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β-Arrestin 1 mediates non-canonical Wnt pathway to regulate convergent extension movements

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ABSTRACT

β-Arrestins are multifaceted proteins that play critical roles in termination of G protein-coupled receptor (GPCR) signaling by inducing its desensitization and internalization as well as in facilitation of many intracellular signaling pathways. Here, we examine using *Xenopus* embryos whether β-arrestin 1 might act as a mediator of β-catenin-independent Wnt (non-canonical) signaling. *Xenopus* β-arrestin 1 (*xβarr*1) is expressed in the tissues undergoing extensive cell rearrangements in early development. Gain- and loss-of-function analyses of *xβarr*1 revealed that it regulates convergent extension (CE) movements of mesodermal tissue with no effect on cell fate specification. In addition, rescue experiments showed that *xβarr*1 controls CE movements downstream of Wnt11/Fz7 signal and via activation of RhoA and JNK. In line with this, xβarr1 associated with key Wnt components including Ryk, Fz, and Dishevelled. Furthermore, we found that *xβarr*1 could recover CE movements inhibited by *xβarr*2 knockdown or its endocytosis defective mutant. Overall, these results suggest that β-arrestin 1 and 2 share interchangeable endocytic activity to regulate CE movements downstream of the non-canonical Wnt pathway.

1. Introduction

The Wnt family of secreted glycoproteins regulates cell proliferation, differentiation and polarity, and cell fate determination during early embryonic development and later tissue homeostasis [1,2]. It has also emerged as an essential signal for the self-renewal of stem cells in the hematopoietic system, the skin and the intestinal epithelium [3]. Currently, three different Wnt signaling pathways have been identified which are activated upon Wnt receptor activation: the canonical Wnt/β-catenin pathway, the non-canonical Wnt signaling or planar cell polarity (PCP) pathway, and the Wnt/Ca^{2+} pathway [2,4–6]. In the canonical Wnt pathway, the binding of Wnts to a cell-surface receptor complex consisting of Frizzled and LRP5/6 induces the stabilization of cytoplasmic β-catenin and its entry into the nucleus, leading to the formation of a complex with the DNA-binding factor, Tcf/Lef and subsequent transcriptional activation of tissue-specific target genes. In the absence of Wnt ligands, β-catenin is bound by the destruction complex composed of the scaffolding proteins Axin and APC, and the serine/threonine kinases, CK1 and GSK3. β-catenin is phosphorylated sequentially by CK1 and GSK3, thereby undergoing the βTrCP-dependent ubiquitination and proteasomal degradation. The Wnt/PCP and Wnt/Ca^{2+} pathways are β-catenin-independent signaling, which regulate convergent extension (CE) movements indispensable for body axis elongation in vertebrates, the orientation of bristles in fly wing, and the induction of epithelial to mesenchymal transition (EMT) in cancer. The Wnt/PCP pathway involves activation of intracellular proteins Dishevelled (Dsh), Daam1, RhoA, Rac1, Rho-associated kinase α, and Jun N-terminal kinase (JNK). The Wnt/Ca^{2+} pathway functions through the activation of trimeric G proteins, which leads to a transient release of intracellular calcium and subsequent activation of protein kinase Cα (PKCα), Cdc42 GTPase, and calcium calmodulin mediated kinase II (CAMKII). The strength and activity of these Wnt pathways are tightly regulated at the multiple levels in signaling cascade from the secretion and spreading of Wnt ligands to the post-translational modifications of cytoplasmic and nuclear signaling mediators [4,7]. Recently, cellular trafficking of Wnt components, including the membrane distribution and endocytosis of Frizzled (Fz) and LRP5/6 receptors and the compartment-specific localization of other signaling mediators, has been shown to play crucial roles in controlling the activity of Wnt pathway [8–10], but the molecular mechanisms underlying this regulation remain to be further investigated.

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β-Arrestins (β-arrestin 1 and 2) are multifaceted scaffolds and adaptors that regulate numerous aspects of G protein-coupled receptor (GPCR) functions [11,12]. Classically, they have been shown to play roles in termination of GPCR signaling by inducing the desensitization and internalization of the receptor. However, recent studies demonstrate that they also serve as signal transducers of a variety of receptor signaling through interaction with various binding partners. β-Arrestins have been implicated as critical mediators of both canonical and non-canonical Wnt signaling [12,13]. β-Arrestin 1 was found to synergistically enhance Lef-mediated transcriptional activity when overexpressed with Dsh [14]. β-Arrestin 2 mediates not only the Wnt5a-induced internalization of Fz4 by binding to phosphorylated Dsh but also Wnt3a-induced signaling downstream of Dsh and casein kinase, but upstream of β-catenin [15,16]. Furthermore, β-arrestin 2 has been shown to regulate CE movements in *Xenopus* by activating RhoA or Rac-1 in a Dsh-dependent manner [17,18]. Knockout studies show that either β-arrestin 1 or 2-deficient mice develop normally, whereas simultaneous ablation of both isoforms in mice results in embryonic lethality [12,19]. These phenotypes suggest that each β-arrestin can functionally substitute for the other isoform in mice, but there are several pieces of evidence that β-arrestin 1 and 2 can also regulate differentially GPCR desensitization and internalization [19,20]. Thus, in this study, we performed the loss-of-function analysis of β-arrestin 1 in *Xenopus* embryo in order to examine whether both isoforms of β-arrestin have distinct or shared activities in regulation of non-canonical Wnt signaling.

2. Materials and methods

2.1. Embryo manipulation and DMZ elongation assay

In vitro fertilization, microinjection and embryo culture were performed as described previously [17]. Developmental stages of embryos were determined according to the Nieuwkoop and Faber's normal table of development [21]. For elongation assay, dorsal marginal zone (DMZ) explants were dissected at stage 10.25 from the injected embryos and then cultured to stage 18 in 1× Modified Ringer's (MR) media containing 10 μg/ml of bovine serum albumin, 50 μg/ml of gentamycin and 5 μg/ml of streptomycin.

2.2. Plasmid constructs and morpholino oligo

The complete coding region of *Xenopus β-arrestin* 1 (GeneBank Accession No. BC082399) was amplified by PCR and subcloned into the *ClaI*/*XbaI* sites of pCS2+(*xβarr*1-*CS2+*), EGFP-pCS2+(*GFP-xβ*arr1) and Myc-pCS2+(*Myc-xβ*arr1) vector or pGEM T vector (*xβarr*1-*T*). For sense *xβarr*1 RNA synthesis, *xβarr*1-*CS2+* construct was linearized with Acc651 and then transcribed with SP6 RNA polymerase. The following constructs were described previously: Myc-Fz7, Myc-Dsh, GFP-*xβarr*2 and *xβarr*2 AAEA [17], XJNK and XRhoA [22], Myc-Ryk [23], DN XWnt11 [24], DN XFz7 [25]. Capped mRNAs were *in vitro* synthesized using the mMessage mMachine kit (Ambion). Anti-sense morpholino oligos were purchased from Gene Tools. *xβarr*1 MO had the following sequence: 5′-CATGATGGGGGACAA-AGGAACCAGA-3′. The sequence for *xβarr*2 MO was described previously [17]. Control MO was a standard morpholino oligo from Gene Tools whose sequence is 5′-CCTCTTACCTCAGTTACAATTTATA-3′.

2.3. In situ hybridization and RT-PCR

Whole-mount *in situ* hybridization was performed as described in [26]. Anti-sense digoxigenin-labelled RNA probes were *in vitro* synthesized using template cDNA encoding *Chordin*, *Goosecoid*, *Xnot* [17], *Xbra* or *XMyoD* [23]. *xβarr*1 *in situ* probe was generated by transcribing the *ClaI*-linearized *xβarr*1-*T* construct with SP6 RNA polymerase. For RT-PCR analysis, total RNA was extracted from whole embryos and tissue explants using TRI Reagent (Molecular Research Center), and RNA was transcribed using M-MLV reverse transcriptase (Promega) at 37 °C for 1 h. PCR products were analyzed on 2% agarose gels. The numbers of PCR cycles for each primer set were determined empirically to maintain amplification in the linear range. The sequences of primers for *xβarr*1 were: 5′-TTGAGATCCCTCCAAACCTG-3′ (Forward); 5′-TCTCCGGAGCATATT-GAACC-3′ (Reverse).

2.4. Immunoprecipitation and western blotting

To observe protein interactions, HEK293T cells were transfected with DNA constructs using Lipofectamine reagent (Invitrogen) and after 48 h, homogenized in Triton X-100 lysis buffer (20 mM Tris–HCl, 1% Triton X-100, 140 mM NaCl, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM sodium orthovanadate, 50 mM NaF, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin). The supernatants were incubated with the indicated antibodies at 4 °C for 6 h and subsequently with Protein A Sepharose (Zymed) at 4 °C for 3 h. The immunocomplexes bound to Protein A beads were washed five times with the lysis buffer. For western blotting, proteins were separated by 10% SDS–PAGE and anti-Myc monoclonal (1:1000, Santa Cruz), anti-GFP monoclonal (1:1000, Santa Cruz), anti-JNK (Cell signaling) and anti-P JNK (Cell signaling) antibodies were used.

3. Results and discussion

3.1. Expression pattern of β-arrestin 1 in Xenopus early development

A full-length cDNA of *Xenopus β-arrestin* 1 (*xβarr*1) was obtained using its putative sequence in the EST database and a PCR-based method. It encodes a protein of 412 amino acids, which shows 91% identity at the amino acid level with human and mouse homologues (Fig. 1A). We next examined the spatiotemporal expression pattern of *xβarr*1 in early embryogenesis. First, *xβarr*1 has both maternal and zygotic transcription throughout early development as analyzed by RT-PCR (Fig. 1B), which is similar to the temporal expression pattern of *Xenopus β-arrestin* 2 (*xβarr*2) [17]. Spatially, *xβarr*1 expression is restricted to the animal hemisphere of the cleavage and blastula stage embryos (Fig. 1C and D). During gastrulation, its message is first observed in the dorsal marginal region of embryo (Fig. 1E) and gradually expands to the lateral and ventral marginal zones (data not shown). At the early neurula stages, it is expressed in the dorsal midline and along the lateral and anterior edges of neural plate (Fig. 1F and G). Unlike *xβarr*2 [17], *xβarr*1 transcripts become detectable in the presumptive heart region from the mid-neurula stages (Fig. 1H) and later localized more markedly to the organ (Fig. 1K and L). At the tadpole stages, *xβarr*1 is also localized to the otic vesicle and specific areas in the brain (Fig. 1I–L). Unlike *xβarr*1, *xβarr*2 is expressed in the eye, branchial arches and broader region in the brain in tadpole [17]. Together, these results suggest that the expressions of the two *β-arrestins* are overlapping before mid-neurulation and become distinct as development proceeds in *Xenopus*.

3.2. β-Arrestin 1 regulates CE movements in gastrulation

As *xβarr*2 has been shown to regulate CE movements in *Xenopus* gastrulation [17], we next tested the potential role of *xβarr*1 in this process. For this, anti-sense morpholino oligo (MO)-mediated knockdown approach was employed to perform the loss-of-function analysis of *xβarr*1. When the injections were targeted toward the dorsal marginal tissue, which undergoes extensive CE

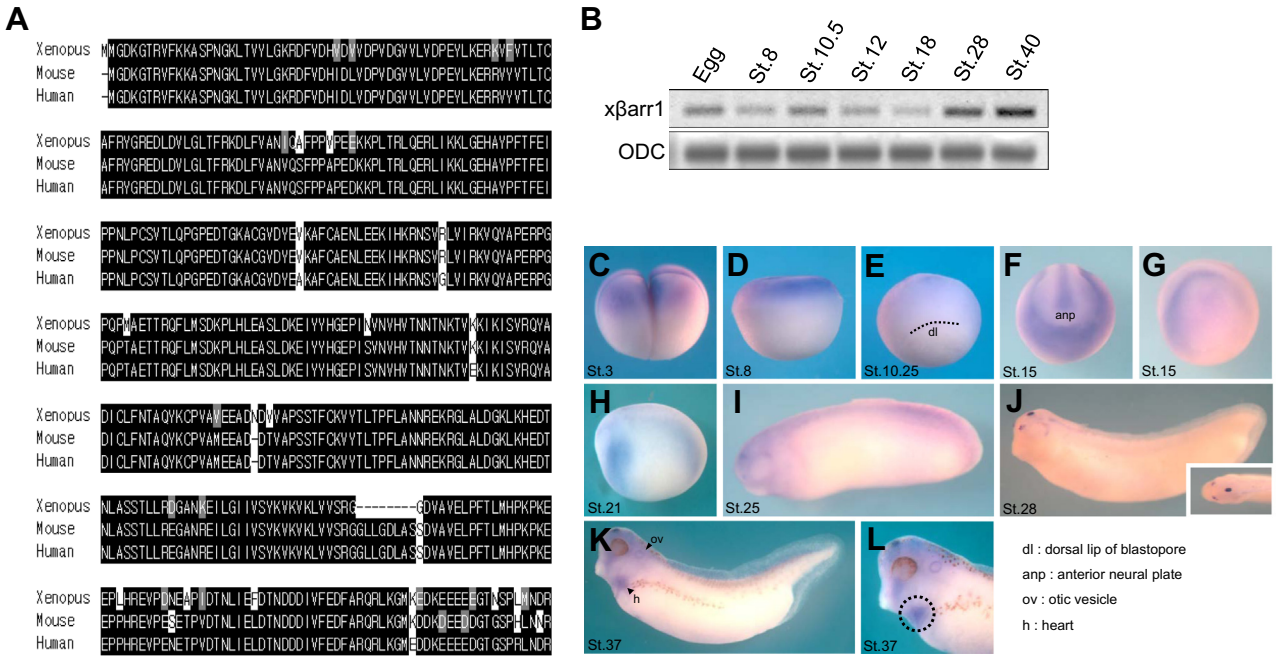

Fig. 1. Expression pattern of *xβarr*1 in *Xenopus* early development. (A) Amino acid sequence comparison of *Xenopus* β-arrestin 1 and its homologues identified in human and mouse. The most conserved residues are indicated by the shaded background. (B) RT-PCR analysis showing the temporal expression pattern of *xβarr*1. ODC serves as a loading control. Stages (St.) are indicated above the lane. (C–L) Whole-mount *in situ* hybridization assays. (C and D) Lateral view with animal to the top. (E) Dorso-vegetal view with animal to the top. (F) Anterior view with dorsal to the top. (G and H) Lateral view with anterior to the left. Inset in (J) shows the dorsal view of head region. A dotted circle in (L) denotes heart region.

movements, *xβarr*1 MO, but not Co MO, caused gastrulation-defective phenotypes including kinked body axis, failure of neural tube closure and spina bifida (96%, $n = 74$; Fig. 2A). These morphant phenotypes could be rescued by co-expression of *xβarr*1 RNA that is resistant to the MO inhibition (66%, $n = 62$; Fig. 2A), thus confirming the specificity of the MO effect. Gain-of-function of *xβarr*1 also produced the morphological defects indistinguishable from those in the morphant (93%, $n = 81$; Fig. 2A). We next examined whether *xβarr*1 regulates CE movements in gastrulation by performing dorsal marginal zone (DMZ) elongation assay, an established measure of CE in *Xenopus* embryo. As shown in Fig. 2B and C, DMZ explants from the Co MO-injected embryos underwent extensive elongation, whereas those from the *xβarr*1 MO-injected embryos displayed a rounded shape, indicative of inhibition of CE. This repressive effect on DMZ elongation of the MO could be reversed to some extent by co-injection of *xβarr*1 RNA (Fig. 2B and C). In addition, overexpression of *xβarr*1 RNA could suppress the elongation of the DMZ explants. Taken together, these results suggest that the optimal levels of *xβarr*1 expression are critical for normal gastrulation including CE movements.

Differentiation and patterning of mesodermal tissue influence indirectly CE movements by changing cell fates in DMZ [27]. Thus, we investigated whether the morphological phenotypes caused by the gain- or loss-of-function of *xβarr*1 might be associated with the altered mesodermal formation. Before mid-gastrulation, embryos overexpressing or depleted of *xβarr*1 exhibited normal blastopore formation and similar expression of early mesodermal markers such as *Chordin*, *Goosecoid* and *Xbra*, compared with uninjected or Co MO-injected embryos (Fig. 2D). During the late gastrula and early neurula stages, *xβarr*1 MO-injected embryos showed markedly both delay of blastopore closure and failure of CE movements of mesodermal tissue as visualized by the inhibition of convergent extended localization of late mesodermal markers, *Xnot* and *MyoD* along the dorsal midline, though these markers are still expressed around the blastopore (Fig. 2E). Overall, these data suggest that *xβarr*1 controls CE movements of dorsal mesodermal tissue without affecting cell fates in *Xenopus* gastrulation.

3.3. β-Arrestin 1 mediates the Wnt/PCP pathway

The non-canonical Wnt/PCP pathway, a key regulator of CE movements in *Xenopus*, involves activation of Wnt11, Fz7, β-arrestin 2, Dsh, RhoA, and JNK [17,22,24]. Therefore, we tested whether like *xβarr*2, *xβarr*1 could be implicated in this pathway to regulate CE. Injection of either dominant negative (DN) *Wnt*11 ligand or *Fz*7 receptor inhibited the elongation of DMZ explants (Fig. 3B, D and F), which could be reverted efficiently by co-expression of *xβarr*1 (Fig. 3C, E and F), indicating that *xβarr*1 mediates CE movements regulated by Wnt11/Fz7 signal. In addition, injection of *xβarr*1 MO suppressed potently the elongation of DMZ explants as shown above (Fig. 3H and K) and this inhibitory effect was rescued by co-injection of *JNK* or *RhoA* RNA (Fig. 3I–K), suggesting that *xβarr*1 acts upstream of these signaling mediators to control CE. In line with this, overexpression of *xβarr*1 increased the level of phospho-JNK, its activated form, in DMZ explants (Fig. 3L). It has been shown that in *Xenopus* CE, xβarr2 cooperates with the receptor tyrosine kinase Ryk to mediate the endocytosis of Fz7 and Dsh upon Wnt stimulation [23]. Thus, we further examined whether β-arrestin 1 would interact physically with these Wnt/PCP components. As shown in Fig. 3M, β-arrestin 1 associated with Fz7, Ryk and Dsh as analyzed by immunoprecipitation assays. Of note, β-arrestin 1 also bound to β-arrestin 2, indicating the possible formation of β-arrestin dimer. Collectively, these results support that *xβarr*1 would function as a mediator of the Wnt/PCP pathway to control CE movements.

3.4. β-Arrestin 1 and 2 have interchangeable activities in CE movements

As demonstrated above, *xβarr*1 regulates CE movements downstream of the Wnt/PCP pathway, which is similar to the activity of

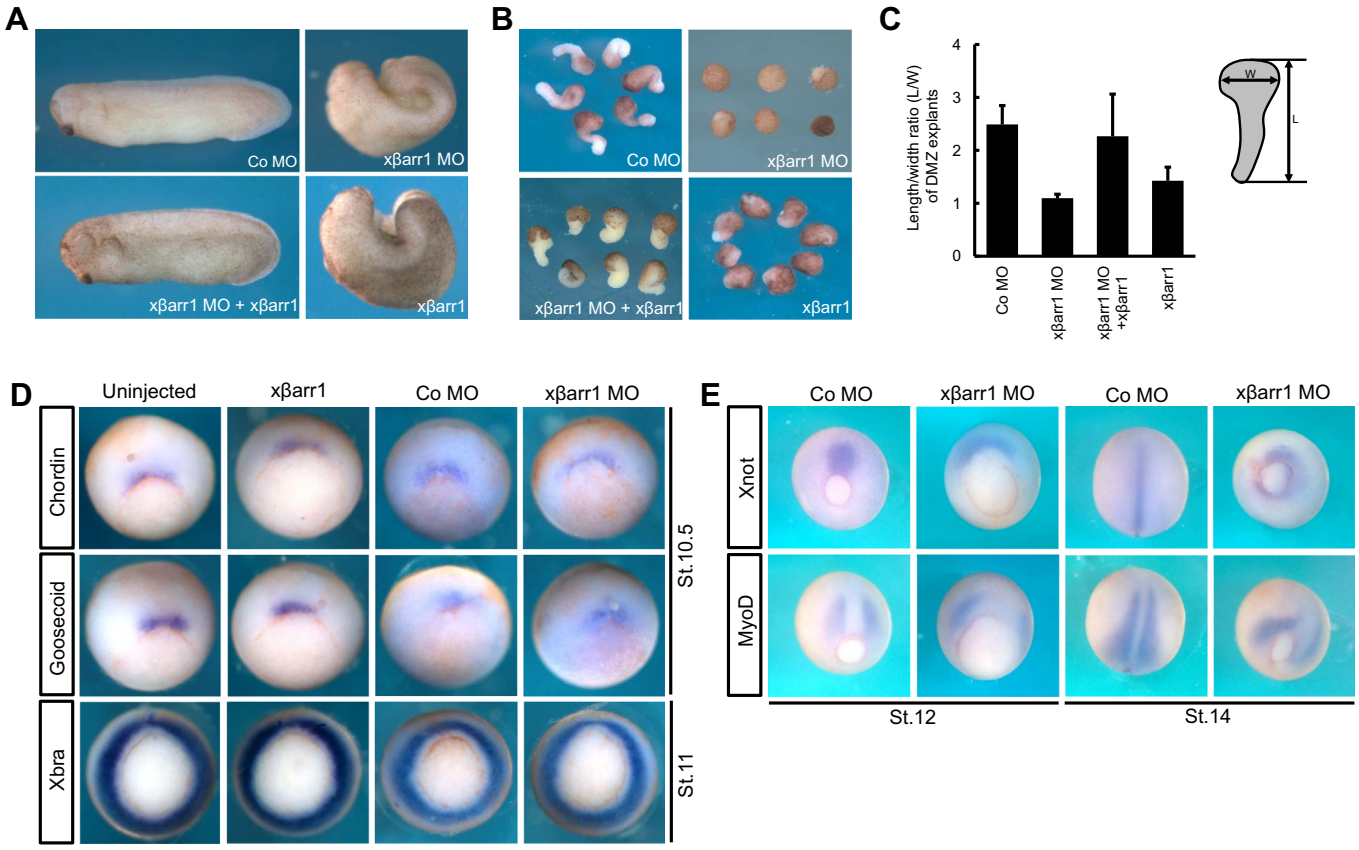

Fig. 2. *xβarr*1 regulates CE movements of dorsal tissue with no effect on cell fates. (A) Gain- and loss-of-function phenotypes of *xβarr*1. (B) DMZ elongation assays. (C) Quantitation of convergent extension of DMZ explants shown in (B). (D and E) Whole-mount *in situ* hybridization analysis. (A–E) Two blastomeres of four-cell stage embryos were injected in the dorsal equatorial region as indicated with *xβarr*1 RNA (1 ng), *xβarr*1 MO (10 ng) and Co MO (10 ng) and then cultured to stage 26 (A), 10.25 for *in vitro* elongation assays (B) or 10.5–14 for *in situ* hybridization (D and E).

*xβarr*2 [17]. Thus, we tested whether *xβarr*1 could functionally compensate for *xβarr*2 in regulation of CE. Control DMZ explants from uninjected embryos exhibited extensive elongation, whereas those from the *xβarr*2 MO-injected embryos remained rounded (Fig. 4A, B and F) as shown previously [17]. Co-injection of *xβarr*1 could reverse this repressive effect of *xβarr*2 MO, resulting in the significant elongation of the explants (Fig. 4C and F). β-Arrestin 2 functions as an adaptor protein to target receptors to clathrin-coated pits for internalization by associating with components of the cellular endocytic machinery [28]. β-Arrestin 2 binds directly to the clathrin through a LIEF motif [29], and mutagenesis of this sequence (LIEF → AAEA) has been performed to investigate its endocytic role. A shown previously [17], injection of *xβarr*2 AAEA mutant interfered with the elongation of DMZ explants (Fig. 4D and F), suggesting that its endocytic activity is critical for CE movements. Moreover, this inhibitory effect of the *xβarr*2 mutant was rescued efficiently by co-injection of *xβarr*1 (Fig. 4E and F). Therefore, these results suggest that β-arrestin 1 and 2 share similar endocytic activities in control of CE movements.

Several pieces of evidence show that β-arrestin 1 and 2 regulate differentially the desensitization, internalization and downstream signaling of some GPCRs, whereas they can also perform interchangeable activities in various signaling pathways [19,20]. In this work, we provide evidence that both isoforms of β-arrestin play similar roles in regulation of CE movements by mediating the Wnt/PCP signaling pathway. The endocytic role of β-arrestin 2 in CE movements and the Wnt/PCP signaling has been clearly demonstrated [17,18,23]. We also show that β-arrestin 1 is able to recover CE movements inhibited by endocytosis-defective β-arrestin 2 mutant and interacts with Ryk and Fz receptors involved in Wnt-mediated endocytosis (Figs. 3 and 4). Given these findings, it seems likely that the two β-arrestins carry out interchangeable endocytic functions in CE movements regulated by the Wnt/PCP pathway. Mice lacking either β-arrestin 1 or 2 have been shown to function normally, whereas the double-knockout is embryonic lethal [12,19], implying their redundant functions in the developing mouse embryo. In contrast, *Xenopus* embryos depleted of either isoform alone exhibited severe gastrulation defects including inhibited CE movements, though the two have similar spatial expression pattern before mid-neurulation. Notably, both gain- and loss-of-function of either isoform of β-arrestin can cause similarly defective gastrulation movements in *Xenopus*, indicating the crucial role of the optimal levels of β-arrestin in this developmental process. Therefore, we hypothesize that in *Xenopus*, both isoforms of β-arrestin should be present to achieve the appropriate levels of their activities that are critical for normal gastrulation, the absence of either isoform leading to the prominent embryonic defects. Probably, in mice, the presence of either isoform might be enough to attain the optimal levels of activity for normal gastrulation, which awaits further investigation. Our immunoprecipitation assay reveals that β-arrestin 1 and 2 interact physically with each other (Fig. 3). Indeed, it has been shown that these β-arrestins exist as homo- and hetero-oligomers in living cells [30]. These oligomerizations of β-arrestins appear to affect their subcellular localizations and control of signaling pathways [30,31]. Thus, it is tempting to speculate that the formation of hetero-oligomers of

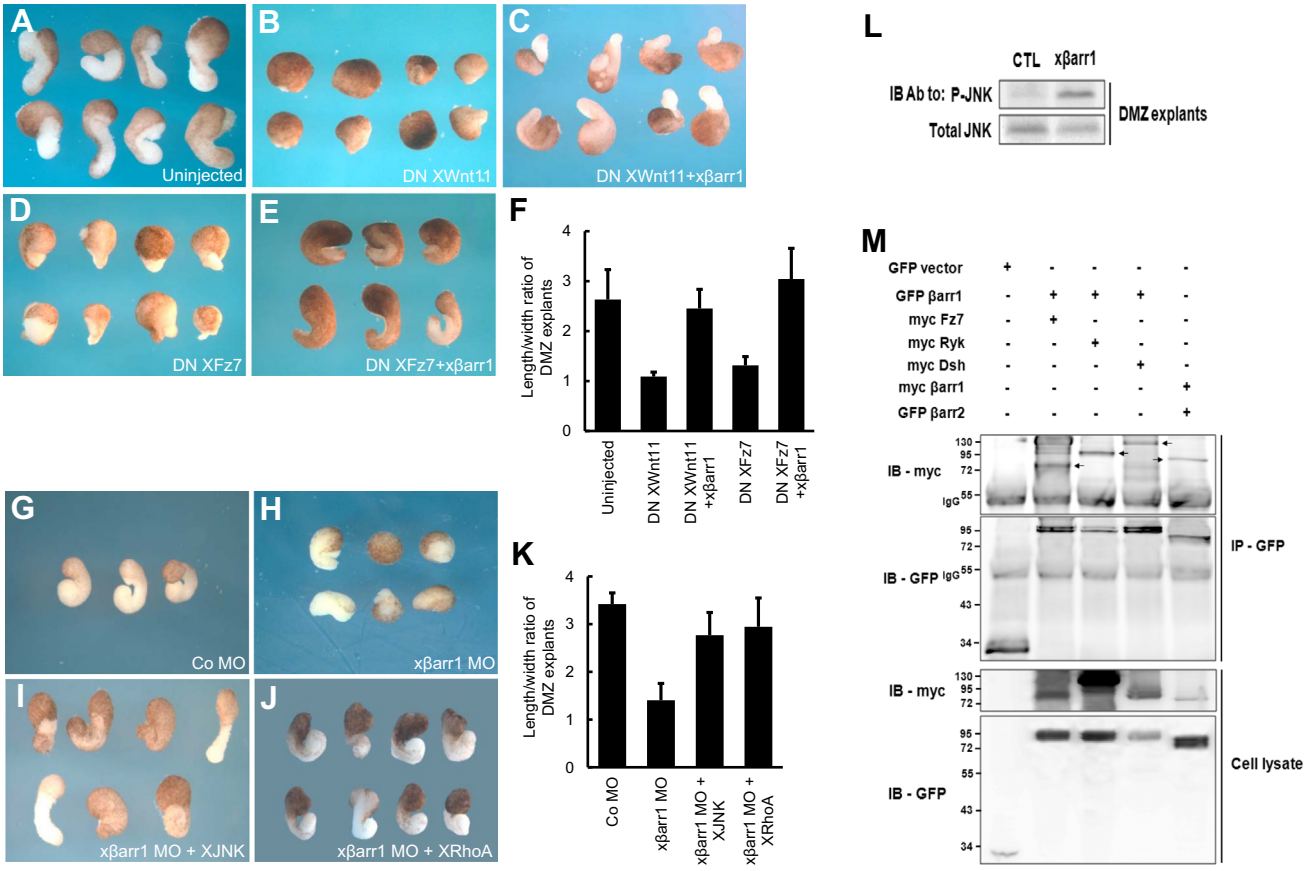

Fig. 3. *xβarr*1 acts downstream of the Wnt/PCP pathway to control CE. (A–K) DMZ elongation assays. (F) and (K) show the quantitation of elongation of DMZ explants in (A–E) and (G–J), respectively. (L) Immunoblotting (IB) analysis of the effect of *xβarr*1 on JNK activation. Total JNK serves as a loading control. (A–L) Two blastomeres of four-cell stage embryos were injected in the dorsal marginal zone with the indicated combination of *DN XWnt*11 (2 ng), *DN XFz*7 (2 ng), *xβarr*1 (1 ng), *XJNK* (200 pg), *XRhoA* (200 pg), *xβarr*1 MO (10 ng) and Co MO (10 ng) and then DMZ explants were excised at stage 10.25 and cultured to stage 18 for observation of CE (A–K) or stage 12 for western blotting (L). CTL, uninjected control DMZ explants. (M) HEK 293T cells were transfected with DNA constructs, either alone or in combination as indicated. Cell lysates were immunoprecipitated with anti-GFP antibody and the immunocomplexes were blotted with anti-myc or anti-GFP antibodies. Arrows denote interacting proteins.

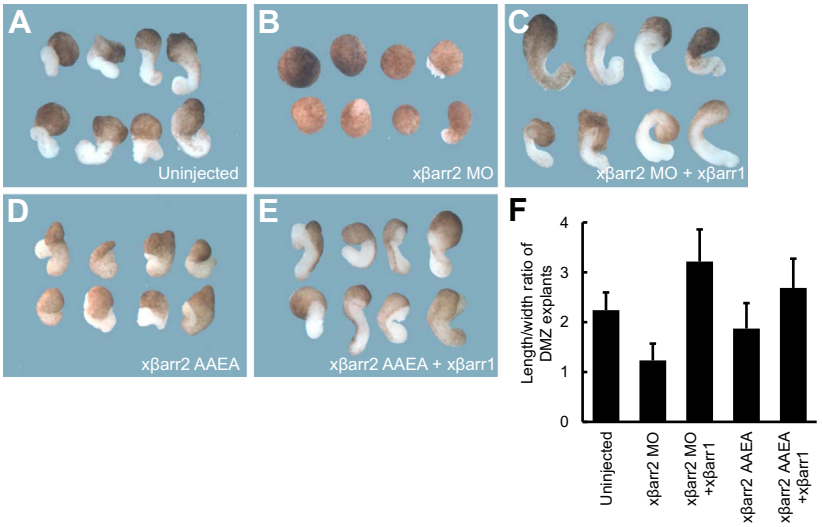

Fig. 4. *xβarr*1 substitutes for *xβarr*2 in regulation of CE. (A–E) *In vitro* elongation assays. (F) Quantitation of convergent extension of DMZ explants shown in (A–E). Four-cell stage embryos were injected in the dorsal marginal region of two blastomeres as indicated with *xβarr*2 MO (10 ng), *xβarr*2 AAEA (1 ng) and *xβarr*1 (1 ng) and DMZ explants were dissected at stage 10.25 and cultured to stage 18.

β-arrestin 1 and 2 plays essential roles in regulation of the Wnt/PCP pathway and CE movements in *Xenopus*. Future studies are warranted to examine whether and how the β-arrestins would function differentially in a species or context dependent manner.

Acknowledgments

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