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Identification of protein domains required for makorin-2-mediated neurogenesis inhibition in *Xenopus* embryos

William K.C. Cheung [a,1], Pai-Hao Yang [a,b,1], Qiu-Hua Huang [c], Zhu Chen [c], Sai-Juan Chen [c], Marie C.M. Lin [a,*], Hsiang-Fu Kung [b,*]

[a] Department of Chemistry and Open Laboratory of Chemical Biology, The University of Hong Kong, Pokfulam Road, Hong Kong SAR, China
[b] Stanley Ho Centre for Emerging Infectious Diseases, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, China
[c] State Key Laboratory of Medical Genomics, Shanghai Institute of Hematology, Rui Jin Hospital affiliated to Shanghai JiaoTong University School of Medicine, Shanghai 200025, China

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ABSTRACT

Makorin-2, consisting of four highly conserved C_3H zinc fingers, a Cys-His motif and a C_3HC_4 RING zinc finger domain, is a putative ribonucleoprotein. We have previously reported that *Xenopus* makorin-2 (mkrn2) is a neurogenesis inhibitor acting upstream of glycogen synthase kinase-3β (GSK-3β) in the phosphatidylinositol 3-kinase/Akt pathway. In an effort to identify the functional domains required for its anti-neurogenic activity, we designed and constructed a series of N- and C-terminal truncation mutants of mkrn2. Concurred with the full-length mkrn2, we showed that overexpression of one of the truncation mutants mkrn2(s)-7, which consists of only the third C_3H zinc finger, Cys-His motif and C_3HC_4 RING zinc finger, is essential and sufficient to produce the phenotypical dorso-posterior deficiencies and small-head/short-tail phenotype in tadpoles. In animal cap explant assay, we further demonstrated that mkrn2(s)-7 not only inhibits activin and retinoic acid-induced animal cap neuralization and the expression of a pan-neural marker neural cell adhesion molecule, but also induces GSK-3β expression. These results collectively suggest that the third C_3H zinc finger, Cys-His motif and C_3HC_4 RING zinc finger are indispensable for the anti-neurogenic activity of mkrn2.

Introduction

Makorin-2, a member of makorin RING zinc finger (MKRN) gene family, was first isolated from human CD34+ hematopoietic stem/progenitor cells [1]. This gene is located on chromosome 3p25, and its nucleotide sequence partially overlaps with the locus of RAF1 oncogene in reversed transcription orientation. MKRN2 is ubiquitously expressed in various tissues and cell lines [1,2].

Members of the MKRN gene family are featured by a distinctive array of zinc fingers. In particular, MKRN2 possesses four C_3H zinc fingers and a signature C_3HC_4 RING zinc finger domain. Three of the C_3H zinc fingers are consecutively resided in the N-terminal region of MKRN2 protein. These three C_3H zinc fingers are followed by a Cys-His motif and the C_3HC_4 RING zinc finger, with the fourth C_3H zinc finger located on the C-terminal end of MKRN2 protein.

We have previously employed *Xenopus laevis* as a model system to characterize the functional role of *Xenopus* makorin-2 (mkrn2) in inhibiting neurogenesis during early embryonic development [3]. mkrn2 works specifically through the phosphatidylinositol 3-kinase (PI3K)/Akt-mediated neurogenesis pathway discovered by our group [4], in which mkrn2 positively regulates the mRNA expression of glycogen synthase kinase-3β (GSK-3β). To identify the domains that are required for neurogenesis inhibition, we constructed a series of N- and C-terminal truncation mutants of mkrn2. The effects of these truncation mutants on neurogenesis were examined by morphological observations, animal cap (AC) assay and reverse transcription-polymerase chain reaction (RT-PCR) analysis of the expression of a pan-neural marker, neural cell adhesion molecule (NCAM). Our results demonstrated that only the third C_3H zinc finger, Cys-His motif and the C_3HC_4 RING zinc finger of mkrn2 are essential and sufficient to inhibit *Xenopus* neurogenesis.

Materials and methods

Cloning of mkrn2 truncation mutants. Truncation mutants of mkrn2 were produced by PCR using wild-type mkrn2 cDNA (GenBank accession number EF626804) as a template. For C-terminal

* Corresponding authors. Addresses: Department of Chemistry, 8/F, Kadoorie Biological Sciences Building, The University of Hong Kong, Pokfulam Road, Hong Kong SAR, China. Fax: +852 28171006 (M.C.M. Lin); Stanley Ho Centre for Emerging Infectious Diseases, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, China. Fax: +852 29944988 (H.-F. Kung).
E-mail addresses: mcllin@hkusua.hku.hk (M.C.M. Lin), hkung@cuhk.edu.hk (H.-F. Kung).
[1] WKCC and PHY contributed equally to this work.

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truncation mutants mkrn2(s)-1–mkrn2(s)-4, a common forward primer 5′-GTA GAA TTC ATG AGT CCC AAG CAG-3′ was designed, while the reverse primers used were as follows: mkrn2(s)-1, 5′-GTA GAA TTC TTA ATG GAG ATA GGG GCA-3′; mkrn2(s)-2, 5′-GTA GAA TTC TTA GTC CAG CTC AAA GTT-3′; mkrn2(s)-3, 5′-GTA GAA TTC TTA ACA TTC TGG GCA CGA-3′; mkrn2(s)-4, 5′-GTA GAA TTC TTA ATG ATC ATA CCT GCA-3′. The common reversed primer used for N-terminal truncation mutants mkrn2(s)-5–mkrn2(s)-7 was 5′-GTA GAA TTC TTA ACA TTC TGG GCA CG-3′, and the forward primers were as follows: mkrn2(s)-5, 5′-GTA GAA TTC ATG TGC AGC ATC TGT A-3′; mkrn2(s)-6, 5′-GTA GAA TTC ATG TGT GAG ATC TGT GGC-3′; mkrn2(s)-7, 5′-GTA GAA TTC ATG TGC CCT TTT GCC-3′. The amplified fragments were digested with EcoRI restriction enzyme (New England Biolabs, Ipswich, MA) and ligated into pCS2 plasmid. The sequences of all mutant constructs were verified by sequencing.

Embryo microinjection. *Xenopus* embryos obtained by artificial insemination were cultured as previously described [4]. Embryos were staged according to Nieuwkoop and Faber [5]. The sequence and efficacy of morpholino antisense oligonucleotides specifically targeting *Xenopus* mkrn2 mRNA (MOa) were reported previously [3]. Embryos at two-cell stage were injected into the animal pole area with 1 ng mRNA or 15 ng MOa or both. Injected embryos were maintained up to stage 33 in 30% Marc's modified Ringer's solution for morphological observations.

Animal cap assay. Following the microinjection of embryos at two-cell stage, ACs were dissected at stages 8.5–9 and cultured at 22 °C in 67% Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA) with 7 mM Tris–HCl (pH 7.5) and gentamicin (50 μg/ml), in the presence or absence of activin (10 ng/ml) and retinoic acid (10^{-5} M). For each injection sample, eight ACs were pooled at stage 22 equivalent for RT-PCR analysis.

RT-PCR. Total RNA was extracted from ACs with the use of TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RT-PCR amplification was performed using Superscript first-strand synthesis system (Invitrogen) and HotStarTaq DNA polymerase (Qiagen, Valencia, CA). Primer pairs for NCAM, EF-1α and GSK-3β have already been described [3,6]. PCR conditions were as follows: 15 min at 95 °C followed by 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C for 29 cycles (or 27 cycles for EF-1α). PCR products were subjected to electrophoresis on 1% ethidium bromide-stained agarose gel. The band intensity was quantitated by ImageQuant software (GE Healthcare, Piscataway, NJ) and expressed as mean ± standard error of mean (SEM).

Statistical analysis. All RT-PCR results were confirmed by three independent experiments, in which only the representative bands were shown. Differences, as compared with control, were considered to be statistically significant at $p < 0.05$ in two-tailed Student's *t* test (GraphPad Prism 5.0, GraphPad Software, San Diego, CA).

Results

Amino acid sequence and phylogenetic analyses of mkrn2

mkrn2 cDNA has an open reading frame extending from nucleotide +1 to +1227, encoding mkrn2 protein of 408 amino acid residues. Amino acid sequence alignment of 10 makorin-2 orthologs constructed by T-coffee manifested that mkrn2 shares 63% sequence identity to human MKRN2 [7] (Supplementary Fig. 1). Four C_3H zinc fingers, a Cys-His motif and a C_3HC_4 RING zinc finger, which are the hallmark of MKRN family, were identified in mkrn2. These domains were found to be highly conserved among species, for example, the four C_3H zinc fingers were of 68%, 63%, 37%, and 71% sequence identities, whereas the Cys-His motif and the C_3HC_4 RING zinc finger were of 48% and 50% sequence identities, respectively. In addition, all cysteine and histidine residues critical for maintaining the zinc finger structures were strictly retained, excepting that the third critical residue histidine was substituted by aspartic acid in the Cys-His motif of pufferfish (*Takifugu rubripes*) makorin-2.

Using neighbor-joining method in MEGA software [8], we also performed phylogenetic analysis to reveal the evolutionary relationships of makorin-2 identified in mammals, chicken, frogs, and fishes (Supplementary Fig. 2). From the phylogenetic tree, mkrn2 was found evolutionarily closer to chicken and mammalian makorin-2 rather than to fish makorin-2, consistent with that expected from the evolutionary distances between these species.

The fourth C_3H zinc finger is not required for neurogenesis inhibition in Xenopus embryos

To identify the protein domains of mkrn2 required for neurogenesis inhibition, we constructed truncation mutants (mkrn2(s)-1 to mkrn2(s)-4) by deleting each of its domains from the C-terminal end progressively (Fig. 1A). We observed that embryos microinjected with mkrn2(s)-3, which lacks the fourth C_3H zinc finger domain, displayed dorso-posterior deficiencies with small-head/short-tail phenotype at stage 33 equivalent (Fig. 1B, 41%; Fig. 2A, *bottom left*), concurring with those injected with full-length mkrn2(s) (Fig. 1B, 50%; Fig. 2A, *top right*). However, further truncation (mkrn2(s)-1/2/4) resulted in phenotype similar to that of β-galactosidase (β-gal) injection control, implicating the importance of C_3HC_4 RING zinc finger domain.

We validated the anti-neurogenic activity of mkrn2(s)-3 by AC assay and RT-PCR analysis of NCAM expression. In the absence of mesoderm-inducing factor activin and neural-inducing factor retinoic acid, only mkrn2(s) and mkrn2(s)-3, but not mkrn2(s)-1/2/4, suppressed NCAM expression (Fig. 2B). Consistent results were obtained by AC assay in the presence of activin and retinoic acid. Neural-like extension of AC induced by activin and retinoic acid (Fig. 2C, *β-gal*) was completely abrogated by overexpression of mkrn2(s) and mkrn2(s)-3, resulting in ACs with round shaped epidermal-like phenotype (Fig. 2C). In addition, mkrn2(s)-3 inhibited activin and retinoic acid-induced NCAM expression (Fig. 2D, *lane* 5), and co-injection of morpholino antisense oligonucleotides specifically targeting mkrn2 mRNA (MOa) could fully restored mkrn2(s)-3-inhibited NCAM expression (Fig. 2D, *lane* 11). These results suggested that the fourth C_3H zinc finger is indeed dispensable for the anti-neurogenic activity of mkrn2.

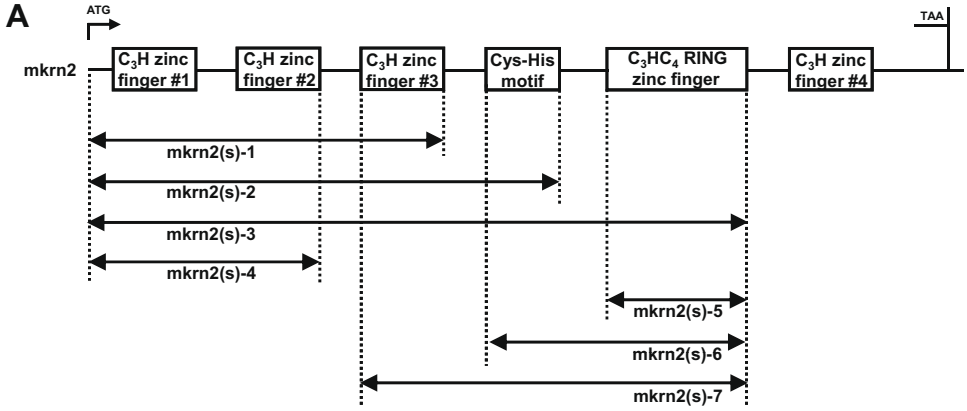
The first and second C_3H zinc finger domains are inessential for mkrn2 anti-neurogenic activity

We further specified the essential domains by constructing another three N-terminal truncation mutants of mkrn2 (mkrn2(s)-5 to mkrn2(s)-7) (Fig. 1A). Since the fourth C_3H zinc finger was shown to be inessential, it was disregarded in these N-terminal truncation mutants. Using the same approaches, we found that microinjection of mkrn2(s)-7 resulted in tadpoles with diminished head structure and short-tail phenotype (Fig. 1B, 36%; Fig. 3A, *bottom*), and suppressed NCAM expression in AC (Fig. 3B). Moreover, mkrn2(s)-7 alone could abolish activin and retinoic acid-induced AC neuralization (Fig. 3C) and NCAM expression (Fig. 3D, *lane* 5), indicating that the first two C_3H zinc fingers are also unnecessary.

The third C_3H zinc finger, Cys-His motif and C_3HC_4 RING zinc finger are sufficient to induce GSK-3β expression

As we have recently reported that mkrn2 positively regulates the mRNA expression of GSK-3β [3], we tested whether mkrn2(s)-7 preserves the same regulation. We found by RT-PCR

A



B

Injection samples (n = 60)	Normal	Defective phenotype	Dead
Animal pole: stage 30-33			
1 ng β-gal RNA	97%(58)	0%(0)[a]	3%(2)
1 ng mkrn2(s)	37%(22)	50%(30)[a]	13%(8)
1 ng mkrn2(s)-1	87%(52)	5%(3)[a]	8%(5)
1 ng mkrn2(s)-2	90%(54)	2%(1)[a]	8%(5)
1 ng mkrn2(s)-3	48%(29)	41%(25)[a]	11%(6)
1 ng mkrn2(s)-4	90%(54)	0%(0)[a]	10%(6)
1 ng mkrn2(s)-5	84%(50)	3%(2)[a]	3%(8)
1 ng mkrn2(s)-6	87%(52)	1%(1)[a]	12%(7)
1 ng mkrn2(s)-7	52%(31)	36%(22)[a]	12%(7)
Animal pole: stage 13			
15 ng MOa	19%(11)	75%(45)[b]	6%(4)
1 ng mkrn2(s) + 15 ng MOa	78%(47)	13%(8)[b]	9%(5)
1 ng mkrn2(s)-1 + 15 ng MOa	16%(10)	70%(42)[b]	14%(8)
1 ng mkrn2(s)-2 + 15 ng MOa	20%(12)	67%(40)[b]	13%(8)
1 ng mkrn2(s)-3 + 15 ng MOa	56%(34)	33%(20)[b]	11%(6)
1 ng mkrn2(s)-4 + 15 ng MOa	12%(7)	72%(43)[b]	16%(10)
1 ng mkrn2(s)-5 + 15 ng MOa	10%(6)	67%(40)[b]	23%(14)
1 ng mkrn2(s)-6 + 15 ng MOa	15%(9)	60%(36)[b]	25%(15)
1 ng mkrn2(s)-7 + 15 ng MOa	60%(36)	37%(22)[b]	3%(2)

[a]Dorso-posterior deficiencies (small head and short tail phenotype)
[b]Defect in dorsal closure and embryonic death at gastrulation stage phenotype

Fig. 1. Abnormal phenotypes induced by microinjection of mkrn2 truncation mutants and MOa in *Xenopus* embryos. (A) Schematic illustration of the protein domains harboured by mkrn2 truncation mutants. (B) Summary of normal, defective, and dead phenotypes in different injection groups. Values were the combined data of three independent experiments with each experiment consisting of 20 embryos. Bracketed figures indicated the number of embryos with specified phenotype.

that mkrn2(s)-7 significantly induced GSK-3β expression (Fig. 4, *lane* 2), and co-injection of MOa could partially abolish mkrn2(s)-7-induced GSK-3β expression (Fig. 4, *lane* 4). Taken together, our results demonstrated that the third C_3H zinc finger, Cys-His motif and C_3HC_4 RING zinc finger of mkrn2 are necessary and sufficient for neurogenesis inhibition and GSK-3β regulation.

Discussion

MKRN2 is being regarded as a putative ribonucleoprotein ever since its discovery in 2000. This speculation is intuitively based on the presence of a distinctive array of zinc finger domains. These domains are highly conserved throughout evolution (Supplementary Fig. 1), implicating their strong resistance against evolutionary changes as well as their biological importance for the molecular function of MKRN2. We previously reported the first biological role of MKNR2 in inhibiting neurogenesis during *Xenopus* early embryonic development [3]. In this study, we are eager to know which domains indeed confer mkrn2 with such anti-neurogenic activity. To address this question, we constructed a series of mkrn2 truncation mutants by deleting each domain from the N- or C-terminal end sequentially. Among all truncation mutants, we found that mkrn2(s)-7, an internal fragment of mkrn2 consisting of the third C_3H zinc finger, Cys-His motif and C_3HC_4 RING zinc finger, is capable of inducing dorso-posterior deficiencies and small-head/short-tail phenotype, as well as inhibiting AC neuralization and NCAM

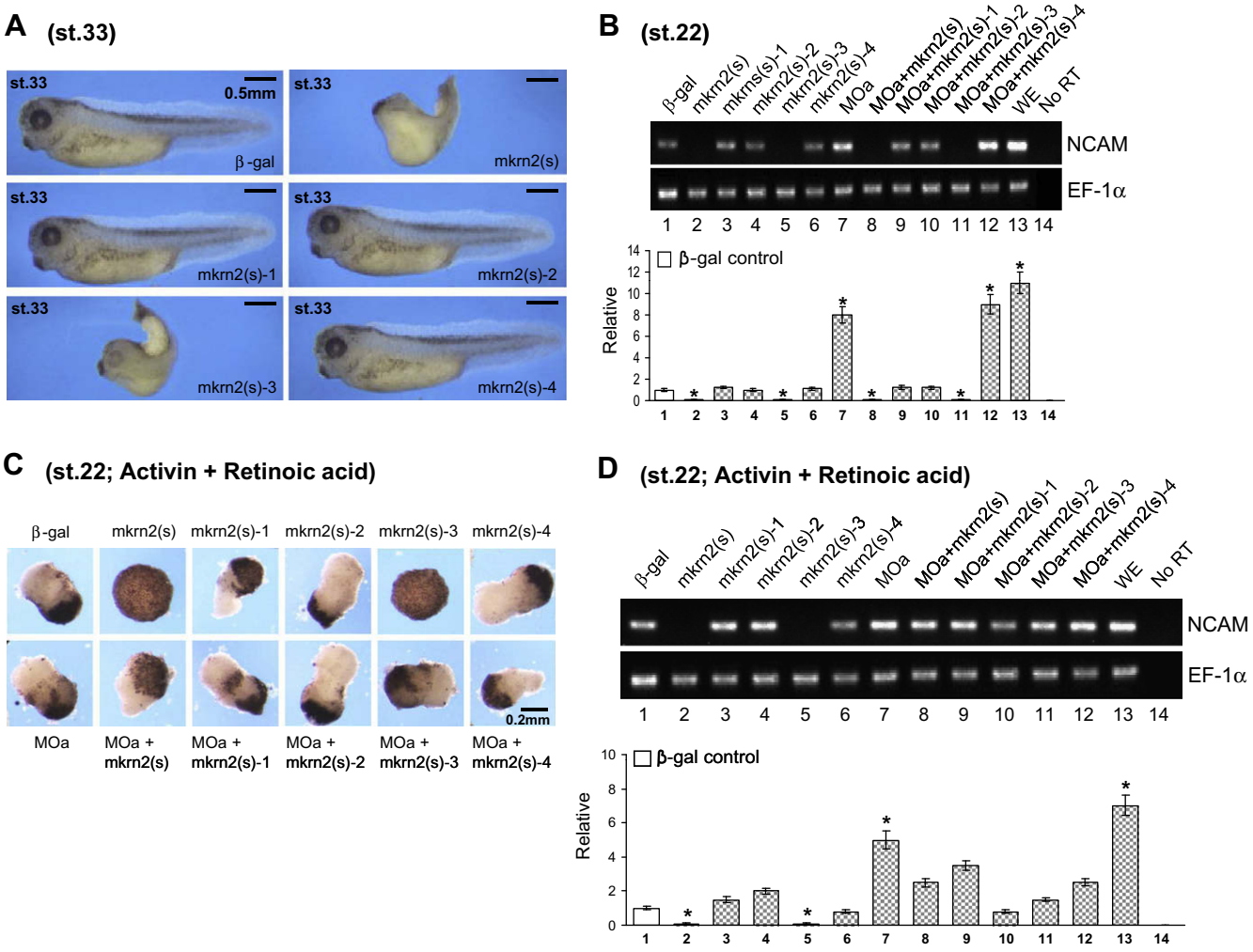


Fig. 2. mkrn2(s)-3 affects neural development in *Xenopus* embryos and suppresses NCAM expression in animal cap. Embryos at two-cell stage were injected into the animal pole areas with mRNAs encoding β-gal (control), mkrn2(s), C-terminal truncation mutants and MOa. (A) Embryos injected with mkrn2(s) and mkrn2(s)-3 showed dorso-posterior deficiencies with small head and short-tail phenotype at stage 33. (B) Dissected ACs were cultured in the absence of activin and retinoic acid to stage 22 equivalent for RT-PCR analysis of NCAM expression. NCAM expression in whole embryos (*WE*) was used as positive control. Reaction without the addition of reverse transcriptase (*No RT*) served as a negative control to confirm the absence of contaminating genomic DNA. Elongation factor 1α (*EF-1α*) expression was used as a loading control. (C) and (D) Dissected ACs were cultured in the presence of activin and retinoic acid until the equivalent of stage 22 for photography or RT-PCR analysis.

expression (Figs. 2 and 3). mkrn2(s)-7 can also induce the mRNA expression of GSK-3β (Fig. 4), suggesting that the third C_3H zinc finger, Cys-His motif and C_3HC_4 RING zinc finger constitute a functional fragment that are essential and sufficient to mimic the anti-neurogenic activity of mkrn2.

Interestingly, the inessential domains are all C_3H zinc fingers having high sequence identity. The first three C_3H zinc fingers located on the N-terminal end of MKRN2 are organised in $Cx_3Cx_5Cx_3H$ configuration, whereas the C-terminal C_3H zinc finger is in $Cx_9Cx_5Cx_3H$ configuration. It has been reported that the C_3H zinc fingers are RNA binding domains. For example, the two C_3H zinc fingers in tristetraprolin are involved in the binding to the sequence 5′-UUAUUUAUU-3′ within the class II AU-rich element of the 3′-untranslated region of tumor necrosis factor-α mRNA to promote its deadenylation and degradation [9,10]. The inessentiality of the first two and the fourth C_3H zinc fingers in mkrn2 may imply that the third C_3H zinc finger alone is sufficient to recognize its RNA targets.

On the other hand, the domains required for mkrn2-mediated neurogenesis inhibition are clustered closely together, in the sense that these three crucial domains are separated by 3 and 15 amino acids only (Supplementary Fig. 1). Being one of the essential domains, C_3HC_4 RING zinc finger can also be found in several proteins which have proven to be E3 ubiquitin-protein ligase within the ubiquitylation pathway. For example, C_3HC_4 RING zinc finger containing protein, c-Cbl, acts as an E3 ubiquitin-protein ligase to recognize tyrosine-phosphorylated substrates through its SH2 domain. The roles of this C_3HC_4 RING finger are to bind to and activate E2 ubiquitin-conjugating enzyme [11].

Using yeast two-hybrid system, we found that four-and-a-half-LIM protein 2 (FHL2) is one of the interacting proteins of MKRN2 (unpublished result). FHL2 is a transcriptional modulator that regulates multiple signaling pathways, including androgen receptor (AR) [12], activator protein-1 (AP-1) [13], cyclic AMP response element binding protein (CREB), cyclic AMP response element modulator (CREM) [14], breast cancer 1 (BRCA1) [15], Wilms' tumor 1 (WT-1) [16], nuclear factor-κB (NF-κB) [17], and β-catenin signalings [18]. Based on our identification of the functional domains required for mkrn2-mediated neurogenesis inhibition and the potential protein–protein interactions with FHL2, it is conceivable that mkrn2 may regulate the expression of GSK-3β via two different mechanisms. One possibility is that mkrn2 may stabilize the GSK-3β mRNA through binding with the third C_3H zinc finger. Alternatively, mkrn2 may bind to and modulate the transcriptional activity of FHL2, in turn affecting the expression of GSK-3β as a putative FHL2-regulated gene.

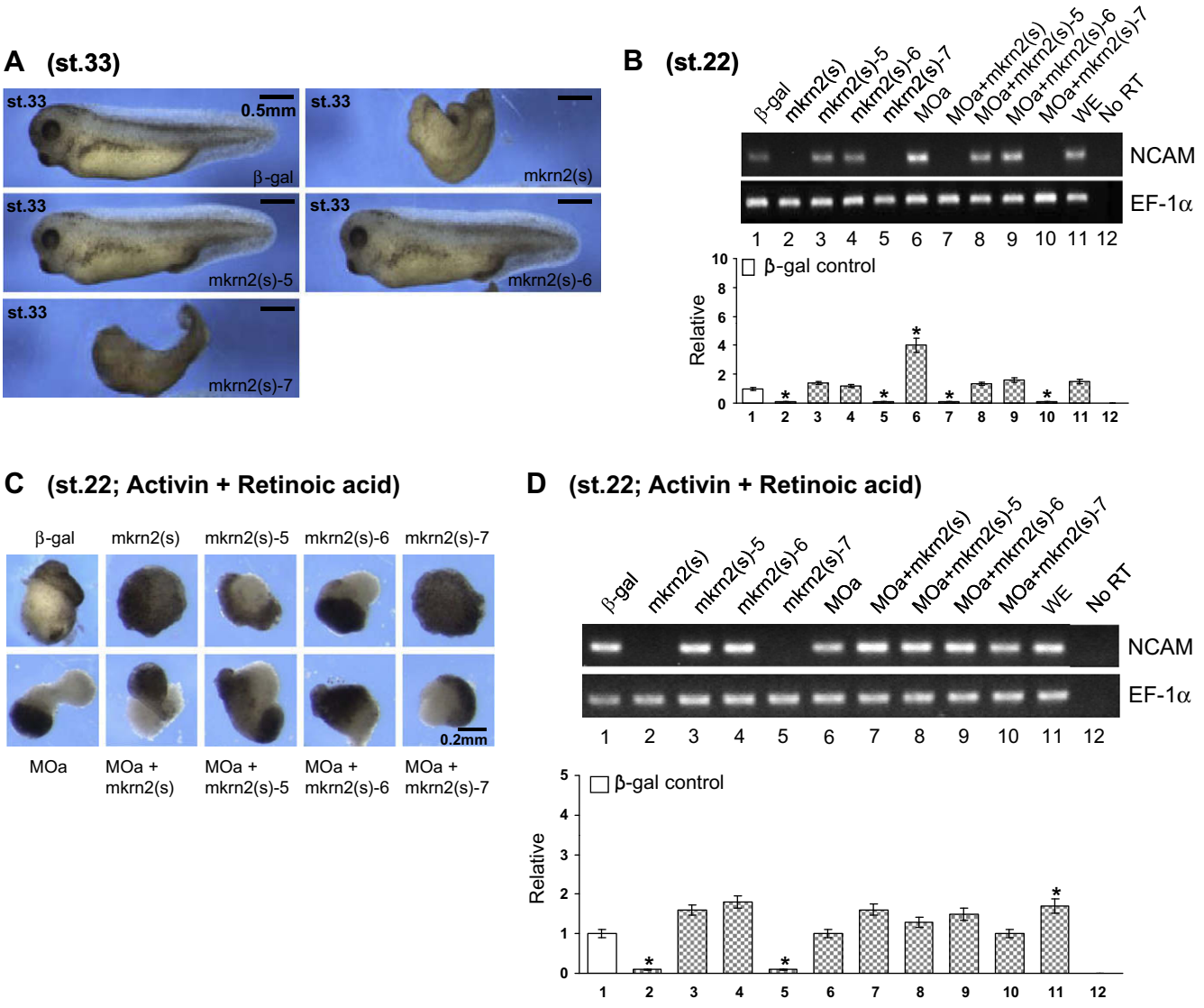


Fig. 3. mkrn2(s)-7 inhibits neurogenesis, animal cap neuralization and NCAM expression. Embryos were injected with mRNA encoding β-gal (control), mkrn2(s), N-terminal truncation mutants and MOa. (A) Morphological observations of tadpoles at stage 33. (B) NCAM expression in ACs cultured in the absence of activin and retinoic acid. (C) and (D) Neural tissue induction and NCAM expression in ACs cultured in the presence of activin and retinoic acid. *WE*, whole embryo; *No RT*, reaction without the addition of reverse transcriptase.

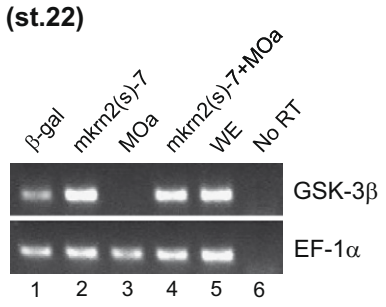


Fig. 4. mkrn2(s)-7 induces GSK-3β mRNA expression. *WE*, whole embryo; *No RT*, reaction without the addition of reverse transcriptase.

To conclude, we report for the first time that the third C_3H zinc finger, Cys-His motif and C_3HC_4 RING zinc finger of mkrn2 are both necessary and sufficient for neurogenesis inhibition and GSK-3β regulation. These results provide invaluable information for understanding the molecular function of MKRN2.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.02.041.

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