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# Myogenic repressor I-mfa interferes with the function of Zic family proteins☆

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**Abstract**

Zinc finger proteins belonging to the Zic family control several developmental processes such as patterning of the axial skeleton. Here we mapped the transcriptional regulatory domains in Zic2 protein and identified a protein which specifically binds to one of them. In the mapping experiments, an amino-terminal region was identified as transcriptional regulatory domains. A search for proteins binding to the amino terminal domain of Zic2 revealed that inhibitor of MyoD family (I-mfa) protein, which has been identified as a repressor of myogenic helix–loop–helix class transcription factors, can physically interact with the amino terminal domain. When Zic1-3 and I-mfa proteins were co-expressed in cultured cells, nuclear import of the Zic proteins was inhibited. Consequently, I-mfa inhibited transcriptional activation by the Zic proteins in cultured cells. These results suggest that the physical and functional interaction between Zic and I-mfa proteins can play a role in the vertebrate development.


*Keywords:* Zic; I-mfa; Protein-to-protein interaction; Transcriptional regulation; Subcellular translocalization; Skeletal development

Zic family zinc finger proteins play critical roles in a variety of developmental processes. For example, *Zic1*, *Zic2*, and *Zic3* mutant mice manifest deformities of the vertebrae and ribs [1–3], and overexpression of *Xenopus Zic1*, *Zic2*, *Zic3*, and *Zic5* in frog embryos enhances neural crest tissue generation [4–6]. In addition, human *ZIC2* mutation causes a congenital forebrain anomaly (holoprosencephaly) [7] while human *ZIC3* is a causal gene of the left–right axis disturbance (heterotaxy) [8].

In human and mouse, Zic family consists of five structurally related proteins (Zic1–5) sharing five tandemly repeated C2H2 motifs [7–13]. Their homologues have been identified in various species including *Drosophila melanogaster* (Odd-paired (Opa), [14]) and *Caenorhabditis elegans* (REF-2, [15]), both of which have essential roles in the invertebrate development. Interestingly, the zinc finger domains of Zic proteins are also conserved in those of Gli and NKL/Glis families, and Zic and Gli genes appear to be cooperating or counteracting in some developmental processes [1,16]. These findings raise a possibility of functional interaction within the Zic/Gli/NKL zinc finger protein superfamily.

Although a number of evidences suggest the biological importance of the Zic family proteins, the molecular mechanisms underlying the developmental processes are largely unknown. In previous studies we found that Zic proteins are located in cell nuclei and can bind a consensus nonamer Gli binding sequence, 5′-TGGGT GGTC-3′, through their zinc finger domains [9,17] although their binding affinity to the target sequence was weaker than that of Gli proteins [9,17]. In terms of transcriptional regulation, they activate transcription from various promoters in reporter gene co-transfection assays [17]. We also found that Zic proteins physically

☆ *Abbreviations:* DBD, DNA binding domain; FL, full-length; IB, immuno-blotted; *I-mfa*, inhibitor of MyoD family; IP, immuno-precipitated; MLP, major late promoter of adenovirus; *opa*, odd-paired; TK, thymidine kinase; ZF, zinc finger; *Zic*, zinc finger protein of the cerebellum; ZOC, Zic-Opa conserved.

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and functionally interact with Gli proteins through their zinc finger domains and affect the subcellular distribution of Gli proteins and the sequence-dependent Gli-mediated transactivation [18]. These results led us to hypothesize that Zic proteins coordinate transcriptional status suited for each developmental context in concert with other transcription regulatory factors.

In this study, we first identified the transcription regulatory domain of the Zic2 protein to determine how Zic proteins regulate the specific developmental processes. Next, we performed a yeast two-hybrid assay to characterize molecules that specifically interact with Zic proteins, and found that inhibitor of MyoD family (I-mfa) protein, which was known to repress myogenic transcription factors such as MyoD, Myf5, and myogenin, binds the amino terminal domain of the Zic2 protein. I-mfa functions as a transcriptional repressor that inhibits the transactivation activity of Zic proteins by retaining them in the cytoplasm.

## Materials and methods

*Yeast two-hybrid screening*. The pLexA-Zic2 (1–255) fusion vector, Zic2 bait, was constructed by inserting the region encoding amino acids 1–255 of mouse *Zic2* in-frame into the *Eco*RI–*Sal*I site of the yeast expression plasmid pBTM116 (a gift from Dr. P. Bartel and Dr. S. Fields). The region of *Zic2* introduced into this vector encompasses the entire N-terminal domain of *Zic2*. Expression of the resulting LexA-Zic2 (1–255) fusion protein was confirmed by Western blotting (data not shown). The mouse embryo PCR library in pVP16, constructed from cDNA from embryonic day (E) 10.5 CD1 mouse embryonic poly(A)$^+$ RNA, was kindly provided by Dr. S. Hollenberg. The yeast strain L40 (MATa his3Δ200 trp1-901 leu2-3, 112 ade2 LYS2::(lexAop)$_4$-HIS3 URA3::(lexAop)$_8$-lacZ GAL4 gal80) was used in the screening of $\sim 5 \times 10^6$ colonies. The yeast transformation was performed by the usual lithium acetate method with some modifications. The lexA-fusion plasmid was first introduced into L40 by selecting for growth on Trp-, Ura-plates. After that, 100 μg of library DNA was sequentially transformed under Leu selection. The transformants were grown on Trp-, Ura-, Leu-, Ade-, and His-selection plates, supplemented with 5 mM of 3-amino-1,2,4-triazole (Sigma). Colonies selected for strong interactions with the bait under His selection were further assayed for β-galactosidase activity. Plasmids from β-galactosidase-positive colonies were isolated and transformed into *Escherichia coli* HB101 (Takara Bio), and cDNA inserts were sequenced.

*Plasmid constructs*. To express proteins containing hemagglutinin epitopes (HA), HA-Zic1 was constructed by inserting the cDNA fragment containing the entire open reading frame of mouse *Zic1* into the *Eco*RI site of pHM6 (Roche). Similarly, HA-Zic2 and HA-Zic3 were produced by inserting the entire protein coding region of mouse *Zic2* and *Zic3* into the *Bam*HI–*Eco*RI site and *Xho*I site of pcDNA3HA (a gift from Dr. T. Nakajima), respectively. The HA-tagged *Zic2* deletion series was generated by inserting the relevant amino acid regions in-frame into pcDNA3HA. These deletions were as follows: HA-Zic2-(1–100), -(1–140), -(1–255), -(1–333), -(1–363), -(1–393), -(1–419), -(1–485), -(57–531), -(102–531), -(141–531), and -(256–531). The deletion series for *Zic2* fused with the GAL4 DNA binding domain (GAL4DBD) was constructed by inserting the relevant amino acid regions in-frame into the *Bam*HI–*Xba*I site of pM vector (Clontech).

Full-length mouse *I-mfa* was cloned from an E10.5 mouse embryo lambda cDNA library (Stratagene) by polymerase chain reaction (PCR). The expression vector Flag-I-mfa was constructed by inserting the full-length *I-mfa* in-frame into the *Eco*RV site of pCMVtag2 (Stratagene). In addition to the full-length constructs, two deletion constructs, Flag-I-mfa-(1–161) and -(141–247), were produced, by inserting the PCR-amplified fragments containing amino acids 1–161 and 141–247, respectively, in-frame into the *Eco*RV site of pCMVtag2.

A luciferase reporter plasmid driven by a TK promoter, TK-Luc, was constructed by ligating a herpes simplex virus thymidine kinase promoter (TK promoter) derived from plasmid pRL-TK (Promega) into the *Hin*dIII and *Bgl*II sites of the pGL2-Basic vector (Promega). A luciferase reporter plasmid driven by an SV40 promoter, SV40-Luc (pGL2-Promoter Vector), and pGL3-Basic reporter plasmid that lacked promoter and enhancer sequences were purchased from Promega. A luciferase reporter plasmid driven by a major late promoter of adenovirus (MLP), pG5Luc, was kindly provided by Dr. Y. Makino. The internal standard, pRL-EF in which *Renilla* luciferase was driven by elongation factor 1 promoter, was constructed by inserting the *Renilla* luciferase gene of a pRL-TK vector (Promega) into a pEF-BOS vector [19].

*Transfection*. 293T cells and NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), while C3H10T1/2 cells were maintained in basal medium Eagle supplemented with 10% FBS. At 70–80% confluence, the cells were transfected with Lipofectamine Plus transfection reagent (Invitrogen) according to the manufacturer's instructions. Cells were harvested 24 h after transfection and processed for immunoprecipitation, reporter assay, and immunofluorescence staining.

*Immunoprecipitation*. 293T cells were cotransfected with expression vectors encoding Flag-I-mfa and HA-Zic, and 24 h later, cells were lysed in immunoprecipitation buffer, containing 25 mM Hepes (pH 7.2), 0.5% NP-40, 150 mM NaCl, 50 mM NaF, 2 mM $Na_3VO_4$, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml aprotinin. A 5 μl volume of anti-HA Y-11 polyclonal antibody (Santa Cruz Biotechnology) and 20 μl of a 50% suspension of protein G (Pierce) were added to the appropriate aliquots of the cell extract. The protein G complex was washed five times with the immunoprecipitation buffer after 2 h incubation. Protein complexes were then eluted with SDS sample buffer and analyzed by Western blotting using anti-Flag M2 monoclonal antibody (Sigma) and an ECL detection system (Amersham). Some immunoprecipitation experiments were carried out by using anti-HA agarose conjugate (clone HA-7, Sigma) instead of HA Y-11 and protein G.

*Reporter assay*. NIH3T3 and C3H10T1/2 cells were transfected with 100 or 180 ng each of the luciferase reporter plasmids and 100, 200 or 400 ng each of the expression vectors, together with 10 ng of pRL-EF as an internal standard. After 24 h, luciferase activity was measured as described [17]. All the results were based on the three independent experiments that showed reproducible results.

*Immunofluorescence staining*. NIH3T3 cells were transfected with appropriate expression vectors. Twenty-four hours after transfection, cells were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 10 min at room temperature. The cells were then incubated in blocking buffer containing 2% goat serum and 0.1% Triton X-100 for 1 h at room temperature, and stained with anti-HA antibody and anti-Flag antibody. Alexa 568-conjugated anti-rabbit IgG and Alexa 488-conjugated anti-mouse IgG (Molecular Probes) were subsequently used for fluorescence staining.

## Results

### *Identification of the Zic2 transcriptional activation domain*

In a previous study, we showed that Zic proteins bound the Gli binding sequence and activated tran-

scription [17]. To better understand the mechanism of Zic-induced transcriptional activation in the present study, we conducted reporter assays to identify transcriptional activation domains. First, we produced a series of C-terminally and N-terminally deleted constructs of HA-tagged *Zic2* as expression vectors (Fig. 1A). As reporter genes, we used the firefly luciferase genes under the regulation of the thymidine kinase (TK) promoter. C3H10T1/2 cells were co-transfected with 200 ng of the expression vectors and 180 ng of the luciferase reporter plasmids. As a result, C-terminally deleted series showed that deletion of the ZF3 most

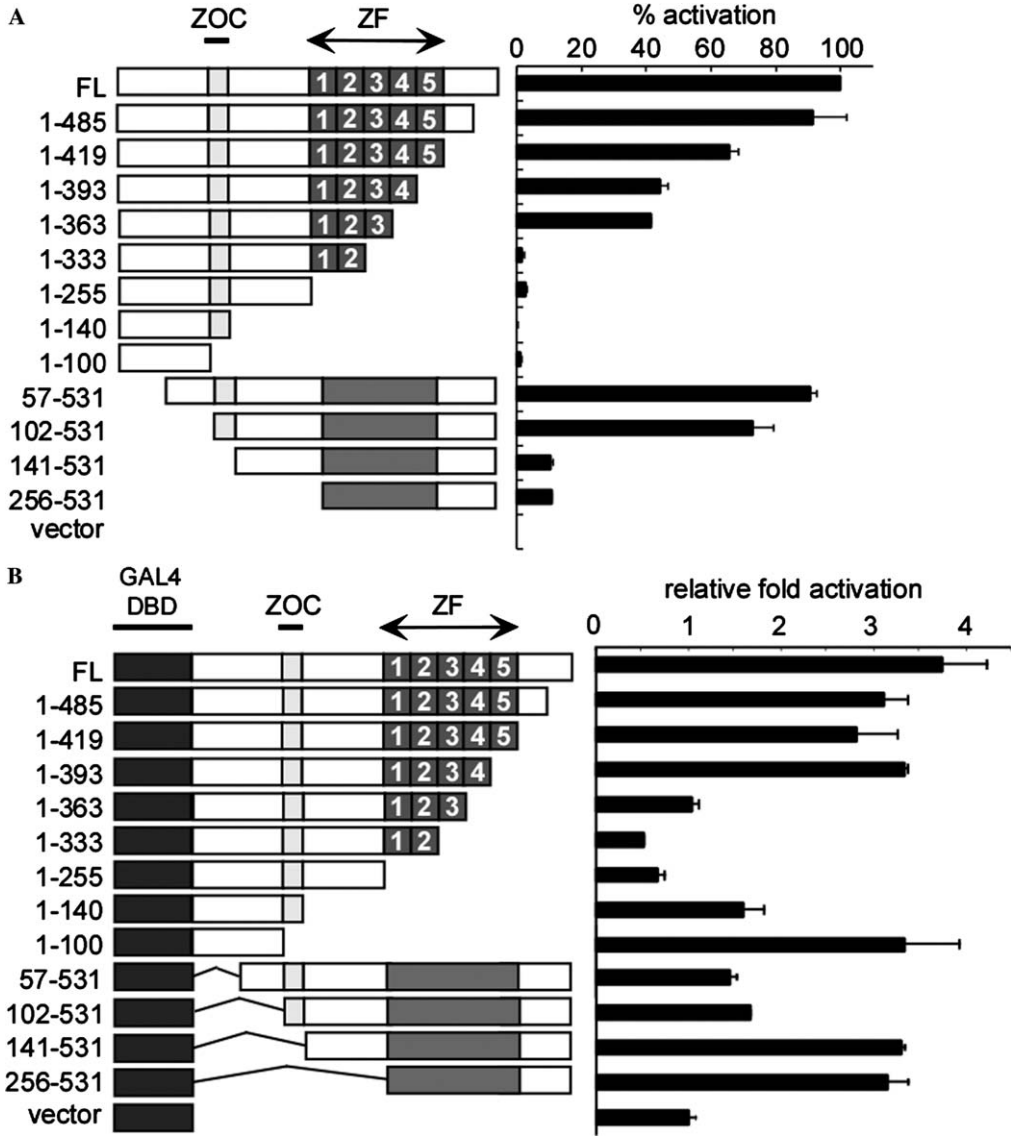


Fig. 1. (A) Identification of Zic2 activation domains by using C-terminally and N-terminally deleted constructs. Left, schematic diagrams of HA-Zic2 expression vectors. Various regions of *Zic2* (amino acid number indicated at the left of the bar) were subcloned into the pcDNA3HA expression vector. C3H10T1/2 cells were transfected with 200 ng of HA-Zic2 expression vectors along with 180 ng of reporter genes (TK-Luc), and luciferase activity was measured. Each luciferase activity value was normalized to the activity of an internal control (*Renilla* luciferase). Right graph, means of relative transactivation ability of each construct in the reporter activity from duplicate samples. Luciferase activity from full-length construct is represented by 100%, and 0% is defined as luciferase activity with the empty vector (pcDNA3HA). (B) Identification of activation domains by means of GAL4-Zic2 constructs. Left, schematic diagrams of GAL4-Zic2 expression vectors. Various regions of *Zic2* (amino acid number indicated at the left of the diagrams) were subcloned into the pM expression vector that contains the coding region of the GAL4 DNA-binding domain (GAL4DBD). Right graph, means of fold activation of reporter activity from duplicate samples. C3H10T1/2 cells were transfected with 200 ng of GAL4-Zic2 expression vectors along with 180 ng of reporter genes (GAL4-MLP-Luc) containing GAL4 DNA-binding sequences, and the luciferase activity was measured. Each luciferase activity value was normalized to the activity of an internal control (*Renilla* luciferase). Normalized luciferase activity was divided by the activity obtained by transfection of the vector control (pM). Error bars represent standard deviations. FL, full length; ZF, zinc finger; and ZOC, Zic-Opa conserved motif. In the diagrams, ZOC is indicated by a light gray box and ZF as a dark gray box, with the numbers 1–5 corresponding to each C2H2 unit.

drastically reduced the transcription activation capacity. Deletion of ZF5 and C-terminal flanking the ZF5 also reduced the extent of activation. In the N-terminally deleted series, the region between amino acids 102 and 141, which contains the Zic-Opa conserved motif (ZOC motif) (amino acids 113–124, [10]), showed a transcriptional activation capacity. However, the ZOC motif alone was insufficient to cause transcriptional activation. These findings suggest that there are two regions required for transcriptional activation, one in the ZF region and the other in the ZOC motif-containing region (ZOC domain).

Next, we used GAL4 fusion protein reporter system to evaluate the transactivation properties of Zic2. We produced GAL4-Zic2 fusion proteins consisting of the GAL4 DNA binding domain (GAL4DBD) linked to a series of C-terminally and N-terminally deleted fragments of Zic2 (Fig. 1B). These chimeric GAL4-Zic2 fusion proteins were co-transfected into C3H10T1/2 cells with the pG5Luc reporter gene containing five tandem copies of the GAL4 DNA binding motif linked to the luciferase gene under the regulation of the major late promoter of adenovirus (MLP). In the C-terminally deleted series, deletion of ZF3 and ZF4 significantly reduced the activation capacity. However, the extreme N-terminus (amino acids 1–100) of Zic2 fused to the GAL4DBD revealed unexpected transcriptional activation activity in contrast to the comparable region of HA-Zic2. The extent of the activation was almost the same to that of full-length Zic2. The N-terminus half fragment of the Zic2 protein (amino acids 1–255) almost completely masked the N-terminal activator domain, suggesting that the region between amino acids 100 and 255, which contains ZOC, constitutes a transcriptional repression domain. These results were further verified in the experiments using the N-terminally deleted constructs, where the ZOC motif was shown to play a key role in the repressor activity.

In summary, we found that the region between amino acids 333 and 485 containing zinc fingers three to five (ZF3–5) and a part of the C-terminus of Zic2 functioned as a transcriptional activator domain, whereas the ZOC domain functioned as either an activator domain or a repressor domain, depending on the assay system used. The extreme N-terminus (amino acids 1–100) was adequate to cause transcriptional activation only when linked to the GAL4DBD.

### *Zic family members specifically interact with I-mfa*

To search for factors that functionally interact with Zic family proteins, we performed yeast two-hybrid screening, which is based on protein-to-protein interaction to restore the transcriptional activation function of two separate proteins one of which carries a DNA-binding domain and the other of which carries a transcriptional activation domain. The entire N-terminal half of the mouse Zic2 protein (1–255) was used as bait in this screening, since the region contained a critical domain in the transcriptional regulation by Zic2 protein and the binding molecules for it have never been characterized. The screening identified I-mfa, a MyoD family inhibitor, as a binding partner that can be predicted to have a biologically significant relationship to Zic proteins. The Zic2/I-mfa binding was reproducible in mammalian cells. When expression vectors encoding HA-tagged *Zic1*, *Zic2*, and *Zic3* (HA-Zic1, HA-Zic2, and HA-Zic3), and Flag-tagged *I-mfa* (Flag-I-mfa) were transfected into 293T cells, Flag-I-mfa was immunoprecipitated with the anti-HA antibody in the presence of HA-Zic1, HA-Zic2, and HA-Zic3 (Fig. 2A). These results indicate that I-mfa binds to all the three Zic proteins.

To determine which domain of I-mfa interacts with Zic2, we used two deletion constructs, Flag-I-mfa-(1–161) and Flag-I-mfa-(141–247), in combination with full-length HA-Zic2 in immunoprecipitation assays (Fig. 2B). The results showed that the region corresponding to amino acids 161–247, which contains a cysteine-rich C-terminus, a characteristic structure of I-mfa, was sufficient to bind to Zic2. Subsequently, the I-mfa-binding domain of Zic2 was mapped to a region between amino acids 100 and 255 by immunoprecipitation assay using full-length Flag-I-mfa and a series of N-terminally deleted (Fig. 2C) and C-terminally deleted (Fig. 2D) Zic2. The I-mfa binding domain may consist of two separable elements, one in the ZOC region and the other in the remaining region (141–255), because either of the two regions was sufficient to bind I-mfa.

### *I-mfa inhibits transcriptional activities of Zic family proteins*

In a direct attempt to confirm a role for I-mfa in Zic activity, we used reporter assays to analyze the effect of co-transfection of Zics and I-mfa on Zic-activated transcription. We used the firefly luciferase gene controlled by the thymidine kinase (TK) or by the SV40 promoter (SV40-Luc) as reporter genes, and HA-Zic1, HA-Zic2, HA-Zic3, and Flag-I-mfa were prepared as expression vectors. Zic proteins activated all reporter genes, whereas I-mfa suppressed the baseline transcription a little. When the Zic and I-mfa expression vectors were transfected into NIH3T3 cells in different ratios, I-mfa was found to efficiently suppress transcriptional activation by Zic1, Zic2, and Zic3 in an I-mfa dose-dependent manner (Figs. 3A and B). Similarly, the I-mfa repression of the transcriptional activity of Zic proteins was also observed for all reporter gene constructs introduced into C3H10T1/2 cells (data not shown). Next, we examined if the transcriptional repression was caused by the Zic/I-mfa interaction itself. For this purpose,

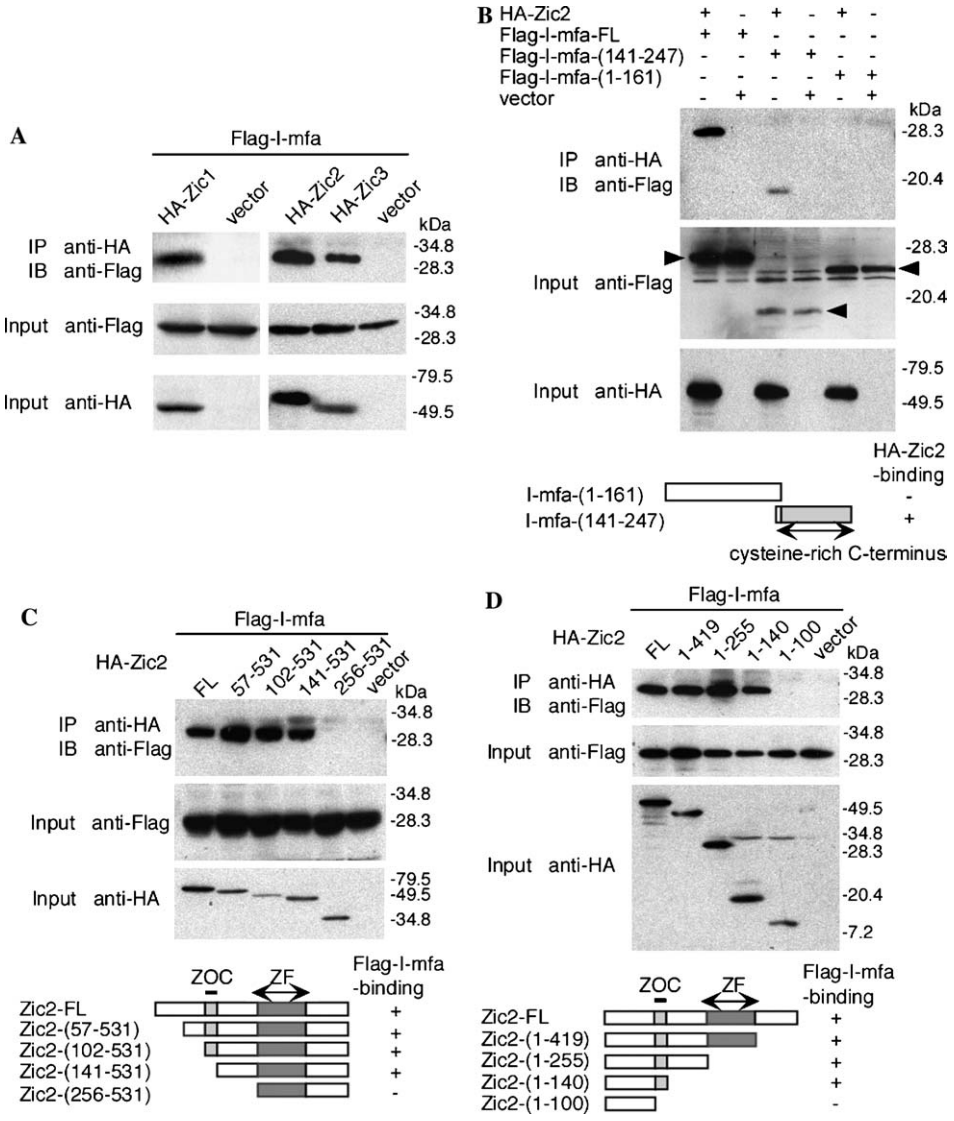


Fig. 2. I-mfa specifically interacts with Zic proteins. (A) Flag-I-mfa was cotransfected with HA-Zic1, HA-Zic2, HA-Zic3, and HA-tagged empty vector into 293T cells. Cell lysates were subjected to immunoprecipitation and immunoblotting using the antibodies indicated (top). Expression of Flag-tagged (middle) and HA-tagged (bottom) proteins was confirmed by immunoblotting. (B) Mapping the Zic2-binding domain in I-mfa. The two regions of I-mfa protein indicated were prepared as Flag-tagged proteins (Flag-I-mfa-(1–161) and Flag-I-mfa-(141–247)). Flag-tagged I-mfa expression vectors were co-transfected with HA-Zic2 or HA-tagged empty vector into 293T cells. Cell lysates were subjected to immunoprecipitation and immunoblotting using the antibodies indicated (top). Expression of Flag-tagged (middle, indicated by closed arrowheads) and HA-tagged (bottom) proteins was confirmed by immunoblotting. (C,D) Mapping the I-mfa-binding domain in Zic2. A series of N-terminally deleted (C) and C-terminally deleted (D) fragments of Zic2 were prepared as HA-tagged proteins. Flag-tagged full-length I-mfa expression vector was co-transfected with a series of HA-tagged Zic2 mutants or HA-tagged empty vector into 293T cells. Cell lysates were subjected to immunoprecipitation and immunoblotting using the antibodies indicated (top). Expression of Flag-tagged (middle) and HA-tagged (bottom) proteins was confirmed by immunoblotting. The numbers refer to amino acids. FL, full length; ZF, zinc finger; and ZOC, Zic-Opa conserved motif.

Flag-I-mfa and HA-Zic2-(256–531), which lacks the I-mfa-interacting region of Zic2 (amino acids; 100–255), were co-transfected into NIH3T3 cells. Although Flag-I-mfa suppressed the HA-Zic2-(256–531)-activated transcription to some extent, the extent of the inhibition was almost same as that of baseline inhibition by I-mfa, revealing that the significant transcriptional repression depends on the specific interaction between Zic proteins and I-mfa (Fig. 3C).

### *I-mfa can retain nuclear Zic proteins in the cytoplasm*

In previous studies, we showed that Zic proteins are localized in the nucleus of cultured cells and cerebellar granule cells ([9,18] Figs. 4A and B). I-mfa protein, on the other hand, has been found to be localized predominantly in the cytoplasm, although weak staining has been detected in the nucleus ([20], Figs. 4C and D). To determine whether I-mfa affects the localization of

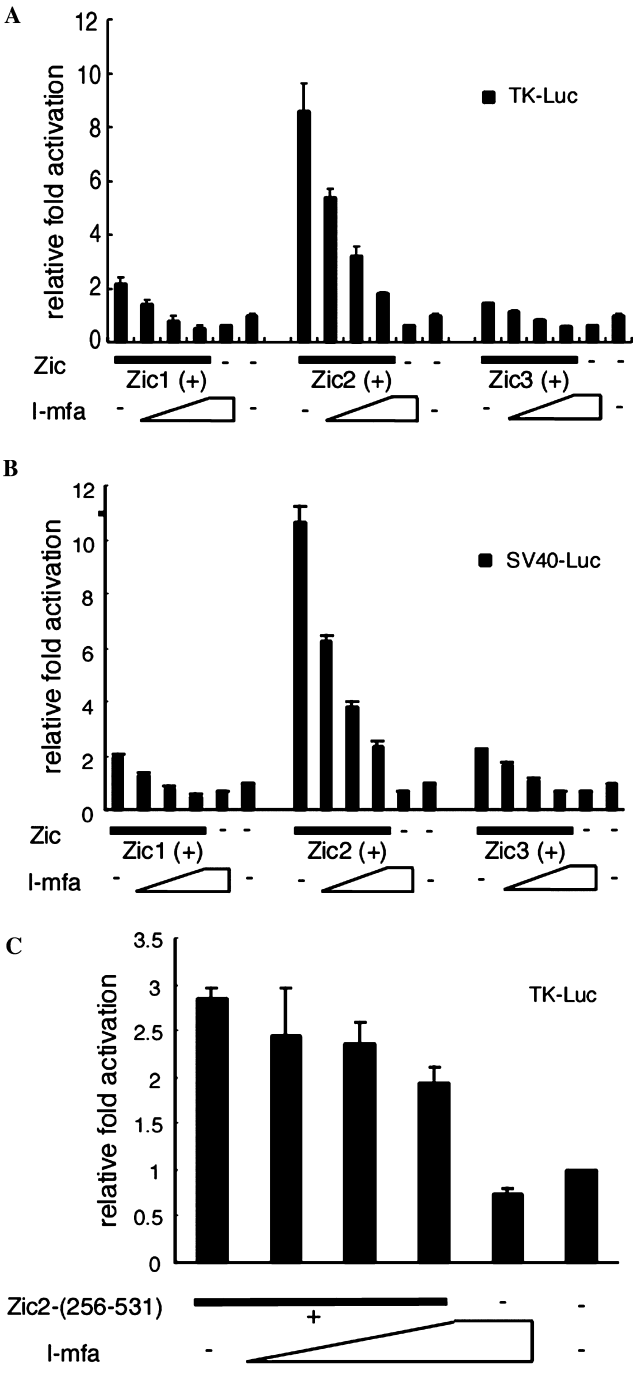


Fig. 3. Repression of transcriptional activity of Zic family proteins by I-mfa. (A) NIH3T3 cells were co-transfected with 100 ng of Zic expression vectors and increasing amounts (100–300 ng) of I-mfa expression vector along with reporter genes (TK-Luc). Total amounts of expression vector were kept constant by adding pcDNA3HA vector. Each luciferase activity value was normalized to the activity of an internal control (*Renilla* luciferase). Normalized luciferase activity from duplicate samples is shown relative to the empty vector, pcDNA3HA. (B) NIH3T3 cells were co-transfected with 100 ng of Zic expression vectors and increasing amounts (100–300 ng) of I-mfa expression vector along with a reporter gene, SV40-Luc. (C) NIH3T3 cells were co-transfected with 100 ng of truncated Zic2 expression vector (amino acids; 256–531) and increasing amounts (100–300 ng) of I-mfa expression vector along with a reporter gene, TK-Luc. Error bars represent standard deviations.

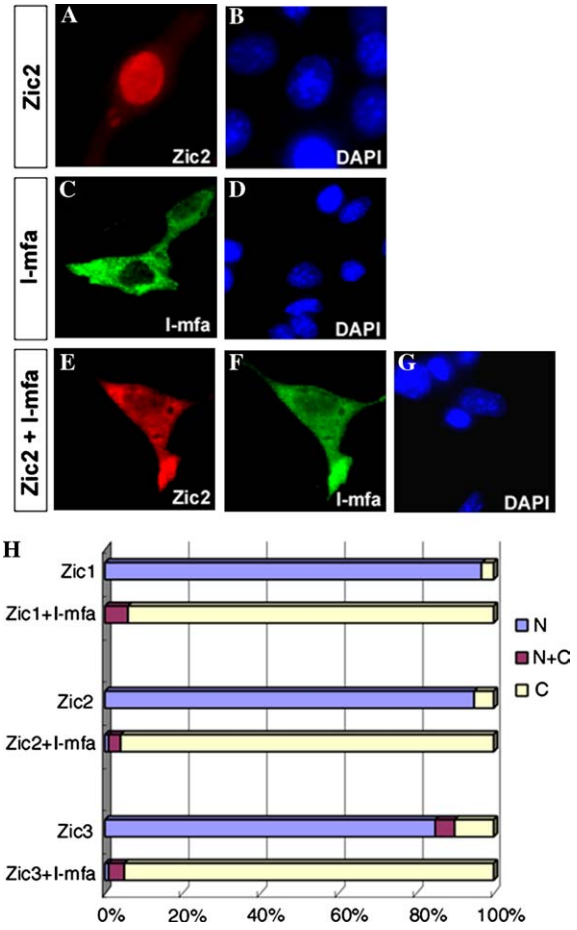


Fig. 4. Zic proteins are retained in the cytoplasm when co-expressed with I-mfa. (A–G) Subcellular localization of Zic and I-mfa in NIH3T3 cells. The cells were transfected with either HA-Zic (A,B), Flag-I-mfa (C,D), or both (E–G). After that, the cells were stained with anti-HA antibody (red; A,E) or anti-Flag antibody (green; C,F). Nuclei were counterstained with DAPI (blue; B,D,G). In the single transfection experiments, Zic proteins were localized exclusively in the nucleus (A,B), whereas I-mfa was predominantly localized in the cytoplasm (C,D). When the cells were co-transfected with HA-Zic and Flag-I-mfa in a 1:1 ratio (E–G), Zic2 protein was retained in the cytoplasm. The localization of I-mfa was not significantly changed by the presence of Zic proteins. (H) Summary of the subcellular localization of Zic1-3 proteins in NIH3T3 cells expressing Zic alone (*Zic1*, *Zic2*, and *Zic3*) and both Zic and I-mfa (*Zic1* + *I-mfa*, *Zic2* + *I-mfa*, and *Zic3* + *I-mfa*). More than 100 cells with each combination were counted according to the classification: nucleus-dominant (N, blue), cytoplasm-dominant (C, yellow), or nucleus/cytoplasm-equivalent (N + C, red), and the percentages of cells are shown.

Zic proteins, we used anti-HA and anti-Flag antibodies to investigate the subcellular localization of transfected HA-Zic1, HA-Zic2, HA-Zic3, and Flag-I-mfa in NIH3T3 cells. When Zic proteins and I-mfa were co-expressed in NIH3T3 cells, almost all Zic proteins were retained in the cytoplasm (Figs. 4E and H), but the subcellular localization of I-mfa was unaltered (Figs. 4F and H). Similar inhibition of nuclear transport of the three Zic proteins was observed in 293T cells (data not shown).

## Discussion

Our findings were that (1) an amino terminal domain including the ZOC motif can act as a transcription regulatory domain, (2) the amino terminal domain of Zic2 physically interacts with the carboxy-terminal cysteine-rich region of I-mfa, (3) I-mfa inhibits the nuclear import of Zic1-3 in cultured cells, and (4) I-mfa inhibits the transcriptional activation by Zic proteins. These results suggest that I-mfa can act as a functional repressor of the Zic proteins. The molecular mechanism of the Zic/I-mfa antagonism and the role of Zic/I-mfa interactions in vertebrate development attract our attention towards the next step of this study.

I-mfa inhibited the function of Zic proteins in the reporter-gene assay in cultured cells. Blockage of nuclear import of Zic is the most likely molecular basis for these functional inhibitions, since Zic proteins are almost always detected in cell nuclei without I-mfa and they have molecular properties of transcription regulatory factors [17]. The functional inhibition and nuclear import blockage by I-mfa are common to the MyoD family, which also binds to the carboxy-terminal cysteine-rich region of I-mfa [20]. I-mfa is predominantly localized throughout the cytoplasm, although it is detectable in the nucleus. MyoD family factors are retained in the cytoplasm because their nuclear localization signal is masked [20]. Similar mechanism is unlikely as to Zic2 because nuclear localization signal of the Zic proteins is mapped in a region other than I-mfa-binding domain (Y. Koyabu, K. Mizugishi, J. Aruga, unpublished observation). Therefore, we postulate that I-mfa provides a strong cytoplasmic anchoring site for Zic2 protein. In addition, it is possible that intra-nuclear I-mfa protein plays a role in functional inhibition of Zic2 protein. Chen et al. [20] showed that I-mfa inhibited the DNA binding of MyoD family factors. Although the DNA-binding domain (ZF3-5) is separable from the I-mfa-binding domain, the effect of I-mfa binding on the function of the zinc finger domain should be examined further.

I-mfa and Zic expression is partially overlapping in sclerotome, limb bud ([20–22], data not shown). It is possible that Zic/I-mfa interactions have a biological role in each tissue development. Sclerotome, which is a ventromedial component of differentiating somites, is known to generate axial skeletons. The importance of the I-mfa in somite development has been demonstrated by an analysis of I-mfa deficient mice, which show abnormal fusion of vertebral arches and fusion and bifurcation of ribs [22]. Some of these skeletal phenotypes are similar to those observed in the Zic mutants [1–3]. However, the apparent similarity does not always mean that both genes have the same role in skeletal development since the skeletal patterning depends on a number of positive and negative signals. Considering the Zic2/I-mfa antagonism observed in this study, it is tempting to speculate that the antagonism is required for the compartment or boundary formation in the skeletal patterning. Additional analysis in both mutant mice and their compound mutants would be an effective means to reveal the biological significance of the Zic/I-mfa interactions.

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