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Original Article

XMam1, the *Xenopus* homologue of *mastermind*, is essential to primary neurogenesis in *Xenopus laevis* embryos

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ABSTRACT Notch signaling is involved in cell fate determination and is evolutionally highly conserved in vertebrates and invertebrates. Mastermind is a nuclear protein which participates in Notch signaling and is involved in direct transactivation of target genes. Here we analyzed the expression and the function of *Xenopus mastermind1* (*XMam1*) in the process of primary neurogenesis. *XMam1* is 3,425 bp and encodes 1,139 amino acids. Overall, Mastermind proteins consist of a basic domain, two acidic domains and a glutamine-rich domain, which are highly conserved among species. The ubiquitous expression of *XMam1* was observed in both maternal and zygotic stages. Whole-mount *in situ* hybridization showed that *XMam1* mRNA was present in the ectoderm by the gastrula stage and localized at the anterior neural region in the neurula stage. Thereafter, *XMam1* expression was restricted to the eye and otic vesicle in the tailbud-stage embryo. XMam1 overexpression caused the repression of primary neural formation. The truncated form of XMam1 (lacking the C-terminus of XMam1; XMam1ΔC) led to excess formation of primary neurons. Furthermore, XMam1ΔC strongly repressed *XESR-1* transactivation. These results show that XMam1 is involved in primary neurogenesis by way of Notch signaling and is an essential component for transactivation of *XESR-1* in *Xenopus laevis* embryos.

KEY WORDS: *mastermind, primary neurogenesis, Xenopus laevis, Notch signaling*

Introduction

The developmental process of multicellular organism proceeds under the strict program that the cell differentiation and repression of the cell differentiation are regulated accurately. In this process, signal transduction between the cells is essential for the cell fate determination (Gurdon 1992). Although a number of signal transduction systems are known during development, the signal transduction by direct contact of cells particularly plays an important role in for the juxtacrine signal transduction.

Notch signaling pathway is known as signal transduction system which functions between the neighboring cells and widely conserved in almost all animal species. Notch signaling determines the alternative cell fate through lateral inhibition, boundary formation and asymmetric cell division. Originally, the genetic analysis of the mutant that showed the neurogenic phenotype in *Drosophila* revealed the existence of Notch signaling during primary neurogenesis (Artavanis-Tsakonas *et al.,* 1995; Greenwald 1998; Artavanis-Tsakonas *et al.,* 1999; Mumm and Kopan 2000). Thereafter, many components involved in this system are identified to date. Not only in *Drosophila* but also in vertebrates, homologues of components involved in Notch signaling are isolated (Blaumueller and Artavanis-Tsakonas 1997; Artavanis-Tsakonas *et al.,* 1999).

The mechanism of Notch signaling is thought to be follows: the Notch ligands, Delta and Serrate, interact Notch receptor expressed on neighboring cells through EGF-like repeats domain in extracellular region, then the Notch intracellular domain (NICD) is cleaved by gamma-secretase, Presenilin (De Strooper *et al.,* 1999; Struhl and Greenwald 1999). NICD translocates into the nucleus and binds to CSL (CBF1, Suppressor of Hairless, LAG-1) family protein and Mastermind to transactivate Notch target genes (Fortini and Artavanis-Tsakonas 1994; Christensen *et al.,* 1996; Petcherski and Kimble 2000; Wu *et al.,* 2000; Kitagawa *et al.,* 2001). The identified Notch target genes belong to bHLH genes such as *Enhancer of split* in *Drosophila melanogaster* and *HES* (*Hairy enhancer of split*) in mammals (Jarriault *et al.,* 1995; Kageyama and Ohtsuka 1999; Mumm and Kopan 2000; Davis and Turner 2001).

Mastermind is a nuclear protein that is identified by analysis of the neurogenic phenotype of *Drosophila* mutants (Yedvobnick *et*

Abbreviations used in this paper: NICD, notch intracellular domain; XMam, *Xenopus* mastermind gene.

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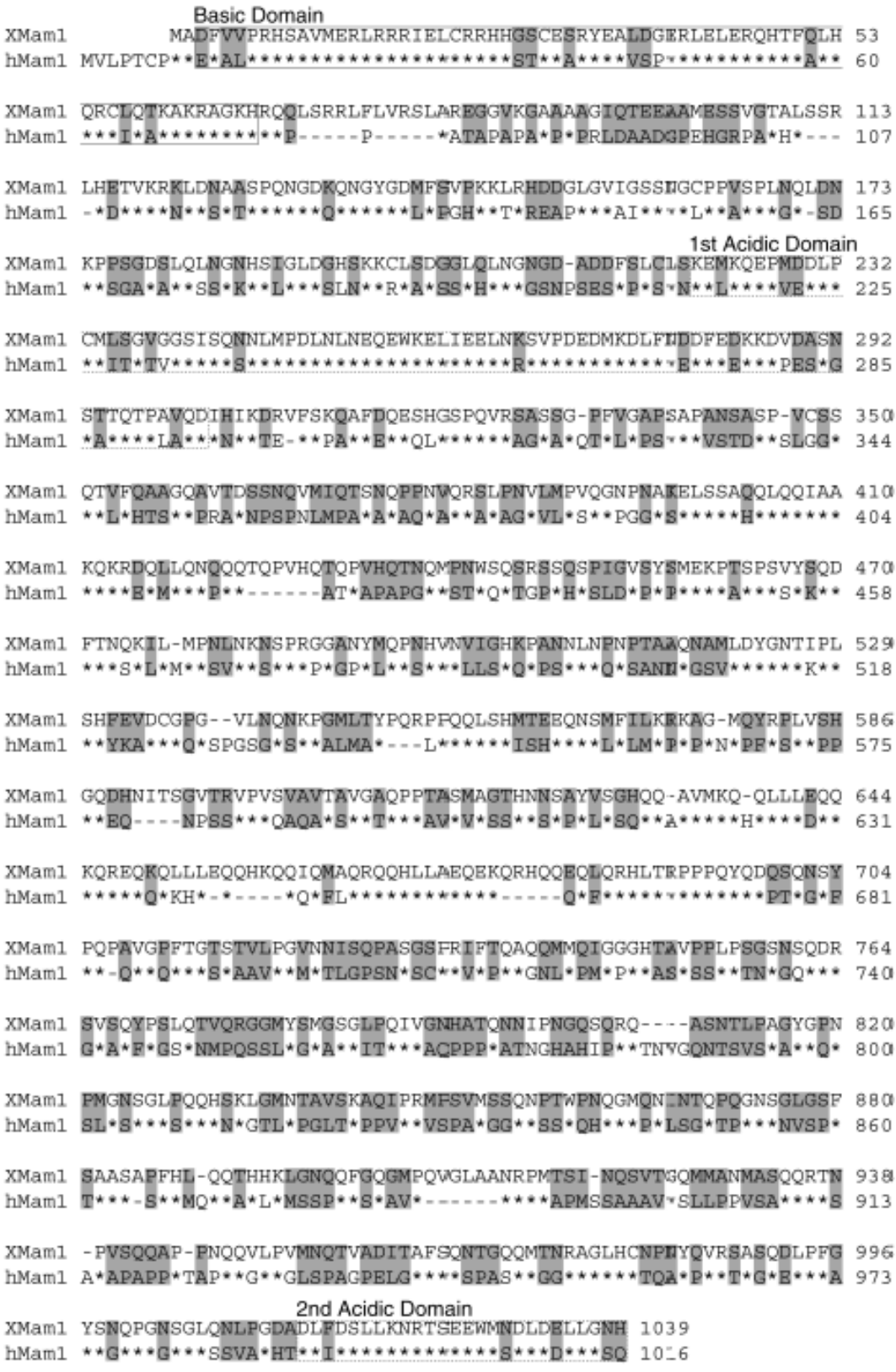


Fig. 1. Amino acid sequence of XMam1 aligned with hMam1. *Bars represent gaps for maximal alignment and asterisks show identical residues. Homologous amino acids are gray-shadowed. Mastermind-specific domain, basic domain and two acidic domains are boxed by solid line and broken line, respectively.*

al., 1988; Smoller *et al.,* 1990; Xu *et al.,* 1990; Bettler *et al.,* 1996; Go and Artavanis-Tsakonas 1998). To date, *mastermind* gene is identified in *Drosophila melanogaster*, *Drosophila virilis* and human (Smoller *et al.,* 1990; Newfeld *et al.,* 1993; Wu *et al.,* 2000; Kitagawa *et al.,* 2001; Lin *et al.,* 2002; Wu *et al.,* 2002). In human, *mastermind* consists of the gene family, *hMam1, 2* and *3* (Lin *et al.,* 2002; Wu *et al.,* 2002).

Mastermind has three distinctive domains in its amino acid sequences; the basic domain in N-terminus, two acidic domains in central part and in C-terminus and glutamine-rich domain (Smoller *et al.,* 1990; Newfeld *et al.,* 1993; Wu *et al.,* 2000; Kitagawa *et al.,* 2001; Lin *et al.,* 2002; Wu *et al.,* 2002). The experiments using various truncated forms of Mastermind showed that the basic domain existed in N-terminus is thought to be involved in binding to the complex of NICD and CSL protein (Petcherski and Kimble 2000). The C-terminal region including the acidic domain is thought to be involved in mediating the transactivation of Notch target genes (Wu *et al.,* 2000; Kitagawa *et al.,* 2001; Fryer *et al.,* 2002; Lin *et al.,* 2002; Wallberg *et al.,* 2002; Wu *et al.,* 2002). Additionally, it is reported that Mastermind interacts a histone acetyltransferase, CBP/p300 (Fryer *et al.,* 2002; Wallberg *et al.,* 2002). Therefore, Mastermind is an important molecule that involved in transactivation of target genes in Notch signaling. In addition to this new evidence, experimental systems of model animals in vertebrates are required for further functional analysis of *mastermind* genes.

In this report, we analyzed the expression pattern and function of *mastermind* in *Xenopus laevis* (*Xenopus mastermind1; XMam1*) during early embryogenesis, especially in primary neurogenesis. The transcripts of *XMam1* already existed in maternal stage ubiquitously, and its ubiquitous expression maintained in zygotic stage. Whole-mount *in situ* hybridization showed that *XMam1* expression was restricted in animal hemisphere by gastrula stage and localized in anterior neural structure in neurula-stage embryos. In tailbud-stage, the strong expression of *XMam1* was observed in the eye, thereafter, *XMam1* was expressed in otic vesicle in addition to head region.

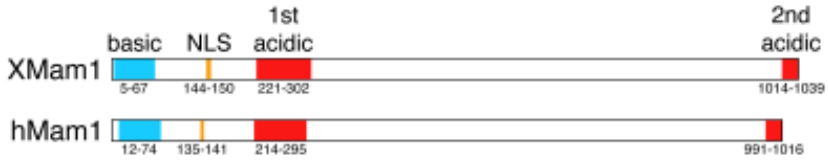
Overexpression of XMam1 caused the repression of primary neural formation, whereas the form lacking the C-terminus of XMam1, XMam1ΔC, overproduces primary neurons reversely. Moreover, XMam1ΔC inhibited the transcription of target gene, *XESR-1*. Taken together, it is suggested that XMam1 is an essential component to transactivation of *XESR-1* and to primary neurogenesis.

Results

Sequence analysis of Xenopus *Mastermind1 (XMam1)*

To investigate the function of Mastermind in early development, we searched out the clones in *Xenopus laevis* which show high homology to the amino acid sequence of human Mastermind in EST database. As a result of that, we found that the clone registered as AW765543 in GenBank accession number had high homology to human Mastermind1 (hMam1). This cDNA clone was isolated from st. 19-23 embryo cDNA library of *Xenopus laevis* constructed by oligo-dT priming method. We obtained this clone from American Type Culture Collection (ATCC) and determined the nucleotide sequence of this cDNA. The insert size of this cDNA

A



B

	hMam1	hMam2	hMam3
overall	51.9%	32.2%	19.2%
basic	83.9%	58.3%	50.0%
1st acidic	78.0%	48.8%	36.7%
2nd acidic	80.8%	56.0%	51.7%

Fig. 2. Structural comparison of XMam1 with hMam1. (A) *Structural scheme of XMam1 and hMam1. NLS, nuclear localization signal.* **(B)** *The overall and domain-specific homologies of XMam1 to hMam1, hMam2 and hMam3 are indicated.*

was 3425bp and included one open reading frame and encoded 1139 amino acids (Fig. 1). The comparison of deduced amino acid sequence of this clone with that of hMam1 showed the 51.9% homology in overall structure (Fig. 2B). The alignment of this clone and hMam1 showed that the homology in Mastermind-specific domains; basic domain and two acidic domains was high especially (Figs. 1, 2B). The values of the homology in each domain were 83.9% (basic domain), 78.0% (1st acidic domain) and 80.8% (2nd acidic domain) respectively (Fig. 2B). The comparison of amino acid sequence of this clone with that of hMam2 and hMam3 showed that the every value of the homology was lower than that of the homology between this clone and hMam1 (Fig. 2B). Therefore, this cDNA clone was designated *Xenopus mastermind1*, *XMam1* (GenBank accession number: AB107103). The more detail analyses of XMam1 and hMam1 sequence showed that each domain, including nuclear localization signal (NLS) region, conserved specifically in Mastermind located the similar position in each amino acid sequence (Fig. 2A). Furthermore, we found that amino acids which constitute the domain except Mastermind-specific domain were not identical but exchanged by the homologous amino acids (Fig. 1).

Expression profile of XMam1 *transcripts*

To make clear the temporal expression of *XMam1* in developmental stages of *Xenopus*, RT-PCR analysis was performed. Total RNAs was extracted from 9 stages of development and *Histone H4* was used as internal control. RT-PCR analysis showed that transcripts of *XMam1* were observed constantly in both maternal and zygotic stage (Fig. 3).

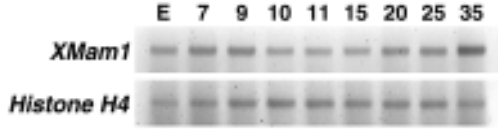


Fig. 3. The temporal expression of *XMam1* analyzed by RT-PCR. *"E" represents unfertilized egg and numbers show stages from which total RNA was extracted.* Xmam1 *transcripts were detected ubiquitously in all examined stages.* Histone H4 *was used as a loading control.*

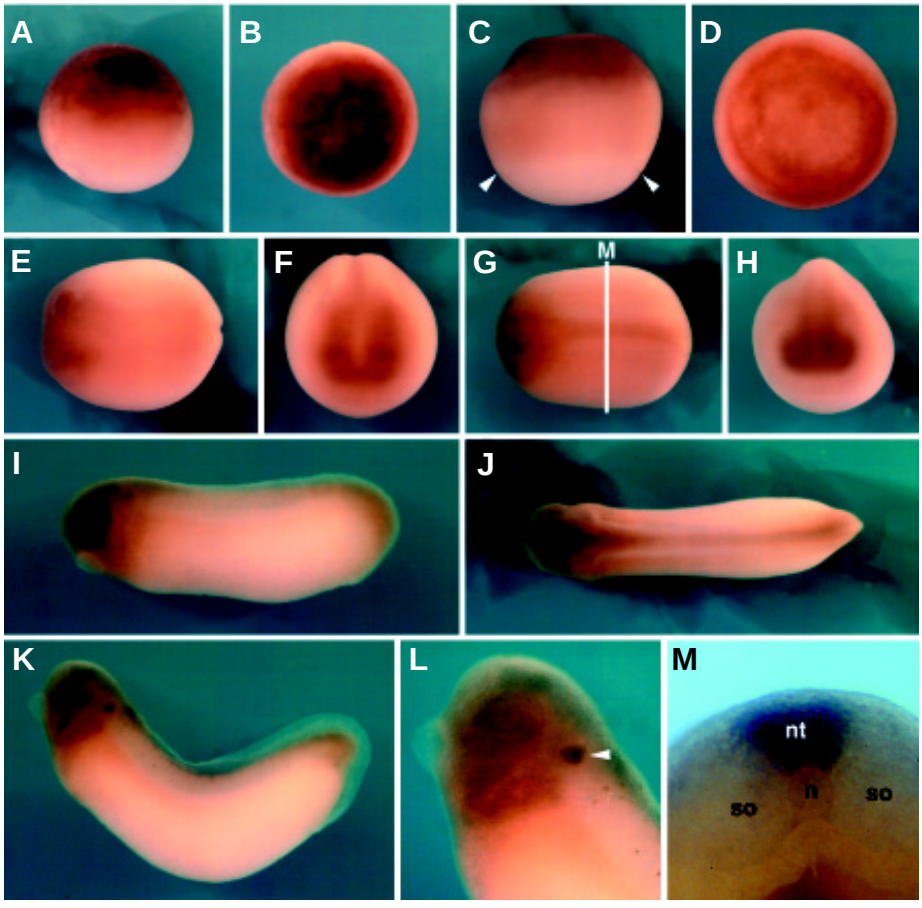


Fig. 4. The spatial expression of *XMam1* analyzed by whole-mount *in situ* hybridization. *The staining in brown shows* XMam1 *expression.* **(A)** *Blastula (st. 7). Lateral view.* XMam1 *is expressed in the animal hemisphere.* **(B)** *Animal view of (A).* **(C)** *Mid-gastrula (st. 11). Lateral view. The expression of* XMam1 *was more intense in the animal half than in the vegetal half. Arrow indicates blastopore.* **(D)** *Animal view of (C).* **(E)** *Mid-neurula (st. 15). Dorsal view.* XMam1 *is expressed in the anterior side of embryos.* **(F)** *Anterior view of (E).* XMam1 *mRNA was observed at the anterior side of the neural plate.* **(G)** *Late neurula (st. 20). Dorsal view. As in mid-neurula, transcripts of* XMam1 *were detected at the anterior side. A weak* XMam1 *mRNA signal was observed in the neural tube. White line indicates the plane of section of panel (M).* **(H)** *Anterior view of (G). In comparison to expression in st. 15 embryos, the* XMam1*-expressing region was somewhat narrower.* **(I)** *st. 25 embryo. Lateral view. Strong expression of* XMam1 *was observed in the eye, in addition to the head regions.* **(J)** *Dorsal view of (I).* **(K)** *Tailbud-stage embryo (st. 35). Lateral view.* XMam1 *expression was observed in the entire region of head. The intensity of* XMam1 *transcripts observed at st. 25 was reduced at this stage.* **(L)** *Magnification of the head in (K).* XMam1 *was strongly expressed in the otic vesicle (arrowhead).* **(M)** *Transverse section of embryo shown in (G).* XMam1 *was expressed in the neural tube. nt, neural tube; n, notochord; so, somite.*

Next, to examine the spatial expression of *XMam1*, whole-mount *in situ* hybridization in different developmental stages was performed. Full length of *XMam1* (3425bp) was used as antisense probe and the staining was performed by NBT/BCIP. In blastula stage (st. 7), *XMam1* mRNA was observed in animal hemisphere (Fig. 4 A,B). As the tendency of the expression was not changed by gastrula stage (st. 11), transcripts of *XMam1* were more localized in animal half than in vegetal half (Fig. 4 C,D). In neurula stage (st. 15), the expression of *XMam1* was detected in anterior side of embryos (Fig. 4 E,F). The expression in this region became narrower in the anterior side during neurula stage (Fig. 4 E-H). Weak signal of *XMam1* mRNA was observed in neural tube in st. 20 embryos (Fig. 4G). To clear this expression more detail, the inner side of stained embryos was observed by cutting with scalpel. As a result, we found that *XMam1* clearly expressed in the neural tube (Fig. 4M). In early tailbud stage embryo (st. 25), the expression was restricted in head region, especially strong expression was observed in the eye (Fig. 4 I,J). The expression was maintained in late tailbud stage embryo (st. 35), the expression in the otic vesicle was remarkable in addition to head region (Fig. 4 K,L).

XMam1 is involved in primary neurogenesis

To investigate the role of XMam1 in early development, the role of XMam1 in primary neurogenesis was examined. At first, the two following constructs were prepared: the pCS2+ vector including entire open reading frame of *XMam1* (construct name is "*XMam1*") and the pCS2+ vector including *XMam1* lacking two acidic domain (construct name is "*XMam1ΔC*"). The construct *XMam1* is for the purpose of overexpression of XMam1, which is expected to activate Notch signaling. XMam1ΔC is for the purpose of down-regulation of Notch signaling because XMam1ΔC is capable of binding CSL and NICD, but can't transactivate target genes owing to lacking to two acidic regions which is thought to be transactivating domain.

Generally, it is known that activation of Notch signaling by X-Delta-1 and X-Serrate-1 causes the repression of primary neurogenesis through the mechanism of lateral inhibition (Chitnis *et al.,* 1995; Kiyota *et al.,* 2001). In the same way, it is thought that XMam1 contributes the activation of Notch signaling and represses primary neurogenesis. It is thought that XMam1ΔC contributes the down-regulation of Notch signaling and overproduce the primary neuron by contraries. To examine this, capped RNA was synthesized using two constructs, *XMam1* and *XMam1ΔC*, as a template, and 1ng of capped RNA was injected into one blastomere of two-cell stage embryo in *Xenopus laevis*. The injected embryos were developed by st. 14, and *N-tubulin* expression analyzed by whole-mount *in situ* hybridization as the marker of primary neuron (Fig. 5). *β-galactosidase* RNA was used as tracer and control sample.

X-Delta-1-injected embryos caused the repression of primary neuron in injected side as reported (Chitnis *et al.,* 1995) (Fig. 5B). The ratio of such embryo was 55% (Table 1). The injection of *XMam1* resulted in the suppression of primary neurogenesis like X-Delta-1 and the ratio of these embryos was 69% (Fig. 5C, Table 1). Therefore, we found that the effect of the repression by XMam1 was somewhat stronger than that by X-Delta-1 (Table 1). On the other hand, *XMam1ΔC*-injected embryos overproduced the primary neuron and the rate was 82% (Fig. 5D, Table 1).

TABLE 1

EFFECT OF XMAM1, XMAM1ΔC AND X-DELTA-1 ON PRIMARY NEUROGENESIS

		N-tubulin expression			
Injected RNA	Amount (ng)	More	Unaffected	Fewer	Total
β-gal	1.0	3 (8)	34 (87)	2 (5)	39
XMam1	1.0	0 (0)	8 (31)	18 (69)	26
XMam1ΔC	1.0	18 (82)	4 (18)	0 (0)	22
X-Delta-1	1.0	1 (3)	14 (42)	18 (55)	33

The indicated amount of *XMam1* or *XMam1ΔC* or *X-Delta-1* RNA was injected into the blastomere of a two-cell embryo and *N-tubulin* expression was examined at st. 14. The numbers in parentheses represent the percentage of the total number.

TABLE 2

EFFECT OF XMAM1, XMAM1ΔC AND X-DELTA-1 ON *XESR-1* EXPRESSION

		XESR-1 expression			
Injected RNA	Amount (ng)	More	Unaffected	Fewer	Total
β-gal	1.0	0 (0)	52 (100)	0 (0)	52
XMam1	1.0	0 (0)	56 (98)	1 (2)	57
XMam1ΔC	1.0	0 (0)	8 (14)	51 (86)	59
X-Delta-1	1.0	21 (72)	7 (24)	1 (4)	29

The indicated amount of *XMam1* or *XMam1ΔC* or *X-Delta-1* RNA was injected into the blastomere of a two-cell embryo and *XESR-1* expression was examined at st. 14. The numbers in parentheses represent the percentage of the total number.

XMam1ΔC strongly represses XESR-1 *transactivation*

It is believed that the regulation of the primary neurogenesis by Notch signaling was carried out by suppressing the function of the proneural genes through transactivation of target genes by Notch pathway. Actually, overexpression of X-Delta-1 and X-Serrate-1, which activate Notch signaling, is proved to be resulted in the up-regulation of transcription of *XESR-1* which is one of target genes of Notch signaling (Wettstein *et al.,* 1997; Kiyota and Kinoshita 2002). Therefore, we examined whether XMam1 activates *XESR-1* transcription in the same pathway of X-Delta-1 and X-Serrate-1 as the regulation of the primary neurogenesis by XMam1. To examine this, 1ng of each capped RNA of *XMam1* and *XMam1ΔC* was injected into one blastomere of two-cell stage embryo in *Xenopus laevis*. The injected embryos were cultured by st. 14, and *XESR-1* expression was analyzed by whole-mount *in situ* hybridization (Fig. 6). *β-galactosidase* was used as tracer and control sample. As a result, *X-Delta-1*-injected embryos caused the ectopic expression of *XESR-1* as reported (Wettstein *et al.,* 1997) (Fig. 6B). The ratio of embryos with *XESR-1* ectopic expression was 72% (Table 2). However, *XMam1*-injected embryos did not show any changes in *XESR-1* expression pattern although we expected that XMam1 caused up-regulation of *XESR-1* or the ectopic expression of *XESR-1* as X-Delta-1 (Fig. 6C). The injection of *XMam1ΔC* resulted in the strong repression of *XESR-1* transcription and caused the disappearance of *XESR-1* expression (Fig. 6D). The rate of these embryos was 86% (Table 2). These results showed that XMam1 is essential molecule to transactivation of *XESR-1*.

Down-regulation of XESR-1 *by XMam1ΔC is an XMam1-specific effect*

To confirm that reduction of *XESR-1* expression by XMam1ΔC was XMam1-specific, the rescue experience was performed.

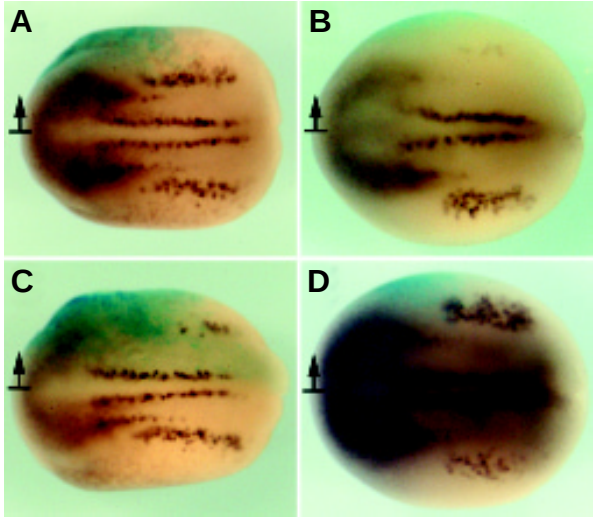


Fig. 5 (Left). The effect of XMam1 on *N-tubulin* expression. N-tubulin *expression was detected by whole-mount* in situ *hybridization (stained in brown). Samples were injected into the blastomere of two-cell stage embryos and* N-tubulin *expression analyzed at st. 14. β-galactosidase was used as tracer (the injected side appeared blue as indicated by arrows).* **(A)** *Control embryo injected with β-galactosidase.* **(B)** X-Delta-1*-injected embryo. The suppression of primary neurons was observed in the injected side.* **(C)** XMam1*-injected embryo. Primary neurogenesis was repressed in the injected side.* **(D)** XMam1ΔC*-injected embryo. The overproduction of primary neurons was observed in the injected side.*

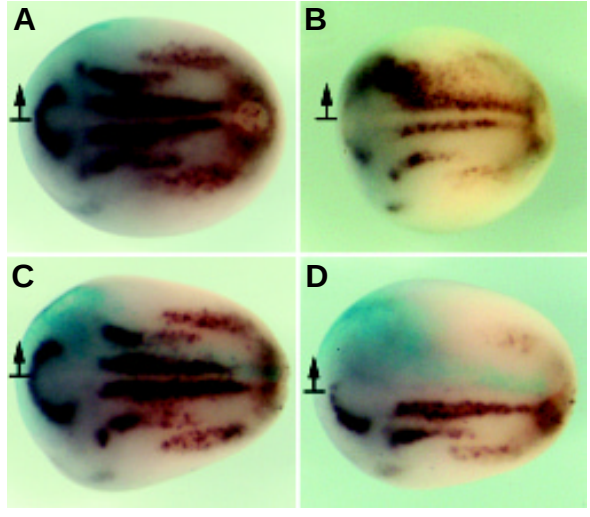


Fig. 6 (Right). The effect of XMam1 on *XESR-1* expression. XESR-1 *expression was detected by whole-mount* in situ *hybridization (stained in brown). Samples were injected at one blastomere of two-cell stage embryos and* XESR-1 *expression analyzed at st. 14. β-galactosidase was used as tracer (injected side was appeared in blue as indicated by arrows).* **(A)** *Control embryo injected with β-galactosidase.* **(B)** X-Delta-1*-injected embryo. The ectopic expression was observed in injected side.* **(C)** XMam1*-injected embryo. Overexpression of XMam1 caused no change of* XESR-1 *expression pattern.* **(D)** XMam1ΔC*-injected embryo. The remarkable reduction of* XESR-1 *expression was observed in injected side.*

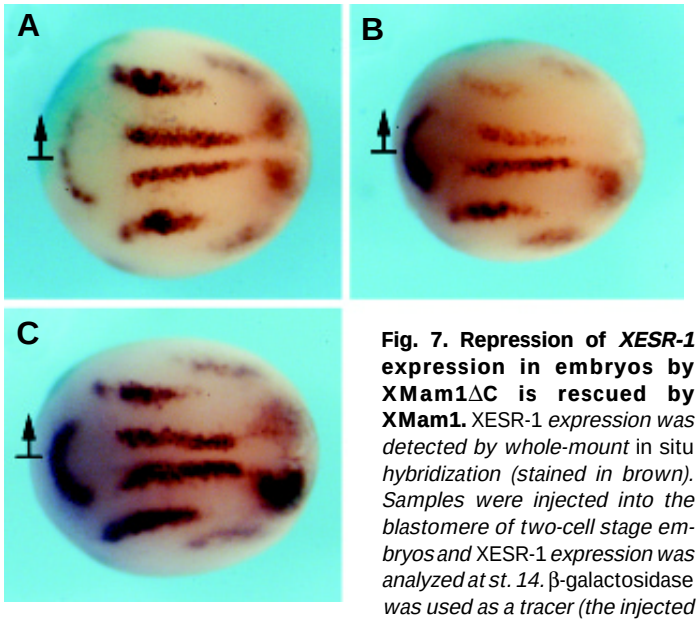


Fig. 7. Repression of *XESR-1* expression in embryos by XMam1ΔC is rescued by XMam1. XESR-1 *expression was detected by whole-mount* in situ *hybridization (stained in brown). Samples were injected into the blastomere of two-cell stage embryos and* XESR-1 *expression was analyzed at st. 14.* β-galactosidase *was used as a tracer (the injected side appeared in blue as indicated by arrows).* **(A)** *Control embryo injected with* β-galactosidase. **(B)** *Embryo injected with 1ng of* XMam1ΔC. *Down-regulation of* XESR-1 *expression was observed in the injected side.* **(C)** *Embryo co-injected with 1ng of* XMam1ΔC *and 3 ng of* XMam1. XESR-1 *expression was restored.*

1ng of *XMam1ΔC* capped RNA and various doses of *XMam1* were co-injected into one blastomere of two-cell stage embryos. Thereafter, injected-embryos were cultured by st. 14 and examined whether *XESR-1* expression was recovered by whole-mount *in situ* hybridization (Fig. 7, Table 3). As a result, in case of co-injection of 1ng or 2ng of *XMam1*, *XESR-1* expression was rescued to some extent. Furthermore, 3ng of *XMam1* co-injection rescued *XESR-1* expression to extent in the ratio of half of all embryos (Table 3). These data shows that down-regulation of *XESR-1* by XMam1ΔC is XMam1-specific effect.

TABLE 3

REPRESSION OF *XESR-1* EXPRESSION IN EMBRYOS BY XMAM1ΔC IS RESCUED BY XMAM1

		XESR-1 expression			
Injected RNA	Amount (ng)	More	Unaffected	Fewer	Total
β-gal	1.0	0 (0)	23 (100)	0 (0)	23
XMam1ΔC	1.0	0 (0)	3 (12)	23 (88)	26
XMam1ΔC	1.0	0 (0)	12 (29)	29 (71)	41
XMam1	1.0				
XMam1ΔC	1.0	0 (0)	14 (29)	34 (71)	48
XMam1	2.0				
XMam1ΔC	1.0	0 (0)	11 (50)	11 (50)	22
XMam1	3.0				

The indicated amount of *XMam1* or *XMam1ΔC* RNA was injected into one blastomere of a two-cell embryo and *XESR-1* expression was examined at st. 14. The numbers in parentheses represent the percentage of the total number.

Discussion

In this report, we identified *Xenopus* homologue of *mastermind*, *XMam1*, which is involved in transactivation of target genes by Notch signaling and analyzed the structure, expression profile and the role of XMam1 in the process of primary neurogenesis. In vertebrates, the function of Mastermind is not understood yet, especially in developmental process. Therefore, our findings are valuable on analyzing the function of Mastermind in primary neurogenesis of vertebrate development.

Identification of XMam1, *the* Xenopus *homologue of* mastermind1

The EST clone (AW765543) we identified included one open reading frame and encoded 1139 amino acids. The comparison of this clone with three kinds of human Mastermind in amino acid sequence revealed that this clone had the highest homology to human Mastermind1 (hMam1). Furthermore, The homology in Mastermind-specific domain, basic domain and two acidic domains between these two, was also highest. Therefore, this clone AW765543 was designated as *Xenopus* homologue of *mastermind*, *XMam1*. Amino acids in the other domain except Mastermind-specific domain were not identical but still similar. This evidence will also support that this clone is *Xenopus* homologue of *mastermind1*.

In human Mastermind, it is reported that there exists three kinds of Mastermind, but the homology of each molecule is not so high in amino acid level (Lin *et al.,* 2002; Wu *et al.,* 2002). In Mastermind-specific domain, hMam1 and hMam2 shares with high homology relatively, but hMam3 has little homology to hMam1 and hMam2. These suggest that *XMam1* form a new gene family. Different types of Mastermind may have different regulatory mechanism by different types of Notch signals in various developmental stages and places.

The role of XMam1 in transactivation of the Notch *target gene*

In our experiments, we found that XMam1ΔC, XMam1 lacking of two acidic domains, intensely repressed transactivation of *XESR-1*. Additionally, we found that *XESR-1* repression by XMam1ΔC was rescued by XMam1. Therefore, it is thought that this effect is XMam1-specific. These results show that XMam1 is essential molecule on transactivation of *XESR-1*.

Consistent with this result, Fryer reported that Mastermind is essential to *in vitro* transactivation in complex of Notch-ICD (NICD) and CBF1 on chromatin (Fryer *et al.,* 2002). These data strongly support our experimental result and Mastermind is essential to transactivation of target genes by Notch signaling *in vivo*. In addition to this, the idea that two acidic domains in XMam1 contribute transactivation of Notch target gene coincides with the experimental data reported previously (Wu *et al.,* 2000; Kitagawa *et al.,* 2001; Fryer *et al.,* 2002; Lin *et al.,* 2002; Wu *et al.,* 2002). Therefore, It is thought that Mastermind recognizes and binds to NICD-CSL complex through basic domain, and regulates transcription of target genes through acidic domain.

As additional interest, in this experiment, XMam1 overexpression resulted in the repression of primary neurogenesis although XMam1 did not enhance *XESR-1* transcription. We discuss about this in next section.

The regulatory mechanism of primary neurogenesis

It is reported that Notch signaling is deeply involved in primary neurogenesis and overexpression of components of Notch signal-

ing causes transactivation of target genes which repress the function of proneural genes to repress primary neuron in *Xenopus* (Wettstein *et al.,* 1997; Kiyota and Kinoshita 2002). For instance, it is known that overexpression of the ligands (X-Delta-1 and X-Serrate-1) and NICD (the active form of X-Notch-1) causes reduction of *N-tubulin* expression which is a marker for primary neuron (Chitnis *et al.,* 1995; Chitnis and Kintner 1996; Kiyota *et al.,* 2001). Also, X-Su(H)1/Ank, the active form of Su(H), repress primary neurogenesis (Wettstein 1997). On the contrary, X-Delta-1^{Stu} (Chitnis *et al.,* 1995), X-Serrate-1^{Eco} (Kiyota *et al.,* 2001) and NΔICD (Greenwald 1994; Kiyota and Kinoshita 2002), which are truncated forms lacking intracellular domain, causes overproduction of primary neuron. X-Su(H)1^{DBM}, which lacks in DNA binding ability, also overproduce primary neuron (Wettstein 1997). It is obvious that these all molecules related to Notch signaling contribute primary neurogenesis through transactivation of *XESR-1*. Activation of Notch signaling causes the increase of *XESR-1* transcription and finally represses primary neurogenesis.

In this experiment, XMam1-overexpressed embryos showed the reduction of *N-tubulin* expression. However, these embryos did not show the any change of *XESR-1* expression in spite that we expected that this resulted from the increase of *XESR-1* transcription. That is, it is thought that the decrease of *N-tubulin* expression by XMam1 overexpression was not due to up-regulation. *XESR-1*, one of target gene of Notch signaling, forms an *ESR* gene family. ESR family includes XESR-2 (Turner, unpublished data), 4, 5 (Jen *et al.,* 1999), 6e, 7 (Deblandre *et al.,* 1999) in addition to XESR-1. It is possible that XMam1 regulates primary neurogenesis by transactivating other *ESR* genes. It remains to be examined that ESRs except XESR-1 is involved in primary neurogenesis. Also, it is thought that XMam1 functions in signal transduction system independent of Notch signaling.

In future, it is necessary that the regulatory mechanism of primary neurogenesis is examined in more detail, considering the existence of *Xenopus mastermind* gene family.

Materials and Methods

Eggs and embryos

Xenopus eggs were obtained by injecting *Xenopus laevis* females with 200 units of human chorionic gonadotropin, gestron (Denka Seiyaku, Japan) and were fertilized with the testis isolated from *Xenopus laevis* male by surgical operation. Embryos were dejellied with 1% sodium thioglycollate and cultured in 0.1xMMR (Marc's Modified Ringers, [10mM NaCl; 0.2mM KCl; 0.1mM $MgCl_2$; 0.2mM $CaCl_2$; 0.5mM HEPES, pH 7.5]). The developmental stages of embryos were determined according to Normal table of *Xenopus laevis* (Nieuwkoop and Faber, 1967).

Sequencing

XMam1 cDNA was provided by ATCC (American Type Culture Collection) as EST clone. The nucleotide sequence of *XMam1* cDNA was determined by an ALFred DNA sequencer (Amarsham Bioscience) using a thermosequenase kit (Amarsham Bioscience) and Cy5-labeled primers. A homology search was carried out using FASTA and BLAST search on the GenBank database.

RT-PCR analysis

Total RNA was extracted from various stages according to Normal table of *Xenopus laevis* (Nieuwkoop and Faber, 1967). Oligo (dT)-primed first strand cDNA was prepared from 0.5 μg of total RNA using Reverscript I (Wako, Japan). Each polymerase chain reaction was performed with this cDNA as a template. The program for XMam1 was: 94˚C for 2 min 30 s, 57˚C for 2 min 30 s, 24 cycles of 72˚C 1 min, 94˚C 30 s, 57˚C 30 s and final extension 72˚C 5 min. Primers of *XMam1* were: upstream primer, 5'-TAG ATC AGC ATC ATC TGG GC-3'; downstream primer, 5'-GTC TGG TGA ACT GGT TGA GT-3'. As a loading control, *Histone H4* was used. The program for Histone H4 was: 94˚C for 2 min 30 s, 55˚C for 2 min 30 s, 20 cycles of 72˚C 1 min, 94˚C 30 s, 55˚C 30 s and final extension 72˚C 5 min. Primers of *Histone H4* were: upstream primer, 5'-CGG GAT AAC ATT CAG GGT ATC ACT-3'; downstream primer, 5'-ATC CAT GGC GGT AAC TGT CTT CCT-3'. Negative control (-RT) was performed in the same way without reverse transcriptase. All cycle numbers are within the linear range of amplification.

Whole-mount in situ *hybridization*

Whole-mount *in situ* hybridization was performed according to the improved method of Shain and Zuber (Shain and Zuber, 1996). Hybridized probes were visualized according to the Röche Diagnostics DIG protocol, with the minor alteration that 0.45 μl NBT (75 mg/ml in dimethyl formamide) and 3.5 μl BCIP (Röche Diagnostics) were added to 1 ml AP buffer [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgSO_4$, 0.1% Tween 20, 2.5 mM levamisole]. The antisense probes for *XMam1* cDNA were generated by linearizing with *SalI* the pBluescript SKII(+) vector containing the coding region (about 3.4 kb) *XMam1* cDNA transcribing with T3 RNA polymerase in the presence of digoxygenin-UTP (Röche Diagnostics). Antisense probe of *XMam1* (about 3.4 kb) was used without hydrolysis. The antisense RNA probe of *N-tubulin* was prepared as described (Oschwald *et al*., 1991) and *XESR-1* probe (A kind gift from Prof. Turner DL) was prepared by linearizing with *KpnI* and transcribed with T7 RNA polymerase.

RNA synthesis and microinjection

Full length of *XMam1* was subcloned into pCS2+ vector (Turner and Weintraub, 1994) at the *ClaI/XbaI* site and prepared by linearizing with *NotI* and transcribing into capped mRNA *in vitro* using SP6 RNA polymerase. Capped mRNA was made using the mCAP RNA synthesis kit (Gibco BRL) according to the manufacturer's instructions. The C-terminus-lacking *XMam1* was constructed by removing about 2.5 kb of 3' region using the *SacI* site in *XMam1* and self-ligation. *X-Delta-1* was subcloned into pCS2+ vector at the *EcoRI* site and capped mRNA was prepared by linearizing with *NotI* and transcribing with SP6 RNA polymerase. *β–galactosidase* RNA was produced from pCMV-SPORT β-gal (Stratagene). Fertilization, culture and microinjection were performed as described previously (Moon and Christian, 1989; Asashima *et al.,* 1990). One blastomere of a two-cell-stage embryo was injected with 5 nl mRNA solution (see text and tables).

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