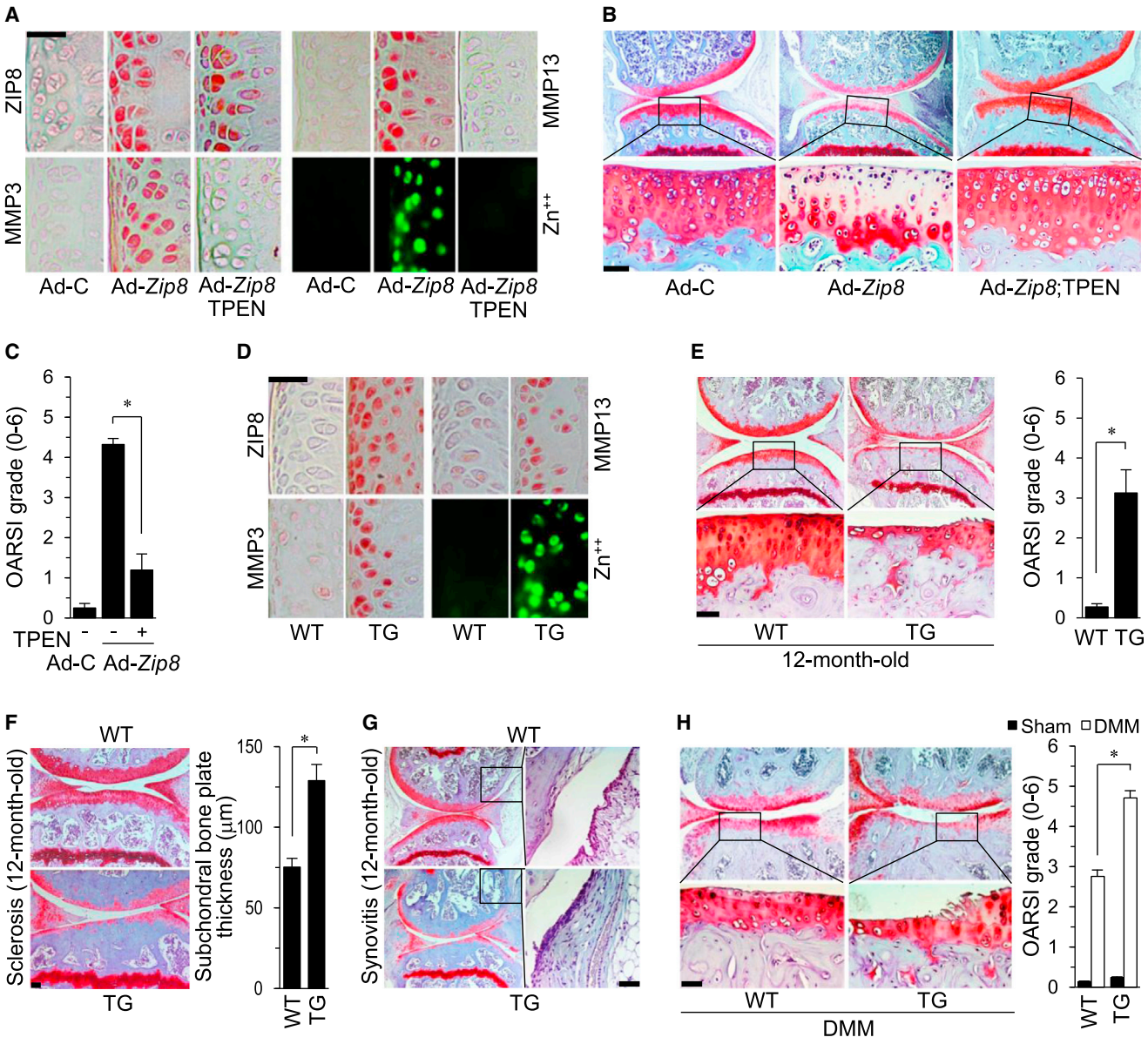

Figure 3. ZIP8 Overexpression in Cartilage Tissue Causes OA Pathogenesis in Mice

(A–C) Mice were IA injected with Ad-C or Ad-*Zip8* with or without TPEN. ZIP8, MMP3, and MMP13 in cartilage were detected by immunostaining, and Zn^{2+} was imaged using a fluorophore (A). Cartilage destruction was detected by safranin-O staining (B) and quantified by OARSI grade ($n \geq 13$) (C).

(D) Cartilage sections from 12-week-old WT and *Zip8* TG mice were immunostained for ZIP8, MMP3, and MMP13. Zn^{2+} was imaged using a fluorophore.

(E) Spontaneous cartilage destruction in aged (12-month-old) WT and *Zip8* TG mice (n = 16).

(F and G) Subchondral bone sclerosis (F) and synovitis (G) in 12-month-old WT and *Zip8* TG mice (n = 16).

(H) Cartilage destruction in 18- to 20-week-old DMM-operated WT and *Zip8* TG mice (n = 12).

Scale bar, 50 μm. Values are presented as means ± SEM (*p < 0.001). See also Figures S1 and S2.

on Ad-*Zip8*-induced upregulation of matrix-degrading enzymes (Figures S3C and S3D). However, knockdown of *Mtf1* with siRNA or inhibition of SP1 with mithramycin-A significantly attenuated ZIP8-induced upregulation of matrix-degrading enzymes (Figures S3E and S3G). Based on these observations and the fact that MTF1 is a master transcriptional regulator of cellular adaptation under conditions of exposure to heavy metals (Laity and Andrews, 2007; Günther et al., 2012), we selected MTF1 for functional characterization in ZIP8-induced OA pathogenesis. Ad-*Zip8* infection promoted the nuclear localization of MTF1 (Figure 5A) and significantly increased its transcriptional activity (Figure 5B) without modulating its expression (Figure S4A). Notably, adenoviruses expressing mouse *Mtf1* (Ad-*Mtf1*) infection significantly upregulated expression of matrix-degrading

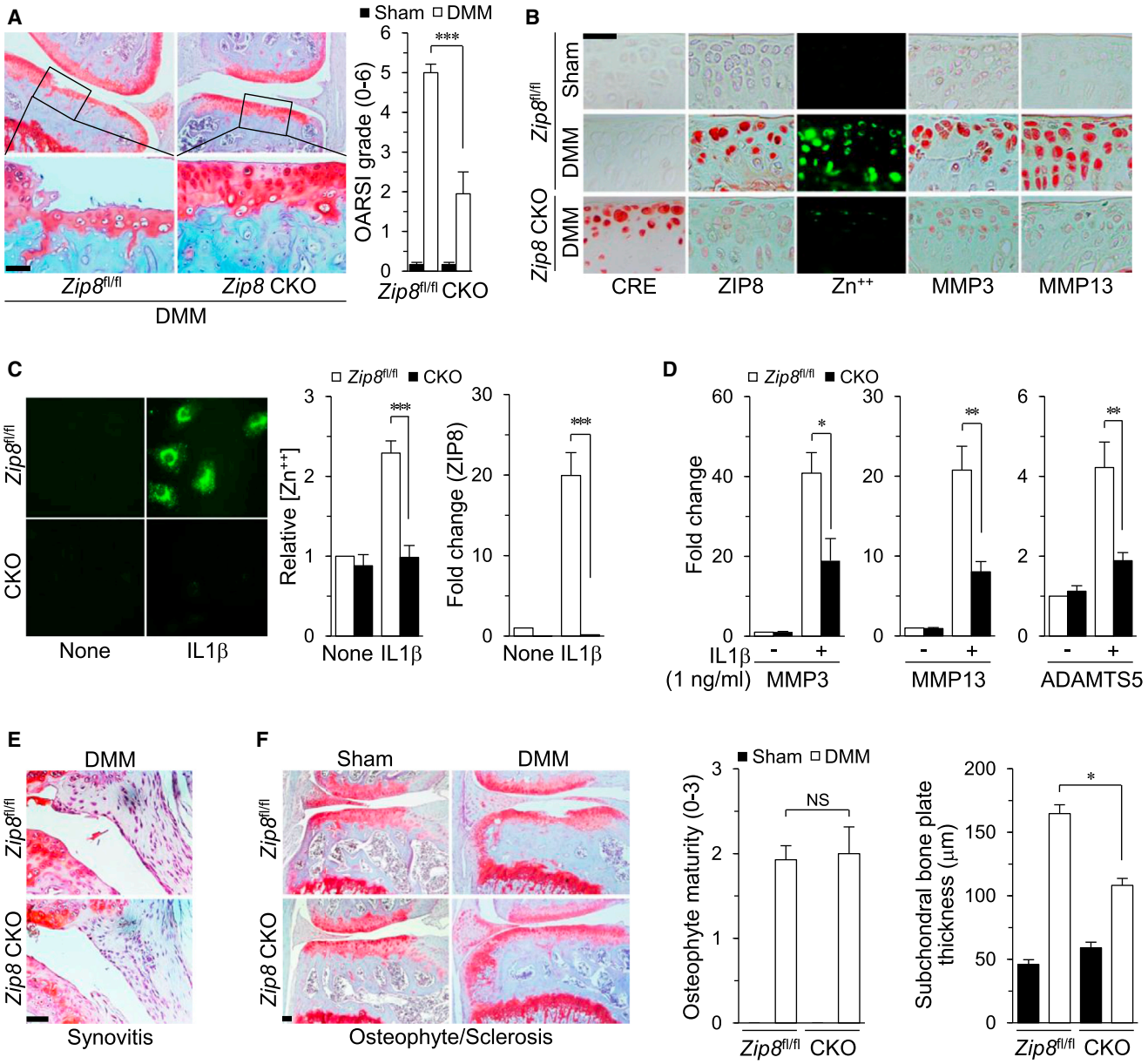

Figure 4. Genetic Deletion of *Zip8* in Mice Inhibits OA pathogenesis

(A) Cartilage sections from sham- and DMM-operated *Zip8*$^{fl/fl}$ and chondrocyte-specific CKO mice (B) were stained with safranin-O. Cartilage destruction was quantified by OARSI grade (n = 10).

(B) CRE, ZIP8, MMP3, and MMP13 were detected by immunostaining, and Zn^{2+} was imaged in cartilage sections from *Zip8*$^{fl/fl}$ and *Zip8*-CKO mice after DMM surgery or sham operation.

(C and D) Primary cultured chondrocytes isolated from *Zip8*$^{fl/fl}$ and *Zip8*-CKO mice were treated with IL1β. Zn^{2+} was imaged and quantified using a fluorophore. mRNA levels of ZIP8 (C) and matrix-degrading enzymes (D) were determined by qRT-PCR (n ≥ 4).

(E and F) Synovitis (E) and subchondral bone sclerosis/osteophyte formation (F) in sham- and DMM-operated *Zip8*$^{fl/fl}$ and *Zip8*-CKO mice (n = 10).

Scale bar, 50 μm. Values are presented as means ± SEM (*p < 0.01, **p < 0.005, ***p < 0.001). See also Table S1.

enzymes (MMP3, MMP9, MMP12, MMP13, and ADAMTS5), an effect that was abolished by CaEDTA or TPEN (Figures 5C and 5D). Additionally, ZIP8 or Zn^{2+}-induced upregulation of matrix-degrading enzymes was significantly reduced by siRNA-mediated knockdown of *Mtf1* (Figures S3E and S3F). Among the ZIP8-regulated metal ions, Zn^{2+} was the most effective in regulating nuclear localization and transcriptional activation of MTF1 and Fe^{2+} and Mn^{2+} had no effect on MTF1 activity (Figures S4B and S4C). Although Cd^{2+} caused a moderate activation of MTF1 (Figure S4C), it had no significant effect on the expression of matrix-degrading enzymes, except for a modest induction of MMP12 expression (Figure 2B). This suggests that, unlike the

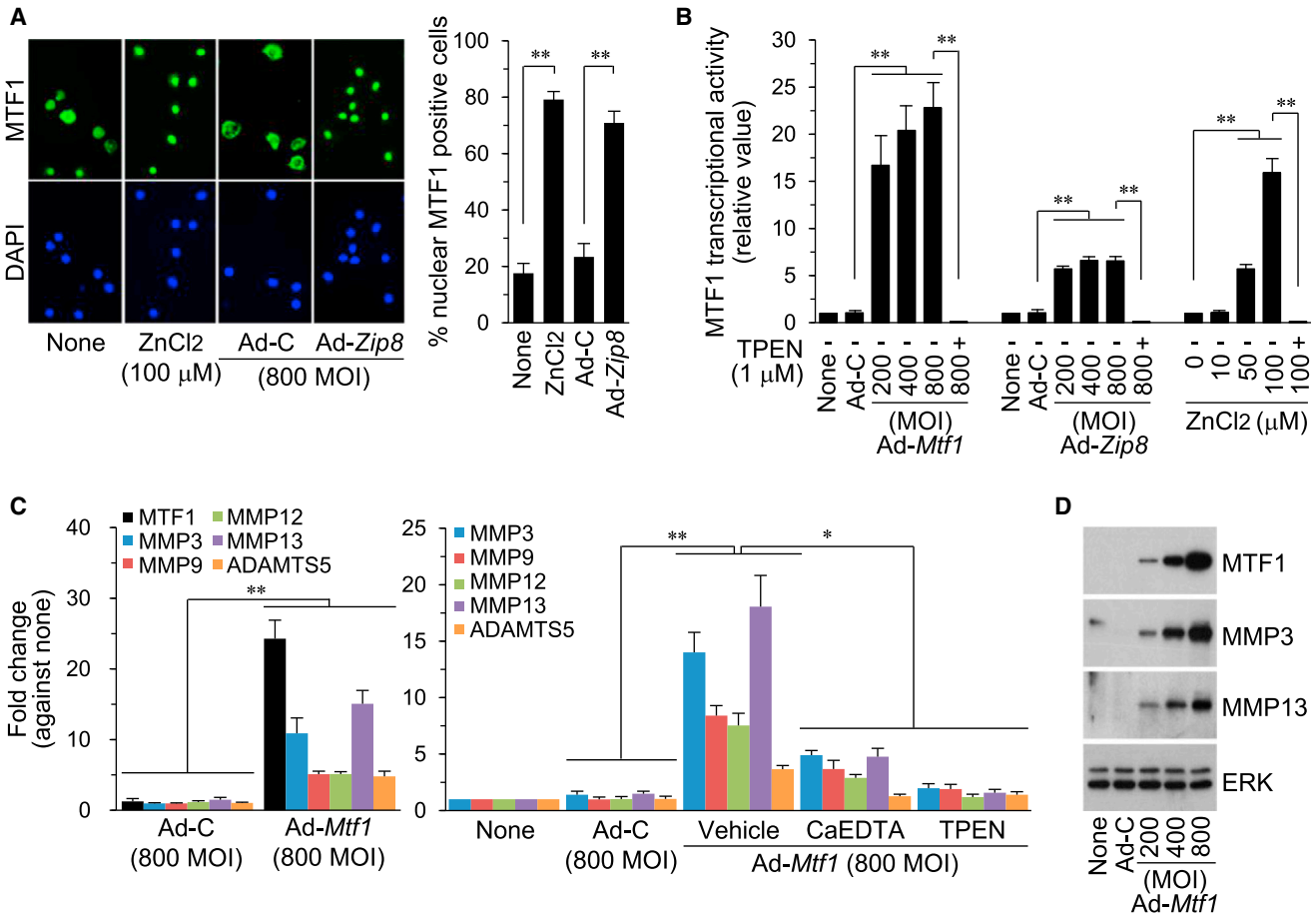

Figure 5. ZIP8-Mediated Zn^{2+} Influx Upregulates Matrix-Degrading Enzymes through Activation of MTF1

(A) Immunostaining for MTF1 and quantification of cells with nuclear-localized MTF1.

(B) MTF1 transcriptional activity quantified by reporter gene assay in chondrocytes treated with $ZnCl_2$ or infected with Ad-C, Ad-*Zip8*, or Ad-*Mtf1* with or without TPEN (n = 9).

(C) mRNA levels in chondrocytes infected with Ad-C or Ad-*Mtf1* (n ≥ 5) in the absence or presence of CaEDTA or TPEN.

(D) Protein levels of MTF1 and matrix-degrading enzymes.

Values are presented as means ± SEM (*p < 0.01, **p < 0.001). See also Figures S3 and S4 and Table S1.

effects of Zn^{2+}-dependent activation of MTF1, Cd^{2+}-induced MTF1 activation does not cause expression of matrix-degrading enzymes. This discrepancy may reflect the fact that MTF1 regulates target-gene expression in a metal-specific manner (Günther et al., 2012). Additionally, preincubation of TPEN with $ZnCl_2$, but not $FeCl_2$, $CdCl_2$, or $MnCl_2$, abolished the inhibitory effects of TPEN on MTF1 transcriptional activity (Figure S4D). These results collectively suggest that ZIP8-mediated Zn^{2+} influx promotes MTF1 transcriptional activity, which in turn upregulates expression of matrix-degrading enzymes in chondrocytes.

MTF1 Mediates ZIP8-Induced OA Pathogenesis in Mice

We next examined the in vivo role of MTF1 in OA pathogenesis. In human cartilage tissues, both MTF1 mRNA and protein were detected at high levels in OA-affected cartilage tissue compared with undamaged regions of arthritic cartilage (Figure 6A). The in vivo function of MTF1 was evaluated by IA injection of Ad-*Mtf1* in mice, which caused MTF1 overexpression in the joint tissues (Figures 6B and S5A). Expression levels of MTF1, MMP3, and MMP13 in the chondrocytes of cartilage tissue were markedly enhanced at 3 weeks after Ad-*Mtf1* injection (Figure 6B) with concomitant cartilage destruction (Figure 6C). IA injection of Ad-*Mtf1* also caused synovial inflammation (Figure S5B), and overexpression of MTF1 in fibroblast-like synoviocytes caused upregulation of various catabolic factors, including matrix-degrading enzymes, cytokines, and chemokines (Figure S5C). Similar to the effects of Ad-*Zip8* injection, Ad-*Mtf1* injection in mice caused osteophyte formation and subchondral bone sclerosis at 8 weeks after IA injection, with more severe cartilage destruction (Figure S5D).

We also confirmed MTF1 function in OA pathogenesis using *Mtf1*-KO mice. Chondrocyte-specific CKO mice (*Mtf1*$^{fl/fl}$; *Col2a1-Cre*) exhibited significantly reduced OA cartilage destruction following DMM surgery (Figure 6D), with concomitant reduction of the expression MTF1, MMP3, and MMP13

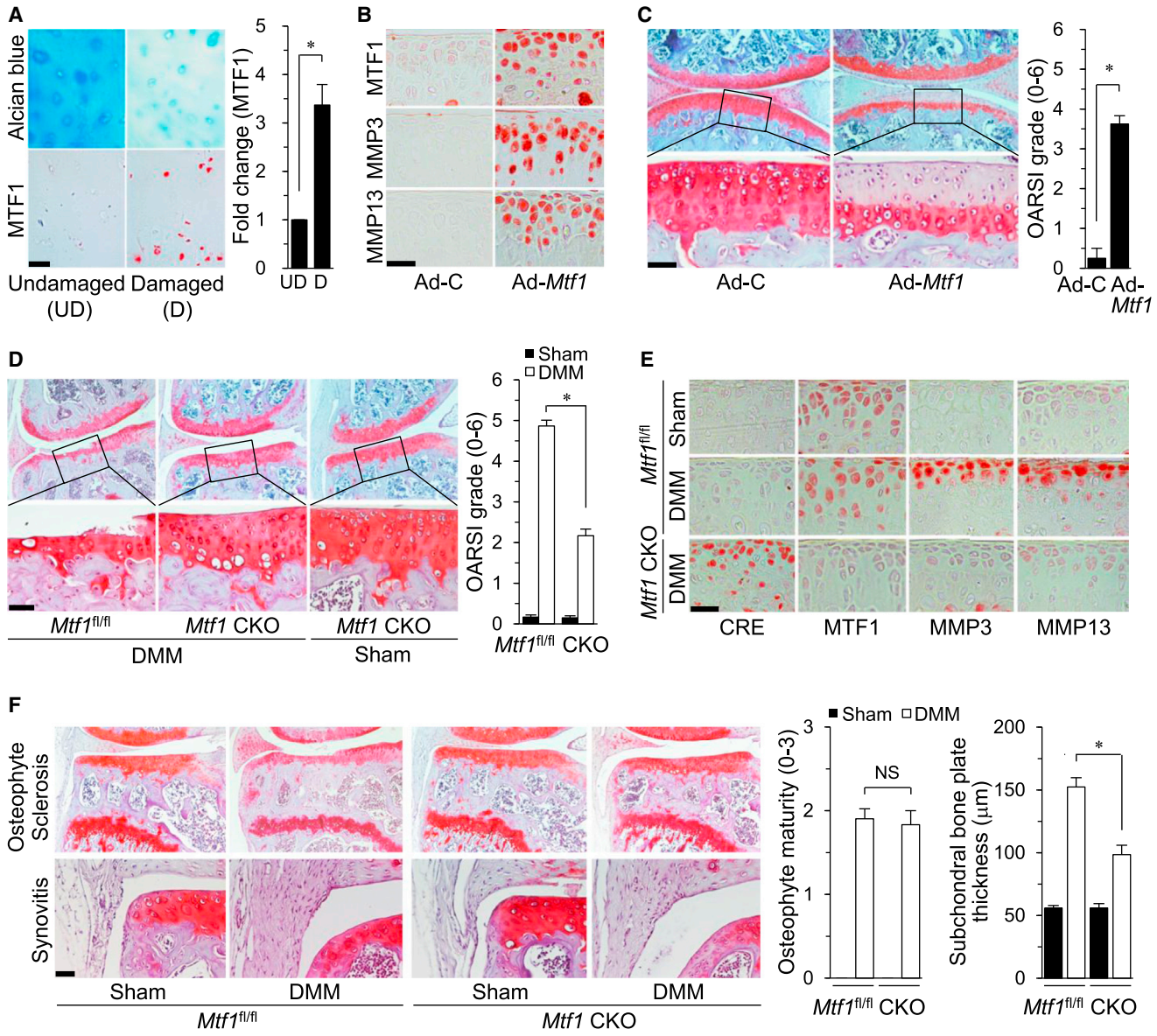

Figure 6. MTF1 Is a Catabolic Regulator of OA Pathogenesis in Mice

(A) MTF1 protein and transcript levels in human OA cartilage determined by immunostaining and qRT-PCR, respectively (n = 10).

(B and C) Mice were IA injected with Ad-C or Ad-*Mtf1*. MTF1, MMP3, and MMP13 immunostaining in cartilage sections (B). Safranin-O staining and scoring of cartilage destruction (n ≥ 14) (C).

(D and E) Cartilage sections from sham- and DMM-operated *Mtf1*$^{fl/fl}$ and *Mtf1*-CKO mice were stained with safranin-O, and cartilage destruction was scored (n = 10) (D) and immunostained for CRE, MTF1, MMP3, and MMP13 (E).

(F) Subchondral bone sclerosis/osteophyte formation and synovitis in sham- and DMM-operated *Mtf1*$^{fl/fl}$ and *Mtf1*-CKO (n = 10).

Scale bar, 50 μm. Values are presented as means ± SEM (*p < 0.001). See also Figures S5, S6, and S7 and Table S1.

(Figure 6E). DMM-operated *Mtf1*-CKO mice also exhibited inhibition of subchondral bone sclerosis without significant effects on synovitis or osteophyte formation (Figure 6F). Additionally, DMM-operated *Mtf1*$^{+/-}$ mice (homozygous deletion of *Mtf1* is embryonic lethal; Wang et al., 2004) also exhibited reduced cartilage destruction, subchondral bone sclerosis, and expression of MMP3 and MMP13 (data not shown). To further elucidate MTF1 as the mediator of ZIP8 functions, we IA coinjected *Mtf1*$^{fl/fl}$ mice with Ad-*Zip8* and Ad-*Cre* to locally delete *Mtf1* in joint tissues. This local deletion of *Mtf1* significantly inhibited Ad-*Zip8*-induced cartilage destruction and synovitis (Figure S6A). In contrast, local deletion of *Zip8* by Ad-*Cre* injection in *Zip8*$^{fl/fl}$ mice did not affect Ad-*Mtf1*-induced cartilage destruction or synovitis (Figure S6B). This clearly supports the notion that MTF1 is a downstream mediator of ZIP8 in OA pathogenesis and ZIP8-activated MTF1 acts as a catabolic regulator of OA

cartilage destruction by upregulating matrix-degrading enzymes in chondrocytes.

Finally, we explored the effects of dietary Zn^{2+} supplementation on surgically induced OA. Low-Zn^{2+} diet did not affect DMM-induced OA cartilage destruction, presumably due to trace amount of Zn^{2+} (<0.5 mg zinc/kg). However, mice fed a high-Zn^{2+} diet exhibited enhanced cartilage destruction following DMM surgery (Figure S7A). Consistent with this, intraperitoneal administration of $ZnCl_2$ augmented DMM-induced cartilage destruction (Figure S7B). Taken together, these observations indicate that Zn^{2+} levels are positively correlated with OA pathogenesis, consistent with our findings that ZIP8-mediated Zn^{2+} influx and MTF1 activation favor OA development.

Knockout of MT Genes Enhances OA Cartilage Destruction in Mice

MTs, which are well-known targets of MTF1, regulate Zn^{2+} homeostasis by virtue of their function as Zn^{2+}-storage proteins, and they protect cells from oxidative stress by acting as antioxidants (Laity and Andrews, 2007; Colvin et al., 2010; Fukada et al., 2011). We therefore elucidated possible functions of MTs in OA pathogenesis. Expression of *Mt1* and *Mt2*, which encode the closely related MT1 and MT2 proteins, was significantly increased in primary cultured chondrocytes infected with Ad-*Zip8* or Ad-*Mtf1* or treated with $ZnCl_2$ (Figures 7A and 7B). The in vivo significance of MT1/MT2 in OA pathogenesis was evaluated using $Mt1^{-/-}$;$Mt2^{-/-}$ double-KO mice. Compared with WT mice, DMM-operated $Mt1^{-/-}$;$Mt2^{-/-}$ double-KO mice exhibited significantly enhanced OA cartilage destruction (Figure 7C) and subchondral bone sclerosis without significant effects on synovitis and osteophyte formation (Figures 7D and 7E). These results collectively suggest that MTF1 activation caused by ZIP8-mediated Zn^{2+} influx activates a catabolic cascade by upregulating matrix-degrading enzymes, whereas upregulation of MT1 and MT2 proteins by MTF1 forms a negative feedback loop that alleviates zinc-cascade-induced OA pathogenesis.

DISCUSSION

The hallmark of OA is the accelerated catabolism elicited by the increased expression of MMPs and ADAMTSs. These matrix-degrading enzymes require Zn^{2+} for their maturation and catalytic activity, suggesting an association of intracellular Zn^{2+} homeostasis with OA pathogenesis. However, no evidence available to date clearly indicates the involvement of Zn^{2+} homeostasis and the genes that regulate it in OA pathogenesis. We demonstrated here that the zinc-ZIP8-MTF1 axis, comprising the Zn^{2+} importer ZIP8, Zn^{2+} influx, and the Zn^{2+}-dependent transcription factor MTF1, is an essential catabolic regulator of experimental OA pathogenesis in mice. We additionally demonstrated that MT1 and MT2 proteins, which are targets of MTF1, form a negative feedback loop that inhibits zinc-cascade-induced OA pathogenesis.

Zn^{2+} acts as an activator or coactivator of proteins by providing a structural scaffold, such as zinc fingers and zinc clusters (Prasad, 1995). Indeed, there are more than 100 Zn^{2+}-dependent enzymes and more than 2,000 Zn^{2+}-dependent transcription factors in mammals (Andreini et al., 2006). The matrix-degrading enzymes involved in OA cartilage destruction also require Zn^{2+} as a structural component (Page-McCaw et al., 2007). In this study, we revealed that Zn^{2+} influx is involved in the transcriptional upregulation of MMP3, MMP9, MMP12, MMP13, and ADAMTS5 in articular chondrocytes. Among these, MMP3, MMP13, and ADAMTS5 are crucial effectors of OA cartilage destruction (Blom et al., 2007; Glasson et al., 2005; Little et al., 2009). These results clearly support the notion that increases in cellular Zn^{2+} play a catabolic role in OA pathogenesis by upregulating matrix-degrading enzymes in articular chondrocytes. Zn^{2+} homeostasis is regulated by the complex interplay of uptake and efflux transporter proteins coupled with metal-dependent transcriptional control of transporters and storage proteins (Cousins et al., 2006; Laity and Andrews, 2007; Günther et al., 2012). Membrane Zn^{2+} transporters are primarily responsible for the regulation of Zn^{2+} homeostasis. We found that ZIP8, among 24 examined importers and exporters, is specifically upregulated in chondrocytes under pathological condition and is responsible for Zn^{2+} influx. We additionally demonstrated that, among the ZIP8-transported metal ions (Zn^{2+}, Fe^{2+}, Mn^{2+}, and Cd^{2+}), Zn^{2+} influx is the essential event that initiates the activation of catabolic cascades in OA chondrocytes. Consistent with this, blocking ZIP8-mediated Zn^{2+} influx effectively suppressed catabolic factor expression and experimental OA pathogenesis.

Zn^{2+} influx causes activation of Zn^{2+}-sensing, metal-dependent transcription factors (Laity and Andrews, 2007; Günther et al., 2012). In articular chondrocytes, ZIP8-mediated Zn^{2+} influx activated numerous transcription factors, including MTF1, NRF1/NRF2, NF-κB, SP1, p53, C/EBP, and AP1. Among these activated transcription factors, MTF1 and SP1 were associated with the ZIP8-mediated expression of matrix-degrading enzymes. MTF1 is involved in cellular adaptation to various stress conditions, primarily exposure to heavy metals such as Zn^{2+} but also hypoxia and oxidative stress. This function of MTF1 is associated with its ability to regulate expression of a variety of target genes (Laity and Andrews, 2007; Günther et al., 2012). We found that MTF1 activation in chondrocytes is sufficient to induce expression of the matrix-degrading enzymes, MMP3, MMP9, MMP12, MMP13, and ADAMTS5. These enzymes were also upregulated by ZIP8-mediated Zn^{2+} influx, suggesting that ZIP8 acts through MTF1 to induce expression of matrix-degrading enzymes. Indeed, the OA pathogenesis caused by Ad-*Zip8* injection was significantly inhibited in *Mtf1*-deficient mice, whereas Ad-*Mtf1*-caused OA pathogenesis was not affected in *Zip8*-deficient mice. This clearly suggests that MTF1 is a downstream mediator of ZIP8 in OA pathogenesis. However, siRNA-mediated knockdown of *Mtf1* or genetic deletion of *Mtf1* in mice did not completely block ZIP8-mediated catabolic factor expression and OA pathogenesis, respectively. This suggests that other factors in addition to MTF1 are likely involved in ZIP8-mediated expression of matrix-degrading enzymes and, hence, OA pathogenesis. It is well appreciated that regulatory pathways of gene expression are very complex, with multiple factors in each regulatory step. Indeed, our current results revealed that inhibition of SP1, which is activated by ZIP8-mediated Zn^{2+} influx, blocked the upregulation of matrix-degrading

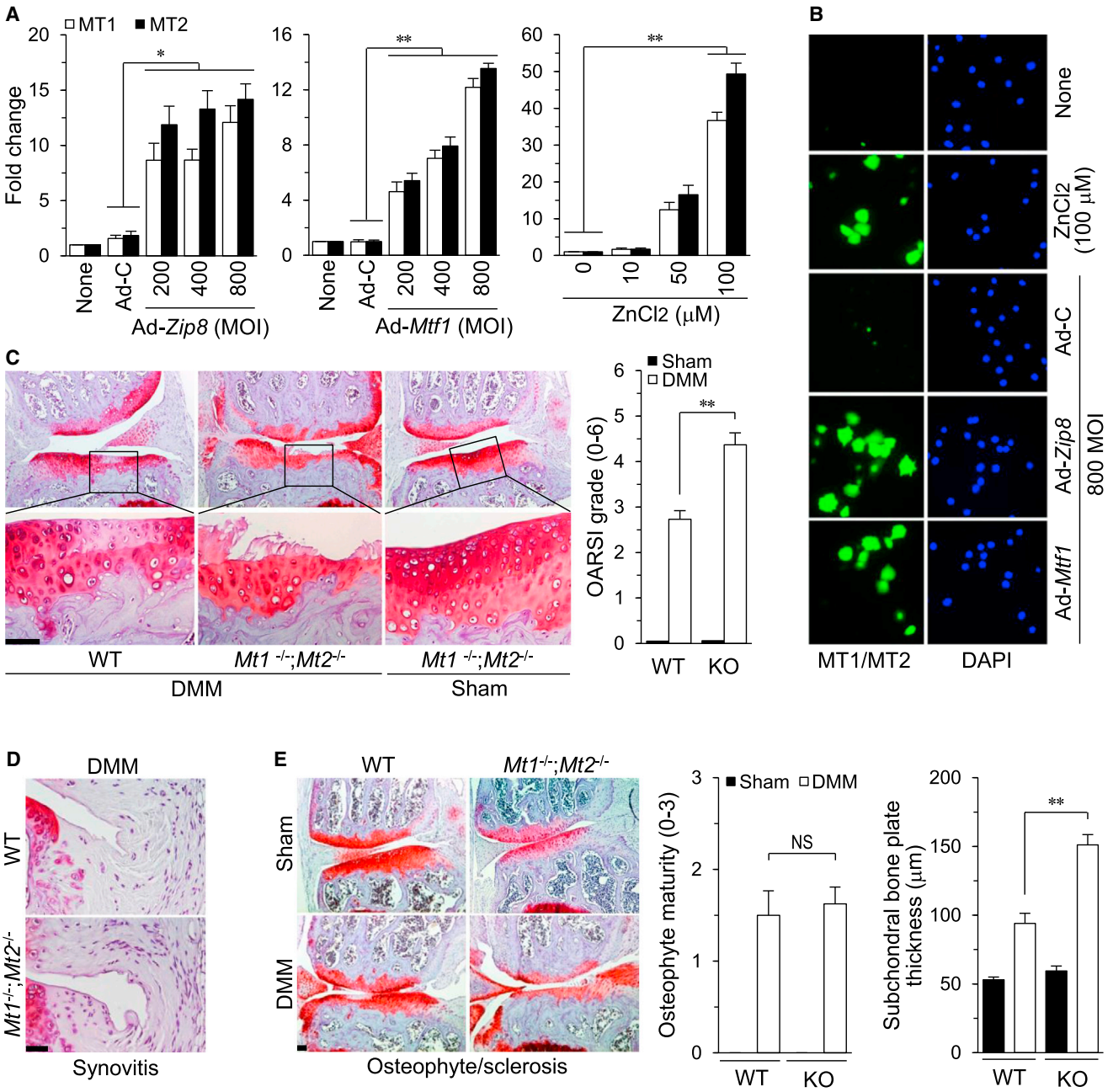

Figure 7. Double Knockout of *Mt1* and *Mt2* Enhances OA Pathogenesis in Mice

(A) mRNA levels of MT1 and MT2 in chondrocytes infected with Ad-*Zip8* or Ad-*Mtf1* or treated with $ZnCl_2$ ($n \geq 6$).

(B) Detection of MT1/MT2 proteins in chondrocytes treated with $ZnCl_2$ or infected with Ad-*Zip8*, Ad-*Mtf1*.

(C–E) Cartilage destruction (C), synovitis (D), osteophyte formation, and subchondral bone sclerosis (E) in sham- and DMM-operated WT and $Mt1^{-/-};Mt2^{-/-}$ double-KO mice ($n = 13$).

Scale bar, 50 μm. Values are presented as means ± SEM (*$p < 0.005$, **$p < 0.001$). See also Table S1.

enzymes induced by Ad-*Zip8* infection, suggesting that SP1 is also associated with ZIP8-induced OA pathogenesis. Nevertheless, IA injection of Ad-*Mtf1* caused OA pathogenesis, whereas *Mtf1*-KO mice exhibited suppressed DMM-induced OA pathogenesis. This clearly supports the notion that MTF1 functions as an essential downstream mediator of ZIP8-induced OA pathogenesis by virtue of its role in upregulating matrix-degrading enzymes.

OA is primarily characterized by cartilage destruction. However, OA is considered to be a whole-joint disease, with pathological changes, including cartilage destruction, synovial inflammation, osteophyte development, and subchondral bone

sclerosis occurring in all tissues of the joint (Bian et al., 2012; Loeser et al., 2012; Little and Hunter, 2013). Therefore, biochemical and biomechanical crosstalk among different joint tissue compartments likely plays an important role in the onset and progression of OA. In this context, synovial inflammation in the joint tissue might contribute to OA pathogenesis by promoting the production of various OA-regulating factors (i.e., cytokines and chemokines) by synovial cells (Scanzello and Goldring, 2012). Indeed, we found that IA injection of Ad-*Zip8* or Ad-*Mtf1* promoted the expression of various matrix-degrading enzymes, cytokines, and chemokines in fibroblast-like synoviocytes and caused synovial inflammation, effects that were blocked by coinjection of the metal chelator TPEN. Although a contribution of this synovitis to cartilage destruction cannot be ruled out, the spontaneous cartilage destruction observed upon aging and the enhanced cartilage destruction induced by DMM surgery in *Col2a1-Zip8* TG mice, without apparent synovial inflammation, suggests that activation of the zinc cascade in chondrocytes is sufficient to cause OA pathogenesis. In addition to cartilage destruction and synovitis, IA injection of Ad-*Zip8* or Ad-*Mtf1* caused subchondral bone sclerosis and osteophyte formation. Consistent with this, genetic deletion of *Zip8* or *Mtf1* in mice effectively inhibited cartilage destruction and subchondral bone sclerosis, whereas overexpression of *Zip8* in *Col2a1-Zip8* TG mice enhanced these manifestations. Therefore, changes in cartilage destruction and subchondral bone sclerosis caused by genetic modulation of *Zip8* and *Mtf1* are highly correlated. However, whereas these correlations have been noted in various OA models, it is still unclear how these two compartments interact each other and which of these two events is more critically associated with the initiation of OA. Given the nature of *Col2a1* promoter-driven expression in the TG and CKO mice used in this study, it is likely that subchondral bone sclerosis is a consequence of the altered functions of chondrocytes in articular cartilage. For example, accelerated cartilage damage in *Zip8* TG mice would augment the mechanical stress imposed on the underlying bone, whereas reduced cartilage degeneration in *Zip8*- or *Mtf1*-CKO mice would alleviate the loading caused by joint instability in the subchondral bone, thereby ameliorating subchondral bone sclerosis. In contrast, genetic modulation of *Zip8* and *Mtf1* in mice did not affect osteophyte formation, indicating that catabolic pathways regulated by ZIP8 and MTF1 are not associated with this process. This notion is further supported by reports that deletion of either *Mmp13* or *Adamts5*, which are the key effectors of OA catabolism, does not prevent osteophyte development in a surgically induced OA model (Botter et al., 2009; Little et al., 2009).

Metal-sensing MTF1 also regulates Zn^{2+} homeostasis by modulating selective Zn^{2+} transporters and MTs (Laity and Andrews, 2007; Günther et al., 2012). MTs, which are small cysteine-rich proteins, bind to Zn^{2+} and thereby regulate cellular Zn^{2+} concentration and intracellular distribution (Blindauer and Leszczyszyn, 2010). In addition to this Zn^{2+}-storage function, MTs also act as antioxidants. This antioxidant potential, as well as the ability of MTs to suppress inflammatory cytokines, including IL1β, protects against allergic inflammation (Penkowa et al., 2001). Because MT1 and MT2 show high structural and functional similarities, we used $Mt1^{-/-}$;$Mt2^{-/-}$ double-KO mice to elucidate MT functions in OA pathogenesis. Our finding that *Mt1*/*Mt2*-deficient mice exhibited enhanced OA cartilage destruction is consistent with the known functions of MTs as Zn^{2+}-storage and antioxidant proteins. Therefore, increased cellular levels of MT1 and MT2 induced by ZIP8-mediated Zn^{2+} influx and MTF1 activation appear to function as a negative feedback loop to alleviate cartilage destruction.

Collectively, our results demonstrate that the zinc-ZIP8-MTF1 axis in chondrocytes forms a catabolic cascade that promotes upregulation of the crucial effector matrix-degrading enzymes, MMP3, MMP13, and ADAMTS5, thereby leading to OA cartilage destruction. Our findings support the notion that local depletion of Zn^{2+}, inhibition of ZIP8 function and Zn^{2+} influx, and/or inhibition of MTF1 activity in cartilage tissue would be effective therapeutic approaches for the treatment of OA.

EXPERIMENTAL PROCEDURES

Human OA Cartilage Tissue

International Cartilage Repair Society grade 4 human OA cartilage was sourced from individuals (age 51–72 years) undergoing arthroplasty. The Institutional Review Board of the Wonkwang University Hospital approved the use of these materials, and all individuals provided full written informed consent before the operative procedure.

Mice and Experimental OA

Male mice (C57BL/6, *Col2a1-Zip8* TG, $Zip8^{+/-}$, $Zip8^{fl/fl}$, $Zip8^{fl/fl}$;*Col2a1-Cre*, $Mtf1^{+/-}$, $Mtf1^{fl/fl}$, $Mtf1^{fl/fl}$;*Col2a1-Cre*, $Mt1^{-/-}$;$Mt2^{-/-}$) were used for experimental OA studies. Chondrocyte-specific *Zip8* TG (*Col2a1-Zip8*) mice were generated using the *Col2a1* promoter and enhancer. $Zip8^{+/-}$ mice were obtained from The European Mouse Mutant Archive. The *Mtf1* mouse strain used for this research project was created from an embryonic stem cell clone generated by the Wellcome Trust Sanger Institute and developed into mice by the Knockout Mouse Project Repository and the Mouse Biology Program at the University of California, Davis. $Zip8^{+/-}$ and $Mtf1^{+/-}$ mice were backcrossed with *Actb-Flp1* TG mice (The Jackson Laboratory) to generate $Zip8^{fl/fl}$ and $Mtf1^{fl/fl}$ mice, respectively. These mice were then backcrossed with *Col2a1-Cre* TG mice (The Jackson Laboratory) to generate chondrocyte-specific CKO mice ($Zip8^{fl/fl}$;*Col2a1-Cre* and $Mtf1^{fl/fl}$;*Col2a1-Cre*). $Mt1^{-/-}$;$Mt2^{-/-}$ double-KO mice were obtained from The Jackson Laboratory. The inbred strain 129S1/SvImJ was used as a control for $Mt1^{-/-}$;$Mt2^{-/-}$ double-KO mice. All mice used in this study showed normal skeletal development (data not shown). Animals were maintained under pathogen-free conditions. All experiments were approved by the Gwangju Institute of Science and Technology Animal Care and Use Committee. Experimental OA was induced by DMM surgery using 10- to 12-week-old male mice; sham-operated mice were used as controls (Glasson et al., 2007). Knee joints were processed for histological analysis 8 weeks after surgery. However, in studies involving *Col2a1-Zip8* TG mice and $Mt1^{-/-}$;$Mt2^{-/-}$ double-KO mice, the duration after surgery was adjusted to 6 weeks. Experimental OA was also induced by IA injection (once weekly for 3 weeks) of Ad-*Zip8* or Ad-*Mtf1* (1×10^9 plaque-forming units in a total volume of 10 μl) into 10- to 12-week-old male mice; IA injection of empty adenovirus (Ad-C) was used as a control. Mice were sacrificed 3 or 8 weeks after the first IA injection for histological and biochemical analyses. Where indicated, the mice were coinjected (IA) with 0.1 mg/kg body weight of TPEN.

Histology and Immunohistochemistry

Human OA cartilage was frozen, sectioned at 10 μm thickness, and fixed in paraformaldehyde. Sulfate proteoglycan was detected with Alcian blue staining. Human ZIP8 was detected by immunostaining. Cartilage destruction in mice was examined using safranin-O staining. Briefly, knee joints were fixed in 4% paraformaldehyde, decalcified in 0.5 M EDTA, and embedded in paraffin. The paraffin blocks were sectioned at a thickness of 5 μm. Serial sections were obtained from the entire joint at 40 μm intervals. Sections were

deparaffinized in xylene, hydrated with graded ethanol, and stained with safranin-O. Cartilage destruction was scored by two blinded observers using the OARSI grading system (Glasson et al., 2010). Synovitis was determined by safranin-O and hematoxylin staining, and synovial inflammation (grade 0–3) was scored as described previously (Yang et al., 2010). Osteophyte development was identified by safranin-O staining, and osteophyte maturity was quantified as described previously (Oh et al., 2012). Subchondral bone sclerosis was determined by measuring the thickness of the subchondral bone plate (Zhen et al., 2013). Antibodies used for immunostaining of cartilage sections are described in detail in Extended Experimental Procedures.

Primary Culture of Articular Chondrocytes

Chondrocytes were isolated from femoral condyles and tibial plateaus of mice, as described previously (Yang et al., 2010; Ryu et al., 2012; Oh et al., 2012). Chondrocytes were maintained as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics, and cells at culture day 2 were treated as indicated in each experiment.

Adenoviruses and Infection of Chondrocytes

Adenoviruses expressing mouse *Zip8* (Ad-*Zip8*) and Ad-*Mtf1* were purchased from Vector Biolabs. Mouse articular chondrocytes were cultured for 2 days, infected with Ad-C, Ad-*Zip8*, or Ad-*Mtf1* for 2 hr at the indicated multiplicity of infection (MOI), and cultured for 24 hr, followed by further analyses.

Transport of Metal Ions

Intracellular Zn^{2+}, Fe^{2+}/Fe^{3+}, Mn^{2+}, and Cd^{2+} were measured by using FluoZin-3 AM, ferrozine assay, cellular Fura-2 manganese extraction assay, and Leadmium Green AM, respectively. Detailed procedures are described in Extended Experimental Procedures.

MTF1 Activity Reporter Gene Assay and Immunostaining

The MTF1 reporter gene assay kit was obtained from SABiosciences. Chondrocytes were transfected for 6 hr with a metal response element reporter construct and a constitutive Renilla luciferase construct using Lipofectamine 2000 (Invitrogen). Transfected cells were infected with Ad-*Mtf1* or Ad-*Zip8* for 2 hr or treated with metal ions. Cells were harvested 24 hr after treatment, and firefly luciferase and Renilla luciferase activities were measured using a Dual Luciferase Assay System (Promega). For immunostaining, cells were fixed in ice-cold 4% paraformaldehyde (pH 7.4), permeabilized with 0.2% Triton X-100 in PBS, and serially blocked with Image-iT FX Signal Enhancer (Invitrogen) and PBS containing 10% goat serum/0.1% bovine serum albumin. MTF1 was detected by immunofluorescence microscopy. Images were acquired using a fluorescence microscope, and at least 200 cells were examined in multiple fields per condition to quantify the percentage of nuclear MTF1-positive cells.

RT-PCR, qRT-PCR, and siRNA

Total RNA was extracted from primary cultured chondrocytes using the TRI reagent (Molecular Research Center). For the isolation of RNA from knee joints, cartilage tissues were scraped with a blade to remove cartilage and RNA was isolated using the TRI reagent and Purelink RNA mini kit (Ambion). The RNA was reverse transcribed, and the resulting cDNA was amplified by PCR. PCR primers and experimental conditions are summarized in Table S1. Transcript levels were quantified by quantitative RT-PCR (qRT-PCR). siRNAs targeting *Zip8* and *Mtf1* were obtained from Dharmacon and transfected using Lipofectamine 2000. Nontargeting (scrambled) siRNA was used as a negative control. Chondrocytes were transfected by incubating for 6 hr with siRNA and Lipofectamine 2000, as described by the manufacturer, and infected with adenoviruses as described above.

Western Blotting

Total cell lysates were prepared in lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, 5 mM NaF) and used to detect cellular proteins (ZIP8, MTF1, and ERK). Secreted proteins (MMP3, MMP13, and ADAMTS5) were detected after trichloroacetic acid (TCA) precipitation from 900 μl of serum-free conditioned medium. All lysis buffers contained a cocktail of protease inhibitors and phosphatase inhibitors (Roche). Source of antibodies are described in detail in Extended Experimental Procedures.

Statistical Analysis

Data quantified based on an ordinal grading system, such as OARSI grade, were analyzed using nonparametric statistical methods. For qRT-PCR data expressed as relative fold changes, Student's t test and ANOVA with post hoc tests were used for pairwise comparisons and multicomparisons, respectively, after confirming a normal distribution using the Shapiro-Wilk test. Significance was accepted at the 0.05 level of probability ($p < 0.05$).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.01.007.

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REFERENCES

Andreini, C., Banci, L., Bertini, I., and Rosato, A. (2006). Counting the zinc-proteins encoded in the human genome. J. Proteome Res. *5*, 196–201.

Bian, Q., Wang, Y.J., Liu, S.F., and Li, Y.P. (2012). Osteoarthritis: genetic factors, animal models, mechanisms, and therapies. Front. Biosci. (Elite Ed.) *4*, 74–100.

Blindauer, C.A., and Leszczyszyn, O.I. (2010). Metallothioneins: unparalleled diversity in structures and functions for metal ion homeostasis and more. Nat. Prod. Rep. *27*, 720–741.

Blom, A.B., van Lent, P.L., Libregts, S., Holthuysen, A.E., van der Kraan, P.M., van Rooijen, N., and van den Berg, W.B. (2007). Crucial role of macrophages in matrix metalloproteinase-mediated cartilage destruction during experimental osteoarthritis: involvement of matrix metalloproteinase 3. Arthritis Rheum. *56*, 147–157.

Botter, S.M., Glasson, S.S., Hopkins, B., Clockaerts, S., Weinans, H., van Leeuwen, J.P., and van Osch, G.J. (2009). ADAMTS5$^{-/-}$ mice have less subchondral bone changes after induction of osteoarthritis through surgical instability: implications for a link between cartilage and subchondral bone changes. Osteoarthritis Cartilage *17*, 636–645.

Colvin, R.A., Holmes, W.R., Fontaine, C.P., and Maret, W. (2010). Cytosolic zinc buffering and muffling: their role in intracellular zinc homeostasis. Metallomics *2*, 306–317.

Cousins, R.J., Liuzzi, J.P., and Lichten, L.A. (2006). Mammalian zinc transport, trafficking, and signals. J. Biol. Chem. *281*, 24085–24089.

Dalton, T.P., He, L., Wang, B., Miller, M.L., Jin, L., Stringer, K.F., Chang, X., Baxter, C.S., and Nebert, D.W. (2005). Identification of mouse SLC39A8 as the transporter responsible for cadmium-induced toxicity in the testis. Proc. Natl. Acad. Sci. USA *102*, 3401–3406.

Fukada, T., Yamasaki, S., Nishida, K., Murakami, M., and Hirano, T. (2011). Zinc homeostasis and signaling in health and diseases: Zinc signaling. J. Biol. Inorg. Chem. *16*, 1123–1134.

Gálvez-Peralta, M., He, L., Jorge-Nebert, L.F., Wang, B., Miller, M.L., Eppert, B.L., Afton, S., and Nebert, D.W. (2012). ZIP8 zinc transporter: indispensable role for both multiple-organ organogenesis and hematopoiesis in utero. PLoS ONE *7*, e36055.

Glasson, S.S., Askew, R., Sheppard, B., Carito, B., Blanchet, T., Ma, H.L., Flannery, C.R., Peluso, D., Kanki, K., Yang, Z., et al. (2005). Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. Nature *434*, 644–648.

Glasson, S.S., Blanchet, T.J., and Morris, E.A. (2007). The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse. Osteoarthritis Cartilage *15*, 1061–1069.

Glasson, S.S., Chambers, M.G., Van Den Berg, W.B., and Little, C.B. (2010). The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the mouse. Osteoarthritis Cartilage *18* (Suppl 3), S17–S23.

Günther, V., Lindert, U., and Schaffner, W. (2012). The taste of heavy metals: gene regulation by MTF-1. Biochim. Biophys. Acta *1823*, 1416–1425.

Kapoor, M., Martel-Pelletier, J., Lajeunesse, D., Pelletier, J.P., and Fahmi, H. (2011). Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. Nat. Rev. Rheumatol. *7*, 33–42.

Kitamura, H., Morikawa, H., Kamon, H., Iguchi, M., Hojyo, S., Fukada, T., Yamashita, S., Kaisho, T., Akira, S., Murakami, M., and Hirano, T. (2006). Toll-like receptor-mediated regulation of zinc homeostasis influences dendritic cell function. Nat. Immunol. *7*, 971–977.

Küry, S., Dréno, B., Bézieau, S., Giraudet, S., Kharfi, M., Kamoun, R., and Moisan, J.P. (2002). Identification of SLC39A4, a gene involved in acrodermatitis enteropathica. Nat. Genet. *31*, 239–240.

Laity, J.H., and Andrews, G.K. (2007). Understanding the mechanisms of zinc-sensing by metal-response element binding transcription factor-1 (MTF-1). Arch. Biochem. Biophys. *463*, 201–210.

Little, C.B., and Hunter, D.J. (2013). Post-traumatic osteoarthritis: from mouse models to clinical trials. Nat. Rev. Rheumatol. *9*, 485–497.

Little, C.B., Barai, A., Burkhardt, D., Smith, S.M., Fosang, A.J., Werb, Z., Shah, M., and Thompson, E.W. (2009). Matrix metalloproteinase 13-deficient mice are resistant to osteoarthritic cartilage erosion but not chondrocyte hypertrophy or osteophyte development. Arthritis Rheum. *60*, 3723–3733.

Liuzzi, J.P., Lichten, L.A., Rivera, S., Blanchard, R.K., Aydemir, T.B., Knutson, M.D., Ganz, T., and Cousins, R.J. (2005). Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response. Proc. Natl. Acad. Sci. USA *102*, 6843–6848.

Loeser, R.F., Goldring, S.R., Scanzello, C.R., and Goldring, M.B. (2012). Osteoarthritis: a disease of the joint as an organ. Arthritis Rheum. *64*, 1697–1707.

Oh, H., Chun, C.H., and Chun, J.S. (2012). Dkk-1 expression in chondrocytes inhibits experimental osteoarthritic cartilage destruction in mice. Arthritis Rheum. *64*, 2568–2578.

Ovesen, J., Møller-Madsen, B., Nielsen, P.T., Christensen, P.H., Simonsen, O., Hoeck, H.C., Laursen, M.B., and Thomsen, J.S. (2009). Differences in zinc status between patients with osteoarthritis and osteoporosis. J. Trace Elem. Med. Biol. *23*, 1–8.

Page-McCaw, A., Ewald, A.J., and Werb, Z. (2007). Matrix metalloproteinases and the regulation of tissue remodelling. Nat. Rev. Mol. Cell Biol. *8*, 221–233.

Penkowa, M., Espejo, C., Martínez-Cáceres, E.M., Poulsen, C.B., Montalban, X., and Hidalgo, J. (2001). Altered inflammatory response and increased neurodegeneration in metallothionein I+II deficient mice during experimental autoimmune encephalomyelitis. J. Neuroimmunol. *119*, 248–260.

Prasad, A.S. (1995). Zinc: an overview. Nutrition *11* (1, Suppl), 93–99.

Roschger, A., Hofstaetter, J.G., Pemmer, B., Zoeger, N., Wobrauschek, P., Falkenberg, G., Simon, R., Berzlanovich, A., Thaler, H.W., Roschger, P., et al. (2013). Differential accumulation of lead and zinc in double-tidemarks of articular cartilage. Osteoarthritis Cartilage *21*, 1707–1715.

Ryu, J.H., Yang, S., Shin, Y., Rhee, J., Chun, C.H., and Chun, J.S. (2011). Interleukin-6 plays an essential role in hypoxia-inducible factor 2α-induced experimental osteoarthritic cartilage destruction in mice. Arthritis Rheum. *63*, 2732–2743.

Ryu, J.H., Shin, Y., Huh, Y.H., Yang, S., Chun, C.H., and Chun, J.S. (2012). Hypoxia-inducible factor-2α regulates Fas-mediated chondrocyte apoptosis during osteoarthritic cartilage destruction. Cell Death Differ. *19*, 440–450.

Scanzello, C.R., and Goldring, S.R. (2012). The role of synovitis in osteoarthritis pathogenesis. Bone *51*, 249–257.

Wang, Y., Wimmer, U., Lichtlen, P., Inderbitzin, D., Stieger, B., Meier, P.J., Hunziker, L., Stallmach, T., Forrer, R., Rülicke, T., et al. (2004). Metal-responsive transcription factor-1 (MTF-1) is essential for embryonic liver development and heavy metal detoxification in the adult liver. FASEB J. *18*, 1071–1079.

Wang, C.Y., Jenkitkasemwong, S., Duarte, S., Sparkman, B.K., Shawki, A., Mackenzie, B., and Knutson, M.D. (2012). ZIP8 is an iron and zinc transporter whose cell-surface expression is up-regulated by cellular iron loading. J. Biol. Chem. *287*, 34032–34043.

Yang, S., Kim, J., Ryu, J.H., Oh, H., Chun, C.H., Kim, B.J., Min, B.H., and Chun, J.S. (2010). Hypoxia-inducible factor-2α is a catabolic regulator of osteoarthritic cartilage destruction. Nat. Med. *16*, 687–693.

Zhen, G., Wen, C., Jia, X., Li, Y., Crane, J.L., Mears, S.C., Askin, F.B., Frassica, F.J., Chang, W., Yao, J., et al. (2013). Inhibition of TGF-β signaling in mesenchymal stem cells of subchondral bone attenuates osteoarthritis. Nat. Med. *19*, 704–712.