CaMKK2 Facilitates Sox9 Turnover in Inflamed Chondrocytes

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INTRODUCTION: Osteoarthritis (OA) is a severely debilitating degenerative joint disease with no cures that is rising in incidence globally. Previously, we reported Ca^{2+}/calmodulin-dependent protein kinase kinase 2 (CaMKK2) to be elevated in murine and human OA cartilage and to play a role in OA pathogenesis by coordinating chondrocyte inflammatory responses and apoptosis[1,2]. CaMKK2-deficient chondrocytes are protected against interleukin (IL)-1β induced activation of the IL-6-Stat3-MMP13 pathway and CaMKK2 inhibition or its genetic ablation protected mice against destabilization of medial meniscus (DMM)-induced OA[1,2]. However, the exact role played by CaMKK2 in OA pathogenesis and inflamed chondrocytes remains unknown. The transcription factor Sox9 is the master regulator of chondrocyte differentiation, and it plays an essential role in cartilage formation by directly regulating the expression of chondrocyte-specific genes such as type II collagen (*Col2a1*)[3]. Sox9 is regulated transcriptionally and through post-translational modification including ubiquitination and proteasomal degradation[4,5]. Sox9 levels are downregulated in inflamed chondrocytes and its overexpression protects against OA pathogenesis. We found Sox9 levels to be elevated in DMM-operated mice in which CaMKK2 activity was pharmacologically inhibited. Based on this finding, we **hypothesized** that the kinase plays a role in Sox9 turnover in inflamed chondrocytes.

METHODS: Animal studies were performed with prior approval from Indiana University School of Medicine (IUSM) Institutional Animal Care and Use Committee (IACUC). Unilateral sham or DMM surgeries were performed on 10-week-old (w/o) C57BL6 male wild type (WT) or *Camkk2*$^{-/-}$ mice; WT-DMM mice were administered tri-weekly *i.p.* injections of saline or CaMKK2 inhibitor STO-609 (0.033 mg/kg body weight) for 12 weeks(w) post-surgery (n=6/group). At termination, operated and contralateral knee joints were harvested, fixed in 4% paraformaldehyde, decalcified, paraffin-embedded, sectioned, and evaluated for Sox9 levels via immunohistochemistry (IHC). Murine articular chondrocytes isolated from newborn WT and *Camkk2*$^{-/-}$ mice were stimulated with or without 10 ng/ml of IL-1β and Sox9 levels assessed by immunoblotting (IB) or immunofluorescence (IF). WT chondrocytes were infected with Lentiviruses expressing intact CaMKK2 or its kinase-dead mutant to assess Sox9 levels. CaMKK2 and Sox9 were immunoprecipitated using respective antibodies and bound proteins were identified by IB and/or mass spectrometry. Statistical comparisons were performed using 2-tailed Student's *t* test or One-Way ANOVA, following normality tests. *P*-values <0.05 were deemed significant.

RESULTS: Sox-9 levels were lower in the articular cartilage of saline-treated WT-DMM mice but not in those treated with STO-609 **(Fig. 1A)**. Sham and DMM-operated *Camkk2*$^{-/-}$ mice possessed higher Sox9 levels than the respective controls **(not shown)**. Naïve *Camkk2*$^{-/-}$ primary chondrocytes possessed 1.7-fold ($p<0.05$; n=3) higher Sox9 compared to naïve WT chondrocytes **(Fig. 1B**, not shown**)**. Sox9 levels diminished 2.5-fold and 2-fold respectively in WT and *Camkk2*$^{-/-}$ chondrocytes by 24 h of IL-1β treatment, but its overall levels remained 2-fold higher in CaMKK2 null chondrocytes than WT **(Fig. 1B**, not shown**)**. IF data also revealed higher Sox9 levels in naïve and IL-1β-treated *Camkk2*$^{-/-}$ primary chondrocytes **(Fig. 1C)**. Overexpression of enzymatically intact (WT) CaMKK2, but not its kinase-defective mutant, decreased Sox9 levels by 1.7-fold in WT primary chondrocytes **(Fig. 1D)**. Phosphorylation of AMPK, a CaMKK2 substrate[1], was observed only in the presence of WT CaMKK2 **(Fig. 1D)**. Further, CaMKK2 immunoprecipitates (IPs) from naïve and IL-1β-treated WT chondrocytes contained ubiquitin and a slowly migrating (85 KD) form of Sox9, whereas Sox9-IPs from the same cells contained not only CaMKK2 and ubiquitin but also two forms of Sox9, the usual 65 KD and the 85 KD species also found in CaMKK2-IPs **(Fig. 1E-F)**. Mass spectrometry analysis of CaMKK2 immunocomplexes revealed the presence of ubiquitin ligases Ubr4 and Trim21 **(**validated in **Fig 1e-F)**, as well as 26S proteasome regulatory subunits 2, 4, 6A, 7, 8 and 10B **(not shown)**. Sox9 immunocomplexes from *Camkk2*$^{-/-}$ chondrocytes treated for 1 h with IL-1β contained lower levels of ubiquitin and 85 KD Sox9, but higher overall Sox9 **(Fig. 1G-H)**.

DISCUSSION: Our findings indicate that CaMKK2 interacts with Sox9 as well as components of the ubiquitin-proteasome complex in naïve and inflamed chondrocytes, such that its absence protects Sox9 turnover.

SIGNIFICANCE/CLINICAL RELEVANCE: No FDA-approved curative therapies are currently available to treat OA. Our studies highlight the potential for CaMKK2 as a novel therapeutic target against OA to preserve Sox9 levels in chondrocytes and prevent cartilage degradation.

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IMAGES AND TABLES:

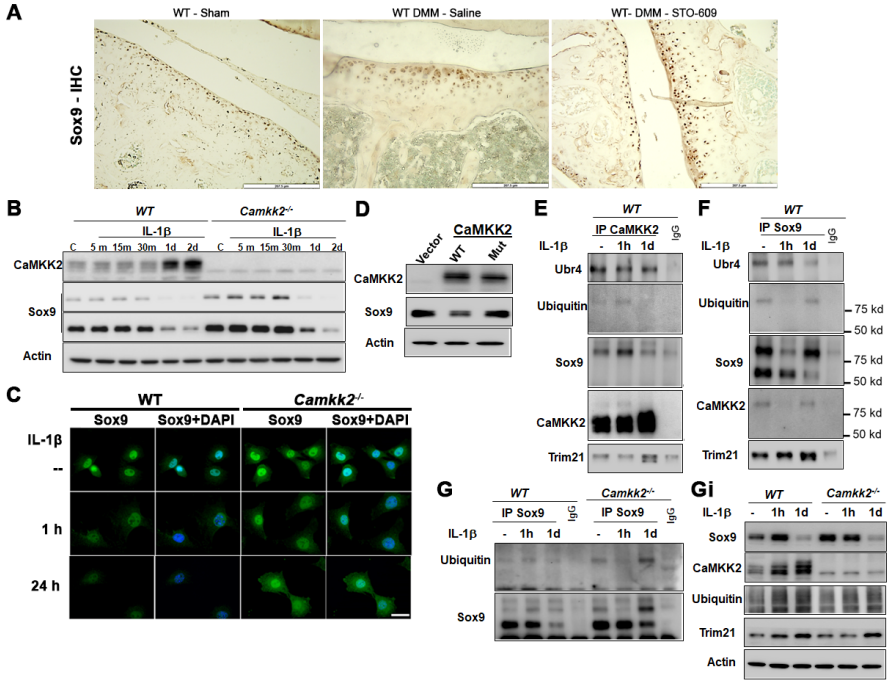

Figure 1. CaMKK2 modulates Sox9 levels in naïve and IL-1β-treated chondrocytes through its interaction with the ubiquitin-proteosome machinery. (A) Representative images showing Sox9 immunoreactivity in articular cartilage of indicated cohorts, 20X, scale bar = 207.5 µm. **(B)** Immunoblots (IBs) of naïve or IL-1β-treated (for indicated timepoints) WT and *Camkk2*$^{-/-}$ primary chondrocytes probed for CaMKK2, Sox9 and Actin levels. **(C)** IF images showing Sox9 (green) and DAPI/Sox9 overlay in WT and *Camkk2*$^{-/-}$ chondrocytes +/- IL-1β-treatment for 1 h or 24h, 40X. **(D)** IBs of WT chondrocytes containing Lentivirus-Vector or Lentivirus-WT CaMKK2 or D311A mutant, probed as shown. **(E-F)** IBs of CaMKK2-IP **(E)** or Sox9-IP **(F)** from WT primary chondrocytes treated with or without IL-1β for 1 h or 1 day (d), probed for the presence of Ubr4, Ubiquitin, Sox9, CaMKK2 and Trim21. **(G)** Immunoblots of Sox9-IP from WT and *Camkk2*$^{-/-}$ primary chondrocytes treated with or without IL-1β for 1 h or 1 d, probed for Ubiquitin and Sox9. **(H)** IB of cell lysates (input) used in Sox9-IP shown in **G** probed as indicated for Sox9, CaMKK2, Ubiquitin, Trim21 and Actin.