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G protein-coupled receptors Flop1 and Flop2 inhibit Wnt/β-catenin signaling and are essential for head formation in *Xenopus*

Asuka Miyagi [a,b], Takefumi Negishi [a], Takamasa S. Yamamoto [a], Naoto Ueno [a,b,*]

[a] Division of Morphogenesis, Department of Developmental Biology, National Institute for Basic Biology, 38 Nishigonaka, Myodaiji, Okazaki, Aichi 444-8585, Japan

[b] Department of Basic Biology, School of Life Science, SOKENDAI (The Graduate University for Advanced Studies), 38 Nishigonaka, Myodaiji, Okazaki, Aichi 444-8585, Japan

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ABSTRACT

Patterning of the vertebrate anterior–posterior axis is regulated by the coordinated action of growth factors whose effects can be further modulated by upstream and downstream mediators and the cross-talk of different intracellular pathways. In particular, the inhibition of the Wnt/β-catenin signaling pathway by various factors is critically required for anterior specification. Here, we report that Flop1 and Flop2 (Flop1/2), G protein-coupled receptors related to Gpr4, contribute to the regulation of head formation by inhibiting Wnt/β-catenin signaling in *Xenopus* embryos. Using whole-mount *in situ* hybridization, we showed that *flop1* and *flop2* mRNAs were expressed in the neural ectoderm during early gastrulation. Both the overexpression and knockdown of Flop1/2 resulted in altered embryonic head phenotypes, while the overexpression of either Flop1/2 or the small GTPase RhoA in the absence of bone morphogenetic protein (BMP) signaling resulted in ectopic head induction. Examination of the Flops' function in *Xenopus* embryo animal cap cells showed that they inhibited Wnt/β-catenin signaling by promoting β-catenin degradation through both RhoA-dependent and -independent pathways in a cell-autonomous manner. These results suggest that Flop1 and Flop2 are essential regulators of *Xenopus* head formation that act as novel inhibitory components of the Wnt/β-catenin signaling pathway.

1. Introduction

Head formation is one of the most critical and complex steps of animal embryogenesis, involving the interplay of a multitude of signaling pathways that impact neural fate and development of the central nervous system (CNS). Head formation in *Xenopus* is achieved by a series of cell-to-cell communications, as first demonstrated by the landmark experiment performed by Spemann and Mangold (1924). In that experiment, the organizer, a small region on the dorsal lip of the early gastrula, was transferred from a host embryo to the ventral side of a recipient embryo and shown to induce a secondary body axis with completely developed head structures (Spemann and Mangold, 1924, 2001). It is now well understood that the functions of growth factors secreted during early embryogenesis must be suppressed to allow for the induction of neural fate in the ectoderm as well as head formation, and that the organizer secretes various inhibitors of growth factor signaling (De Robertis and Kuroda, 2004; Hikasa and Sokol, 2013). Bone morphogenetic proteins (BMPs) play crucial roles in establishing epidermal fate in the presumptive ectoderm, thereby inhibiting neural fate. Therefore, for the induction of neural fate, BMP activities must be inhibited by proteins such as Chordin, Noggin, and Follistatin, all of which are secreted from the organizer of *Xenopus* and physically bind BMPs to prevent the activation of their receptors (Fainsod et al., 1997; Lemura et al., 1998; Sasai et al., 1994; Smith and Harland, 1992). The induced neural ectoderm is then patterned along the anterior–posterior axis. During this patterning event, Wnt/β-catenin signaling is inhibited by sFRP, Dkk, and Cerberus, which are secreted Wnt antagonists (Glinka et al., 1998; Leyns et al., 1997; Pera and De Robertis, 2000; Piccolo et al., 1999), and by Shisa, Apcdd1, and Tiki1, which are transmembrane Wnt antagonists (Shimomura et al., 2010; Yamamoto et al., 2005; Zhang et al., 2012). All of these Wnt/β-catenin signaling inhibitors are implicated to be essential regulators for anterior specification. In addition, RhoA, a small GTPase, has the capacity to induce ectopic head structures when co-expressed with a dominant-negative BMP receptor (tBR), which alone only induces secondary trunk without obvious head structures (Wünnenberg-Stapleton et al., 1999). However, it has been unclear how RhoA is integrated into the regulatory pathway of head formation.

* Corresponding author at: Division of Morphogenesis, Department of Developmental Biology, National Institute for Basic Biology, 38 Nishigonaka, Myodaiji, Okazaki, Aichi 444-8585, Japan. Fax: +81 564 55 7571.
E-mail address: nueno@nibb.ac.jp (N. Ueno).

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In this study, we found that Flop1 and Flop2, G protein-coupled receptors (GPCRs) related to Gpr4 (Chung et al., 2004; Tao et al., 2005) contribute to head formation in *Xenopus* by inhibiting Wnt/β-catenin signaling through both RhoA-dependent and -independent pathways. The Flops-mediated RhoA-independent pathway induced the phosphorylation and proteasome-dependent degradation of β-catenin, disabling β-catenin-mediated transcription. In contrast, the Flops-mediated RhoA-dependent pathway inhibited Wnt/β-catenin signaling upstream of Disheveled (Dvl). Consistent with the reported mechanism for transmembrane Wnt antagonists, such as Shisa and Apcdd1, Flops inhibited Wnt/β-catenin signaling in a cell-autonomous manner. This is the first report demonstrating that GPCRs have essential functions in head formation and act as novel negative regulators of the Wnt/β-catenin pathway.

2. Materials and methods

2.1. Embryo handling and microinjection

Xenopus laevis (*X. laevis*) embryos were obtained by standard methods (Morita et al., 2010). mRNAs or morpholino antisense oligonucleotides (MOs) were injected into the appropriate region of two-, four-, or 16-cell-stage embryos. The injected embryos were cultured in 3% Ficoll/0.1x Steinberg's Solution until stage (St.) 9 and then cultured in 0.3x Marc's Modified Ringer's Solution until the desired stage (Nieuwkoop and Faber, 1967).

2.2. DNA constructs, mRNA preparation, and MOs

The following *X. laevis* cDNA clones were obtained from the EST database (XDB3, http://xenopus.nibb.ac.jp) and cloned into the pCS2p+ vector: XL072l08 (*gpr4*), XL506k06ex (*flop1*), XL155m04 (*flop2*), XL255j14ex (*rhoA*), XL281d07ex (*frzb2*), XL330o07ex (*cerberus*), XL301p20ex (*dishevelled3*), XL285b09ex (*β-catenin*), XL280g21ex (*smad2*), XL011e23 (*claudin4*), XL109a23 (*hyaluronan synthase1*), XL085m19 (*slug*), and XL250b20ex (*sonic hedgehog*). *bf1* was subcloned into pCS2p+ vector from the *X. laevis* cDNA and *en2* construct was used as reported previously (Hemmati-Brivanlou et al., 1991). Expression constructs for Wnt8 (Christian et al., 1991), dnWnt8 (Hoppler et al., 1996), tBR (Suzuki et al., 1995), Xnr1 (Jones et al., 1995), eFGF (Lombardo and Slack, 1997), and BMP4 (Nishimatsu et al., 1992) were reported previously. Gsk3β, 6xMyc-β-catenin, and 6xMyc-caβ-catenin (Δaa 1–53) expression constructs were kindly provided by Dr. Noriyuki Kinoshita. The dnRhoA (T19N) and Flops DRY motif mutants (Flop1; R113N, Flop2; R112N) were generated by PCR. Flop1 and Flop2 rescue constructs were generated by changing the nucleotides at the MO target site as follows (changed nucleotides are indicated in small letters): Flop1 (−2) 5′-GAATGTGcAAtCAatcCGTcagcTG-3′ (+23) and Flop2 (−1) 5′-TATGGCaTGcAAcCAatctTGcGAg-3′ (+24). Capped mRNAs were synthesized using the mMESSAGE mMACHINE® SP6 kit (AM1340; Ambion) and purified on NICK columns (17-0855; GE Healthcare). MOs were purchased from Gene Tools. PCR primers for expression constructs, and MO sequences are shown in Tables S1 and S2 (Chung et al., 2004).

2.3. Whole-mount in situ hybridization (WISH) and quantitative RT-PCR (QRT-PCR)

WISH was performed as described previously (Goda et al., 2009). As the endogenous expression signals of *flop1/2* were very weak, we used albino embryos to avoid the bleaching process, which might further reduce their signals. Pigmented embryos used in other experiments were bleached before WISH. To visualize the injected regions, a GFP tracer was injected and immunostained with an anti-GFP antibody after WISH. For the temporal expression analysis of *gpr4* and *flops*, the total RNA from one whole embryo at each stage of interest was isolated as described previously (Yamamoto et al., 2001). For marker gene analysis, the total RNA was isolated from five anterior or posterior halves of St. 20 embryos, five animal caps from St. 9 embryos, or five St. 9 animal caps cultured until reaching St. 12, and five explants of the dorsal or ventral marginal zone (DMZ, VMZ) of St. 10 embryos. QRT-PCR was performed with the SYBR® premix ExTaq (Tli RNaseH plus) (RR420; TaKaRa) on a Thermal Cycler Dice Real Time System Single (TP850; TaKaRa) according to the manufacturer's instructions, and the relative gene expression was calculated with the ΔΔCt method. The primer sets used in this experiment are shown in Table S3 (Agius et al., 2000; Onichtchouk et al., 1996).

2.4. Immunostaining

Embryos or animal caps excised from St. 9 embryos were fixed in MEMFA for 2 h at room temperature and then placed in PBS. The fixed embryos were embedded in fish gelatin (G7765; Sigma), and 16-μm cryosections were generated (Morita et al., 2010; Suzuki et al., 2010). The sections, fixed animal caps, or embryos previously analyzed by WISH were incubated with the following primary antibodies: rabbit anti-GFP (1:500, #598; MBL) or rabbit anti-β-catenin (1:500, C2206; Sigma), or with Alexa Fluor® 546 phalloidin (1:50, A22283; Molecular Probes) for F-actin staining. The following secondary antibody was used: Alexa Fluor® 488 goat anti-rabbit IgG (1:1000, A11017; Molecular Probes). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (5 μg/ml, 049-18801; Wako).

2.5. Western blotting and RhoA pull-down assay

Prior to western blotting, mRNAs and/or MOs were injected into the animal pole of two-cell-stage embryos. Subsequently, 30 animal caps were excised from St. 9 embryos and lysed in 50 μl PBST (PBS with 0.3% TritonX-100) containing a protease inhibitor cocktail (1:50, 25955-11; Nacalai Tesque). After incubating on ice for 5 min, the lysates were centrifuged at 17,400xg for 5 min at 4 °C, and the supernatants were collected, diluted in 2x sample buffer, and boiled for 5 min. The samples were then subjected to SDS-PAGE (13.5% gel for Histone and 10% gel for all other proteins) and then blotted onto a PVDF membrane (162-0177; Bio-Rad). The membranes were incubated with the following primary antibodies: rabbit anti-GFP (1:2000, #598; MBL), mouse anti-Histone H2B (1:1000, #2934; Cell Signaling Technology), mouse anti-Flag (1:1000, F3165; Sigma), mouse anti-cMyc (1:1000, 9E10 #sc-40; Santa Cruz), and mouse anti-C-cadherin (1:50, 6B6 supernatant; DSHB). The following secondary antibodies were used: HRP-conjugated sheep anti-mouse IgG (1:10,000, NA931; GE Healthcare) and HRP-conjugated donkey anti-rabbit IgG (1:10,000, NA934; GE Healthcare), and the signals were detected using ECL™ Prime or ECL™ Select Western Blotting Detection Reagent (RPN2232, RPN2235; GE Healthcare).

The RhoA pull-down assay was performed as described previously (Hara et al., 2013). Prior to the assay, mRNAs were injected into the animal pole of two-cell-stage embryos, and 100 animal caps were excised from the St. 9 embryos and analyzed.

2.6. cAMP assay

The cAMP assay was performed with the Cyclic AMP XP® Assay Kit (#4339; Cell Signaling Technology). mRNAs were injected into the animal pole of two-cell-stage embryos, and the animal caps