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Zbtb7c is a molecular 'off' and 'on' switch of *Mmp* gene transcription

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ABSTRACT

Matrix metalloproteinases (MMPs) are zinc-containing endopeptidases that play roles in cell proliferation, migration, differentiation, angiogenesis, and apoptosis. The expression of *MMP* gene is tightly regulated and shows cell- and tissue-specific expression patterns. Despite their differential expression, *MMP* genes have AP-1 (activator protein-1) binding elements within their promoters. Interestingly, c-JUN phosphorylation by cytokine signaling decreased its interaction with NCoR, but increased its interaction with p300, resulting in activation of *MMP* gene transcription. Here, we found that Zbtb7c (Kr-pok) is a critical component of a transcriptional repressor complex containing c-Jun and NCoR. c-Jun, bound at AP-1, interacts with Zbtb7c, which in turn recruits an NCoR/Hdac3 complex to repress several *Mmp* (-8, -10, -13, and -16) genes. The molecular interaction between c-Jun and Zbtb7c also prevents phosphorylation of c-Jun by p-Jnk, However, Zbtb7c phosphorylation by p-Jnk (induced by TNFα), and its (Zbtb7c) subsequent degradation by the ubiquitin-mediated proteasomal pathway, leads to c-Jun phosphorylation by p-Jnk. Promoter-bound p-c-Jun then recruits the coactivator p300 to upregulate *Mmp* gene. Overall, these findings show that Zbtb7c is a key molecule that recruits an NCoR/Hdac3 complex to inhibit phosphorylation of c-Jun, and thereby repress *Mmp* gene expression.

1. Introduction

Matrix metalloproteinases (MMPs) are calcium-dependent, zinc-containing endopeptidases that can degrade various extracellular matrix (ECM) proteins. MMPs are strongly involved in the cleavage of cell surface receptors, the release of apoptotic ligands (*e.g.*, FAS ligand), and chemokine/cytokine inactivation. MMPs are also thought to play a major role in various cellular processes, such as cell proliferation, migration, differentiation, angiogenesis, apoptosis, and host defense [1–5]. Currently, MMP family proteins consist of structurally related 23 proteinases in humans, and 24 in mice [2–4]. MMPs can be classified according to their substrate specificity: collagenases, gelatinases, stromelysins, and matrilysins [3,4]. Collagenases (MMPs-1, -8, and -13) degrade triple-helical fibrillar collagens, which are the main components of bone and cartilage [6,7]. Gelatinases (MMPs-2 and -9) degrade molecules in the basal lamina around capillaries, facilitate angiogenesis and neurogenesis, and contribute to cell death [6,8]. Stromelysins (MMPs-3, -7, -10, and -11) are small proteases that degrade components of the ECM. Another type of MMPs, known as MT-MMPs (membrane-type matrix metalloproteinases), localize to the cell surface. MMPs-14, -15, -16, and -24 contain hydrophobic transmembrane domains, followed by a cytoplasmic domain. MMPs-17 and -25 lack cytoplasmic domains, and are thought to be GPI (glycophosphatidylinositol)-anchored to the cell surface [6].

MMPs expression was reported to be upregulated in diverse human diseases, including rheumatoid arthritis, emphysema, and cancer [9–11]. However, recent clinical data indicate that the relationship between MMP and disease is complicated. For example, increased MMP activity (and its inhibiting TIMPs) can enhance or inhibit tumor progression [12]. This complex relationship between MMP expression and cancer has triggered basic and clinical interest in the pathological function of MMP *in vivo*, but relatively less attention on the normal roles of these enzymes. Historically, MMPs were thought to function mainly as enzymes that degrade structural components of the ECM. However, MMP proteolysis can create space for cells to migrate, produce specific substrate-cleavage fragments with independent biological activity, regulate tissue architecture through effects on the ECM and intercellular junctions, and modify and activate specific signaling molecules [13]. Because cells have receptors (integrins) for structural ECM components, MMPs can also affect cellular functions by regulating the

Abbreviations: MMP, matrix metalloproteinase; TNFα, tumor necrosis factor alpha; Kr-pok, kidney cancer related POZ domain and Krüppel-like protein; Jnk, c-Jun N-terminal kinase.

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composition of the ECM [14]. For example, type I collagen degradation is necessary for epithelial cell migration and wound healing in culture models. Cleavage of ECM proteins by MMPs can also release ECM-bound growth factors, such as insulin-like growth factors and fibroblast growth factors [15,16].

Analysis of genetic knockouts of *Mmps* or of genes affecting MMP expression offers opportunities to study functions of MMPs and relevant genes [17–20]. At least 15 mouse MMP mutants have been generated. The initial characterizations have revealed subtle phenotypes, seemingly dispensable with each other during embryonic development. MMPs have many overlapping substrates *in vitro*, which indicates functional redundancy *in vivo*.

The expression of *MMP* gene is tightly regulated, and shows cell- and tissue-specific expression patterns. Despite their differential expression, *MMP* gene promoters share a similar AP-1 (activator protein-1) binding element [21]. AP-1 (c-JUN) interacts with NCoR to repress target gene expression, and the loss of NCoR results in derepression of, *MMPs* (AP-1 target genes). The molecular interaction can be relieved by TPA/LPS signal-dependent phosphorylation of c-Jun, in which the NCoR/Hdac3 complex is dissociated from the AP-1 binding site and the coactivator p300 HAT complex is recruited instead [22–24]. The molecular switch of coregulatory complex at AP-1 site triggered by c-JUN phosphorylation activates *MMP* expression. Inflammatory signaling in RAW264.7 macrophages was shown to phosphorylate c-Jun, and eventually activate Mmps expression *via* this mechanism.

Recently, we found that ZBTB7C (KR-POK) is a proto-oncoprotein that interacts with p53, MIZ-1 and NCoR, which represses transcription of $p21^{WAF1/CDKN1A}$ and stimulates cell proliferation [25,26]. Zbtb7c was also shown to modulate the DNA binding of SREBP-1c and Sp1 at the proximal promoter of *FASN*, and thereby regulate lipid metabolism [27]. To identify the various target genes regulated by the transcription factor Zbtb7c, we analyzed differential mRNA expression in stable, doxycycline-inducible HEK293Trex-Zbtb7c cells, finding several *MMP* genes to be downregulated by doxycycline. We then investigated the molecular mechanism(s) of Zbtb7c regulation of *Mmp* gene expression, and how Zbtb7c expression can be dysregulated by cytokine signaling.

2. Material and methods

2.1. Cell cultures/stable cell lines and animals

Cells were cultured in media recommended by ATCC (Manassas, VA, USA). Stable NIH/3T3 cells overexpressing Zbtb7c were prepared by infection with a recombinant lentivirus (LentiM1.4-Zbtb7c tagged with His and Myc), and selected with puromycin. $Zbtb7c^{+/+}$ and $Zbtb7c^{-/-}$ mouse embryonic fibroblasts were prepared by a standard protocol [25,27].

2.2. Plasmids, antibodies, and reagents

pcDNA3.0-FLAG-Zbtb7c, pcDNA3.1-Zbtb7c-Myc-His, pcDNA3.1-c-Jun-Myc-His, and pcDNA3.1-cFos-Myc-His constructs were prepared by cloning cDNA fragments into pcDNA3.0 or pcDNA3.1 (Invitrogen, Carlsbad, *CA*, USA). Mutant Zbtb7c-expressing plasmids were prepared using a site-directed mutagenesis kit (Stratagene, CA, USA). To prepare recombinant GST-POZ Zbtb7c or GST-ZF Zbtb7c proteins, cDNA fragments encoding the Zbtb7c POZ (a.a 1–132) or zinc finger (a.a 365–468) domains were cloned into pGEX4T3 (Amersham Biosciences, NJ, USA). GFP-NCoR and His-Ubiqutin expression plasmids were kindly provided by Dr. Ho-Geun Yoon (Yonsei University School of Medicine, Seoul, Korea). All plasmid constructs were verified by sequencing. The following antibodies were used: GAPDH (FL-335, sc-25778), c-Jun (G-4, sc-74,543), p-c-Jun (Ser 63/73, sc-16,312), NCoR (C-20, sc-1609), HDAC3 (H-99, sc-11417), Myc-tag (N-262, sc-764); Santa Cruz Biotechnology (Santa Cruz, CA, USA), Mmps-8 (ab81286), -10 (ab59437), -16 (ab73877); Abcam (Cambridge, MA, USA), Mmp-13 (Mab13424; lv1583450), p300 (05-257); Millipore (Billerica, MA, USA), Jnk (#9252), p-Jnk (#9251), Ac-*H*3 (#9649), Ac-H4 (#9672), His-tag (#2365); Cell Signaling Technology (Danvers, MA, USA); and FLAG-tag (F3165); Sigma (St. Louis, MO, USA). To obtain a rabbit polyclonal antibody against Zbtb7c, a white rabbit was immunized by subcutaneous injection with recombinant polypeptide GST-POZ (a.a. 1–120) eight times, at 2-week intervals. Blood was collected, incubated at 37 °C for 90 min, and centrifuged. The supernatant was incubated with Affi-Gel 10 beads cross-linked to a recombinant Zbtb7c POZ domain (Bio-Rad, Hercules, CA, USA.). The precipitated beads were washed with PBS, and the antibody was eluted (1.0 M Tris pH 7.6). Most of the chemical reagents were purchased from Sigma.

2.3. Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was isolated from the cells using TRIzol reagent (Invitrogen). cDNA was prepared using total RNA, random hexamers, and Superscript reverse transcriptase II (Promega, Madison, WI, USA). RT-qPCR was conducted in an ABI PRISM 7300 RT-PCR System using a SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) and gene-specific primers (see Supplementary Table). GAPDH mRNA was used as a control.

2.4. Western blot analysis

Cells were washed, pelleted, and resuspended in RIPA buffer supplemented with protease inhibitors. Cell extracts were separated by 12% SDS-PAGE gel electrophoresis, transferred to Immun-Blot™ PVDF membranes (Bio-Rad) and blocked with 5% skim milk (BD Biosciences, Franklin Lakes, NJ, USA) or BSA. Blotted membranes were then incubated with primary antibodies overnight at 4 °C, followed by incubation with secondary antibodies conjugated to HRP (Thermo Scientific, Rockford, IL, USA) at RT for 2 h. Protein bands were visualized by ECL solution (Thermo Scientific). GAPDH protein was used as a normalization control.

2.5. GST-fusion protein purification and GST-fusion protein pull-down assays: in vitro transcription and translation (TNT) of c-Jun and c-Fos

Recombinant GST, GST-Zbtb7c-POZ and GST-Zbtb7c-ZF fusion proteins were prepared from *E. coli* BL21 (DE3) cells by glutathione-agarose 4-bead affinity chromatography (Peptron, Daejeon, Korea). [^{35}S]-methionine-labeled c-Jun and c-Fos polypeptides were prepared using *in vitro* TNT kit (Promega). GST-fusion ZF or POZ domain protein-agarose beads were incubated with [^{35}S]-labeled c-Jun and c-Fos polypeptides in HEMG buffer, centrifuged, and the pellets then washed and separated by 12% SDS-PAGE. The gels were then exposed to X-ray film (Kodak, Rochester, NY, USA).

2.6. Immunoprecipitation (IP) and ubiquitination assays

Cell lysates were precleared, and supernatants incubated overnight with antibodies at 4 °C, followed by incubation with protein A/G agarose beads. Beads were collected, washed, and resuspended in equal volumes of 5× SDS loading buffer. Immunoprecipitated proteins were separated by 12% SDS-PAGE and analyzed by western blotting, as described above.

For ubiquitination, cells were transfected with Zbtb7c and/or His-tagged-ubiquitin expression vectors in the presence or absence of drugs (TNFα and MG132). Overnight-cultured cells were lysed, and the lysates were immunoprecipitated using *anti*-His or anti-Myc antibodies, followed by western blot analysis.