INTRODUCTION

Iron is crucially involved in many of the essential physiological processes of the human body, such as oxygen delivery by red blood cells, energy processes in muscles and enzymatic catalysis of various metabolic processes. Intestinal cells release absorbed iron from the diet into the plasma, and iron binds with transferrin, an iron carrier protein, in the plasma to be distributed to the target organ or cells. However, excessive iron release into the plasma can saturate the binding capacity of transferrin and result

Runx3 regulates iron metabolism via modulation of BMP signalling

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Abstract

Objectives: Runx3, a member of the Runx family of transcription factors, has been studied as a tumour suppressor and key player of organ development. In a previous study, we reported differentiation failure and excessive angiogenesis in the liver of Runx3 knock-out (KO) mice. Here, we examined a function of the Runx3 in liver, especially in iron metabolism.

Methods: We performed histological and immunohistological analyses of the Runx3 KO mouse liver. RNA-sequencing analyses were performed on primary hepatocytes isolated from Runx3 conditional KO (cKO) mice. The effect of Runx3 knock-down (KD) was also investigated using siRNA-mediated KD in functional human hepatocytes and human hepatocellular carcinoma cells.

Result: We observed an iron-overloaded liver with decreased expression of hepcidin in Runx3 KO mice. Expression of BMP6, a regulator of hepcidin transcription, and activity of the BMP pathway were decreased in the liver tissue of Runx3 KO mice. Transcriptome analysis on primary hepatocytes isolated from Runx3 cKO mice also revealed that iron-induced increase in BMP6 was mediated by Runx3. Similar results were observed in Runx3 knock-down experiments using HepaRG cells and HepG2 cells. Finally, we showed that Runx3 enhanced the activity of the BMP6 promoter by responding to iron stimuli in the hepatocytes.

Conclusion: In conclusion, we suggest that Runx3 plays important roles in iron metabolism of the liver through regulation of BMP signalling.

1 | INTRODUCTION

Iron is crucially involved in many of the essential physiological processes of the human body, such as oxygen delivery by red blood cells, energy processes in muscles and enzymatic catalysis of various metabolic processes. Intestinal cells release absorbed iron from the diet into the plasma, and iron binds with transferrin, an iron carrier protein, in the plasma to be distributed to the target organ or cells. However, excessive iron release into the plasma can saturate the binding capacity of transferrin and result
in non-transferrin-bound iron in the blood, which is a highly reactive form that can cause cellular and visceral damage. Therefore, tight regulation of plasma iron is required to avoid iron-related toxicity in the body.

Hepcidin, a key regulator of iron transport, suppresses the release of iron from macrophages or intestinal cells into the plasma via binding to ferroportin, which induces internalization and degradation of the cellular iron exporter. Genetic deficiency of hepcidin causes excessive iron in blood, which is followed by the deposition of iron and consequent functional failure in the liver and other tissues. The bone morphogenetic protein (BMP) signalling pathway is a major regulatory pathway of hepcidin expression in the liver. In hepatocytes, the pathway is initiated by the binding of BMP6 with the BMP receptor (BMPR) complex and a membrane-anchor coreceptor hemojuelin (HJV) at the cell surface. The binding elevates kinase activity of the BMPR complex and results in phosphorylation of Smad1, Smad5 and Smad8, the cytoplasmic effectors of the BMP pathway. Phosphorylated Smad1, 5 and 8 form heteromeric complexes with the common mediator Smad4, and they then translocate into the nucleus to induce the transcription of target genes. A deficiency of the BMP pathway-related genes causes low hepcidin expression, excessive iron in the blood and iron-overloaded organs in mice.

The activity of the BMP signalling pathway in the liver should be associated with the plasma iron concentration to maintain iron homeostasis. In the liver of mice fed high-iron diet, transcriptional activation of BMP6 has been observed. Recent studies suggested liver sinusoidal endothelial cells (LSECs) as main sources of hepatic BMP6 responding to the iron stimuli. Hepatocytes, once considered to serve dual roles as iron-sensor and autocrine sources of BMP6, revealed as passive producers of hepcidin regulated by paracrine BMP6 from non-parenchymal cells. However, high expression of transferrin receptor 2 (TfR2) and its unveiled function in hepatocytes implies possible mechanism of direct sensing of iron by the hepcidin producer.

Here, we demonstrated that Runx3 is an upstream regulator of BMP6 in the liver. Prussian blue staining revealed an iron-overloaded liver at postnatal day 1 (PN1) in Runx3 knock-out (KO) mice. Hepcidin was decreased in the liver of Runx3 KO mice. Interestingly, a similar iron-overloaded liver was reported in Bmp6 KO mice. To reveal the possible engagement of BMP signalling with Runx3 deficiency-induced iron overload in the liver, we detected BMP6 expression in the liver tissue. The results showed a decrease in BMP6 and BMP signalling in the liver of Runx3 KO mice. A systematic approach using RNA sequencing of primary hepatocytes isolated from Runx3 conditional KO (cKO) mice revealed that BMP6 was specifically induced by iron stimuli, and Runx3 KO using Cre recombinase-expressing adenovirus aborted the iron-induced BMP6 expression in the hepatocytes. Down-regulation of these genes was also observed in Runx3 knock-down (KD) in both HepaRG cells, which are functional human hepatocytes, and HepG2 cells, which are human hepatocellular carcinoma cells.

The Runx3 KD abolished iron-induced BMP6 transcription and the resultant activation of BMP signalling in both cells. Furthermore, we found that Runx3 activated the promoter of BMP6 to trigger the BMP signalling-mediated hepcidin regulation by iron stimulation. Taken together, Runx3 plays important roles in the iron metabolism of the liver through regulation of BMP signalling.

## Methods

### 2.1 Runx3 KO and cKO mice

Runx3 knock-out (Runx3<sup>−/−</sup> FVB) and Runx3 cKO (Runx3<sup>fl</sup> /<sup>fl</sup>) mice were generated and maintained as described previously. The animals were maintained in pathogen-free conditions and monitored daily. All experiments were performed according to the guidelines of the Yonsei University College of Dentistry, Intramural Animal Use and Care Committee.

### 2.2 Histology and immunohistochemistry

Samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and then embedded in paraffin using standard procedures. Serial paraffin sections (4-μm thickness) were prepared, and individual slides were stained with haematoxylin and eosin. Antigen retrieval was achieved by citrate buffer, pH 6.0. After antigen retrieval, immunohistochemical analyses were performed using following primary antibodies: Hepcidin (ab-75883; AbCam, Cambridge, UK), L-Ferritin (ab-69090; AbCam), Ferroportin-1 (sc-49668; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), BMP-6 (bs-10090R; Bioss Antibodies, Bioss Antibodies, Woburn, MA) and Smad-4 (sc-7966; Santa Cruz Biotechnology, Inc.). Immunostainings were performed using the DakoCytomation Envision System (DAKO, Glostrup, Denmark) according to the manufacturer’s instructions. Alexa Fluor-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) were used for immunofluorescent staining. The stained sections were examined with a stereomicroscope (MD5500D; Leica Microsystems, Wetzlar, Germany) and a confocal microscope (LSM700; Carl Zeiss, Jena, Germany).

### 2.3 Western blotting analyses

Liver tissue and hepaRG cells underwent lysis by sonication (Next Advance Inc., Averill Park, NY) in radio-immunoprecipitation assay (RIPA) buffer (50 nM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100). Anti-L-Ferritin or anti-α-Tubulin (T6199; Sigma-Aldrich, St. Louis, MO) antibody was used. Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) were used, and the protein bands were visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).
2.4 | Primary hepatocyte isolation and RNA preparation

Primary hepatocytes of Runx3 cKO mice were isolated as described previously.24 The isolated hepatocytes were infected with Cre recombinase-expressing adenovirus (Ad-Cre-GFP, #1700; Vector Biolabs, Philadelphia, PA) to induce knock-out of Runx3. After 24 h of the virus treatment, 60 mM of holo-transferrin (hTF, #616424, Millipore Corp., Bedford, MA) was treated to the hepatocytes for 24 h. Total RNAs were isolated using TRIzol reagent as a manufacturer’s instruction (#10296010; Invitrogen). RNA concentrations were quantified using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE), and the 260/280 nm ratio was confirmed to be between 1.7 and 2.0. The integrity of the total RNA samples was evaluated using the Agilent 2100 (Agilent Technologies, Inc., Santa Clara, CA) and Tecan F2000 (Tecan Group Ltd., Männedorf, Switzerland) devices, and only samples with an RNA integrity number (RIN) >7.0 and high-quality RNA (28S/18S > 1) were used for the subsequent experiments.

2.5 | RNA-sequencing and data analysis

Reverse transcription was performed, and cDNA was synthesized using 5’ adaptor forward and 3’ adaptor reverse primers. Libraries for Illumina sequencing were constructed from cDNA as described.25 High-throughput RNA sequencing was performed by Theragen Bio Institute (Suwon, Korea) on an Illumina HiSeq 2000 high-throughput sequencer (Illumina, Inc. San Diego, CA) according to the manufacturer’s specifications. RNA-sequencing data were analysed according to the method described. Briefly, reads were mapped to the Mus musculus reference genome obtained from the University of California, Santa Cruz (UCSC) database using TopHat and Bowtie from Illumina iGenomes. Gene expression values were measured for each gene from the Ensembl database by fragments per kilobase of exon per million mapped reads (FPKM) calculated using Cufflinks.26 Differentially expressed genes were considered in a given library when the p-value was less than 0.05 and a greater-than-or-equal to twofold change in expression across libraries was observed and used to identify the genes differentially expressed between two samples. Clustered heat maps and volcano plots were drawn using a statistical computing software, R (https://www.R-project.org/).

2.6 | Cell culture, transfection and analysis

HepG2 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen) supplemented with 10% foetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Cells were transfected with Runx3 siRNA (Santa Cruz Biotechnology, sc-37679) or a luciferase reporter plasmid containing BMP6 promoter (S710500; Switchgear Genomics, Menlo Park, CA) using Lipofectamine (Invitrogen) as a manufacturer’s instruction. For real-time PCR, total RNA of cells were extracted using TRIzol reagent. The extracts were reverse-transcribed using Maxime RT PreMix (#25081; iNtRON, Daejon, Korea). The products were subjected to real-time PCR analyses with primer sets designed using Primer Express software (Applied Biosystems, Foster City, CA) and StepOnePlus Real-Time PCR System (Applied Biosystems). For reporter assay, cells were lysed and reacted using Luciferase Assay System (Promega, Madison, WI) and the luciferase activities were measured using Centro XS3 Microplate Luminometer LB 960 (Berthold Technologies, Oak Ridge, TN).

3 | RESULTS

3.1 | Iron overload in Runx3 KO mouse liver hepatocytes at PN1

Runx3 KO mice showed lethality soon after birth, as reported previously.27 At postnatal day 1, skin pigmentation was observed in the KO mice (Figure 1A), which revealed a possible abnormality in iron metabolism.28 Depletion of Runx3 in the liver tissue of Runx3 KO mice was confirmed using immunohistochemistry and immunoblotting (Figure 1B-1E). Using Perls’ Prussian blue staining, substantial iron accumulation was visualized in liver parenchymal cells (hepatocytes) of Runx3 KO mice at PN1 (Figure 1F). The accumulation of iron was significantly higher in the central lobule region than the peripheral region of the liver (Figure 1F). In addition, an increase in ferritin protein, indicating accumulation of iron in the cytosol, was observed in the hepatocytes of Runx3 KO mice compared to those of WT mice (Figures S1A, S1B).

The iron accumulation in the centrallobular region of liver tissue is a typical pattern of iron overload caused by hepcidin deficiency.29 Therefore, we examined hepcidin expression in the liver tissue of Runx3 KO mice. The expression of hepcidin decreased in the liver tissue of Runx3 KO mice compared to that of WT mice (Figure 1G, 1H). On the contrary, ferroportin expression was increased in the KO mouse liver, indicating escape of the iron exporter from hepcidin-mediated degradation (Figure 1I, 1J).

To investigate possible engagement of BMP signalling in the iron-overloaded liver of Runx3 KO mice, BMP signalling activation in the liver tissue. Nuclear Smad4, an indicator of BMP signalling activation, was decreased in the hepatocytes of Runx3 KO mice compared with those of WT mice (Figure 1K, 1L).

3.2 | Systemic regulation of BMP signalling- and iron metabolism-related genes by Runx3 KO in primary hepatocytes

The lethality of Runx3 KO mice set limitations on the systemic analysis of the Runx3 effect on iron metabolism. We introduced cKO mice of Runx3 to avoid the limitations.22 Primary hepatocytes of Runx3 cKO mice were isolated, and Runx3 was knocked out by exogenous
expression of Cre recombinase (Cre) using an adenoviral expression system (Figure 2A). The Runx3 KO or control hepatocytes were incubated with or without hTF, a complex of iron and its carrier protein (Figure 2A). The alternation of transcriptomes by iron stimuli in the cells was monitored by RNA-sequencing analysis (Figure 2B–2H).

The analysis result showed that Runx3 was knocked out in Cre overexpressed hepatocytes (Figure 2B). We examined expressions of the direct target genes of Runx3, which are known to be positively (Claudin1, angiotensin II type 1 receptor-associated protein [Agtrap] and Bim)30-32 or negatively (Hes1, Jagged1 [Jag1] and Akt1)33-35 regulated by Runx3 (Figure 2B). As expected, Claudin1, Agtrap and Bim were decreased and Hes1, Jag1 and Akt1 were increased by the Runx3 KO (Figure 2B).

Differentially expressed gene (DEG) analysis revealed that 182 (up 99 and down 83), 98 (up 33 and down 65) and 170 (up 82 and down 88) genes were significantly regulated by Cre overexpression,
hTF treatment or both respectively (Figure 2A and Tables S1 and Table S2). The expression patterns of 294 genes, which were significantly regulated in at least one treated group, were visualized as a heat map (Figure 2C). The clustered heat map showed a cluster of genes that were highly responsive to hTF treatment (Figure 2C, dashed-line box). Cre-mediated Runx3 KO suppressed the hTF-induced expression of the 19 genes in this cluster (Figure 2C, 2D). BMP6 was identified as one of the 19 genes (Figure 2D). This cluster also included genes that encode for cytochrome P450s, which are the liver-specific heme-containing enzymes (Cyp2a5, Cyp2a26, Cyp3a25 and Cyp17a1); a sodium-independent cystine-glutamate antiporter involving ferroptosis (Slc7a11)36; and proteins related to gastric secretion and iron absorption in the stomach, Adrenomedullin (Adm)37 and Cholecystokinin (Cck)38 (Figure 2D).

To investigate the specificity of BMP6 regulation by hTF treatment and Cre expression, we displayed the DEG analysis results on volcano plots and indicated all identified BMPs on the plots (Figure 2E, 2F). Most of the BMP members were identified in both control and hTF-treated hepatocytes; however, only BMP6 was significantly increased by the iron stimuli (Figure 2E). The iron-induced expression of BMP6 was not observed in Runx3 KO hepatocytes (Figure 2F). Interestingly, BMP7, a potent substitute for BMP6 in iron metabolism,39 was significantly increased by hTF treatment in the Runx3 deficient hepatocytes; however, the expression of other BMPs was not significant (Figure 2F). Id1 and Hepcidin, target genes of the BMP signalling pathway, were not significantly changed by hTF treatment or Cre overexpression (Figures S2A, S2B). The opposite expression pattern of BMP7 compared with BMP6 possibly compensates for the BMP6 deficiency by Runx3 KO. The increase in BMP6 was confirmed by immunohistochemistry of BMP6 in liver tissue of WT and Runx3 KO mice (Figure 2G).

3.3 | Regulation of BMP signalling- and iron metabolism-related genes by Runx3 KD in human hepatocytes and hepatocellular carcinoma cells

Here, we aimed to confirm the role of Runx3 on the expression of BMP signalling- and iron metabolism-related genes using established hepatocyte cell lines. Firstly, we used HepaRG cells, which are functional human hepatocytes. Transfection of Runx3 siRNA successfully decreased the mRNA level of Runx3 in the hepatocytes (Figure 3A). The knock-down effect also confirmed in protein level using immunoblotting (Figure 3B). Similar to the observation in the KO mice, the mRNA level of hepcidin decreased by the knock-down (KD) of Runx3 (Figure 3C). The Runx3 KD effect also confirmed using HepG2 cells, which are human hepatocellular carcinoma cells (Figure 3D). Immunohistochemistry results revealed a decrease in hepcidin expression in the Runx3 KD hepatocellular carcinoma cells (Figure 3D).

Hepcidin regulates ferroportin expression at the protein level, rather than at the transcription level.3 We found that Runx3 KD cannot alter the mRNA level of ferroportin (Figure 3E). The mRNA level of transferrin, a liver-originated iron carrier protein, increased by Runx3 KD in the HepaRG cells (Figure 3F). To monitor the activity of the BMP pathway in Runx3 KD hepatocytes, we detected the mRNA levels of the BMP ligand and BMP pathway target genes. The quantitative real-time PCR results showed that mRNA levels of BMP6 decreased by Runx3 KD (Figure 3G). The mRNA levels of well-known target genes of the pathway Id1, Smad7 and Atoh8 also decreased in the Runx3 KD hepatocytes (Figure 3H–3J).

3.4 | Direct transcriptional regulation of BMP6 by Runx3

BMP6 induction and activation of the BMP pathway by iron stimuli in hepatocytes were reported previously.40 To investigate whether Runx3 functions as a mediator between iron stimuli to BMP pathway activation, HepG2 cells transfected with control siRNA or Runx3 siRNA were starved for 24 h to remove possible effect foetal bovine serum (FBS) in media and then treated with 0, 10 and 50% FBS for other 24 h (Figure 4A). The real-time PCR results showed that the BMP pathway target genes, Id1 and Smad7, were induced in a dose-dependent manner with FBS treatment in control siRNA-transfected cells (Figure 4B, 4C). However, activation of the BMP pathway was abolished in Runx3 siRNA-transfected cells (Figure 4B, 4C).

Holo-transferrin is an iron-bound form of transferrin, which is known to induce BMP6 expression.41 Similar to the experiment using serum, holo-transferrin increases mRNA levels of Id1 and Smad7; however, the effect was cancelled by Runx3 KD (Figure 4D–4F). These results indicate that Runx3 functions as a mediator between iron stimuli and BMP pathway activation.

The Runt domain of the Runx3 has a specificity on a conserved sequence, 5'‑GGYGGT-3'.42 Most of the target genes of Runx3 contain this sequence in their promoter region. We analysed an ~1 Kb upstream sequence from the BMP6 open reading frame to determine if the Runx3 binding sequence (RBS) existed, which was identified as one of the enriched motif in Runx3-bound promoter of natural killer cells.42 The sequence analysis showed two putative RBSs in the BMP6 promoter region that were evolutionally well-conserved (Figure 4G). We used a luciferase reporter plasmid containing the BMP6 promoter to investigate the role of Runx3 on the transcriptional activity of the promoter (Figure 4H). Serum treatment showed a dose-dependent increase of promoter activity in the reporter plasmid-transfected HepG2 cells (Figure 4I). However, Runx3 KD suppressed activation of the BMP6 promoter induced by serum treatment (Figure 4I).

4 | DISCUSSION

Hereditary haemochromatosis (HH) is a term used to describe a group of genetic disorders characterized by increased iron absorption.43 This absorption may lead to a progressive accumulation of iron in tissues and organs, resulting in impairment of organ structure...
and function, especially of the liver, pancreas, heart, pituitary gland and, likely, joints. The prevailing mechanism in most types of HH is deficiency of hepcidin, originally identified as an antimicrobial peptide\(^4\) and then shown to play a major role in iron homeostasis.\(^4\)\(^5\)\(^6\) Hepcidin is synthesized mainly in hepatocytes and controls the plasma iron concentration by binding to ferroportin (also termed SLC40A1), the only known cellular iron exporter. After binding, ferroportin is degraded, reducing both intestinal absorption of iron from enterocytes and iron released from hepatocytes and macrophages. Increased plasma iron or cellular iron stores, as well as inflammation, generate a negative feedback loop that leads to a restriction of iron release into the plasma and blockade of dietary iron absorption through increased hepcidin production. In this study, Runx3 KO mice showed a haemochromatosis-like phenotype. Skin pigmentation and an iron-overloaded liver were observed in the KO mice. Molecular biological analyses showed that BMP6 expression and activity of the BMP pathway were suppressed in the liver of Runx3 KO mice.

Studies have shown that, at least in rodent models, increasing body iron stimulates the production of BMP6, which binds to a complex of type I and II BMP receptors on the plasma membrane.
(A) siRNA transfection 0, 10, 50% FBS Media Harvest
(B) Id1 Relative expression
(C) Smad7 Relative expression
(D) siRNA transfection 0, 30, 60 mM hTFN Harvest
(E) Id1 Relative expression
(F) Smad7 Relative expression
(G) DNA sequence alignment
(H) siRNA Reporter 0, 10, 50% transfection FBS Media Harvest
(I) Luminescence intensity (x10^4)
of hepatocytes (Figure 5). This leads to the phosphorylation of SMAD1, 5 and 8 in the cytoplasm, which allows the binding of SMAD4. The entire complex is then translocated into the nucleus where it binds to BMP responsive elements in the hepcidin promoter, stimulating transcription. However, the regulation mechanism of BMP6 by iron stimuli has not yet been revealed. Here, we showed that Runx3 KO or KD in mouse primary hepatocytes, human hepatocytes and human hepatocellular carcinoma decreased BMP6 expression and inhibited the BMP-Smad pathway in human hepatocytes. Therefore, Runx3 regulates iron metabolism of the liver via modulation of BMP signalling.

The upstream regulatory mechanism of Runx3 is remained to elucidated. Extra- or intra-iron sensing proteins are possible candidates of a Runx3 modulator. The TFR2, a transferrin receptor, is functionally unknown although highly expressed in hepatocytes. Increase or decrease in intracellular iron induces redox change, which is recognized by redox proteins. The iron regulatory proteins, IRP1 and IRP2, possibly modulate Runx3 in a similar way with ferritin. Defining upstream modulator of Runx3 would be a first goal of future study.

Global transcriptome analysis of the Runx3 KO primary hepatocytes with or without hTF treatment showed that the BMP6 induction by iron stimuli was a specific regulation and that Runx3 abolished the regulation. However, regulation of the target genes of the BMP-Smad pathway was not observed in the transcriptome analysis. Of note, BMP7, another member of BMP ligands, was upregulated in the liver tissue of BMP6 null mice treated with iron-dextran, and exogenous BMP7 injected into the null mice induced hepcidin expression and reduced an abnormally high concentration of plasma iron. Therefore, a compensatory effect by BMP7 is a possible explanation for the insensitivity of the BMP-Smad pathway in the experiment using primary hepatocytes.
CONCLUSION

In conclusion, this work depicts Runx3 as a transcription factor of regulating hepcidin expression. Our findings highlight possible role of Runx3 in human iron metabolism disorders, such as haemochromatosis, hemosiderosis and atrasferrinemia.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Hyun-Yi Kim, Jong-Min Lee, Shujin Li and Seung-Jun Lee. carried out in vivo and in vitro experiments; Hyun-Yi Kim, Jong-Min Lee and Han-Sung Jung designed the study and performed data analyses; You-Soub Lee and Suk-Chul Bae provided Runx3 cKO mice; Hyun-Yi Kim, Jong-Min Lee and Han-Sung Jung wrote the manuscript, and Suk-Chul Bae and Han-Sung Jung reviewed the manuscript. All authors approved the author list, had access to the study data, and reviewed and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

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