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Cooperative interaction of Zhangfei and ATF4 in transactivation of the cyclic AMP response element

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Abstract **Zhangfei (ZF) is a basic region-leucine zipper protein that has been implicated in herpesvirus infection cycle and related cellular processes. Here we show both in vivo and in vitro data demonstrating that ZF is a novel cellular binding partner of activating transcription factor 4 (ATF4) (or CREB2). We found that ZF competed with ATF4 to form ATF4-ZF heterodimeric complexes through the bZIP regions. ZF enhanced ATF4 binding to the cAMP response element (CRE), and augmented activation of a CRE reporter by ATF4, in response to MEK1 activation. These results suggest an important role of ZF in the MEK1-ATF4 signaling pathway.**

Keywords: Activating transcription factor 4; Cyclic AMP response element; Leucine zipper; Mitogen-activated protein/extracellular signal-regulated kinase kinase; Protein–protein interaction; Zhangfei

1. Introduction

Zhangfei (ZF) [1] is a basic region-leucine zipper (bZIP) protein identified through its association with the Herpes Simplex Virus-1 related host cell factor protein-1, HCF-1 (or C1) [2,3]. HCF-1 has been shown to be involved in transcriptional regulation during herpesvirus latency as well as and cellular processes such as cell cycle progression (reviewed in [4]). It has been shown that ZF is mimicked by the viral protein VP16 in its ability to bind HCF-1. This observation has since led to the hypothesis that VP16 may be involved in cellular signaling events associated with HSV-1 latency/re-activation and related cellular signaling events [1].

ZF possesses a bZIP region that exhibits significant sequence homology with other members of the CREB/ATF bZIP family. These bZIP transcription factors form homo- and heterodimeric complexes through pairing of their leucine-zipper motifs, creating a DNA contact surface of their adjacent basic regions. Like another bZIP cellular factor CHOP/GADD153 however, ZF can homodimerize but is unable to bind DNA as a homodimer [1]. We have thus postulated that ZF need heterodimerize with other factors in order to bind target promoters and regulate the downstream genes.

Here we present both in vitro and in vivo data demonstrating that ZF interacts with activating transcription factor 4 (ATF4) (or CREB2) [5–7] through their bZIP regions. We found that ZF enhanced ATF4 binding to the cAMP response element (CRE), and augmented the transactivation potential of ATF4 in response to activation of the mitogen-activated protein kinases signaling pathway by MEK1.

2. Materials and methods

Clone construction and site directed mutagenesis. The ATF4 cDNA was amplified by PCR and cloned into pcDNA3.1 (Invitrogen) and pGEX-KG to make pcATF4 and pGEX-ATF4. The plasmids pFLAG-ZF and pHA-C-AE1 are based on pcDNA3.1 which expresses ZF with *N*-terminal FLAG epitope tag, or HA and FLAG tags on both ends. The pRL-SV40 plasmid [8] has the Renilla (*Renilla reniformis*) luciferase gene under the control of the SV40 immediate early promoter. ZFΔbZIP and ATF4ΔbZIP were generated by site-directed mutagenesis using the QuikChange II system (Stratagene).

Cell culture and calcium phosphate transfection. HEK 293, HEK 293-T7, and HeLa cells were cultured in Dulbecco's modified Eagle's medium (high glucose; Sigma) containing 10% (v/v) fetal bovine serum and 1% penicillin and streptomycin at 37 °C in a 5% CO_2 atmosphere. Cell cultures were grown to approximately 50–65% confluency prior to transfection using the calcium phosphate method [9,10].

Glutathione S-transferase (GST)-pulldown assay. Recombinant GST fusion proteins were produced in *Escherichia coli* BL21 (DE3) (Novagen) and purified using glutathione–Sepharose beads (Pharmacia). Proteins were ^{35}S-labeled using the TnT® Coupled Rabbit Reticulocyte Lysate System (Promega), and the pull down assays were performed as previously reported [11]. ZF and ATF4 proteins used in the competition assays were eluted from beads via thrombin cleavage. All protein sample concentrations were quantified by BioRad protein assay and by SDS–PAGE against bovine serum albumin standards using the Image Quant program (Amersham Biosciences).

Electrophoretic mobility shift assay (EMSA). Oligonucleotides representing the consensus cellular CRE sequence (5′-CCGGTGACGTCATCGCA) were annealed and end-labeled using [α-^{32}P]dCTP and the Klenow fragment of the *E. coli* DNA Polymerase (New England Biolabs). The assay was carried out using purified recombinant proteins described above, as reported previously [12].

Luciferase reporter assays. 40 h post-transfection, cell lysates were prepared and dual-luciferase® reporter assays were performed according to the manufacturer's protocol (Promega). Reporter activity was

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Abbreviations: ATF4, activating transcription factor 4; bZIP, basic region-leucine zipper; CRE, cyclic AMP response element; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated protein degradation; ERK, extracellular-regulated kinase; MAPK, mitogen-activated protein kinases; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; PERK, protein kinase-like endoplasmic reticulum kinase; UAS, upstream activation sequence; ZF, Zhangfei

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calculated as relative luciferase activity (firefly luciferase/*Renilla* luciferase) to correct for transfection efficiency. Assays were independently repeated at least three times and shown with standard errors.

Co-immunoprecipitation and immunoblotting. Cells were harvested and lysed 24 h post-transfection in the RIPA buffer for 20 min at 4 °C. Pre-cleared aliquots of lysate were incubated with specific immunoprecipitation antibodies for 12 h, followed by addition of protein G–Sepharose beads for 2 h at 4 °C. Proteins were detected by immunoblotting using ECL Plus (Amersham).

3. Results

3.1. ZF and ATF4 interact through their C-terminal bZIP domains

Recently, a potential association between the bZIP proteins ZF and ATF4 was suggested in a coiled-coil peptide array [13]; however, such high-throughput analyses are often prone to false positives and negatives. To assess if a *bona fide* interaction exists between these factors, we first chose to test it by competitive GST-pulldown assays, with GALAD-GFP and HCF-1 as controls (Fig. 1A). We found that radiolabeled ZF was able to bind ATF4 as well as itself (Fig. 1B). Addition of unbound ATF4 protein drastically reduced ZF binding to ATF4. Reciprocally, ^{35}S-labeled ATF4 could also interact with ZF and itself (result not shown). These results suggest that ZF and ATF4 interact through the same domain that is utilized in their homodimerization, i.e., the bZIP region.

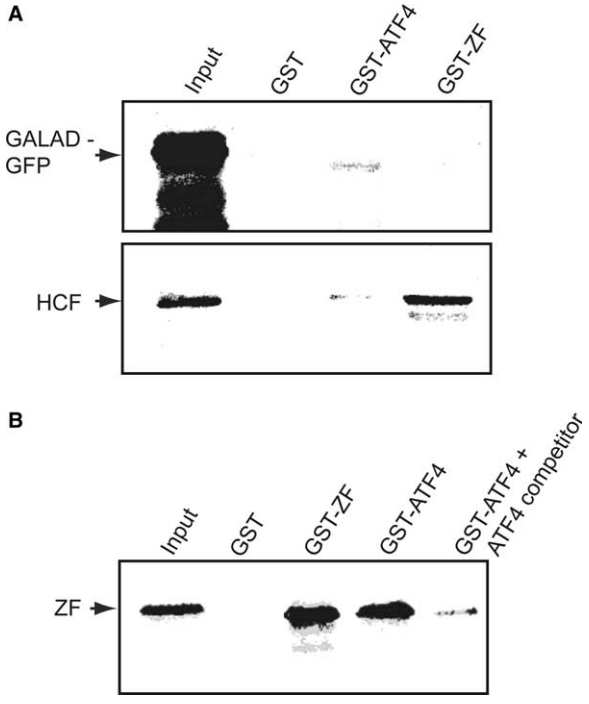

Fig. 1. ZF interacts with ATF4 in vitro as demonstrated by competitive GST-pulldown assays. GALAD-GFP and HCF-1 were used as negative or positive control respectively (A). In the competitive GST-pulldown assays (B) equal amount of bead-bound and eluted proteins were used as indicated. Bound proteins were eluted, resolved by SDS–PAGE and visualized using Typhoon 9400 phosphorimager. The "Input" lane represents 10% of the [^{35}S] methionine-labeled protein added to each respective assay.

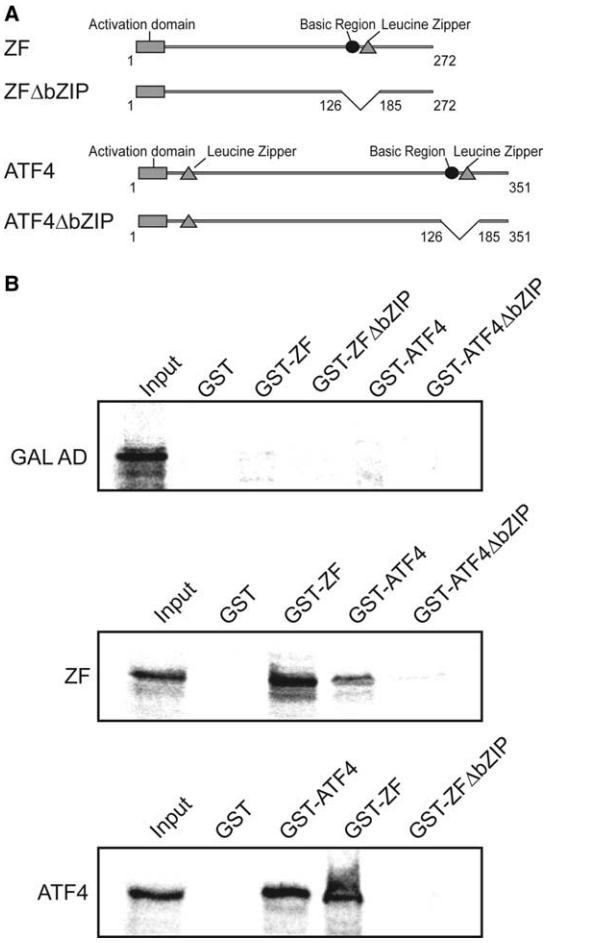

Fig. 2. ZF and ATF4 interact through their C-terminal bZIP domains. (A) Schematic illustration of ZF and ATF4 and their mutants. (B) GST-pulldown assays. Equivalent amount of bead-coupled proteins were used. Input lanes represent 10% of the [^{35}S]-labeled protein added to each pulldown assay.

To verify ZF and ATF4 indeed interact through their bZIP regions, mutants were generated in which the bZIP regions were removed (Fig. 2A). In a GST-pulldown experiment, we found that radiolabeled ZF and ATF4 could bind to the wild-type proteins, but not the bZIP deletion mutants (Fig. 2B). These observations indicate that ZF and ATF4 form classical bZIP dimers by interacting through their bZIP domains.

3.2. ZF associates with ATF4 in vivo

To examine whether ZF interacts with ATF4 within the cell, co-immunoprecipitation experiments were carried out. Endogenous ATF4 was induced in ZF-transfected HeLa cells using the endoplasmic reticulum (ER) stress-inducing agent tunicamycin [14]. We found that ZF could readily co-precipitate with ATF4, as detected by an ATF4 antibody (Fig. 3A). It was noticed that two prominent ATF4 bands were present in the ZF precipitate. These doublet bands likely represent two forms of ATF4 of different phosphorylation status, as reported previously [15]. Reciprocal immunoprecipitation in ATF4 and ZF-transfected cells by the ATF4 antibody also confirmed the result (Fig. 3B).

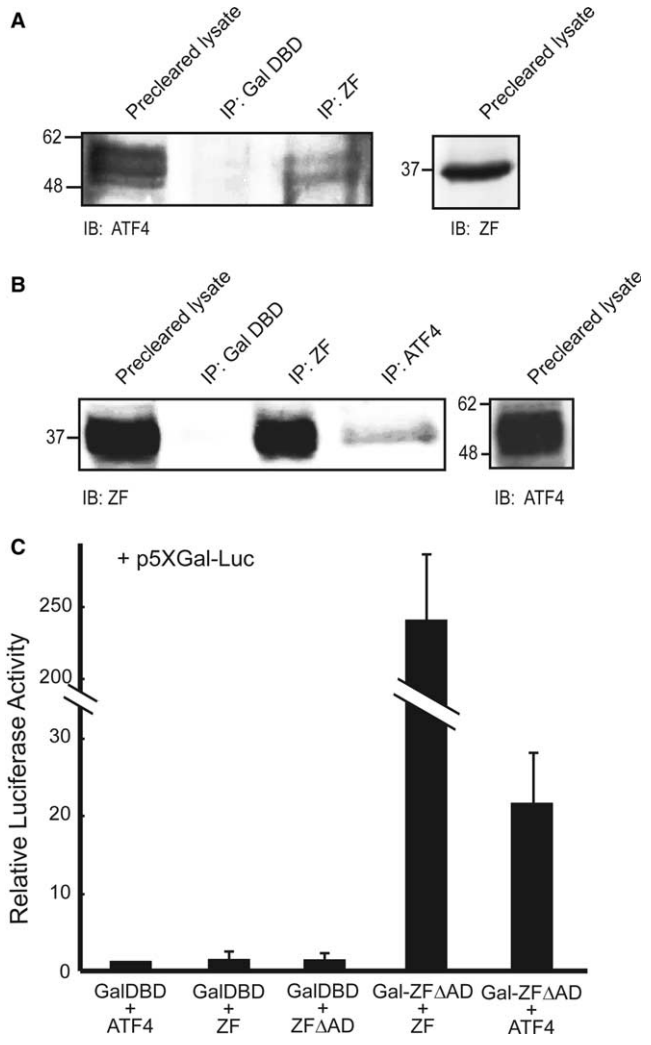

Fig. 3. ZF interacts with ATF4 in vivo. (A) ZF interacts with endogenous ATF4. HEK-293 cells were transfected with ZF-expressing plasmid pHA-C-AE1, treated with 4 μg/mL of tunicamycin for 9 h to induce endogenous ATF4 and with 0.01 mM MG132 for 6 h prior to lysis. Pre-cleared lysate was immunoprecipitated with ZF antibody RB5 [1] or GAL DBD antibody (control). Eluted proteins were detected with ATF4 antibody C-20 (Santa Cruz Biotech) by immunoblot analysis. (B) Reciprocal immunoprecipitation in ATF4 and ZF-transfected HEK 293 cells. (C) Modified mammalian two-hybrid assay. HEK293T cells in 6-well plates were transfected using 1 μg of GAL4-Luciferase reporter, 40 ng of Renilla luciferase reference plasmid pRL-SV40 and 1 μg of each of the various plasmids as indicated.

In order to confirm the observation that ZF and ATF4 interact in vivo, a modified mammalian two-hybrid assay was performed which utilized the strong activation domains (AD) of ZF and ATF4. In this system GALDBD-ZFΔAD cannot activate the GAL-UAS reporter alone; however, if ATF4 is recruited to the GAL4-UAS reporter by the ZF protein, transactivation of the luciferase gene will occur. The ability of ZF to homodimerize was used as a positive control (Fig. 3C, column 4). We observed that the interaction between GALDBD-ZFΔAD and ATF4 resulted in over 20-fold activation over the background, although it was not as high as that produced by ZF homodimerization (Fig. 3C, column 5). Thus we concluded from the coimmunoprecipitation and mammalian two-hybrid experiments that ZF can indeed interact with ATF4 within the cell.

3.3. Enhanced ATF4 binding to CRE in the presence of ZF

After having confirmed the interaction between ZF and ATF4, we were interested in determining the potential outcome of such an interaction. ZF is known to have the unique property that it does not bind DNA by itself. Therefore, we investigated whether the heterodimerization of ZF and ATF4 could change the DNA-binding property of ATF4. It is known that ATF4 can bind CRE enhancer elements for transcriptional regulation of downstream targets. To ascertain if ZF affects ATF4 binding to CRE, we performed an EMSA (Fig. 4A). As reported previously [1], ZF by itself was unable to bind CRE

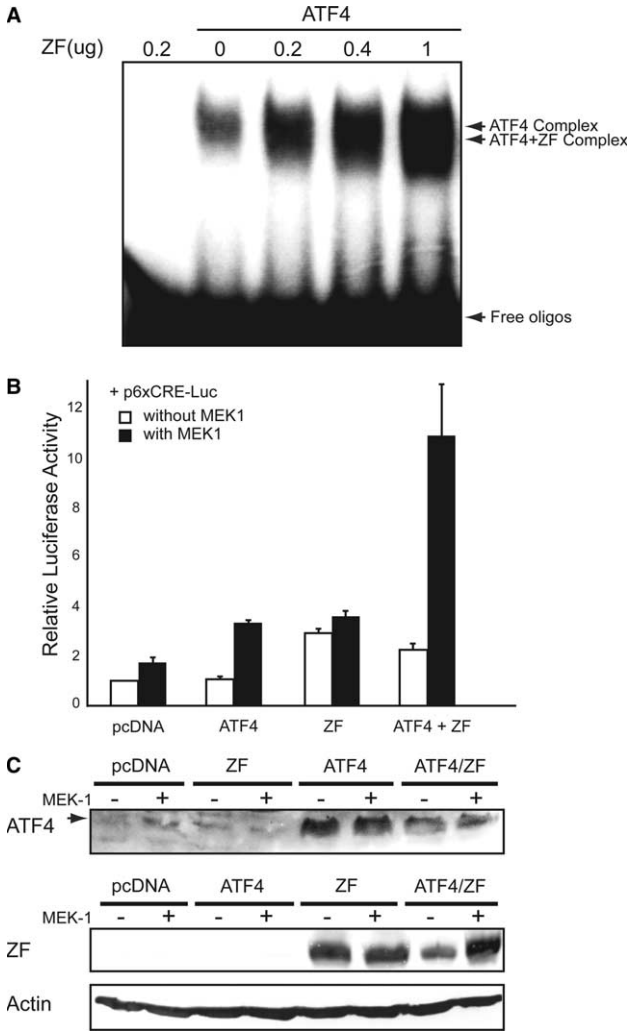

Fig. 4. (A) ZF enhances binding of ATF4 to the consensus CRE. In each EMSA except for lane 1, 200 ng of GST-ATF4 was added to all lanes with indicated amount of GST-ZF protein. (B) ZF enhances ATF4 transactivation of a CRE-containing promoter in response to MEK1 activation. HeLa cells were transfected with 1 μg of 6× CRE-Luc [40], 40 ng of RL-SV40 (and 1 μg of each of pcDNA, pcATF4 and pFLAG-ZF plasmids, with or without the addition of plasmid pFC-MEK1 which constitutively expresses MEK1(CA) (Stratagene). (C) Immunoblot analysis of ATF4 and ZF protein concentrations in the cell lysates as transfected in the dual luciferase assays. β-actin was detected with a monoclonal antibody (AC-15, Sigma) and used as a control.

(Fig. 4A, lane 1). Although ATF4 was able to effectively bind to the radiolabeled CRE probe without ZF (Fig. 4A, lane 2), addition of ZF was found to enhance ATF4 binding to the CRE in a dose-dependent manner (Fig. 4A, lanes 2–5). We noticed that with increasing amount of ZF added to the reaction, the protein–DNA complex band became more intense and smeared further down the gel (migrated faster). As the calculated molecular weight of the ATF4/ZF heterodimer (67.5 kDa) is only 10 kDa smaller than that of ATF4 homodimer (77.2 kDa), this faster-migrating smearing seems to indicate that ATF4/ZF heterodimer formed a complex with CRE at a higher affinity, which was not well resolved in the non-denaturing gel.

3.4. ZF augments ATF4 transactivation potential on CRE-containing promoters in response to MEK1 activation

To assess whether the enhancement of ATF4 CRE binding by ZF impacts the activation of CRE-containing promoters, a reporter assay was carried out. Since it has been reported that ATF4 itself is activated by the mitogen-activated protein/extracellular signal-regulated kinase (MEK/ERK) kinase pathway [15,16], a constitutively active form of MEK1 kinase, MEK1(CA), was included in the assay. Without MEK1(CA), co-transfection of ATF4 and ZF did not cause significant enhancement to the activation level (Fig. 4B, blank columns). In the presence of MEK1(CA), however, co-transfection of ZF and ATF4 led to synergistic activation of the CRE reporter that was significantly higher than that of ZF or ATF4 alone (Fig. 4B, filled columns).

It is notable that transfection of ZF alone led to moderate reporter activation. Since ZF does not bind CRE by itself, we believe that this was due to ZF interaction with endogenous ATF4, as observed in the immunoblotting results (Figs. 3A and 4C). In all, these results suggest that ZF can augment the transactivation potential of ATF4 on CRE-containing promoters in a MEK1-dependent manner.

4. Discussion

In this study we report evidence demonstrating that ATF4 is a novel interacting partner of the cellular protein ZF. Mutagenesis studies showed that the bZIP region of ZF and ATF4 was indispensable for their interaction, suggesting that these two proteins likely form heterodimers through this region. The interaction of ZF enhanced ATF4 binding to the CRE element, and augmented the activation potential of ATF4 in a MEK1-dependent manner. These results implicate a potential role of ZF in ATF4-related regulated cellular processes.

With ZF lacking a consensus DNA-binding basic domain [1,17], the structural mechanism for how ZF enhances ATF4 binding to CRE is unclear. It is known, however, another bZIP protein, CHOP/GADD153 which also does not bind DNA homodimerically, enhances C/EBP binding of a DNA element in a similar fashion [18]. In addition to binding CRE homodimerically, ATF4, like most bZIP factors, can also bind other DNA sequences through formation of heterodimers [19]. Dimerization via its C-terminal bZIP domain with a myriad of bZIP proteins [5,20–26] allows ATF4 to bind to specific enhancers and selectively regulate target genes. One of the first identified binding targets of ATF4 is the CRE enhancer [6,22,27]. Although ATF4 is able to bind to CRE, it is unique from other bZIP factors in that it can both positively and negatively regulate transcription through this enhancer (reviewed in [19]). Phosphorylation of ATF4 at serine 245 by the growth factor regulated kinase, RSK2, has been linked to ATF4 activation, rendering ATF4 a critical role in skeletal formation [15]. In addition to the fact that RSK2 is a direct target of the MEK/ERK pathway [28,29], the significance of the MEK/ERK pathway in activation of ATF4 has also been inadvertently revealed in a study investigating transcriptional regulation of the HO-1 hematopoiesis factor. Instrumentally, it was found that transcriptional activation of the *ho-1* gene is dependent on both ATF4 expression and MEK/ERK activation [30]. It is worth noting that in our transient transfection assays, the activation potential of ATF4 increased over 3-fold in response to MEK1(CA), whereas ZF showed minimal changes upon MEK1 activation (Fig. 4, columns 2 and 3). We therefore believe that the phosphorylation of ATF4 (not ZF) mediated by MEK1 led to the significant increase of CRE-reporter activation by the ATF4-ZF complex.

ATF4 has been implicated in many developmental and cellular processes such as skeletal [15] and eye formation [31–33], and hematopoiesis [32]. Specifically, the functional interaction of ZF and ATF4 may also have relevance in the mammalian unfolded protein response (UPR) pathway of ER stress responses. To date all known signaling pathways of UPR lead to activation of bZIP transcription factors [34], with ATF4 being a key factor in the protein kinase-like endoplasmic reticulum kinase (PERK) pathway of the UPR. Activation of PERK may lead to translational activation of ATF4 expression [35], which in turn regulates its downstream target genes, such as the ones involved in the ER-associated protein degradation (ERAD) [34]. In addition to ATF4, ZF may also interact with other UPR-related bZIP proteins (e.g., ATF6 and XBP1), as suggested by the coiled-coil peptide arrays [13] although still unconfirmed in vivo. The ability of these bZIP proteins to form homo- and heterodimers, as well as their tissue specificity, provides the cell with a complex combinatorial regulatory network during the UPR.

Recently, we have found that CREB3 (previously called as Luman or LZIP), another bZIP protein that binds HCF-1 like ZF and VP16 [9,11,36–38], can bind and activate transcription from the unfolded protein response element (UPRE) and induces the ERAD-related protein, EDEM [12]. Interestingly ZF is also a repressor of CREB3 [39]. All this circumstantial evidence and its interaction with ATF4 suggest a potential role of ZF in the UPR. It would be of interest to confirm and investigate the potential interaction of ZF with these UPR-related bZIP factors, and to study how such interactions might function in the complex gene regulation events during the mammalian UPR.

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