# Nicotinamide Adenine Dinucleotide (NAD) Salvage is Essential for Physeal but not Articular Chondrocyte Survival and Function in Young Adult Mice

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**DISCLOSURES:** None

**INTRODUCTION:** Nicotinamide adenine dinucleotide (NAD) is a critical electron donor for energy production and a substrate for other vital cellular processes. Cellular NAD levels are sustained by *de novo* synthesis from circulating vitamin precursors as well as the recycling of consumed NAD through a salvage pathway. The avascularity of cartilage may increase demand for NAD salvage within this tissue. In previous work, we demonstrated that the rate-limiting enzyme in the salvage pathway, nicotinamide phosphoribosyltransferase (Nampt), is indispensable for perinatal chondrocyte survival and function and, thereby, necessary for murine limb development; in contrast, osteoblast lineage cells do not require Nampt during long bone development [1]. The goal of the current study was to determine whether NAD salvage is similarly important for the postnatal growth plate (physeal) as well as for articular chondrocytes challenged by joint injury.

**METHODS:** Study design: Animal studies were conducted as pre-approved by the local Institutional Animal Care and Use Committee. To define the role of NAD salvage in postnatal chondrocyte function, we deleted Nampt in 3-month-old mice either specifically within articular chondrocytes using Prg4-CreERT2 (Nampt$^{\Delta Prg4ER}$) or within both articular and physeal chondrocytes using Agc1-CreERT2 (Nampt$^{\Delta Agc1ER}$). One week after completion of the tamoxifen regimen (100 mg/kg delivered once daily for 5 consecutive days by intraperitoneal injection), some male and female Nampt cKO or Cre control mice underwent destabilization of the medial meniscus (DMM) or sham surgery in one knee to model post-traumatic osteoarthritis [2]. Groups included a minimum of 8 mice per genotype-sex-surgery combination. Knees were harvested at 4 weeks post-injury to capture early joint degeneration. To assess the impact of Nampt deletion on long term chondrocyte homeostasis, additional Nampt$^{\Delta Agc1ER}$ and Nampt$^{\Delta Prg4ER}$ mice were aged to 12 months (9 months after tamoxifen delivery). Outcome measures: For the PTOA study, knees were imaged by µCT, then embedded in paraffin under RNase-free conditions. Coronal sections taken in the middle third of the knees were stained with Safranin O/Fast Green, and knees were graded for cartilage loss and osteophyte formation [3,4] by two blinded reviewers. Serial sections underwent TUNEL staining, immunostaining for Nampt or activated caspase-3, or *in situ* hybridization for *Col2a1* and *Col10a1*. For the aging study, one knee, one contralateral femur, and a segment of the lumbar vertebrae were collected for µCT and histology. Statistical analysis: For histological scoring, differences between genotypes and interactions between genotype and sex/injury were assessed by 2-way ANOVA on Ranks with Mann Whitney post-hoc analysis. Other measures were analyzed by 2-way ANOVA with Tukey's post-hoc analysis.

**RESULTS:** Nampt immunostaining confirmed efficient deletion in physeal and/or articular chondrocytes for the Nampt$^{\Delta Agc1ER}$ and Nampt$^{\Delta Prg4ER}$ cohorts. At 4 weeks post-DMM, neither cKO strain displayed differences in early articular cartilage loss or osteophyte formation compared to their respective Cre controls (**Fig. 1**). Strikingly, Nampt$^{\Delta Agc1ER}$ but not Nampt$^{\Delta Prg4ER}$ mice exhibited pronounced growth plate dysplasia at the distal femur and proximal tibia by 5 weeks following Nampt deletion, as characterized by extensive proteoglycan depletion, reduced chondrocyte number, and focal ossification traversing the plate. *In situ* hybridization and immunostaining revealed severe suppression of anabolic transcripts (*Col2a1*, *Col10a1*) and increased chondrocyte apoptosis, respectively, in Nampt$^{\Delta Agc1ER}$ compared to Nampt$^{\Delta Prg4ER}$ growth plates (**Fig. 2**). Additional unoperated mice were aged to 12 months. The articular cartilage and growth plates of Nampt$^{\Delta Prg4ER}$ mice were similar to age-matched wild type (WT) mice; conversely, Nampt$^{\Delta Agc1ER}$ mice completely lacked growth plates in the distal femur and proximal tibia (**Fig. 3A**). Femur lengths were decreased in Nampt$^{\Delta Agc1ER}$ relative to WT and Nampt$^{\Delta Prg4ER}$ mice. Similar growth plate loss was observed in the lumbar spine of Nampt$^{\Delta Agc1ER}$ mice (**Fig. 3B**), without any obvious defect within the intervertebral disks. The articular cartilage of Nampt$^{\Delta Agc1ER}$ knees showed reduced proteoglycan staining but no change in tissue volume. While significant osteophyte formation was not evident in the 12-month groups, there was a noted difference in the shape of the peri-articular bones of Nampt$^{\Delta Agc1ER}$ mice (**Fig. 3A**), possibly due to loss of the growth plates.

**DISCUSSION:** Here we show that physeal but not articular chondrocytes within young adult mice require NAD salvage for their anabolic activity and survival. We have not yet determined whether these results indicate higher demand for NAD+/NADH in physeal relative to articular chondrocytes, an insufficient supply of circulating NAD precursors (used for *de novo* synthesis) to the growth plate relative to articular cartilage, or both. It is also not yet clear whether the growth plate dysplasia is attributed to inadequate NAD redox activity, which is essential for cellular metabolism, or to insufficient NAD substrate needed by sirtuins, poly(ADP-ribose) polymerases, and other important cellular enzymes.

**SIGNIFICANCE/CLINICAL RELEVANCE:** Our results demonstrate distinct NAD requirements between physeal and articular chondrocytes within the postnatal skeleton. While genetic variants of Nampt itself have not been linked to skeletal dysplasias, the present findings may have implications for the short stature and other skeletal deficits observed in children with Congenital NAD Deficiency Disorder (CNDD), which involve mutations in certain enzymes of the *de novo* synthesis pathway [5].

**REFERENCES:** [1] Warren et al. *Nat Communications* (2023) 14:3616; [2] Glasson et al. *Osteoarthritis Cartilage* 2007 15:1061; [3] Glasson et al. *Osteoarthritis Cartilage* 2010 18 Suppl 3:S17; [4] Gil Alabarse et al. *Arthritis Rheumatol* 2023 75(3):364; [5] Shi et al. *N Engl J Med* 2017;377:544.

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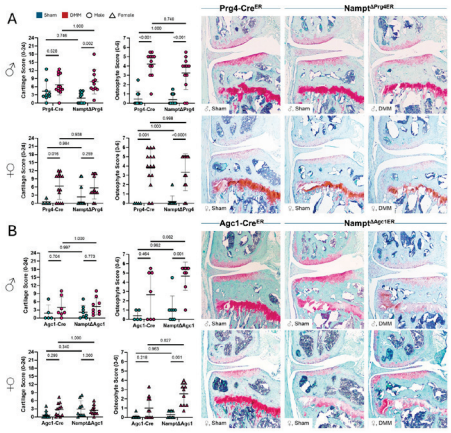

**Figure 1. Nampt is not essential for articular chondrocyte function.** Histological scoring of knees from (**A**) Nampt$^{\Delta Prg4}$ and (**B**) Nampt$^{\Delta Agc1}$ mice and their respective Cre controls 4 weeks after sham or DMM surgery (and 5 weeks after tamoxifen delivery). Left: Scores of cartilage damage and osteophyte formation for male and female mice. Means were analyzed using ANOVA of aligned rank transformed data with Mann-Whitney post-hoc analysis. Right: Representative Safranin O/Fast Green stains are shown for each genotype and sex.

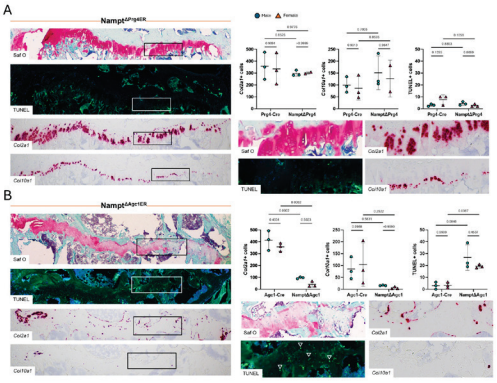

**Figure 2. Nampt is essential for physeal chondrocyte function in young adult mice.** Histological evaluation of tibial growth plates from (**A**) Nampt$^{\Delta Prg4}$ and (**B**) Nampt$^{\Delta Agc1}$ mice five weeks after tamoxifen delivery starting at 11 weeks of age. Left: Scores of cartilage damage and osteophyte formation in male and female mice 4 weeks after sham or DMM surgery. Means were analyzed using 2-way ANOVA with Tukey's multiple comparisons test. Right: Representative Safranin O/Fast Green stains are shown for each genotype and sex.

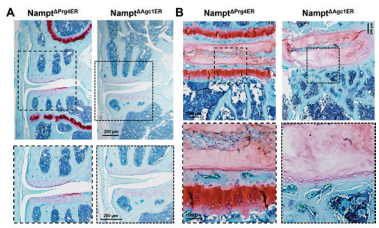

**Figure 3. Nampt deletion within physeal chondrocytes at 3 months leads to complete growth plate loss by 12 months of age.** Safranin O/Fast green staining of coronal sections of the knee (**A**) and lumbar spine (**B**) at 12 months of age after tamoxifen delivery at 3 months. Boxed regions are shown at higher magnification.