Master of science

Wnt 신호전달 과정에서 RING finger 단백질인 RNF152의 역할

Role of RING finger protein, RNF152 in Wnt signaling pathway

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Abstract

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The members of the RING finger protein family regulate diverse biological processes such as growth, differentiation, transcription, signal transduction, oncogenesis, and apoptosis. In this study, I investigated functional relevance of *Xenopus* RNF152 to Wnt signaling in vertebrate early development. Although *XRNF152* plays a role in both maternal and zygotic transcription in *Xenopus* embryogenesis, its maternal mRNA messages are barely detectable. However, the transcripts were detectable early in the animal hemisphere of the embryo from cleavage to gastrula stages and later in the presumptive head region. Overexpression *XRNF152* suppressed the cellular level of β-catenin, a key component of Wnt pathway. The expression of Wnt target genes such as Siamois and Xnr3 in the organizer region of embryo and in Wnt signals stimulated tissue explants, was down-regulated in *XRNF152* injected embryos. Conversely, anti-sense morpholino oligo-mediated knockdown of *XRNF152* resulted in embryonic morphological defects such as shortened and dorsally bent body axis. We found that *XRNF152* suppresses the expression of a neural crest marker genes during *Xenopus* development. Taken together, these results suggest that *XRNF152* regulates neural crest formation, acting as an inhibitor of Wnt signaling pathways.

Keywords: Wnt signaling, Ring finger protein, *Xenopus* laevis

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Ⅰ. Introduction

The Wnt signaling pathway is one of the standard mechanisms that direct cell proliferation, cell polarity, and cell fate determination during embryonic development and tissue homeostasis [1]. The Wnt/β-catenin pathway is activated when a Wnt ligand binds to transmembrane co-receptor, these event lead to inhibition of Axin-mediated β-catenin phosphorylation and thereby to the stabilization of β-catenin. Accumulated β-catenin is translocating to nucleus activates Wnt target gene expression [2]. The canonical Wnt pathway is controls essential development gene expression by regulating the amount of the transcriptional co-activator β-catenin [3]. The canonical Wnt signaling pathway is used to make cell fate decisions in many developmental and cellular activities [3, 4]. Also, It's essential for the embryonic body axis in *Xenopus* development. The localized stabilization of β-catenin of the early *Xenopus* embryo is a crucial event in the formation of the dorsal organizer [5]. Depletion of maternal β-catenin can cause a complete loss of dorsal structures in the embryo and ectopic expression of β-catenin ventrally leads to the formation of a secondary dorsal axis [6]. Therefore, experiments that disrupt β-catenin activity can affect both D-V and A-P axis development in *Xenopus* [7]. Wnt family secreted proteins are implicated in every step of neural development [8]. This process of neural development leads to formation of a neural plate with a defined anterior-posterior axis. Wnt proteins provide

positional information within the embryo for anterior-posterior axis specification of the neural plate [9]. Wnt signals is required for multiple steps of neural plate and neural crest development [10].

RING finger proteins (RNFs) are a family of the RING finger motif [11]. Canonical RING finger domain has CX2-$CX_{[9-39]}$-$CX_{[1-3]}$-$HX_{[2-3]}$-CX2-$CX_{[4-48]}$-CX2-C, and X can be any amino acid [12]. RING finger domain is important for the E3 ubiquitin ligase activity of RNFs [13]. Ubiquitination is an essential post-translational modification that is including ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) for many cellular activities [14]. The RNFs has a various activity of E3 ligase function in various biological processes. The RNFs are important for growth, differentiation, transcription, signal transduction, oncogenesis, and apoptosis and so on [15]. It is polyubiquitinated through its RING finger domain [6]. and has the function of transmembrane RING finger ubiquitin ligases in modulating Wnt signaling in cancer. The RNF152 is a new member of the RNFs family [16]. It is known polyubiquitinated through its RING finger domain [17].

In this study, despite extensive studies on the cellular role for RNFs, the importance of RNF152 in embryonic development has not been tested in *Xenopus*. This protein is expected to be related to the Wnt signaling. In this study, we identify *XRNF152* as a negative regulator of Wnt signaling.

Ⅱ. Materials and Methods

1. Embryos and in vitro fertilization

Eggs were obtained from *Xenopus* laevis frogs primed with 800 units of Human Chorionic Gonadotropin (HCG). They were *in vitro* fertilized using macerated testis, dejellied in 2% cysteine solution (pH 7.8) and cultured in 0.33x Modified Ringer (MR). Developmental stages of embryos were determined according to Nieukoop and Faber's normal table of development [18].

2. Microinjection

Microinjection was carried out in 0.33x MR containing 4% Ficoll PM400 (GE Healthcare) using a Pico Injector (Harvard). Injected embryos were cultured in 0.33x MR until stage 8 and then transferred to 0.1x MR containing gentamycin until they reached the appropriate embryonic stages.

3. Animal cap assay

Animal cap explants are dissected at stage 8-8.5 from the embryos animally injected with RNF152 mRNA or morpholino (MO) and then cultured in 1x MR containing gentamycin until the desired embryonic stages.

4. Western blot

Whole embryos or animal cap explants were incubated in Triton X-100 lysis buffer (1% Triton X-100, 50 mM NaCl, 1 mM EDTA, 50 mM Tris-CI (pH 7.6), 1 mM PMSF, 10 mM NaF, 1 mM Na3VO4, 20 μg/ml aprotinin, 20 μg/ml leupeptin). Equal amounts of protein were separated by 8% SDS-PAGE and then immunoblotting was performed according to standard protocol using antibodies specific for with β-actin (Santa Cruz), β-catenin (Cell signaling), Non-p-β-catenin (Cell signaling).

5. RT-PCR analysis

Total RNA was isolated from whole embryos or animal cap explants using TRIzol Reagent (Ambion) and treated with RNase-free DNase I (Roche) to remove genomic DNA. RNA was transcribed by using M-MLV reverse transcriptase (Promega). PCR amplification was

performed using Taq polymerase (TaKaRa). PCR products were analyzed on 2% agarose gels.

6. In situ hybridization

Whole-mount *in situ* hybridization was performed with embryos that were animally or marginally injected with RNF152 mRNA or MO, and the embryos were incubated with Digoxigenin (DIG)-labeled anti sense probes against key marker genes and visualized with BM purple substrate (Roche), as described by Harland RM's *in situ* hybridization (Harland, 1991).

7. Reporter gene assay

Whole embryos were homogenized in 1x lysis buffer (Promega). Reporter assays were performed using luciferase assay kit (Promega) according to the manufacture's protocol. Three independent experiments were performed.

Ⅲ. Results

1. Spatio-temporal expression pattern of RNF152 in Xenopus embryo

To investigate the biological function of *XRNF152* in vertebrates, we observed its developmental expression pattern in *Xenopus* embryos. We examined by RT-PCR analysis whole embryos harvested at several embryonic stages to observe the temporal expression pattern of *XRNF152* (Fig. 1A).

XRNF152 previously unknown both maternal and zygotic transcription. Its zygotic transcription is abundant but its maternal mRNA messages are barely detectable in embryogenesis. We also performed *in vivo* by whole-mount *in situ* hybridization experiments to determine the spatial expression pattern of *XRNF152* in *Xenopus* embryos. At the early cleavage stages, *XRNF152* maternal transcripts are localized to the animal hemisphere of the embryo, and this pattern persists into the gastrula stages (data not shown). The expression of *XRNF152* is strongly observed in the presumptive neural crest in the neurula stage (Fig. 1B and C). At the early tailbud stages, its express was detectable in the presumptive head region (data not shown).

2. Depletion of XRNF152 disrupts the formation of dorsal axial structures

In order to examine the effects of depletion of *XRNF152* on the early development of vertebrates, *XRNF152* anti-sense morpholino oligo (MO) was microinjected into the dorsal-marginal region of *Xenopus* cleavage stage embryos and the resulting morphological phenotypes were observed at the tadpole stages. These phenotypes showed that injection of *XRNF152* MO caused a failure in gastrulation movements, lack anterior structures and shortened with dorsally bent body axis, but Co MO did not (64%, n=22 for *XRNF152* MO, Fig 2A and B).

In addition, the morphological phenotypes of animal region injection of the *XRNF152* MO caused gastrulation-defective morphologies, including shortened and dorsally bent body axis (62%, n=45 for *XRNF152* MO, Fig 2C and D). These defects are considered that expanded Wnt signal in embryos. This effect of the MO was rescued by co-injection of *XRNF152* mRNA, which contains no target sequence of the MO (48%, n=45 for Res, Fig 2E).

3. Role of XRNF152 in the regulation of Wnt signaling

Western blotting analysis was performed to test the levels of total cellular β-catenin that were increased in Xwnt8-mRNA microinjected whole embryos more than in control whole embryos as compared a lane 1 and lane 3. As shown in Fig. 3A, overexpression of wild-type

of *XRNF152* down-regulated the levels of total cellular β-catenin as compared an lane 3 and lane 4, but not by ΔTm mutant as compared an lane 3 and lane 6. These results indicate that *XRNF152* has a negative effect on the stabilization of β-catenin, thus reducing its total cellular levels.

As *XRNF152* appears to down-regulate Wnt signaling, as shown above, we examined the effects of the gain-of-function and loss-of-function for *XRNF152* function on the activity of Wnt responsive promoter using luciferase reporter assays in *Xenopus* embryos (Fig. 3B and C). To evaluate the activation of siamois expression by β-catenin, we microinjected siamois promoter construct (S01234) and pRL-TK vector (active the luciferase activity). The expression of Wnt signal is induced by Xwnt8 mRNA microinjected animal pole region of whole embryos, and the activities of the Wnt responses were suppressed by co-injection of *XRNF152* (Fig. 3B). By contract, co-injection of depletion of *XRNF152* enhanced the activities of the Wnt responses (Fig. 3C).

4. Overexpression of XRNF152 inhibits the expression of Wnt target genes

RT-PCR analysis revealed that the expression of Wnt target genes marker siamois and xnr3 were induced by ectopically in XWnt8-stimulated animal explants, and their expression levels were down-regulated gradually by co-injection of increasing doses of wild-type of

XRNF152. In addition, co-injection of the C-S mutant more inhibits than did wild-type in Wnt signaling, and ΔTm mutant had no effect (Fig. 4).

5. Knockdown of XRNF152 enhances the expression of Wnt target genes

We also found using RT-PCR analysis that co-injection of *XRNF152* MO A and MO B could up-regulate the ectopic expression of Wnt target genes marker including siamois and xnr3 in animal caps which was induced by stimulation with XWnt8 mRNA, but Co MO had no effect on their expression. These increase effects of *XRNF152* MO could be reversed by co-injection *XRNF152* mRNA (Fig. 5).

6. Overexpression of XRNF152 down-regulates the neural crest formation

Neural crest markers are induced ectopically in animal cap tissue by co-expressing Wnt and BMP inhibitors such as noggin and chordin. We used this animal cap assay to test whether *XRNF152* is required for ectopic neural crest induction. Co-injection of Xwnt8 and noggin RNAs induced the expression of Sox9 and Slug in the animal cap tissues. Overexpression of wild-type of *XRNF152* and C-S mutant could down-regulate this ectopic expression, whereas the co-injection of ΔTm mutant had no effect (Fig. 5A). Next, we investigated the effects of

overexpression of *XRNF152* on the initial steps of neural crest induction by whole-mount *in situ* hybridization. Targeted injection of *XRNF152* inhibited the expression of the neural crest markers, Sox9 (71%, n=28), Slug (61%, n=28) and Sox10 (67%, n=27) in the injected side, compared with the uninjected side of embryos (Fig. 6B-M).

7. Knockdown of XRNF152 up-regulates in the neural crest formation

We used this animal cap assay to test whether *XRNF152* affects ectopic neural crest induction. Co-injection of Xwnt8 and noggin RNAs induced the expression of neural crest markers. This experiment revealed that *XRNF152* MO induces ectopic neural crest induction (Fig. 7A). Additionally, we examined the effects of knockdown of *XRNF152* of neural crest induction by whole-mount *in situ* hybridization in the neurula stage. Targeted injection of *XRNF152* MO, enhanced the expression of the neural crest markers, Sox9 (67%, n=42), Slug (73%, n=30) and Sox10 (64%, n=33) in the injected side, compared with the uninjected side of embryos (Fig. 7C, F and I). These enhancing effects of *XRNF152* MO, which could be decreased by co-injection of *XRNF152* mRNA (61%, n=36 for Sox9; 75%, n=32 for Slug; 61%, n=38 for Sox10, Fig. 7D, G and J).

Ⅳ. Discussion

The RING finger proteins belong to a large protein family [19]. The RNFs are E3 ubiquitin ligases that regulate key cellular functions, such as cell signaling. The RNF146 promotes Wnt signaling by mediating tankyrase-dependent degradation of axin. It is identified as a critical regulator of Axin and acts positive regulator of Wnt signaling [20] [21]. In human colon cancer cell, RNF43 blocks Wnt responses and targets surface expressed frizzled receptors to lysosomes by the RING domains. It is encoded by Wnt target genes and composed a negative Wnt feedback loop [22] [23]. Also RNF152 has function roles in suppression or progression of cancer in tumorigenesis based on their ubiquitination targets [24]. The physiological functions of RNF152 have not yet been handled in embryogenesis.

In this study, we have identified the expression pattern of *XRNF152* during embryos development. The *XRNF152* is zygotic gene, and detectable in the neural crest region (Fig. 1). The Wnt/β-catenin signaling regulates gene transcription by controlling the stability of β-catenin protein [2]. We found *XRNF152* that degradation of β-catenin level in *Xenopus* embryos (Fig. 3). In addition, it is shown speculated that *XRNF152* acts as a negative regulator downstream of Wnt/β-catenin signaling pathways. The Wnt signals can induce neural tissue in the ectoderm and induce a complete axis in the mesoderm or endoderm in *Xenopus* embryos [25]. The overexpression of *XRNF152* was inhibits Wnt target marker (Fig.

4). In addition, unexpectedly C-S mutant is more inhibits than wild-type in Wnt signaling, and ΔTm mutant is not affect. And, the knockdown of *XRNF152* was enhances Wnt target marker (Fig. 5). The Wnt signaling has potent effects on Bmp4 expression suggest that the ability of Wnt to induce neural crest. A Wnt extracellular interaction with BMPs would lead to neural induction, whereas signaling through receptors would lead to the classic Wnt-like responses [26]. To dissect whether neural crest induction by Wnt signaling is the result of affected by *XRNF152*, we examined the neural crest expression during development. In *Xenopus* neurula, we found *XRNF152* interferes with the induction of the neural crest. This expression pattern suggests that *XRNF152* affects neural crest induction (Fig.6 and 7). Together these findings provide important and novel sights into the role of *XRNF152* in Wnt signaling during embryos development. Further experiments will be necessary to test these hypotheses and to elucidate the precise mechanism underlying the activity of *XRNF152*.

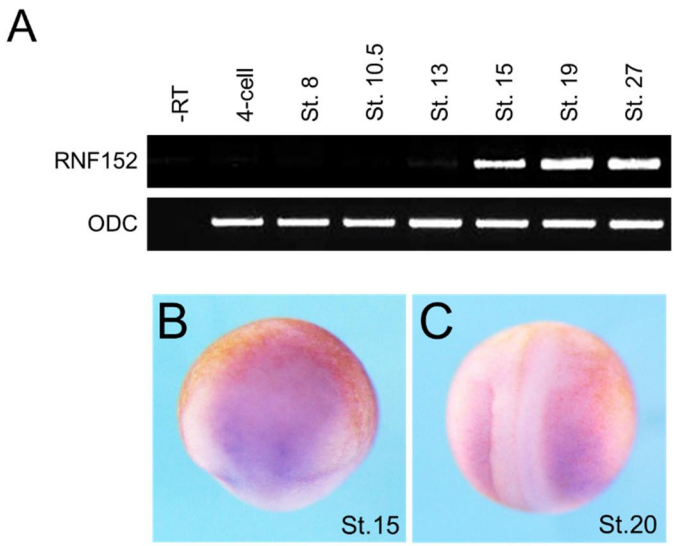


Figure 1. Expression pattern of *XRNF152* in *Xenopus* development

(A) RT-PCR analysis showing the temporal expression of RNF152 during *Xenopus* development. Ornithine decarboxylase (ODC) serves as a loading control. -RT, control in the absence of reverse transcriptase.

(B-D) Whole-mount *in situ* hybridization showing the spatial expression pattern of *XRNF152* during *Xenopus* development.

(B) Anterior view with dorsal to the top.

(C) Dorsal view with anterior to the top.

(D) Lateral view with anterior to the left.

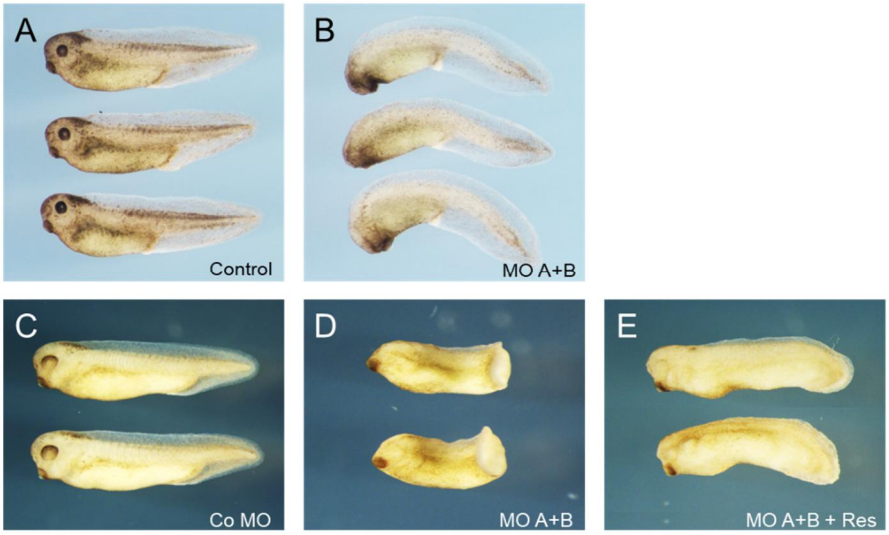


Figure 2. Morphological phenotypes caused by *XRNF152* morphants

The defective phenotype of *XRNF152* morphant.

(B) Embryos were injected in dorso-marginal region of one blastomeres at the 2-cell stage with RNF MO (40 ng, a combination of RNF MO A and RNF MO B), and cultured to tadpole stages.

(C-E) Embryos were injected in animal pole region of four blastomeres at the 4-cell stage with RNF MO (60 ng, a combination of RNF MO A and RNF MO B) or with *XRNF152* mRNA (200 pg), and cultured to tadpole stages. Embryos in (A-E) are shown in lateral views with anterior to the left. Control, an uninjected control embryo.

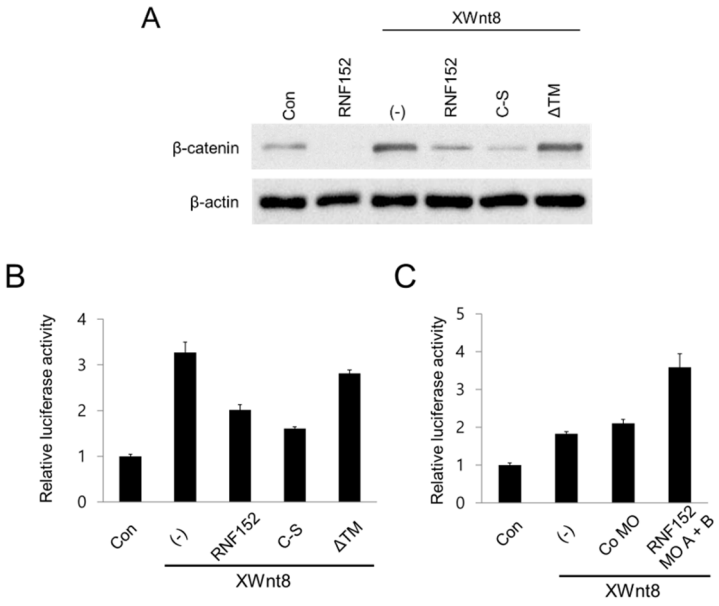


Figure 3. The activity of β-catenin is reduced in embryos overexpressing *XRNF152*

(A) 4-cell stage embryos were injected in the animal pole region with XWnt8 mRNA (100 pg), together with wild-type *XRNF152* or its mutant C-S and ΔTm each (300 pg), and cultured to stage 10.5 for western blotting analysis. β-actin is a loading control.

(B, C) Luciferase reporter assay using 4-cell stage embryos that were injected in the animal pole region with the siamois reporter genes, S01234 (40 pg) and luciferase control reporter vectors, pRL-TK vector (1 pg). Together with XWnt8 mRNA (100 pg) alone or with wild-type *XRNF152* or its mutant C-S and ΔTm each (300 pg), Co MO (40 ng) or combination of RNF MO (40 ng), and cultured to stage 10.5 for measurement of firefly and renilla luciferase activities. Three independent experiments were performed, and a single representative result is shown. Error bars denote standard deviations.

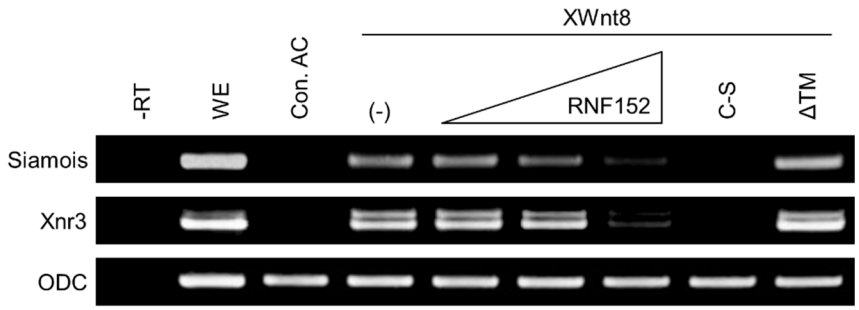


Figure 4. Overexpression of *XRNF152* inhibits Wnt signaling

4-cell stage embryos were injected in the animal pole region with XWnt8 mRNA (100 pg), alone or with wild-type *XRNF152* (50, 100, 300 pg) or its mutant C-S and ΔTm each (300 pg), and the animal cap explants were dissected at stage 8 and cultured to stage 10.5 for RT-PCR analysis. ODC serves as a loading control. -RT, control in the absence of reverse transcriptase. WE, stage 10.5 whole embryos. Control, uninjected control animal caps. (-), injection of only XWnt8 mRNA.

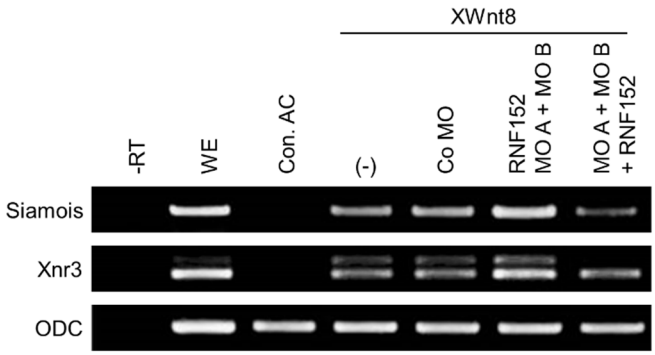


Figure 5. Knockdown of *XRNF152* enhances Wnt signaling

(A) 4-cell stage embryos were injected in the animal pole region with XWnt8 mRNA (100 pg), alone Co MO (40 ng) or combination of RNF MO (40 ng), and the animal cap explants were dissected at stage 8 and cultured to stage 10.5 for RT-PCR analysis. ODC serves as a loading control. -RT, control in the absence of reverse transcriptase. WE, stage 10.5 whole embryos. Control, uninjected control animal caps. (-), injection of only XWnt8 mRNA.

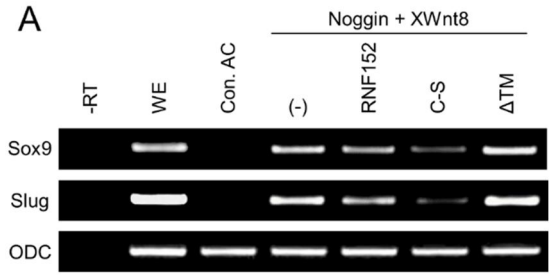
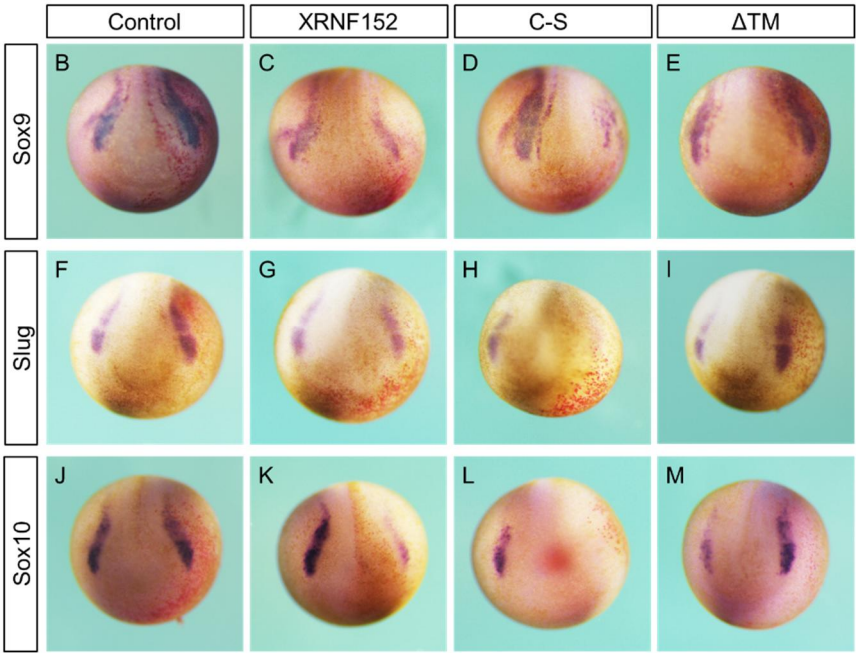


Figure 6. Overexpression of *XRNF152* suppresses of neural crest marker

(A) 4-cell stage embryos were injected in the animal pole region with the indicated combination of Noggin (100 pg), XWnt8 mRNA (100 pg), alone or with wild-type *XRNF152*, mutant C-S and ΔTm (300 pg) and the animal cap explants were dissected at stage 8 and cultured to stage 10.5 for RT-PCR analysis. ODC serves as a loading control. -RT, control in the absence of reverse transcriptase. WE, stage 10.5 whole embryos. Control, uninjected control animal caps. (-), injection of only XWnt8 mRNA.



(B-M) One blastomere of a 2-cell stage embryo was injected in the dorsal-animal region with wild-type *XRNF152*, mutant C-S and ΔTm (300pg) as indicated, and then subjected to *in situ* hybridization for detection of mRNAs specific for Sox9, Slug and Sox10 at stage 16. The injected side was traced by lacZ staining. All embryos are shown in the dorsal-anterior view with posterior to the top.

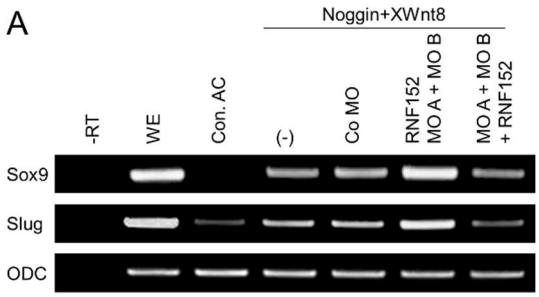
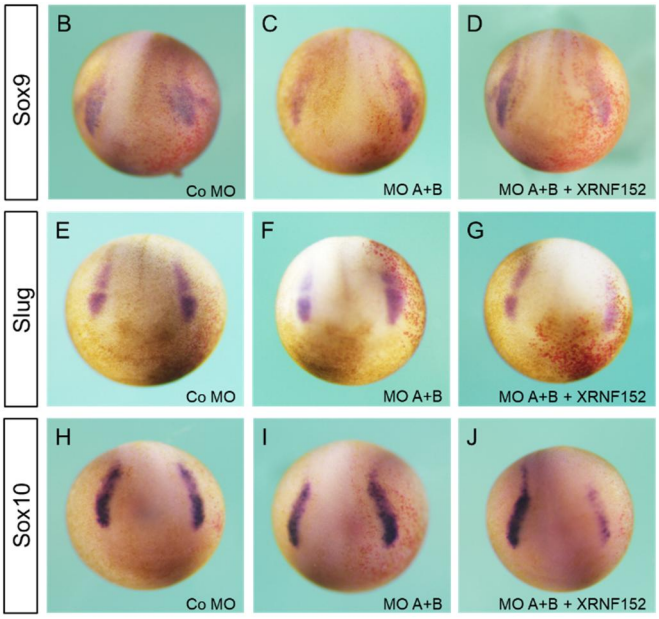


Figure 7. Knockdown of *XRNF152* induces multiple of neural crest marker

(A) 4-cell stage embryos were injected in the animal pole region with the indicated combination of Noggin (100 pg), XWnt8 mRNA (100 pg), alone or Co MO (40 ng) or combination of RNF MO (40 ng), and the animal cap explants were dissected at stage 8 and cultured to stage 10.5 for RT-PCR analysis. ODC serves as a loading control. -RT, control in the absence of reverse transcriptase. WE, stage 10.5 whole embryos. Control, uninjected control animal caps. (-), injection of only XWnt8 mRNA.



(B-J) One blastomere of a 2-cell stage embryo was injected in the dorsal-animal region with Co MO (40 ng) and RNF MO (40 ng, a combination of RNF MO A and RNF MO B) and RNF MO with *XRNF152* mRNA (200 pg) as indicated and then subjected to *in situ* hybridization against Sox9, Slug and Sox10 at stage 16. The injected side was traced by lacZ staining. All embryos are shown in the dorsal-anterior view with posterior to the top.

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국문요약(Summary in Korean)

RING finger 단백질 패밀리의 구성원은 성장, 분화, 전사, 신호 전달, 종양 형성 및 세포자멸사와 같은 다양한 생물학적 과정을 조절한다. 이 연구에서는 척추동물의 초기 개발에서 Wnt 신호 전달과 *Xenopus* RNF152 의 기능적 관련성을 조사했다. XRNF152 는 *Xenopus* embryogenesis 에서 maternal 및 zygotic 전사에 중요한 역할을 하지만, maternal 때 발현에서는 거의 감지할 수 없었다. 그러나, 전사물은 초기의 난할부터 낭배기까지 위축 단계까지 배아의 동물 반구와 나중에 추정 머리 영역에서 검출되었다. 과발현된 XRNF152 는 Wnt 신호전달 경로의 주요 성분인 β-catenin 의 세포 수준을 억제하였다. 또한, 배아의 organizer 영역 및 Wnt 신호 자극된 조직을 체외 배양하여 Siamois 및 Xnr3 과 같은 Wnt 표적 유전자의 발현을 보았을 때, XRNF152 주사된 배아에서 하향-조절되었다. 반대로, XRNF152 의 anti-sense morpholino oligo 로 매개된 knockdown 된 배아는 단축되고 등쪽으로 구부러진 신체 축과 같은 형태학적 결함을 초래하였다. 우리는 XRNF152 가 *Xenopus* 개발하는 동안 신경 능선 표적 유전자의 표현을 억제하는 것을 발견하였다. 종합적으로, 이 결과는 XRNF152 가 Wnt 신호전달 경로의 억제제로서 작용하며 신경 능선 형성을 조절함을 시사한다.

중심단어: Wnt 신호전달, RING finger 단백질, 제노푸스