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Essential role of *AWP1* in neural crest specification in *Xenopus*

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ABSTRACT The neural crest (NC) comprises a transient and multipotent embryonic cell population, which gives rise to a wide variety of cell types, including craniofacial cartilage, melanocytes, and neurons and glia of the peripheral nervous system. The NC is induced by the integrated action of Wnt, FGF, and BMP signaling, and its cell fates are subsequently specified by a genetic cascade of specific transcription factors. Here we describe a critical role of *AWP1* in NC induction during *Xenopus* early development. *Xenopus AWP1* (*XAWP1*) was found to be expressed in the presumptive preplacodal ectoderm, neural tissue, and posterior dorsal mesoderm, but was absent in the neural fold along the anterior-posterior axis of the neurulae. Notably, *XAWP1* was induced by *FGF8a* in naïve ectodermal tissue. *XAWP1*-depleted embryos exhibited defects in pigmentation, craniofacial cartilage, and in the dorsal fin. A knockdown of *XAWP1* impaired both endogenous and the *FGF8a* or *Wnt8*-induced expression of NC markers without affecting mesoderm formation. Furthermore, NC induction inhibited by *XAWP1* depletion was rescued by co-expression of activating forms of β-*catenin* or *TCF3*. In addition, overexpression of *XAWP1*, in concert with BMP inhibition, induced the expression of neural plate border specifiers, *Pax3* and *Msx1*, and these regulatory factors recovered NC induction in the *XAWP1*-depleted embryos. β-catenin stability and Wnt-responsive reporter activity were also impaired in *AWP1*-depleted cells. Taken together, these results suggest that *XAWP1* functions as a mediator of Wnt signaling to regulate NC specification.

KEY WORDS: *AWP1, neural crest induction, Wnt, Xenopus*

Introduction

The neural crest (NC) is a vertebrate multipotent embryonic cell population, which arises at the border between the neural plate and epidermis along the antero-posterior (AP) axis posterior to the diencephalon and migrates to several parts of the embryo to differentiate into a large variety of cell types, including melanocytes, craniofacial cartilage and neurons and glia of the peripheral nervous system (Le Douarin and Dupin, 2003; Sauka-Spengler and Bronner-Fraser, 2008). During gastrulation, NC cells are induced by the combined action of Wnt, fibroblast growth factor (FGF), bone morphogenetic protein (BMP), and Notch signals that originate from the epidermis, neural plate, and underlying mesoderm (Sauka-Spengler and Bronner-Fraser, 2008; Steventon *et al.*, 2005). These NC-inducing signals are integrated at the neural plate border (NPB) to activate a genetic cascade of transcription factors, first inducing the expression of NPB specifiers (*Msx1*, *Pax3*, *Zic1*, *Ap2*, *Dlx3*) that, in turn and in concert with the same signaling pathways, trigger the expression of NC specifiers (*Sox9*, *Sox10*, *Slug*, *FoxD3*) (Heeg-Truesdell and LaBonne, 2004; Prasad *et al.*, 2012; Sauka-Spengler and Bronner-Fraser, 2008). NC specifiers ultimately control NC behaviors such as the epithelial to mesenchymal transition (EMT), delamination, migration and differentiation by regulating the adhesive properties, shape, motility, cell-cycle, cell survival and multipotency of NC progenitors (Sauka-Spengler and Bronner-Fraser, 2008; Theveneau and Mayor, 2012). However, our current understanding of the complex gene regulatory network for NC specification, migration and differentiation into diverse derivatives appears to be preliminary. More connections and the hierarchical organization of the transcription factors involved in NC formation remain to be elucidated.

Canonical Wnt signaling regulates cell fates and cell prolifera-

Abbreviations used in this paper: BMP, bone morphogenetic protein; FGF, fibroblast growth factor; MO, morpholino oligo; NC, neural crest; NPB, neural plate border; ODC, ornithine decarboxylase; XAWP1, *Xenopus* AWP1.

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tion through β-catenin/TCF complex-dependent gene transcription (MacDonald *et al.*, 2009). A large body of evidence suggests that canonical Wnt/β-catenin signaling is implicated in NC induction and subsequent development (Stuhlmiller and Garcia-Castro, 2012). Double-homozygous null mutant mice for *Wnt1*/*Wnt3a* show severe abnormalities in NC derivatives (Ikeya *et al.*, 1997). In *Xenopus* and zebrafish, the morpholino-mediated knockdown of *Wnt8* or *Wnt3a* inhibits the expression of NPB and NC markers (Elkouby *et al.*, 2010; Hong *et al.*, 2008; Lewis *et al.*, 2004). In addition, Wnt6, which is secreted from the chick non-neural ectoderm adjacent to the NPB, is necessary and sufficient to induce NC cells in avian embryos (Garcia-Castro *et al.*, 2002). Gain- and loss-of-function analyses of other Wnt signaling components, including β-*catenin*, *Frizzled7*, *LRP6*, *Dishevelled*, and *Kremen2*, further support the model that NC formation involves the canonical Wnt pathway (Abu-Elmagd *et al.*, 2006; Hassler *et al.*, 2007; Tamai *et al.*, 2000).

In this study, we have identified *AWP1* as a critical regulator of NC specification in *Xenopus* early embryogenesis. AWP1 (also known as zinc finger AN1 type-6; ZFAND6) was originally identified as a protein associated with the serine/threonine kinase PRK1, a lipid-activated kinase belonging to the protein kinase C superfamily (Duan *et al.*, 2000). AWP1 contains A20 and AN1 zinc finger domains at its N- and C-terminal, respectively, and is known as ubiquitin-binding negative modulator of NF-κB activity (Fenner *et al.*, 2009). Recently, AWP1 has also been shown to affect apoptotic cell death as a regulator of NF-κB activity (Chang *et al.*, 2011) and protein export during biogenesis of the peroxisome, a subcellular organelle (Miyata *et al.*, 2012). AWP1 is expressed in several human tissues and also in the early stages of mammalian development (Duan *et al.*, 2000), but its physiological function remains unknown. We have found that *AWP1* affects the expression of NPB and NC markers in the neural fold by mediating Wnt/β-catenin signaling. Notably, it is a target gene of FGF signaling and is also essential for NC induction via this pathway. Our present results thus suggest that *AWP1* functions to mediate the crosstalk of Wnt and FGF signaling in NC induction.

Results

Expression pattern of AWP1 in early Xenopus embryogenesis

To investigate the biological function of *AWP1* in vertebrates, we first observed its developmental expression pattern in *Xenopus laevis* embryos. Temporally, *XAWP1* shows both maternal and zygotic transcription in early embryogenesis as revealed by RT-PCR analysis (Fig. 1A). We also performed *in situ* hybridization to examine its spatial expression pattern. At the early cleavage stages, *XAWP1* maternal transcripts are localized to the animal hemisphere of the embryo (Fig. 1B), and this pattern persists into the blastula stages (data not shown). As gastrulation proceeds, its expression is observed in the mesoderm and overlying ectoderm (Fig. 1 C,D). During neurulation, its zygotic message is detectable in the presumptive preplacodal ectoderm and neural tissue but not in the anterior and lateral neural folds (Fig. 1 E-G). Of note, *XAWP1* is strongly expressed in the posterior dorsal mesoderm in neurulae (Fig. 1 E,H). At the tadpole stages, its specific expression is found in the eyes, as well as in NC derivatives such as branchial arches and otic vesicle in the head region, and in mesodermal derivatives such as somites and kidney tubules along the trunk region of embryo (Fig. 1I). In addition, we examined whether *XAWP1* expression could be induced by signals critical for *Xenopus* development, including FGF, Wnt and Nodal (Kimelman, 2006). Intriguingly, RT-PCR analysis showed that injection of *FGF8a* RNA, but not *Wnt8* and *Xnr-1*, could enhance *XAWP1* expression in the naïve ectodermal tissue (Fig. 1J), suggesting a possible role of *XAWP1* in developmental events involving FGF signaling.

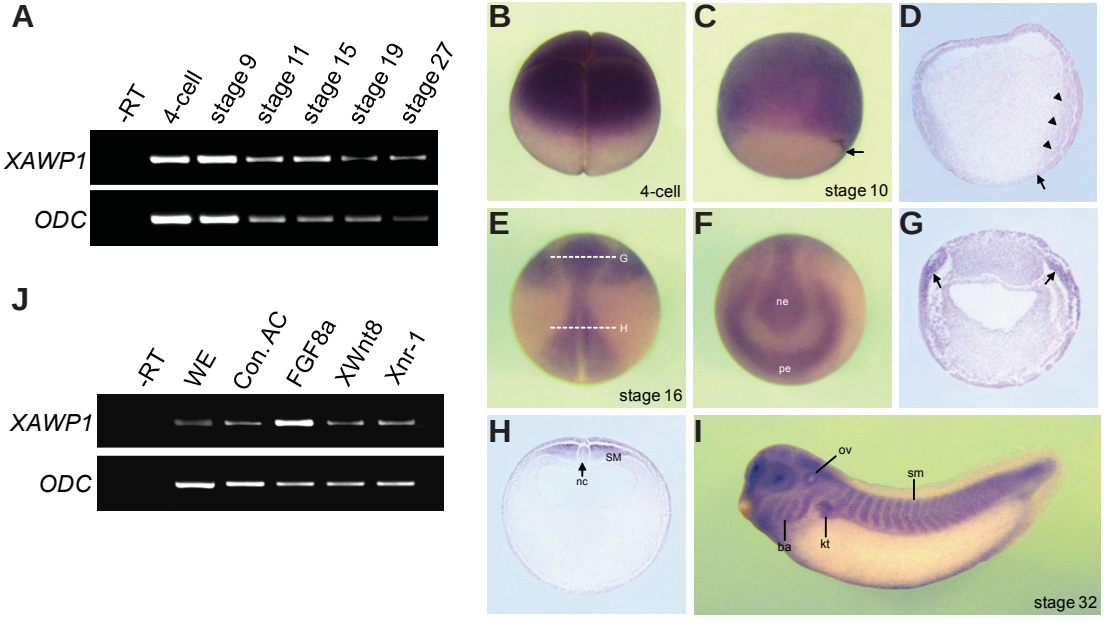

Fig. 1. Expression of the *XAWP1* gene. (A) *RT-PCR analysis showing the temporal expression pattern of* AWP1 *in* Xenopus *early development. Ornithine decarboxylase (ODC) serves as a loading control. –RT, a stage 27 control embryo in the absence of reverse transcriptase.* **(B-I)** *Spatial expression pattern of* XAWP1. **(B)** *Animal-lateral view with the vegetal pole to the bottom.* **(C)** *Vegetal-lateral view with dorsal to the right.* **(D)** *Sagittal section of a stage 10.5 gastrulae. Arrows in (C,D) denote the dorsal blastopore lip. Arrowheads indicate the involuting dorsal mesoderm.* **(E)** *Dorsal view with anterior to the top.* **(F)** *Anterior view of the embryo shown in (E) with dorsal to the top. ne, neural ectoderm; pe, preplacodal ectoderm.* **(G,H)** *Transverse sections of the embryo in (E) at the levels indicated by the dashed lines. Arrows in (G) denote the preplacodal ectoderm. SM, somitic mesoderm; nc, notochord; ov, otic vesicle; ba, branchial arch; kt, kidney tubule; sm, somites.* **(J)** *Four-cell stage embryos were injected in the animal pole region as indicated with* FGF8a *(1 ng),* XWnt8 *(400 pg) and* Xnr-1 *(100 pg) and then animal caps were excised at stage 9 and cultured to stage 10.5 for RT-PCR analysis. WE, a stage 10.5 whole embryo. Con. AC, uninjected control animal caps.*

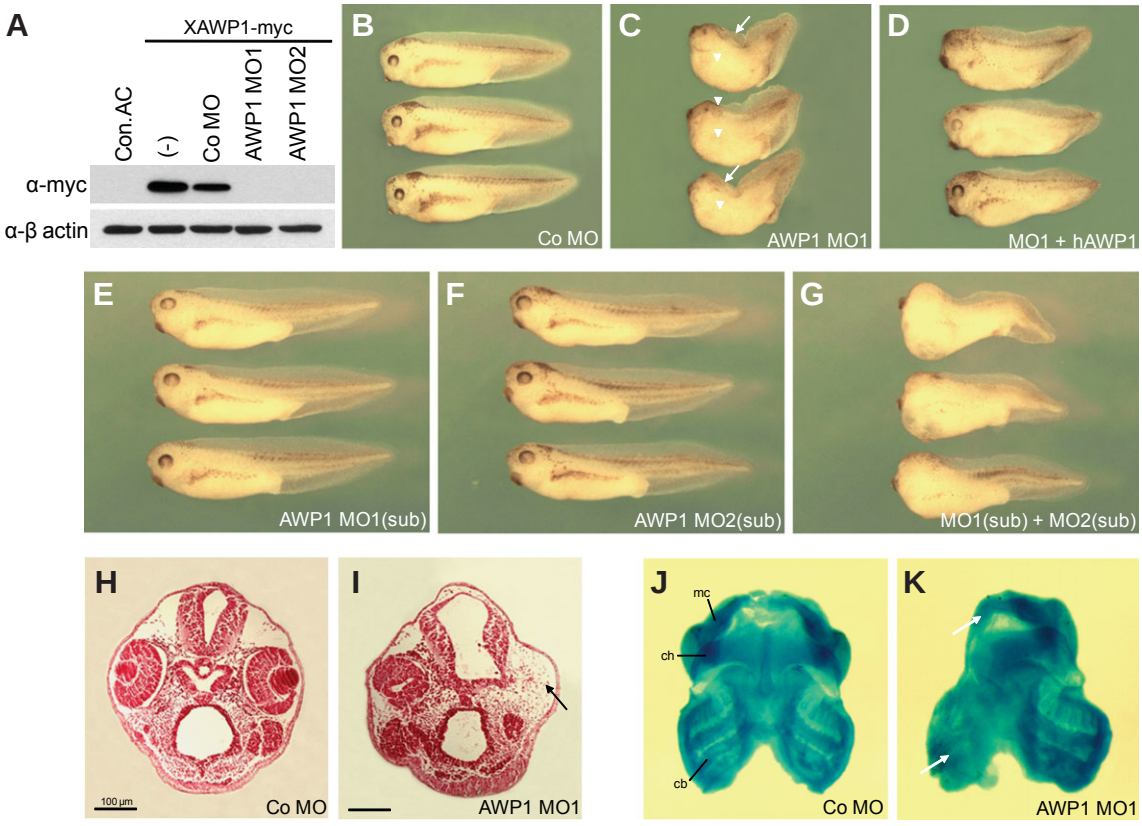

Fig. 2. A knockdown of *XAWP1* disrupts neural crest derivatives. (A) *Western blotting analysis of the specificity and efficiency of MOs. Four-cell stage embryos were injected in the animal pole region with* XAWP1-Myc *(200 pg) alone or with Co MO (30 ng),* AWP1 *MO1 (20 ng) or* AWP1 *MO2 (30 ng) and animal cap explants were then dissected at stage 9 and cultured to stage 11 for western blotting with an anti-myc antibody. β-actin was used as a loading control.* **(B-G)** *Embryos were injected in the dorso-animal region of two blastomeres at the 8-cell stage with Co MO (10 ng),* AWP1 *MO1 (10 ng for C and D, 5 ng for E and G),* AWP1 *MO2 (30 ng) and* hAWP1 *(5 pg) as indicated and cultured to stage 38. Arrows and arrowheads indicate defects in the dorsal fin and pigmentation, respectively. sub, suboptimal.* **(H,I)** *Four-cell stage embryos were injected in the dorso-animal region of one blastomere with* AWP1 *MO1 (5 ng) or Co MO (10 ng) and then subjected to paraffin sectioning and eosin staining at stage 40. An arrow indicates the defective eye in the injected side.* **(J,K)** *One blastomere of a four-cell stage embryo was injected in the dorso-animal region with* AWP1 *MO1 (5 ng) or Co MO (10 ng) and then stained with alcian blue at stage 45. Arrows indicate severely disrupted ceratobranchial and Meckel's cartilage in the injected side. cb, ceratobranchial cartilage; mc, Meckel's cartilage; ch, ceratohyal cartilage.*

Knockdown of XAWP1 causes defective neural crest derivatives

We employed an anti-sense morpholino oligo (MO)-mediated knockdown approach for loss-of-function analysis of *XAWP1*. For this, we designed two different MOs (MO1 and MO2), which target distinct sequences around the translation initiation site of *XAWP1* mRNA. BLAST searches against *Xenopus* EST database revealed that several genes have internal nucleotide substitutions, possibly due to the allotetraploidy, displaying over 93% identity with *XAWP1*. As the 5' regions around the transcription initiation sites of these genes are highly conserved (data not shown), most of them appear to be targeted efficiently by the two MOs. Western blotting also demonstrated that both *AWP1* MO1 and MO2, but not control (Co) MO, could efficiently inhibit the production of XAWP1 protein (Fig. 2A). To examine the morphological phenotypes of *XAWP1*-depleted embryos, we targeted the injection toward the dorsal region of embryos. As shown in Fig. 2C, *AWP1* MO1-injected embryos exhibited severe defects in craniofacial structures such as the eyes, and in pigmentation and fin formation along the trunk region of embryos (89%, n=68), and this malformation could be partially rescued by co-injection of human *AWP1* (*hAWP1*) RNA (Fig. 1D, 58%, n=73), which has no the target sequence of the MO and appears to be resistant to its inhibitory effect. To further confirm the specificity of these MO phenotypes, we also investigated whether the two MOs could synergize to impair the formation of craniofacial structures. Thus, we titrated the MOs down to levels that did not yield the phenotypes on their own. Targeted injection of either MO1 or MO2 at such suboptimal levels did not elicit any defects (Fig. 2E, F), whereas co-injection of the MOs at the same reduced doses interfered with head formation and pigmentation (74%, n=72; Fig. 2G), phenocopying the defective embryos that were injected with a high level of MO1 alone. These results thus corroborate the specific effects of the MOs in disrupting the craniofacial structures. In addition, histological analysis clearly showed that injection of *AWP1* MO1, but not Co MO, could inhibit formation of the lens and multilayered retina in the eye and of head mesenchyme in the injected side of the embryo (Fig. 2 H,I). We further examined the morphology of craniofacial cartilage, a NC derivative, in *XAWP1*-depleted embryos. As visualized by alcian blue staining, unilateral injection of *AWP1* MO1 resulted in a severe loss of craniofacial skeletal elements, including Meckel's, ceratobranchial and ceratohyal cartilage in the injected side compared with the uninjected control side of embryo (Fig. 2K). In contrast, injection of Co MO had no effect on the size or morphology of the craniofacial cartilage (Fig. 2J). Taken together, these data suggest that *XAWP1* plays a critical role in the formation of NC-derived structures.

XAWP1 is required for neural crest specification

We next investigated the effects of depleting *XAWP1* on the initial steps of NC formation. Targeted injection of *AWP1* MO1, but

not Co MO, abrogated the expression of the NC markers, *Sox10* and *Slug* in the injected side, compared with the uninjected side of embryos (86%, n=50 for *Sox10*; 65%, n=65 for *Slug*; Fig. 3 A,B, D,E). These repressive effects of the MO were rescued to some degree by co-expression of *hAWP1* (54%, n=58 for *Sox10*; 67%, n=70 for *Slug*; Fig. 3 C,F). Moreover, while single injection of MO1 or MO2 at below threshold levels did not affect the expression of *Sox10* (Fig. 3 G,H), the co-injection of the MOs at the same reduced concentrations could efficiently repress its expression in the injected side (40%, n=45; Fig. 3I). This confirmed the specificity of the MOs in inhibiting NC specification. NC markers are induced ectopically in animal cap tissue by co-expressing *Wnts* and BMP inhibitors such as *noggin* and *chordin* (Chang and Hemmati-Brivanlou, 1998). We also used this animal cap assay to test whether *XAWP1* is required for ectopic NC induction. Co-injection of *Wnt8* and *noggin* RNAs induced the expression of *Slug* and *Sox9* in the animal cap tissues (Fig. 3J). MO1 or MO2-mediated knockdown of *XAWP1* could down-regulate this ectopic expression, whereas the co-injection of Co MO had no effect (Fig. 3J). These results thus suggest that *XAWP1* is essential for the early specification of NC cells. As *XAWP1* is expressed in the mesoderm at the gastrula and neurula stages as described above, we next examined whether it might be involved in mesoderm induction and/or maintenance. Although the *XAWP1*-depleted embryos exhibited the seemingly disrupted trunk structure as shown above, its knockdown had no effect on the expression of an early pan-mesodermal marker, *Xbra* and late mesodermal markers, *MyoD* and *Xnot*, suggesting that it is not critical for mesoderm formation.

XAWP1 acts upstream of neural plate border specifiers

The expression of NPB specifiers such as *Msx1* and *Pax3*, which function as early factors in the NC genetic cascade, is regulated by Wnt and FGF signals (Monsoro-Burq *et al.*, 2005). We next asked whether *XAWP1* could also control the expression of these NPB markers. To address this, we performed the animal cap assay described above in which NC and NPB markers can be induced by Wnt or FGF signals combined with BMP inhibition. Notably, injection of increasing levels of *XAWP1* RNA could induce the expression of these NPB markers as well as of a NC marker, *FoxD3* in the animal caps (Fig. 4A) in the presence of a dominant negative (DN) *BMP4* receptor, which inhibits BMP signaling, suggesting a possible role for AWP1 as a mediator of Wnt and/or FGF signaling. Thus, we further examined whether *Msx1* and *Pax3* might function downstream of *XAWP1* to regulate NC specification. The targeted injection of *AWP1* MO1 impeded the expression of the marker *Sox10* in the injected side of the embryo (59%, n=55; Fig. 4B), which could be efficiently rescued by co-injection of *Msx1* or *Pax3* RNA (77%, n=52 for *Msx1*; 85%, n=50 for *Pax3*; Fig. 4 C,D). Taken together, these data indicate that *XAWP1* acts upstream of NPB regulators to specify NC cells.

XAWP1 mediates neural crest induction by Wnt and FGF signaling

Wnt/β-catenin and FGF signals have been shown to be crucial for NC induction (Stuhlmiller and Garcia-Castro, 2012). We examined whether NC induction by these signals depends on *XAWP1* activity. The unilateral injection of *FGF8a* RNA strongly enhanced the expression of *Sox10* along the neural fold in the injected side of the embryo (74%, n=60; Fig. 5A), and co-injection of *AWP1* MO1, but not Co MO, could impair this expansion (93%, n=45; Fig. 5B,

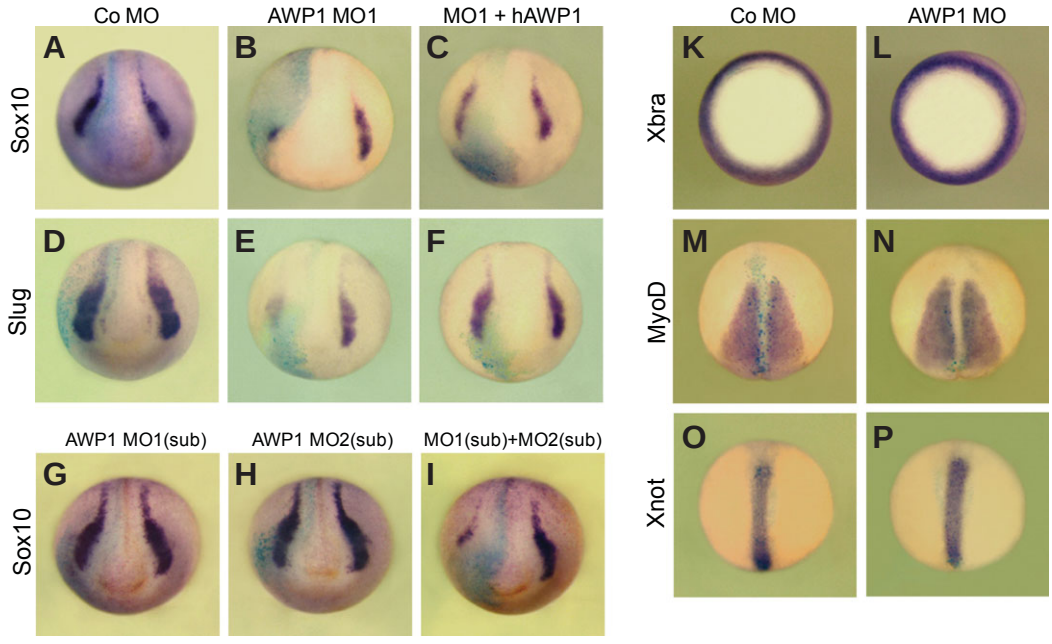

Fig. 3. XAWP1 is required for neural crest induction. (A-I) *One blastomere of a four-cell stage embryo was injected in the dorso-animal region with Co MO (5 ng),* AWP1 *MO1 (5 ng for B,C,E,F; 2 ng for G,I),* AWP1 *MO2 (20 ng) and* hAWP1 *(2.5 pg) as indicated and then subjected to* in situ *hybridization against* Slug *or* Sox10 *at stage 16. The injected side was traced by* lacZ *staining. All embryos are shown in dorso-anterior view with posterior to the top.* **(J)** *Four-cell stage embryos were injected in the animal pole region with the indicated combination of* noggin *(100 pg),* XWnt8-CSKA *(100 pg), Co MO (40 ng),* AWP1 *MO1 (20 ng) and* AWP1 *MO2 (40 ng), and the animal caps were dissected at stage 9 and cultured to stage 16 for RT-PCR analysis.* **(K-P)** *Two blastomeres of a four-cell stage embryo were injected in the dorsal-marginal region with Co MO (10 ng) or* AWP1 *MO1 (10 ng) and subsequently subjected to* in situ *hybridization against* Xbra *at stage 10.5 or* MyoD *or* Xnot *at stage 14. Embryos are shown in vegetal view with dorsal to the top* **(K,L)** *or in dorsal view with anterior to the top* **(M-P)**.

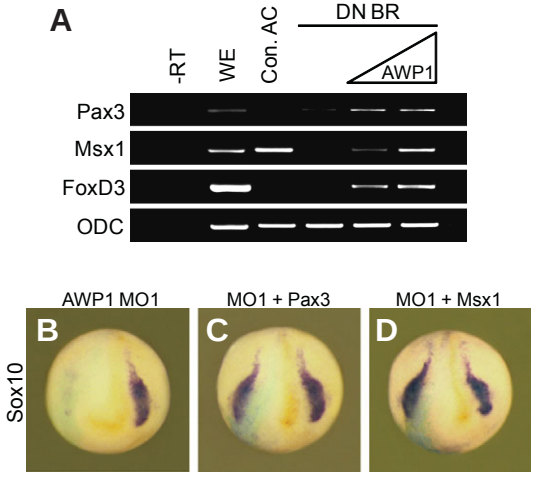

Fig. 4. XAWP1 regulates neural crest induction upstream of Msx1 and Pax3. (A) *Four-cell stage embryos were injected in the animal pole region with DN* BMP4 *receptor (1 ng) with or without* XAWP1 *(0.5, 2 ng), and the animal cap explants were excised at stage 9 and cultured to stage 11 for RT-PCR analysis.* **(B-D)** *Four-cell stage embryos were injected in the dorso-animal region of one blastomere with* AWP1 *MO1 (5 ng) with or without* Msx1 *(100 pg) or* Pax3 *(70 pg) as indicated and then subjected to* in situ *hybridization against* Sox10 *at stage 16.*

C), suggesting a critical role of *XAWP1* in *FGF8a* induction of the NC. In addition, injection of *XWnt8*-expressing plasmid resulted in ectopic rostral expansion of *Sox10* in the injected side (62%, n=71; Fig. 5D), which could be potently inhibited by co-injection of *AWP1* MO1 (89%, n=60) but not by Co MO (Fig. 5 E,F). Similarly, targeted expression of a DN *XWnt8* RNA strongly abrogated the expression of *Sox10* along the lateral neural ridge (71%, n=68; Fig. 5G) and this suppression could be rescued by co-expressing *XAWP1* RNA (55%, n=87; Fig. 5H). Furthermore, the *AWP1* MO1-mediated repression of *Sox10* expression (61%, n=83; Fig. 5I) could be reverted to normal levels by co-injecting an activating form of β-*catenin* (*pt* β-*catenin*) or of *Tcf3* (*VP16-Tcf3*) RNA (67%, n=87 for *pt* β-*catenin*; 65%, n=83 for *VP16-Tcf3*; Fig. 5 J,K). pt β-catenin is a stabilized mutant of β-catenin that lacks the phosphorylation sites required for its degradation, and expression of *pt* β-*catenin* or *VP16-Tcf3* is able to induce strongly the transcription of Wnt-responsive genes in the nucleus (MacDonald *et al.*, 2009). Thus, these data suggest that *XAWP1* controls NC induction downstream of Wnt8 ligand and upstream of the β-catenin/Tcf3 complex in the Wnt signaling pathway. We conclude from this evidence that *XAWP1* functions to mediate the activities of Wnt and FGF signaling in NC induction.

XAWP1 affects the stability of β-catenin

Wnt/β-catenin signaling regulates gene transcription by controlling the stability of β-catenin protein (MacDonald *et al.*, 2009). Since *XAWP1* regulates NC specification by acting in a position between a Wnt ligand and the β-catenin/Tcf3 complex in the Wnt signaling cascade as shown above, we tested whether it could affect the stability of β-catenin. Interestingly, injection of *AWP1* MO1, but not Co MO, reduced the level of β-catenin-myc protein, which could be reversed by co-expression of *hAWP1* RNA (Fig. 6A). Furthermore, co-injection of *XAWP1* up-regulated slightly its level (Fig. 6A). These results suggest that *XAWP1* plays a critical role in the stabilization of β-catenin. Next, we performed reporter assays to confirm the requirement of *AWP1* for Wnt/β-catenin signaling. The expression of a constitutively active form of Wnt receptor, *LRP6* (Δ*N LRP6*) or *Dishevelled* increased the luciferase activity driven by Wnt-responsive elements in a TOPFlash reporter gene (Fig. 6B). However, the siRNA-mediated knockdown of *AWP1* could interfere with the full activation of this reporter by the Wnt signaling components, although a control siRNA had a marginal effect on reporter activation (Fig. 6B). Overall, these results suggest that *AWP1* can affect Wnt signaling by modulating the stability of β-catenin.

Discussion

Although *AWP1* has been shown to be expressed in mouse and human early embryos as well as in several human tissues (Duan *et al.*, 2000), its physiological function remains unknown. In this study, we have identified the pivotal role of *AWP1* in NC formation during vertebrate early development. In *Xenopus* neurulae, *XAWP1* is absent in the neural fold, a neural crest-forming region but is detectable in its surrounding tissues, including the

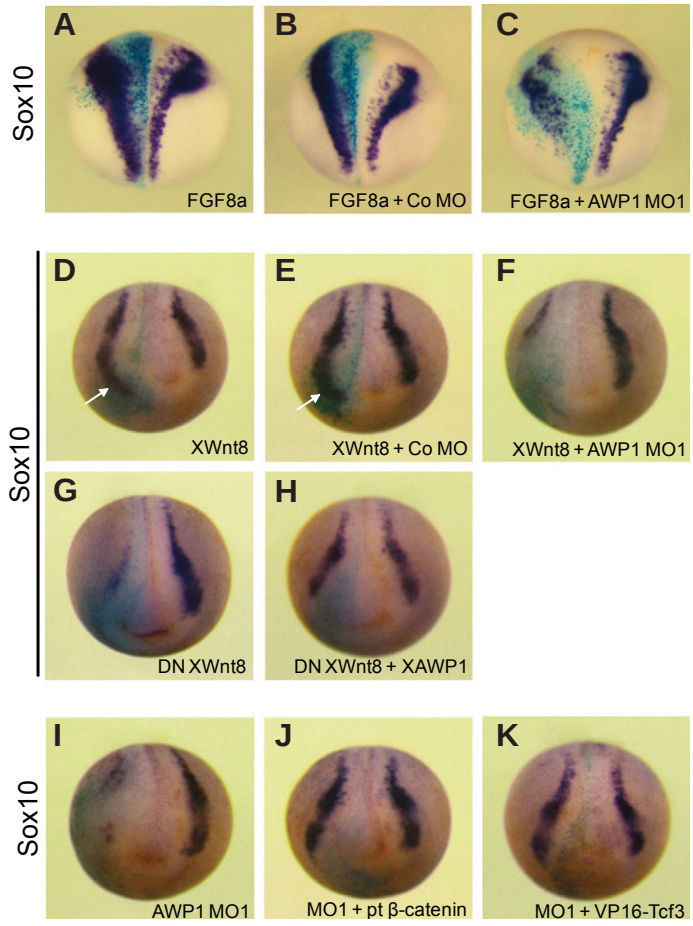

Fig. 5. Neural crest induction by FGF or Wnt signal requires XAWP1 function. (A-K) *One blastomere of a eight-cell stage embryo was injected in the dorso-animal region as indicated with* FGF8a *(20 pg),* XWnt8-CSKA *(100 pg),* DN XWnt8 *(400 pg),* XAWP1 *(300 pg),* pt β-catenin *(100 pg),* VP16-Tcf3 *(50 pg), Co MO (5 ng) and* AWP1 *MO1 (5 ng) and then subjected to* in situ *hybridization against* Sox10 *at stage 16. Arrows indicate the expanded expression of* Sox10.

preplacodal ectoderm, neural ectoderm, and axial and paraxial mesoderm (Fig. 1). This expression pattern suggests that *XAWP1* affects neural crest induction non-autonomously in the adjacent tissues but not in the neural crest progenitors. In support of this, the paraxial somitic mesoderm where the strong expression of *XAWP1* is visible, has been shown to be a key source of neural crest inducing signals such as Wnt and FGF in *Xenopus* (Hong *et al.*, 2008; Monsoro-Burq *et al.*, 2003). Furthermore, *XAWP1* acts as a mediator of Wnt and FGF signaling to specify neural crest cells as shown in this work. In contrast, *XAWP1* appears not to be critical for mesodermal specification as evidenced by no changes in the expression of early and late mesodermal markers in the *XAWP1*-depleted embryos (Fig. 3), which display the seemingly defective trunk structure. In addition to the marked expression of *XAWP1* in the preplacodal ectodermal region, the craniofacial defects caused by its knockdown, in particular, the malformed eyes, suggest that preplacodal development as well as neural crest formation is also disrupted in the *XAWP1*-depleted embryos. The neural crest cells can organize the vertebrate eye by contributing to the alignment of retina and lens via TGF-β and Wnt signaling (Grocott *et al.*, 2011). Moreover, it has been shown that lens develop from the preplacodal ectoderm (PE), requiring the sequential activation of a variety of transcription factors (Ogino *et al.*, 2012). A combination of FGF signal with Wnt inhibition promotes preplacodal development (Streit, 2007). *Xenopus FGF8* is expressed in the placodal region and anterior neural ridge at the neurula stages (Fletcher *et al.*, 2006). Our analysis revealed that *FGF8a* can induce the expression of *XAWP1* in naïve ectodermal tissue (Fig. 1). Taken together, these results suggest that FGF signal may be responsible for the expression of *XAWP1* in the preplacodal ectoderm. *XAWP1* is also detected in developing neural ectoderm. This expression pattern of *XAWP1*, in concert with its assumed mediation of Wnt signaling in the underlying mesoderm, suggests a possibility that it might affect neural induction and/or patterning. Indeed, depletion of *XAWP1* was found to expand the expression of a pan-neural marker, *Sox2* and a forebrain marker, *Otx2* but to repress that of a hindbrain marker, *Krox20* (data not shown). Neural crest induction is independent of the anterior-posterior patterning of the neural tissue (Wu *et al.*, 2005). Therefore, *XAWP1* is likely to affect these two processes separately.

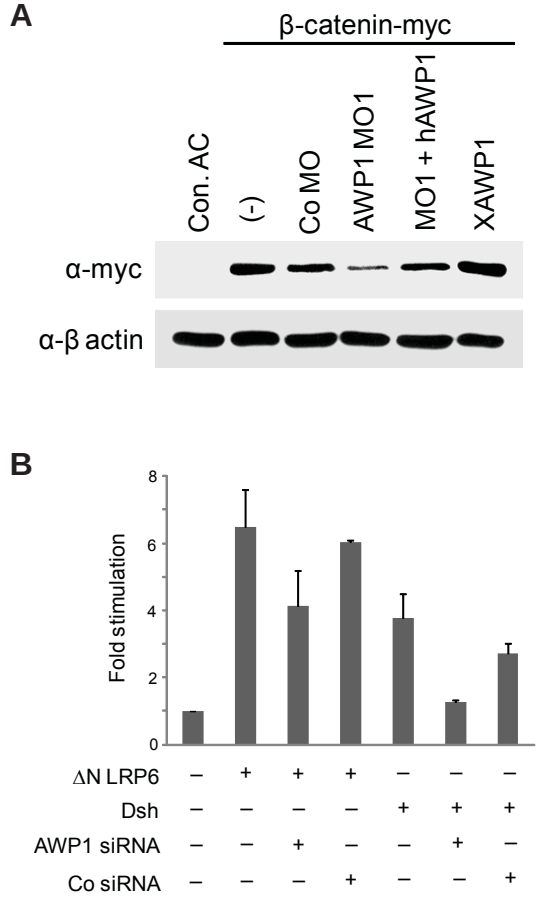

Fig. 6. AWP1 is involved in Wnt/β-catenin signaling. **(A)** *Four-cell stage embryos were injected in the animal pole region with the indicated combinations of β-*catenin-myc *(100 pg), Co MO (40 ng),* AWP1 *MO1 (40 ng),* hAWP1 *(400 pg) and* XAWP1 *(400 pg), and the animal caps were excised at stage 9 and cultured to stage 11 for western blotting.* **(B)** *HEK 293T cell were transfected as indicated with ΔN* LRP6 *(450 ng),* XDsh *(450 ng),* AWP1 *siRNA (50 nM) and Co siRNA (50 nM). Three independent experiments were performed and a single representative result is shown. Error bars denote standard deviations.*

XAWP1 activity is required for NC induction by Wnt8 or FGF8a ligand (Fig. 5). Notably, it acts upstream of the β-catenin/Tcf3 complex to control NC induction. Given that *FGF8a* induces the NC indirectly by activating the expression of *Wnt8* (Hong *et al.*, 2008), it seems likely that *XAWP1* mediates both Wnt and FGF induction of the NC by affecting the Wnt signaling pathway. Supportingly, the depletion of *AWP1* impairs the stability of β-catenin and the activity of a Wnt-responsive reporter gene, indicating its possible role as a Wnt signaling component. However, there is also evidence that Wnt and FGF signals act in parallel to regulate NC specification (Monsoro-Burq *et al.*, 2005). Thus, it is possible that these two signals converge on AWP1 activity during NC induction. Moreover, FGF induction of *AWP1* expression (Fig. 1) suggests that it might have a role in mediating the crosstalk between the Wnt and FGF signals in NC formation.

It appears that the mechanism underlying the activity of *AWP1* in the Wnt signaling pathway involves the control of β-catenin stability as described above. It is well known that GSK3 and CK1-phosphorylated β-catenin undergoes β-Trcp-dependent ubiquitination for proteasome degradation (MacDonald *et al.*, 2009). Since *AWP1* has been shown to mediate polyubiquitination of proteins (Fenner *et al.*, 2009), it is tempting to speculate that it could somehow affect the ubiquitination-mediated degradation of the β-catenin protein. In addition, *AWP1* is implicated in the regulation of NF-κB activity (Chang *et al.*, 2011). A recent study has shown that β-catenin acts as a transcriptional co-activator of NF-κB-dependent transcription (Armstrong *et al.*, 2012). Moreover, NF-κB signaling is also involved in the specification of mouse mesencephalic NC cells (Fujita *et al.*, 2011). Given these findings, it is possible that the loss-of-function of *AWP1* interferes with the cooperation between Wnt and NF-κB signaling, which could lead to the inhibition of NC induction by Wnt signaling. In addition, it has been shown that a balance between the anti-apoptotic and apoptotic activities in the neural fold plays a critical role in the proper formation of the NC (Tribulo *et al.*, 2004). Interestingly, the level of *AWP1* expression affects the incidence of apoptotic cell death by regulating the activity of NF-κB (Chang *et al.*, 2011). We also observed that both gain- and loss-of-function of *XAWP1* could increase the rate of apoptotic cell death in neural

tissue (data not shown). Thus, the effects of *XAWP1* on apoptotic cell death could be relevant to its regulation of NC specification. Further experiments will be necessary to test these hypotheses and to elucidate the precise mechanism underlying the activity of *AWP1*.

Materials and Methods

Embryo manipulation and lineage tracing

In vitro fertilization, embryo culture and microinjection were carried out as described previously (Sive *et al.*, 1989). The developmental stages of the embryos were determined according to the Nieuwkoop and Faber's normal table of development (Nieuwkoop and Faber, 1994). For lineage tracing, β-galactosidase mRNA (LacZ, 50 pg) was co-injected with MOs and other RNAs, and its activity was visualized with the X-Gal substrate (Duchefa Biochemie).

Plasmid constructs, MOs and RNA synthesis

The complete coding region of *Xenopus AWP1* (GeneBank Accession No. BC042359) was amplified by PCR with the primers, 5'-ATGATTATG-GCGCAGGAG-3' (forward) and 5'-TTATATCTTCTGAATCTTTTCTCC-3' (reverse) and then subcloned into a pGEM T vector (*XAWP1-T*). The DNA fragment, which was produced by digesting the *XAWP1-T* construct with *EcoRI* and *SpeI* restriction enzymes, was inserted into the *EcoRI*/*XbaI* sites of a pCS2 vector (*XAWP1-CS2+*). The PCR product encompassing the coding region and MO target sites was generated with the primers, 5'-CGGGATCCCGGCTACGGTTCCCGTTGCATG-3' (forward) and 5'- CGCGGATCCGCGTATCTTCTGAATCTTTTCTC-3' (reverse) and then inserted into the *BamHI* site of a pCS2+-Myc vector to produce a *XAWP1-Myc* construct. For sense mRNA synthesis, *XAWP1-CS2+* and *XAWP1-Myc* constructs were linearized with *NotI* and transcribed with Sp6 RNA polymerase. The coding region of human *AWP1* (Gene-Bank Accession No. AJ251095) was amplified by PCR with the primers, 5'- GGAATTCCATGGCTCAAGAAACTAAT-3' (forward) and 5'-TGCTC-TAGAGCTCAAATCTTTTGGATCTT-3' (reverse) and inserted into the *EcoRI*/*XbaI* sites of a pCS2+-Myc vector to generate the *hAWP1-Myc* construct. Capped mRNAs were *in vitro* synthesized with the mMessage mMachine kit (Ambion). Anti-sense MOs were purchased from Gene Tools. *XAWP1* MOs had the following sequences: MO1, 5'-TTTGTCTCCTGCGC-CATAATCATCC-3'; MO2, 5'-TCCACCATGCAACGGGAACCGTAGC-3'. The control MO was a standard MO from Gene Tools with the sequence 5'-CCTCTTACCTCAGTTACAATTTATA-3'.

In situ hybridization

Whole-mount *in situ* hybridization was performed as described previously (Harland, 1991). Anti-sense RNA probes were *in vitro* synthesized with digoxigenin-labeled nucleotides. An anti-sense *XAWP1* probe was generated by transcribing the *SpeI*-linearized *XAWP1-T* construct with T7 RNA polymerase. BM purple (Roche) was used as a substrate for the alkaline phosphatase.

Western blotting

Animal cap tissues were 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 MgCl2, 1 mM DTT, 1 mM sodium orthovanadate, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Equal amounts of protein were separated by 10% SDS-PAGE. Western blotting was carried out according to a standard protocol with anti-Myc (1:1000, Santa Cruz) and anti-β-actin (1:1000, Santa Cruz) antibodies.

RT-PCR

For RT-PCR analysis, total RNA was extracted from whole embryos and animal cap explants with TRI Reagent (Molecular Research Center) and treated with RNase-free DNase I (Roche Molecular Biochemicals) to remove genomic DNA. RNA was transcribed with M-MLV reverse transcriptase (Promega) at 37°C for 1 hour. 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 the PCR primers used are as follows: *XAWP1*, 5'-TACACAGGCCAGCTC-GTTGCC-3' (forward), 5'-AACGGTGAGTCCCGCAGAATACG-3' (reverse); *Slug* (Mizuseki *et al.*, 1998); *Sox9* (Monsoro-Burq *et al.*, 2003); *Pax3* (de Croze *et al.*, 2011); and *Msx1* (Suzuki *et al.*, 1997).

Histology and cartilage staining

For histology, embryos were fixed in MEMFA at stage 40 and embedded in Paraplast (Leica). Sections were cut at a thickness of 10 μm on a rotary microtome and stained with eosin (Sigma). For cartilage staining, embryos were fixed in MEMFA at stage 45, dehydrated in ethanol and stained for three nights in 0.04% alcian blue/30% acetic acid in ethanol. Embryos were then washed extensively with ethanol and rehydrated in 2% KOH solution. Finally, the embryos were washed in 20% glycerol/2% KOH for at least 1 hour and dehydrated through a glycerol series into 80% glycerol. Cranial cartilage was manually dissected out and photographed.

Luciferase reporter assay

For the TOPflash reporter assay, HEK 293T cells were transfected in 12-well dishes with Lipofectamine (Invitrogen). Approximately 48 hours after siRNA transfection, each well received 50 ng of TOPflash reporter plasmid (Upstate) and 1 ng of pRL-TK Renilla vector (as an internal control). Cells were lysed 24 hours after plasmid transfection and luciferase activity was measured with a dual luciferase assay system (Promega).

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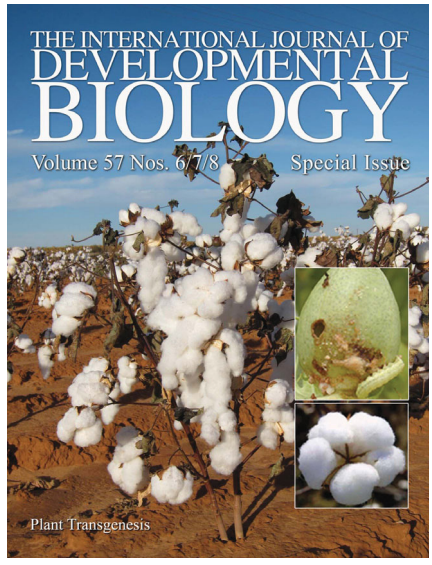

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