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RNF152 negatively regulates Wnt/β-catenin signaling in *Xenopus* embryos

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The Wnt/β-catenin signaling plays crucial roles in early development, tissue homeostasis, stem cells, and cancers. Here, we show that RNF152, an E3 ligase localized to lysosomes, acts as a negative regulator of the Wnt/β-catenin pathway during *Xenopus* early embryogenesis. Overexpression of wild-type (WT) *RNF152* inhibited XWnt8-induced stabilization of β-catenin, ectopic expression of target genes, and activity of a Wnt-responsive promoter. Likewise, an E3 ligase-defective RNF152 had repressive effects on the Wnt-dependent gene responses but not its truncation mutant lacking the transmembrane domain. Conversely, knockdown of *RNF152* further enhanced the transcriptional responses induced by XWnt8. RNF152 morphants exhibited defects in craniofacial structures and pigmentation. In line with this, the gain-of-RNF152 function interfered with the expression of neural crest (NC) markers, whereas its depletion up-regulated NC formation in the early embryo. Mechanistically, RNF152 inhibits the polymerization of Dishevelled, which is key to Wnt signaling, in an E3 ligase-independent manner. Together, these results suggest that RNF152 controls negatively Wnt/β-catenin signaling to fine-tune its activity for NC formation in *Xenopus* embryo. [BMB Reports 2022; 55(5): 232-237]

INTRODUCTION

Signaling by the Wnt family of secreted glycoproteins controls a wide range of cellular processes, including cell growth, cell polarity, cell differentiation and cell movement, during early development and tissue homeostasis (1). The canonical Wnt/β-catenin signaling functions by regulating the levels of transcriptional co-activator β-catenin (2). Without Wnt stimulation, cytoplasmic β-catenin is targeted for destruction by a complex consisting of the scaffold protein, Axin and APC and the serine/threonine kinases, CK1 and GSK3. Within this complex, CK1 and GSK3 sequentially phosphorylate β-catenin, which in turn undergoes β-TrCP-mediated ubiquitination and is degraded by the proteasome. Consequently, cytoplasmic β-catenin is kept at a low level, resulting in the transcription of Wnt target genes being repressed by the TCF/LEF family of proteins. When a Wnt ligand binds to Frizzled receptor and LRP6 co-receptor, GSK3-mediated phosphorylation of β-catenin is inhibited through the action of Dishevelled and the binding of Axin to LRP6. The stabilized β-catenin accumulates and moves to the nucleus, subsequently interacting with TCF/LEF factors and activating the expression of target genes.

Ubiquitination is a reversible posttranslational modification that affects the stability, positioning, and function of modified proteins. This modification is catalyzed by a three-enzyme cascade including a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). E3 ligases are crucial for determining the specificity of the ubiquitinated substrates (3). According to their structural characteristics, E3 ligases are classified into two major categories: the HECT domain family and the RING domain family (4). RNF152 is an E3 ubiquitin ligase with RING domain and single-pass transmembrane (TM) domain, which is mainly localized in lysosomes (5). RNF152 exhibits pro-apoptotic activity when overexpressed (5) and negatively controls mTORC1-mediated signaling by targeting RagA or Rheb GTPases for ubiquitination (6, 7). In addition, RNF152 positively regulates TLR/IL-1R-mediated inflammatory response by facilitating oligomerization of the adaptor protein MyD88, which is independent of its E3 ligase activity (8). In zebrafish embryos, RNF152 is involved in the development of the eyes and brains, and in Notch-Delta signaling (9).

In this study, we observed that RNF152 controls negatively Wnt/β-catenin signaling in *Xenopus* early development. Overexpression of *RNF152* inhibited Wnt-induced β-catenin stabilization and gene expression, whereas its knockdown sensitized the transcriptional responses to the Wnt signal. Depletion of *RNF152* caused morphological defects reminiscent of disrupted neural crest (NC) specification. These results suggest that RNF152 functions to fine-tune the activity of Wnt/β-catenin signaling during NC formation of the early embryo.

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RESULTS

Overexpression of RNF152 represses Wnt/β-catenin-dependent gene expression

It has been shown that RNF152 inhibits cell proliferation and its expression is down-regulated in diverse types of cancers (10). However, not much is known about the biological activities of RNF152 in vertebrate early development. First, as a strategy to explore the developmental functions of RNF152, we overexpressed wild type (WT) *RNF152* or its mutants (Fig. 1A) in stem cell-like ectodermal tissues of *Xenopus* embryo and examined the effects of its gain-of-function on key cellular signaling pathways. Interestingly, injection of WT *RNF152* mRNA resulted in reduced level of endogenous β-catenin in animal cap cells (Fig. 1B, lane 2). Its co-expression also interfered with XWnt8-induced increase in the level of β-catenin (lanes 3 and 4). Of note, co-injection of the E3 ligase-deficient mutant of *RNF152*, *RNF152*(*CS*), inhibited more strongly the up-regulation of β-catenin level in response to XWnt8 signal (lane 5), but its truncation mutant lacking the transmembrane domain, RNF152(dTM), had no effect on it (lane 6). These results indicate that membrane targeting of RNF152, but not its enzymatic activity, is critical for its repressive effect on Wnt/β-catenin signaling. We next investigated the effect of overexpression of *RNF152* on the activity of a Wnt-responsive promoter. As analyzed by luciferase assays (Fig. 1C), co-expression of WT *RNF152* elicited an inhibitory effect on XWnt8-induced activity of a reporter driven by Wnt-responsive siamois promoter (S01234). Co-injection of *RNF152*(*CS*) also reduced the level of reporter activity induced by XWnt8 signaling, but not *RNF152*(*dTM*). We also performed RT-PCR to test whether the gain-of-RNF152 function would down-regulate the transcription of Wnt target genes such as *siamois* and *Xnr3*. Injection of *XWnt8* mRNA caused ectopic expression of these target genes in animal caps, which could be blocked by co-expression of WT *RNF152* (Fig. 1D, lanes 4 and 5; Supplementary Fig. 1A). Co-expression of *RNF152*(*CS*), but not *RNF152*(*dTM*), also inhibited their ectopic expression induced by XWnt8 (Fig. 1D, lanes 6 and 7; Supplementary Fig. 1A). Taken together, these data suggest that RNF152 acts as a membrane protein to regulate negatively Wnt/β-catenin signaling.

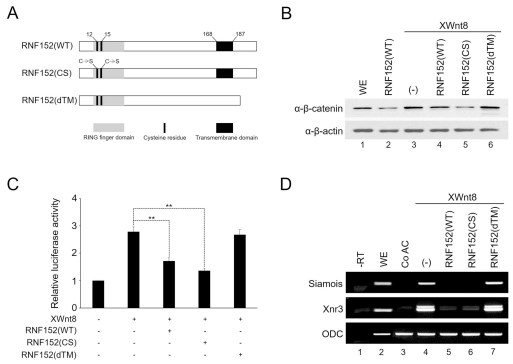

Fig. 1. Overexpression of *RNF152* inhibits Wnt-induced transcriptional responses. (A) Schematic diagram of wild-type (WT) *Xenopus* RNF152 and its mutant proteins, RNF152(CS) with two serine residues substituted for cysteine residues in the RING finger domain and RNF152(dTM) with deletion of the transmembrane domain. (B-D) Four-cell stage embryos were injected in the animal pole region with the indicated combination of mRNAs, cultured to stage 10.5 and harvested for western blotting (B) and reporter assays (C) or animal caps were excised from the injected embryos at stage 8, incubated until sibling embryos reached stage 10.5 and subsequently subjected to RT-PCR analysis (D). The amount of mRNA injected: *XWnt8* (100 pg), WT *RNF152* (500 pg), *RNF152*(*CS*) (500 pg) and *RNF152*(*dTM*) (500 pg). Data in (C) are expressed as the mean ± SEM (n = 3 biological replicates). **P-value < 0.01. β-actin and ODC serve as loading controls. WE, uninjected control whole embryo. Co AC, uninjected control animal caps. (−), no injection of WT *RNF152* or its mutants. −RT, control in the absence of reverse transcriptase.

Knockdown of RNF152 enhances Wnt/β-catenin signaling

Prior to the loss-of-RNF152 function analysis in *Xenopus* embryo, we observed the developmental expression pattern of its transcripts. Temporally, *RNF152* exhibited both maternal and zygotic

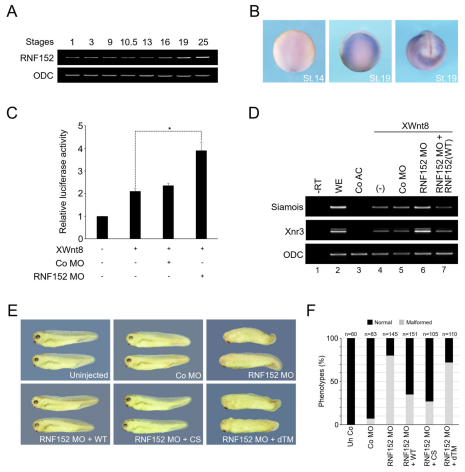

Fig. 2. Knockdown of *RNF152* up-regulates Wnt-dependent gene responses. Temporal (A) and spatial (B) expression patterns of *RNF152* in *Xenopus* early development were analyzed by RT-PCR and *in situ* hybridization, respectively. In (B), the leftmost embryo is shown in dorsal view with anterior to the top, the middle embryo in lateral view with anterior to the left and the rightmost embryo in anterior view with dorsal to the top. (C, D) Four-cell stage embryos were injected in the animal pole area as indicated with *XWnt8* (100 pg), *RNF152* MO (40 ng), Co MO (40 ng) and WT *RNF152* mRNA (300 pg) and cultured to stage 10.5 for reporter assays (C) or animal caps were dissected at stage 8 from the injected embryos and incubated to stage 10.5 for RT-PCR analysis (D). Data in (C) are expressed as the mean ± SEM (n = 3 biological replicates). *P-value < 0.05. (−), no co-injection of MO and/or *RNF152* mRNA. (E) Four-cell stage embryos were injected radially in the marginal zone with *RNF152* MO (60 ng), Co MO (60 ng), WT *RNF152* (300 pg), *RNF152*(*CS*) (300 pg) and *RNF152*(*dTM*) (300 pg) mRNA as indicated and cultured to tadpole stages. Embryos are shown in lateral views with anterior to the left. (F) Quantification of the phenotypes shown in (E).

transcription in early embryogenesis as analyzed by RT-PCR (Fig. 2A; Supplementary Fig. 1B). Notably, its zygotic messages increased after the onset of neurulation. We also performed *in situ* hybridization experiments to examine its spatial expression pattern. From the cleavage stages to late gastrula stages, weak expression of *RNF152* was detected in the animal pole region of the embryo (data not shown). Its faint expression persisted in the neural plate of the early neurulae (Fig. 2B, leftmost). At the mid- and late neurula stages, *RNF152* was strongly expressed in the preplacodal ectoderm and neural crest (NC) area lateral to the neural fold (Fig. 2B, middle and rightmost).

For loss-of-function analysis of RNF152, we adopted an antisense morpholino oligo (MO)-mediated knockdown approach. For this, two different MOs (MO-a and MO-b) were designed to target distinct sequences around translation initiation sites of *RNF152* mRNA, thus blocking its translation. Western blotting demonstrated that MO-a and MO-b each, but not control (Co) MO, could inhibit the production of RNF152 protein with no effect on the level of actin (Supplementary Fig. 2), confirming the efficacy and specificity of both MOs. In further analysis, the two MOs were mixed at an equimolar ratio and co-injected (the mixed MOs will be designated as *RNF152* MO hereafter).

Since overexpression of *RNF152* regulated negatively Wnt/β-catenin signaling, its knockdown was expected to promote gene responses induced by Wnt signals. In support of this assumption, co-injection of *RNF152* MO, but not Co MO, further enhanced XWnt8-induced activity of the reporter under the control of a Wnt-responsive element (Fig. 2C). Consistently, depletion of *RNF152* augmented the ectopic expression of target genes caused by XWnt8 stimulation (Fig. 2D, lanes 4-6; Supplementary Fig. 1C). This promoting effect of *RNF152* MO was reverted by co-expression of WT *RNF152* mRNA resistant to MO inhibition (lane 7). We also examined the morphological phenotypes of RNF152 morphants by targeting the injection toward the marginal region of embryos. Radial injection of *RNF152* MO resulted in defects in craniofacial structures such as the eyes, lateral pigmentation and dorsal fin (Fig. 2E, F). This malformation could be partially rescued by co-expression of WT *RNF152* mRNA lacking the MO target sequence, thus supporting the specific effects of MO. Co-injection of *RNF152*(*CS*) could efficiently reverse the defective phenotypes of RNF152-depleted embryos but not *RNF152*(*dTM*) (Fig. 2E, F). These morphological defects of RNF152 morphants resemble those caused by the disruption of NC formation in *Xenopus* embryo (11). Wnt/β-catenin signaling is critical for NC development in the early embryo (11). Given the expression pattern of RNF152, these data suggest that its knockdown enhances Wnt/β-catenin signaling, leading to disrupted NC specification.

The gain-of-RNF152 function causes defective neural crest formation

We further tested whether the function of RNF152 is relevant to NC formation. As shown in Supplementary Fig. 3A, B, co-expression of *XWnt8* and a BMP antagonist, *noggin* induced ectopic expression of NC markers including *Slug* and *Sox9* in animal caps, which could be reversed by co-injection of WT *RNF152* (lanes 4 and 5). Co-expression of *RNF152*(*CS*) also abrogated their ectopic expression, whereas *RNF152*(*dTM*) had no effect (lanes 6 and 7). These results indicate that the increased levels of RNF152 have an inhibitory influence on NC specification. We next examined the effects of overexpression of *RNF152* on the *in vivo* expression of NC markers in neurulae. Targeted injection of WT *RNF152* repressed the expression of the NC markers *Slug*, *Sox9*, and *Sox10* in the injected side, compared with the uninjected side of embryos (Supplementary Fig. 3C, D). Abrogated expression of these markers was also observed in embryos injected with *RNF152*(*CS*). In contrast, RNF152(dTM) had no suppressive effect but appeared to act in a dominant-negative manner, resulting in an expanded expression of the markers. Together, these results suggest that the gain-of-RNF152 function down-regulates Wnt/β-catenin signaling, thereby negatively affecting NC formation.

Depletion of RNF152 up-regulates NC formation

Using the animal cap assays, we also investigated the effects of knockdown of *RNF152* on NC specification. Stimulation with both XWnt8 and noggin induced ectopically the expression of NC markers in the animal caps, which could further be enhanced by co-injection of *RNF152* MO (Fig. 3A, lanes 4-6;

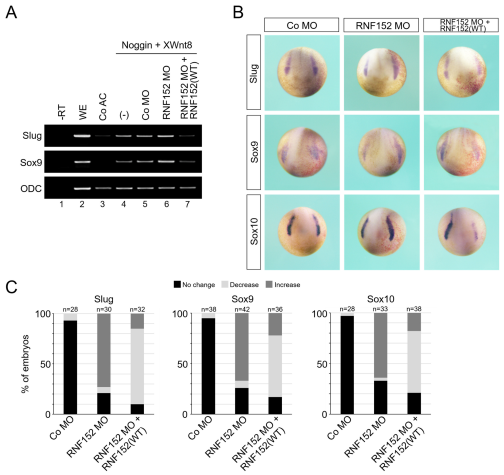

Fig. 3. Depletion of *RNF152* causes increased expression of neural crest markers. (A) Four-cell stage embryos were injected in the animal pole region with *noggin* (100 pg) and *XWnt8* (100 pg) with or without *RNF152* MO (40 ng), Co MO (40 ng) and WT *RNF152* mRNA (300 pg), and animal caps excised from the injected embryos were processed for RT-PCR analysis. (B) Embryos were injected in one blastomere at two-cell stage with *LacZ* (100 pg) along with the same amount of MOs and mRNA as in (A), and subjected to *in situ* hybridization at stage 16. Embryos are shown in dorso-anterior view with posterior to the top. The injected sides are on the right as indicated by red LacZ staining. (C) Quantification of the results from *in situ* hybridization experiments in (B).

Supplementary Fig. 1D). This promoting effect of *RNF152* MO was reverted by co-expression of WT *RNF152* mRNA immune to MO inhibition (lane 7). Consistently, the *in vivo* expression of NC markers including *Slug Sox9* and *Sox10* was augmented in embryos injected with *RNF152* MO, but not with Co MO (Fig. 3B, C). Co-expression of WT *RNF152* interfered with the enhancing effect of MO, indicative of the specificity of MO effects. Therefore, these results suggest that depletion of *RNF152* up-regulates Wnt/β-catenin signaling, leading to expansion of NC marker-positive area in the neurulae.

RNF152 interferes with the oligomerization of Dishevelled

Dishevelled (Dsh), a key mediator of Wnt/β-catenin signaling, undergoes dynamic oligomerization, resulting in formation of cytoplasmic puncta (12, 13). These puncta contain head-to-tail polymers, which correlate with the ability of Dsh to activate this pathway. The polymerization of Dsh at the plasma membrane provides a platform for the clustering of Wnt-LRP5/6-Fz to form signalosomes. It has been shown that RNF152 facilitates oligomerization of the adaptor protein MyD88 independently of its E3 ligase activity, resulting in activation of TLR/IL-1R-mediated inflammatory response (8). Given these findings, it is tempting to speculate that RNF152 might be involved in Dsh polymerization to negatively control Wnt/β-catenin signaling. To test this hypothesis, we first examined whether RNF152 would interact physically with Dsh. Notably, co-immunoprecipitation experiments demonstrated that the former associates with the latter in an E3 ligase-independent manner (Fig. 4A). In addition, we found that WT RNF152 markedly reduced the association of Myc-tagged and GFP-tagged Dsh in a dose-dependent fashion (Fig. 4B). RNF152(CS) also inhibited more strongly their interaction, indicating its E3 ligase-independent effect on Dsh oligomerization. We next tested the effects of overexpression of RNF152 on the formation of Dsh puncta in cells. As shown in Fig. 4C, GFP-Dsh exhibited a punctate cytoplasmic pattern in control animal cap cells. Co-expression of *RNF152*(*WT*) led to the diffused distribution of GFP-Dsh throughout the cytoplasm. RNF152(CS), but not RNF152(dTM), also disrupted the punctate distribution of GFP-Dsh. Together, these data suggest that RNF152 interferes with Dsh polymerization, resulting in its negative control of the Wnt signaling pathway.

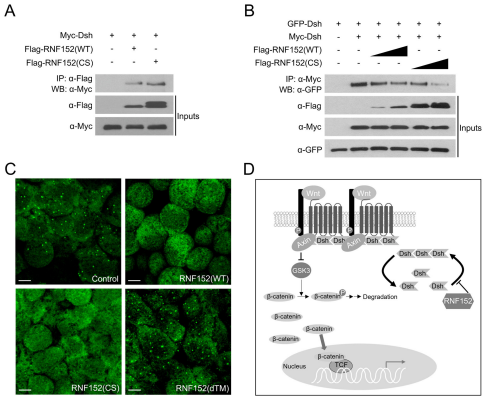

Fig. 4. RNF152 interferes with the polymerization of Dsh. (A) Physical interaction between RNF152 and Dsh. (B) Effects of overexpression of either WT RNF152 or its E3 ligase-defective mutant on the oligomerization of Dsh. (A, B) HEK293T cells were transfected with the indicated constructs, and then coimmunoprecipitation and western blotting were performed. (C) RNF152(WT) and RNF152(CS), but not RNF152(dTM), disrupted the punctate distribution of ectopic GFP-Dsh in animal cap cells. Control, animal cap cells from embryos injected with GFP-Dsh only. Scale bar, 20 μm. (D) Proposed model of the mechanism by which RNF152 controls negatively Wnt/β signaling. See the discussion for details.

DISCUSSION

In this study, we have demonstrated that RNF152 functions as a negative regulator of Wnt/β-catenin signaling in *Xenopus* early development. In support of this, overexpression of *RNF152* interfered with the Wnt stabilization of β-catenin. Wnt-induced ectopic expression of target genes and the activity of a reporter driven by Wnt-responsive promoter were also down-regulated in cells overexpressing RNF152. Conversely, knockdown of *RNF152* sensitized the transcriptional responses to the Wnt signal. Morphological phenotypes of RNF152 morphants recapitulate aspects of disrupted NC specification in *Xenopus* embryos. Consistent with this, overexpression of *RNF152* abrogated the ectopic and endogenous expressions of NC markers that are regulated by Wnt/β-catenin signaling, whereas its depletion further enhanced their expression. Therefore, these results suggest that RNF152 controls negatively to fine-tune the activity of Wnt/β-catenin signaling, thereby contributing to proper NC formation.

RNF152 is an E3 ubiquitin ligase with a transmembrane (TM) domain which mediates its localization to lysosomes (5). Notably, the RNF152(CS) mutant but not the RNF152(dTM) can inhibit Wnt/β-catenin signaling (Fig. 1), suggesting that the membrane association but not the E3 ligase activity of RNF152 is critical for this inhibitory function. Similarly, the TM domain-mediated membrane localization of RNF152 but not its enzymatic activity is indispensable for its role in TLR/IL-1R-mediated signaling (8). In contrast, RNF152 acts on RagA and Rheb as a direct E3 ubiquitin ligase anchored to the lysosomal surface to negatively regulate mTORC1 activity (6, 7). As such, it is likely that RNF152 has E3 ligase-dependent and -independent functions, both of which require its membrane localization. Our results showed that RNF152(CS) mutant could inhibit Wnt-dependent transcriptions more strongly than WT RNF152 (Supplementary Fig. 3), even though their respective mRNAs were injected at the same concentration. RNF152 has been shown to ubiquitinate itself, which leads to its short half-life (14). The steady-state protein level of E3 ligase-defective RNF152 was increased compared to that of WT RNF152 (Fig. 4B). Thus, the stronger effects of RNF152(CS) on Wnt/β-catenin signaling may be due to abrogation of its autoubiquitination activity. RNF152 appears not

to act as an E3 ligase to ubiquitinate a signaling mediator in the Wnt/β-catenin pathway. However, since the E3 ligase activity of RNF152 functions to shorten its own half-life, this enzymatic activity of RNF152 can affect the kinetics of Wnt/β-catenin signaling.

As shown in this work, the self-association of Dsh proteins and the formation of their cytoplasmic puncta were impeded in RNF152-overexpressed cells. This inhibitory effect on Dsh polymerization could also be seen in cells overexpressing RNF152(CS). These results suggest that RNF152 might control negatively Dsh polymerization in an E3 ligase-independent fashion. Dsh polymerization provides a dynamic scaffold with a high concentration of binding sites for its partners such as Frizzled receptors and Axin to form signalosome (13). This signalosome assembly leads to the inhibition of GSK3-mediated phosphorylation of β-catenin and, as a consequence, to stabilization of β-catenin. Thus, high levels of RNF152 activity would disrupt signalosome formation, thereby repressing Wnt/β-catenin signaling. As a mechanism of action, RNF152 might regulate the equilibrium between diffuse Dsh monomers and Dsh polymers in the cytoplasm or the intermolecular interactions between Dsh proteins, which remains to be investigated in the future. It has been shown that Dsh puncta do not colocalize with endocytic markers or lipid dyes or associate with membranes (15). In contrast, RNF152 colocalizes with early endosomes and late endosomes as well as lysosomes as revealed by confocal microscopy (8). Future experiments are warranted to elucidate how the formation of Dsh puncta is relevant to membrane-anchored RNF152.

MATERIALS AND METHODS

Embryos and lineage tracing

This study was compliant with all relevant ethical regulations regarding animal research. All animal experiments were approved by the Institutional Animal Care and Use Committee at the Asan Institute for Life Sciences, Asan Medical Center. *In vitro* fertilization, microinjection and embryo culture were performed as described previously (16). Developmental stages of embryos were determined according to the Nieuwkoop and Faber's normal table of development (17). For lineage tracing, β-galactosidase mRNA (LacZ) was co-injected with MOs and other mRNAs, and its activity was visualized with the Red-Gal substrate.

RT-PCR and *in situ* hybridization

For RT-PCR analysis, total RNA was extracted from embryos or animal caps with TRIzol reagent and treated with RNase-free DNase I to remove genomic DNA. Approximately 5 μg of total RNA was reverse-transcribed using random hexamers and M-MLV reverse transcriptase (Promega). PCRs were carried out in a standard 50 μl of PCR with Taq polymerase. The numbers of PCR cycles for each primer set were determined empirically to maintain amplification in the linear range. The following primers were used: *RNF152*, (forward) 5′-TGTCCCAGGACTCCCTACTG-3′, (reverse) 5′-ACCGGGTAACAATGCCCTTT-3′; *Siamois*, *Xnr3* and *ODC* (16); *Slug* and *Sox9* (11). Whole-mount *in situ* hybridization was performed as described previously (18). Anti-sense digoxigenin (DIG)-labeled RNA probes were *in vitro* synthesized using template cDNA encoding *Slug*, *Sox9* or *Sox10* (11). For anti-sense *RNF152* probe, RNF152-T construct was linearized with *Nco*I and transcribed with SP6 RNA polymerase. Hybridization was detected with an alkaline phosphatase (AP)-conjugated anti-DIG antibody and visualized using BM purple as a substrate (Roche).

Immunoprecipitation and Western blot analysis

For coimmunoprecipitation of proteins, cultured cells were lysed in Triton X-100 lysis buffer (50 mM Tris (pH 7.6), 1% Triton X-100, 50 mM NaCl, 1 mM EDTA, 1 mM sodium vanadate, 10 mM NaF, 1 mM PMSF, 20 μg/ml aprotinin, 20 μg/ml leupeptin). Proteins were collected from cell lysates using Protein G Mag Sepharose (GE Healthcare)-bound antibodies overnight at 4°C on a rotating platform. The eluted immunoprecipitates and protein lysates were separated by 6-10% SDS-PAGE. Western blotting was carried out according to a standard protocol with anti-β-catenin (1:1000, Santa Cruz), anti-Myc (1:1000, Santa Cruz), anti-Flag (1:1000, Sigma), anti-GFP (1:1000, Santa Cruz) and anti-β-actin (1:1000, Santa Cruz) antibodies.

Luciferase reporter assay

For reporter assays in *Xenopus* embryos, a reporter gene (S01234, 40 pg) was co-injected with or without mRNAs and/or MOs, and the harvested embryos were homogenized in 1x Passive Lysis Buffer. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. All values were normalized against the activity of pRL-TK Renilla luciferase as an internal control. All experiments were carried out in triplicate and repeated three times. A single representative result is shown in Fig. 1C and 2C.

Fluorescence microscopy

The subcellular localization of GFP-Dsh was examined using an assay described in (19). Briefly, four-cell stage embryos were injected into the animal pole region of all blastomeres with GFP-Dsh (1 ng) with or without mRNAs as indicated. Animal cap explants were excised at late blastula stages, fixed in 4% paraformaldehyde in PBS for 2 h, rinsed in PBS and directly mounted for GFP-tagged proteins. The image was acquired on a confocal laser-scanning microscope (Zeiss).

Statistical analysis

Statistical significance was determined by using *t*-test in Microsoft Excel software. A P-value of less than 0.05 was considered as significant.

Additional materials and methods can be found in the Supplementary data.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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