A comparative analysis of *TonEBP/Nfat5* conditional KO mouse models reveals the inter-dependency between compartments of the intervertebral disc.

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Introduction: *TonEBP/Nfat5* is a transcription factor that allows cells to adapt to a rise in extracellular osmotic pressure by promoting the expression of transporters for non-charged osmolytes. The proteoglycan-rich nature of all cartilaginous tissues in the axial and appendicular skeleton implies that these tissues expose resident cells to high osmotic pressure. We thus hypothesized that these cells require TonEBP to form or maintain all skeletal tissues characterized by a high proteoglycan content and we focused our study on the maturation of the intervertebral disc (IVD).

Methods: A new *TonEBP* floxed allele was generated by CRISPR/Cas9 and crossed with a panel of Cre transgenic mice targeting different IVD compartments. To target all IVD compartments at embryonic stage we used the constitutive Col2-Cre strain (Col2^{cKO}), whereas we used the inducible Acan-CreERT2 to target all IVD compartment at postnatal stage (iAcan^{cKO}), with tamoxifen injection (100ug/gram body weight at postnatal day 3 (P3)) to induce Cre activity. To target specifically the nucleus pulposus (NP) or the cartilage end plate (CEP)/growth plate (GP)/annulus fibrosus (AF) compartments at postnatal stages, we used the K19-CreERT (iK19^{cKO}) and the Col2-CreERT transgenic mice (iCol2^{cKO}), respectively, using tamoxifen injection at P3 and P5. All IVD analyses were performed in the lumbar area (L4-L3) with a sample size varying from n=4 mice for histological parameters and n=8-10 mice for X-ray and micro–computed tomography studies (number per genotype and time point studied). All mouse procedures were approved by the institutional BCM IACUC board.

Results: The embryonic loss of *TonEBP* in all IVD compartments in Col2^{cKO} mice caused a 40% decrease of vertebral height compared WT controls, and a doubling of the intervertebral space (DHI) at 8 months of age (n=8, $p<0.05$). We observed a loss of vertebral GP structural continuity and GP cellularity in mutant mice, concomitant with a larger NP and loss of AF lamellar structure organization following Safranin-O staining on coronal sections. Staining at P21 also revealed a disorganization of the CEP/GP, a larger NP, but no obvious change in the AF. Immunohistochemistry for the main ECM markers (Aggrecan, type II and type X collagen) revealed that TonEBP is required for their expression in the CEP/GP but not in the NP. Deletion of *TonEBP* in the whole embryonic IVD led to chondrocyte cell death but did not affect NP cell survival, nor cell proliferation (n=4, $p<0.05$). To determine the tissue of origin of the phenotypes observed upon IVD-global loss of *TonEBP* in the Col2^{cKO} mice, we compared the same histological parameters between the IVD from iK19^{cKO} (*TonEBP* deleted only in NP) and iCol2cKO (*TonEBP* deleted in all IVD compartments except the NP). By safranin-O staining, we observed that NPs from iCol2^{cKO} were larger, as observed in Col2^{cKO} mice, despite absence of *TonEBP* recombination in NP cells, whereas NP height from iK19^{cKO} mice was not different from control littermates (P21, n=4, $p<0.05$). The CEP/GP from iCol2^{cKO} mice showed similar structural defects as in Col2^{cKO} mice, but surprisingly in iK19^{cKO} mice, the height of the CEP/GP was increased compared to WT, despite absence of *TonEBP* recombination in the CEP/GP of these mice. In iK19^{cKO} mice, ECM markers were unchanged, whereas in iCol2^{cKO} mice, Type II and X collagen expression was decreased in the CEP/GP compartments, whereas Aggrecan was not affected in any IVD compartment compared to WT controls. Cell death analyses revealed that TonEBP is not required for NP cell survival in iK19^{cKO} mice, whereas it is required for chondrocyte survival in the GP compartment of iCol2^{cKO} mice compared to WT controls (P10, n=4, $p<0.05$). Unexpectedly, we observed an increase of cell proliferation in the NP and CEP/GP of iK19^{cKO} mice compared to WT controls, despite absence of *TonEBP* deletion in CEP/GP (P10, n=4, $p<0.05$). *TonEBP* deletion specifically in the NP did not impact the expression of the NP markers Brachyury and Keratin 19, however, deletion of *TonEBP* specifically in the CEP/GP compartments decreased their expression in the NP (n=4, $p<0.05$). We next sought to determine when deletion of *TonEBP* was required to induce the IVD phenotypes observed in Col2^{cKO} mice. To do so, we compared the IVD phenotypes of 4-month-old Col2^{cKO} mice (IVD-global embryonic deletion) with iAcan^{cKO} mice (postnatal IVD-global deletion following tamoxifen injection at P3). Results showed similar short stature, short vertebrae, high DHI, low vertebral trabecular bone mass (n=8, $p<0.05$), enlarged NPs and CEP/GP loss of structural and cellular integrity, indicating that TonEBP has a predominant role in the postnatal maturation of the IVD.

Discussion: Our study is the first to assess the role of *TonEBP* during the maturation of the IVD and its role in the different compartments of the IVD. Unexpectedly, deletion of *TonEBP* specifically in the NP compartment did not lead to NP cell death nor to a reduction in aggrecan level, as expected from *in vitro* studies. In the opposite, we observed an increase of cell proliferation in the NP and CEP/GP compartments in iK19^{cKO} mice. We conclude from these results that TonEBP in postnatal NP cells is not required for their survival or fate maintenance but governs the proliferation of NP and CEP/GP cells in a likely autocrine and paracrine manner, respectively. In contrast to NP cells, the loss of *TonEBP* in chondrocytes revealed that TonEBP is required in this lineage, in a cell-autonomous manner, for cell survival. The data presented herein are experimental evidence of a crosstalk between IVD compartments that is necessary for postnatal IVD maturation, which implies the existence of mediators from NP toward CEP/GP and CEP/GP toward NP, whose identity remain to be determined.

Significance/clinical relevance: Although *TonEBP* cKO mice were not analyzed at a timepoint relevant to aging, these results have potential relevance to IVD degeneration. The vast majority of studies related to IVD degeneration and low back pain have been, for good reasons, NP-centric. Our results in this comparative analysis of *TonEBP* cKO mice, along with pioneering data by others, support the notion that disc degeneration and low back pain may stem from abnormalities in any of the IVD compartments (not only the NP), and that variability in clinical presentation may stem from the tissue-of-disease origin within the IVD. This implies, acknowledging possible differences between mice and humans, that CEP/GP/AF could be targeted for a more personalized management of IVD degeneration.