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Apolipoprotein C-I mediates Wnt/Ctnnb1 signaling during neural border formation and is required for neural crest development

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ABSTRACT In vertebrates, the neural crest and placodes originate in the neural border, which is located between the neural plate and epidermal ectoderm. The neural crest and placodes give rise to a vast array of cell types. Formation of neural crest is a multi-step process, in which Wnt signals are used reiteratively, but it is currently not clear if a Wnt signal is required for neural border formation. Here, we have identified *apolipoprotein C-I* (*apoc1*) in a screen for genes regulated by Wnt/Ctnnb1 signaling in late blastula stage *Xenopus tropicalis* embryos. We show that *Xenopus laevis apoc1* encodes a small, secreted protein, and is induced by Wnt/Ctnnb1 signaling. Depletion of Apoc1 protein results in a neural border formation defect and loss of border fates, including neural crest cells. However, unlike another Wnt/Ctnnb1 target, *gbx2.2*, *apoc1* is not required for patterning of the neural border. We further show that *gbx2.2* and *apoc1* are independently regulated by Wnt signaling. Our results thus suggest that Wnt regulates border formation and patterning by distinct genetic mechanisms.

KEY WORDS: *apolipoprotein, Xenopus, neural crest, neural plate border, wnt, ctnnb1*

Introduction

Neural crest cells constitute a transient cell population that is unique to vertebrate embryos. Together with the placodes, this multipotent and self-renewing cell population forms the glia and neurons of the peripheral nervous system. In addition, the neural crest contributes a vast array of other cell types to the developing embryo, such as blood vessels, melanocytes, and craniofacial cartilage and bones (Sauka-Spengler and Bronner-Fraser 2008). Development of neural crest cells has been suggested to occur in five sequential steps: neural border induction, neural crest induction, neural crest specification, neural crest migration and neural crest differentiation (Pegoraro and Monsoro-Burq 2013). In *Xenpous*, Wnt, Bmp, and Fgf signals begin patterning the ectoderm already during the blastula stage resulting in the formation of neural ectoderm, epidermal ectoderm, and the neural border (hereafter referred to as the border) from which both neural crest and placodal cells originate (Pegoraro and Monsoro-Burq 2013).

Conflicting reports exist on the involvement of Wnt signals in border formation. While one study proposed that an initial Wnt signal is required to initiate Bmp-mediated border formation (Patthey *et al.,* 2009), another suggested that Wnt activity initially need to be repressed for border formation (Steventon and Mayor 2012). Induced border cells are characterized by expression of border specifiers, e.g. members of the *dlx*, *msx*, *pax* and *zic* families, which stabilize the border fate (Patthey and Gunhaga 2011; Milet and Monsoro-Burq 2012). At gastrula stages, regionalization of the border then allows induction of neural crest cells and placodal cells from the posterior and anterior border, respectively. Later, at neurula stages, the posterior border is further regionalized resulting in formation of neural crest from the medial border and posterior placodes from the lateral border. Wnt is required for posteriorizing the border, and *gbx2.2* mediates this activity (Patthey *et al.,* 2008;

Abbreviations used in this paper: Apoc1, apolipoprotein C-I; BMP, bone morphogenetic protein; Chrd, chordin; Ctnnb1, catenin beta1; Fgf, fibroblast growth factor; Gbx2.2, gastrulation brain homeobox2, gene2; MO, morpholino oligo; WISH, whole mount *in situ* hybridization.

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Li *et al.,* 2009). Induction of neural crest requires the combined action of the border specifiers and signals, including Wnt, Bmp, and Fgf, produced by surrounding tissues (Hong *et al.,* 2008). For example, *pax3* and *zic1* are capable of inducing neural crest in the ventral *Xenopus* embryo, but only if Wnt signaling is not blocked (Sato *et al.,* 2005). Neural crest induction results in the activation of neural crest specifiers, including *snai2*, *foxd3*, and *sox9,* which initiate epithelial-to-mesenchymal transition, neural crest cell migration and differentiation (Duband 2010; Milet and Monsoro-Burq 2012). The timing of differentiation is correlated with the final fate/destination of the specific cell type (Theveneau and Mayor 2012).

Members of the Wnt family are secreted glycoproteins, which can activate at least three intracellular signaling pathways. The most intensively studied pathway is the Wnt/Ctnnb1 pathway (formerly "canonical pathway") that regulates cellular processes by Ctnnb1-mediated transcriptional activation of downstream target genes. In Ctnnb1-depleted embryos, the neural plate is dramatically expanded and border fates suppressed, suggesting that *ctnnb1* is required to inhibit neural induction and to promote the specification of border fates, possibly by regulating neural border

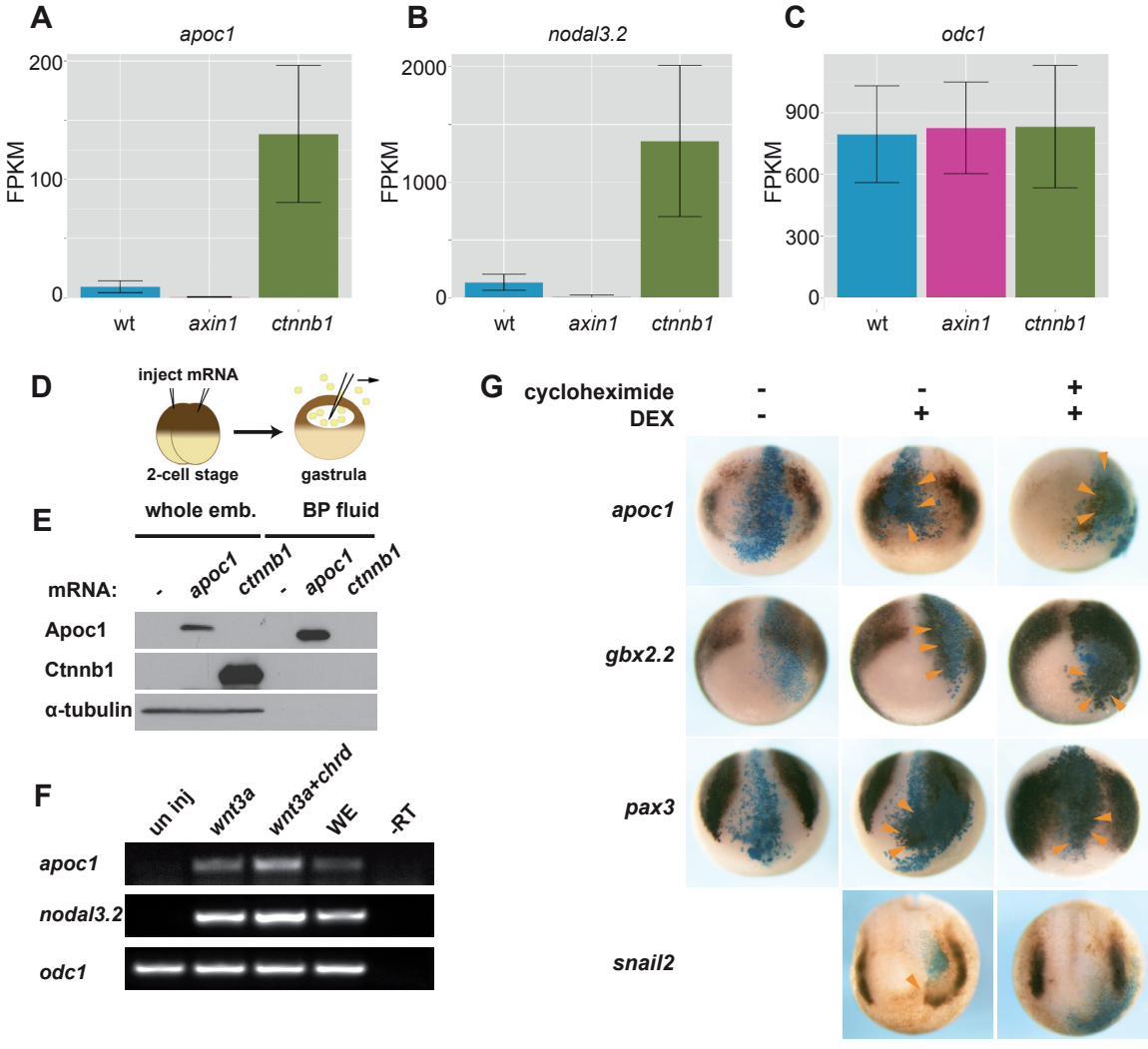

Fig. 1. Identification, characterization and regulation of Xenopus apoc1. (A-C) *Bar charts showing the expression levels, displayed as fragments per kilobase of transcript per million fragments mapped (FPKM), for* apoc1 **(A)**, *the Wnt regulated gene* nodal3.2 **(B)**, *and the non-Wnt regulated gene* odc1 **(C)** *in* Xenopus tropicalis *embryos that were either uninjected (blue), injected with 105 pg* axin1 *mRNA (pink) or 24 pg* <N-HA-ctnnb1 *mRNA (green).* **(D)** *Schematic drawing of the Apoc1 secretion experiment. mRNA was injected at the two-cell stage. The blastopore fluid was collected at the gastrula stage and subjected to western blot (WB) analysis to determine whether flag-tagged Apoc1 protein is secreted from cells during embryonic development.* **(E)** *Apoc1, but not Ctnnb1, protein was detected by WB in the blastocoel fluid after injection of flag-tagged* apoc1 *(500 pg) and flag-tagged* ctnnb1 *(1 ng). The tubulin blot shows that there is no cellular contamination in the blastopore fluid.* **(F)** *Wnt regulation of* apoc1 *in* Xenopus laevis *embryos.* **(F)** *300 pg of* wnt3a *or 200 pg of* chrd *and 300 pg* wnt3a *were injected into the animal side of two blastomeres at the two-cell stage. Animal cap explants were dissected from stage 9 embryos, cultured until stage 10, and processed for RT-PCR analysis. Expression of* apoc1, *and the known Wnt target gene* nodal3.2, *was induced by* wnt3a *alone, and* chrd+wnt3a. Odc1 *was used as loading control.* **(G)** Apoc1, gbx2.2 *and* pax3 *are immediate-early target gene of Wnt/Ctnnb1 signaling. 200 pg of* GR-ctnnb1 *and 100 pg* lacZ *mRNA were injected into one side of a dorsoanimal blastomere of 8-cell stage embryos and the embryos were treated with cycloheximide 30 min prior to DEX treatment at stage 11.5. The expression of* apoc1, gbx2.2, pax3 *and* snail2 *were examined by whole mount* in situ *hybridization. Orange arrows indicate ectopic localization of the tested genes.*

formation (Heeg-Truesdell and LaBonne 2006). In addition, ectopic expression of *ctnnb1* will posteriorize the anterior border (Li *et al.,* 2009). Thus, available data suggests that both Wnt ligands and Ctnnb1 transcriptional activity is instrumental for the developmental program that ultimately results in formation of neural crest cells.

In adult mice and humans, members of the apoliporotein family bind lipids to form lipoprotein particles. They function in lipid transport, but also as coenzymes. In addition, they can interact with lipoprotein receptors (Bolanos-Garcia and Miguel 2003). Mouse and human *apolipoprotein c1 (apoc1)* may influence memory functions and contribute to the pathogenesis of Alzheimer's disease (Berbee *et al.,* 2011). In adult *Xenopus laevis*, *apoc1* is predominantly expressed in the liver, but also in the stomach, gonads, intestines, and ovarian follicles during late oogenesis (Gohin *et al.,* 2010). However, a role for *apoc1* during early vertebrate development has not been reported.

In a screen for Wnt/Ctnnb1-regulated genes in late blastula stage *Xenopus tropicalis* embryos, we identified a strongly regulated transcript, which encodes for *apoc1*. Using *Xenopus laevis*, we demonstrate that Wnt can activate *apoc1* in both embryos and ecodermal explants. Furthermore, we show that *apoc1* is required for development of border cells fates, including neural crest cells. Previously, *gbx2.2* has been suggested to be the earliest Wnt/Ctnnb1 target in neural crest formation (Li *et al.,* 2009). However, we show that *gbx2.2* and *apoc1* are independently regulated by Wnt and mediate separate activities. Our data suggest a model where *apoc1* functions as a critical mediator of Wnt in border formation. However, *apoc1* is not required for patterning of the border along the anteroposterior axis, a function performed rather by *gbx2.2*.

Results

Identification, characterization, and regulation of Xenopus apoc1

To better understand the role of Wnt/Ctnnb1 signaling in early development, e.g. in ectoderm patterning, we attempted to discover novel Wnt target genes by comparing the transcriptional profiles of late blastula (Nieuwkoop and Faber stage 9.5) *Xenopus tropicalis* embryos where Wnt/Ctnnb1 signaling is normal (uninjected embryos), decreased (embryos injected with *axin1* mRNA) or increased (embryos injected with a constitutively active form of *ctnnb1* mRNA; Fig. 1 A-C). We identified an unannotated genomic region, scaffold_701:348314-352838 (JGI v4.1), predicted by TopHat/Cufflinks to encode a transcript that exhibits strong Wnt/Ctnnb1 regulation (uninjected: 9,44 Fragments Per Kilobase of transcript per Million fragments mapped (FPKM), *axin1* injected 0,70 FPKM, *ctnnb1* injected: 138,33 FPKM; Fig. 1A). Inspection of the genomic region in the UCSC genome browser revealed numerous unspliced ESTs with 3 or 4 exons. A nucleotide BLAST analysis of the genomic region identified a RefSeq predicted mRNA (XM_002941032.1) with four exons, encoding an 85 amino acid protein (XP_002941078) containing an Apolipoprotein C-I conserved domain (pfam04691). Further analysis using BLASTP of the predicted *Xenopus tropicalis* protein identified the 89 amino acid *Xenopus laevis* Apolipoprotein C-I (Apoc1) protein (ADD51575.1) as the closest ortholog with 66/89 (74%) identical and 78/89 (87%) positive amino acids.

We used SignalP 4.1 to analyze the *Xenopus laevis* and *tropicalis* Apoc1 protein sequences for possible signal peptides (www.cbs.dtu.dk/services/SignalP). Both proteins contain a putative signal

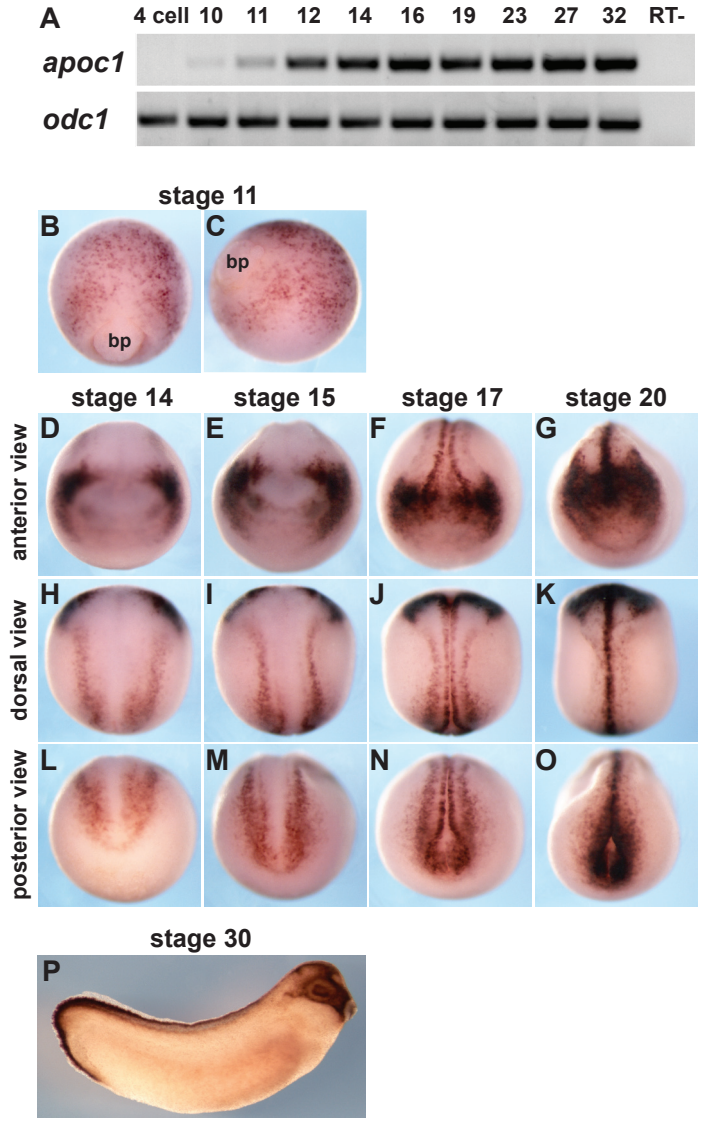

Fig. 2. Spatiotemporal expression pattern of *Xenopus laevis* apoc1. (A) *The temporal expression pattern of* apoc1 *during* Xenopus *development (4-cell stage, stages 10-32) was determined by RT-PCR (RT- is without reverse transcriptase from stage 32).* **(B-P)** *The spatiotemporal expression pattern of* apoc1 *determined by whole mount* in situ *hybridization.* **(B,C)** Apoc1 *expression at stage 11; bp - the closing blastopore. Anterior view* **(D-G)**, *dorsal view* **(H-K)**, *posterior view* **(L-O)** *of stage 14* **(D,H,L)**, *stage 15* **(E,I,M)**, *stage 17* **(F,J,N)** *and stage 20* **(G,K,O)** *embryos.* **(P)** *Lateral view of a stage 30 embryo.*

peptide, but no transmembrane domain, suggesting that they are secreted proteins. To test whether Apoc1 is secreted in *Xenopus* embryos, we injected C-terminally flag-tagged *Xenopus laevis apoc1* or flag-tagged *ctnnb1* mRNA at the 4-cell stage and collected the blastocoel fluid from stage 10 (early gastrula) embryos (Fig. 1D). As predicted, Apoc1, but not Ctnnb1, was detected by Western blot analysis in the blastocoel fluid (Fig. 1E).

Our screen established that *apoc1* is Wnt/Ctnnb1 regulated in *Xenopus tropicalis*. To further test whether Wnt/Ctnnb1 signaling regulates *apoc1* expression also in *Xenopus laevis*, we injected *wnt3a,* the BMP antagonist *chordin*/*wnt3a* mRNA into embryos

and performed RT-PCR on dissected animal caps at stage 10. As predicted, both *apoc1* and the well-established direct Wnt/Ctnnb1 target gene *nodal3.2* were strongly upregulated (Fig. 1F). We then asked if *apoc1* is a direct target of Wnt/Ctnnb1 signaling in *Xenopus laevis* (Fig. 1G). Previous studies have shown that both *gbx2.2* and *pax3* but not *snail2* were direct targets of Wnt/Ctnnb1 signaling (de Croze *et al.,* 2011; Li *et al.,* 2009), and our result in Fig 1G confirmed this. Strong ectopic expression of *apoc1, gbx2.2 and pax3* were observed overlapping area where *GR-Ctnnb1* was expressed even in the cycloheximide treated embryos. These data suggested that *apoc1* is a direct target of Wnt/Ctnnb1 signaling.

Apoc1 *is expressed at the neural plate border and in neural crest cells*

We next determined the temporal expression profile of *apoc1* in *Xenopus laevis* embryos (Fig. 2A). While we could not detect any expression by RT-PCR analysis in 4-cell stage embryos, we found low levels of *apoc1* in early gastrula (stage 10) embryos, consistent with our screen data, detecting low-level expression in late blastula (stage 9.5) *Xenopus tropicalis* embryos. The expression level increased dramatically by late gastrula (stage 12) and remained high during embryonic development (until stage 32; tadpole stage).

To determine the spatiotemporal distribution of *apoc1* mRNAs, we performed whole mount *in situ* hybridization (WISH) on stages 11 to 30 (gastrula to tadpole) *Xenopus laevis* embryos (Fig. 2 B-P). Robust *Apoc1* expression was first detected by WISH at stage 10.5 in the dorsal animal region (data not shown). At stage 11, the area of expression stretched towards the dorso-anterior and dorso-lateral part of the embryo along the gastrulation movement (Fig. 2 B,C). At the neurula stage, *apoc1* was highly expressed in parts of the preplacodal ectoderm, cranial neural crest cells, posterior neural plate border cells, the neural folds, and migrating neural crest cells (Fig. 2D-O). In stage 30 embryos, expression remained high in the dorsal fin, which consists of neural crest derived cell types (Fig. 2P). At this stage, *apoc1* expression was also detected in cranial placodes, including the lens placode, and in the mandibular neural crest. In summary, the *apoc1* expression pattern suggests a function for *apoc1* in neural crest and cranial placode development.

Apoc1 *is required for neural crest development*

To determine whether *apoc1* is required for neural crest development *in vivo*, we designed a translation blocking morpholino antisense oligo (tbMO) to prevent Apoc1 protein synthesis (Fig. 3A). As the genome sequence for *Xenopus laevis* was not yet available, an analysis of more than 50 expressed sequence tags (ESTs) was performed to identify a tbMO binding site that was conserved in the 5 prime untranslated sequence (5'UTR) of all ESTs. To determine the efficiency of the Apoc1 tbMO, we co-injected it (or control MO) with +/-5'UTR flag-tagged *apoc1* mRNA (Fig. 3A) and analyzed the Apoc1 protein expression levels in gastrula stage embryos. The Apoc1 tbMO efficiently blocked translation of *apoc1* mRNA only when it contained the tbMO target sequence (Fig. 3B). We also designed a splice blocking MO (spMO) against *apoc1* and assayed its efficiency by RT-PCR (Fig. 3C). Expression of *apoc1* mRNA was detected in stage 10 embryos that had been injected with control MO, but not in embryos injected with the spMO suggesting that it efficiently blocked the splicing of *apoc1* pre-mRNA (Fig. 3C).

We next analyzed the consequences of Apoc1 depletion in *Xenopus* embryos by co-injecting the Apoc1 tbMO with *lacZ* mRNA, encoding for beta-galactosidase, as a lineage tracer (Fig. 3D). The tbMO was first targeted to the presumptive cranial neural crest region from which craniofacial cartilage and bone forms (Fig. 3F, G). We

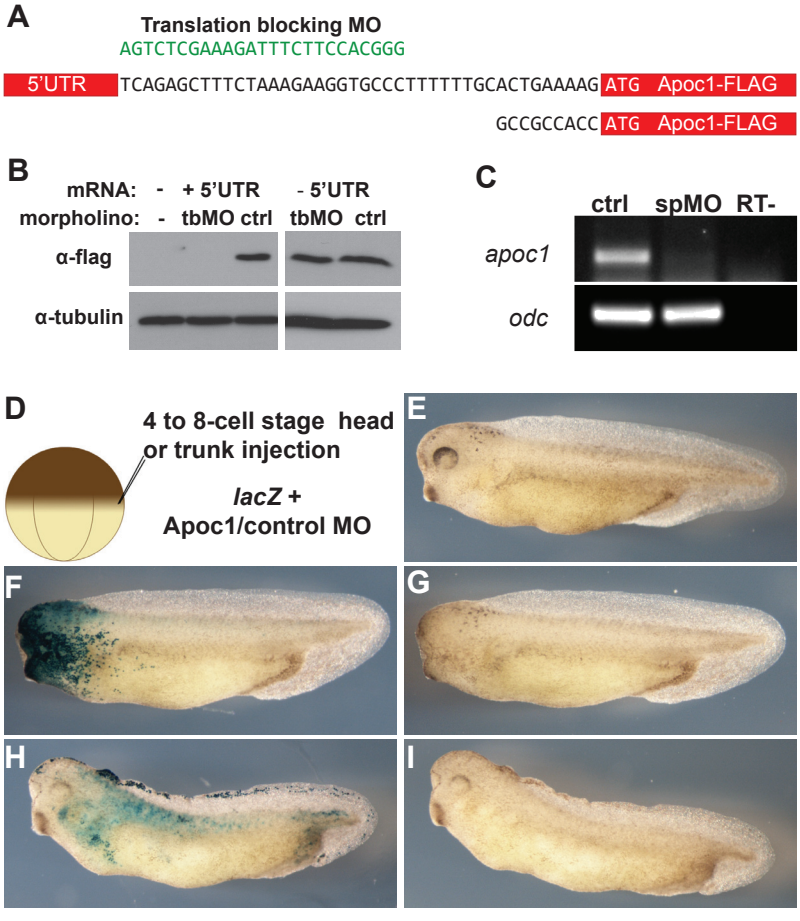

Fig. 3. Loss of neural crest derivatives in Apoc1 depleted embryos. (A) *The Apoc1 translational blocking MO (tbMO) targets the 5' UTR region of the* Xenopus laevis apoc1 *gene. To determine the efficiency of the tbMO, two flag-tagged* apoc1 *constructs were used differing only in the region upstream of the ATG start site.* **(B)** *Western blot using an anti-flag antibody on lysates from embryos injected with 300 pg 5'UTR-*apoc1*-flag or* apoc1*-flag mRNA and 40 ng Apoc1 tbMO or 40 ng control MO (ctrl).* **(C)** *30 ng of the splice blocking morpholino (spMO) or control MO (ctrl) was injected into the animal side of two blastomeres at the two-cell stage and the embryos were assayed by RT-PCR at stage 10.5.* Odc *is used as loading control.* **(D)** *Schematic drawing of the experiment. 40 ng Apoc1 MO was injected into either the presumptive head* **(F,G)** *or trunk/tail* **(H,I)** *region together with 200 pg* lacZ *mRNA.* **(E)** *Embryos injected with 40 ng control MO were normal.* **(F,G)** *Injections targeted to the head region resulted in 76.1% (n=134) of the embryos displaying small or missing eyes and head deformation.* **(H,I)** *Injections targeted to the trunk region resulted in perturbed dorsal fin development in 55.2% (n=38) of the embryos. The pictures in* **(G,I)** *were taken prior to X-gal staining* **(F,H)**.

allowed the embryos to develop until they reached the tadpole stage. Apoc1 depleted embryos exhibited clear head developmental defects including smaller or missing eyes (76.1%, n=134; supplementary material Table S1). Next, we targeted the tbMO to the presumptive trunk neural crest region from which cells of the dorsal fin derives (Fig. 3 H,I). Clear defects in dorsal fin formation were observed (55.3%, n=38; Fig. 3 H,I; supplementary material Table S1), with the heads unperturbed. Control MO injected embryos exhibited no developmental defects (Fig. 3E). To further strengthen these observations, we injected the spMO into the presumptive cranial or trunk neural crest regions, and, as expected, we observed head developmental (56.4%, n = 55) or dorsal fin formation defects (45.2%, n = 31) respectively (Fig. S1; supplementary material Table S1). As both the tbMO and spMO efficiently depleted the Apoc1 protein levels and resulted in identical phenotypes, we mainly used the tbMO in subsequent experiments.

Given that *apoc1* expression was detected prior to neural crest induction, we next wanted to determine whether *apoc1* is essential for neural crest emergence. Thus, we co-injected Apoc1 tbMO together with *lacZ* mRNA, and analyzed the expression of the neural crest markers *c-myc*, *sox9*, *snai2* and *twist1* by *in situ* hybridization (Fig. 4A). Consistent with an early role for *apoc1* in neural crest development, expression of these genes was missing or significantly downregulated on the injected side of early neurula stage embryos (supplementary material Table S2). Furthermore, depletion of *apoc1* mRNA with the spMO also resulted in reduced *snail2*, *sox9*, *twist1*, *pax3* and *zic1* expression (Fig. S2; supplementary material Table S2). The reduction/loss of neural crest markers could be rescued by injecting tbMO resistant *apoc1* mRNA (Fig. 4B upper panel; supplementary material Table S2), but not by injecting *apoc1* mutant mRNA lacking signal sequence (Fig. 4B lower panel; Fig. S3; supplementary material Table S2). These results demonstrate that the morpholino phenotype is indeed caused by a specific depletion of Apoc1 protein, and secretion is required for its function.

Bellmeyer and colleagues have shown that inhibition of Wnt signaling results in lost *c-myc* expression already in gastrula stage embryos, and that *c-myc* is required for expression of definitive neural crest markers (Bellmeyer *et al.,* 2003). Apoc1 depletion resulted in reduced or completely lost *c-myc* expression, placing *apoc1* downstream of *wnt*, but upstream of *c-myc*. Furthermore, *c-myc* has been demonstrated to regulate *id3* (Light *et al.,* 2005). We therefore examined *id3*, which controls the maintenance of neural crest stem cell behavior, in Apoc1-depleted embryos. Indeed, *id3* expression was downregulated or missing (Fig. 4A; supplementary material Table S2).

Neural crest cells originate from the border between the neural and epidermal ectoderm. The expression of neural crest markers is preceded by expression of border specifiers, such as *msx1*, *pax3* and *zic1* (Fig. 4C). C-myc depleted embryos also exhibit border defects, suggesting that *c-myc* also can be considered a border specfier. More specifically, in C-myc morphants, *zic1* and the neural precursor marker *sox2* are both expanded laterally. In Apoc1 depleted embryos, *zic1* and *sox2* are both expanded laterally (Fig. 4C; supplementary material Table S2), and the medial boundary of *xk81a1* (*keratin*), which is expressed in epider-

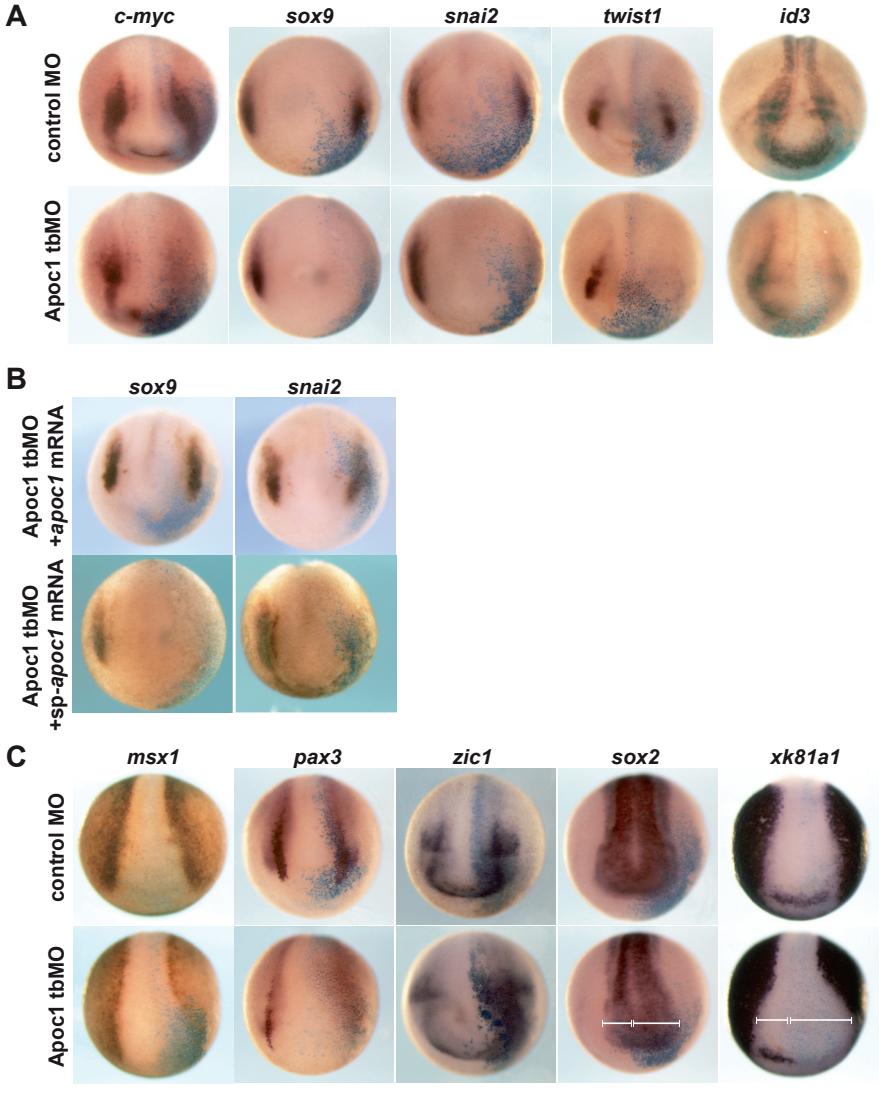

Fig. 4. Apoc1 is required for neural border formation and neural crest emergence. (A) *Whole mount* in situ *hybridization for the neural crest (NC) markers* c-myc, sox9, snai2, twist1, id3. *Control MO injected embryos are shown in upper panel and Apoc1 tbMO injected ones are shown in lower panel.* **(B)** *The reduction of* sox9 *and* snai2 *expression by the Apoc1 tbMO could be rescued by coinjection of 400 pg of* apoc1 *mRNA (upper panel) but not by coinjection of 400 pg of sp-*apoc1 *mRNA (lower panel).* **(C)** *Whole mount* in situ *hybridization for the neural plate border specifiers* msx1, pax3, zic1, *the neural marker* sox2, *and the non-neural ectoderm marker* xk81a1. *Control MO injected embryos are shown in upper panel and Apoc1 tbMO injected ones are shown in lower panel. One of the dorsal blastomeres was injected with 30 ng of either control MO (upper panels in A, C) or Apoc1 tbMO (lower panels in A, C) together with 100 pg* lacZ *mRNA. Embryos were fixed at stage 15. The injected side was traced by X-gal staining (blue).*

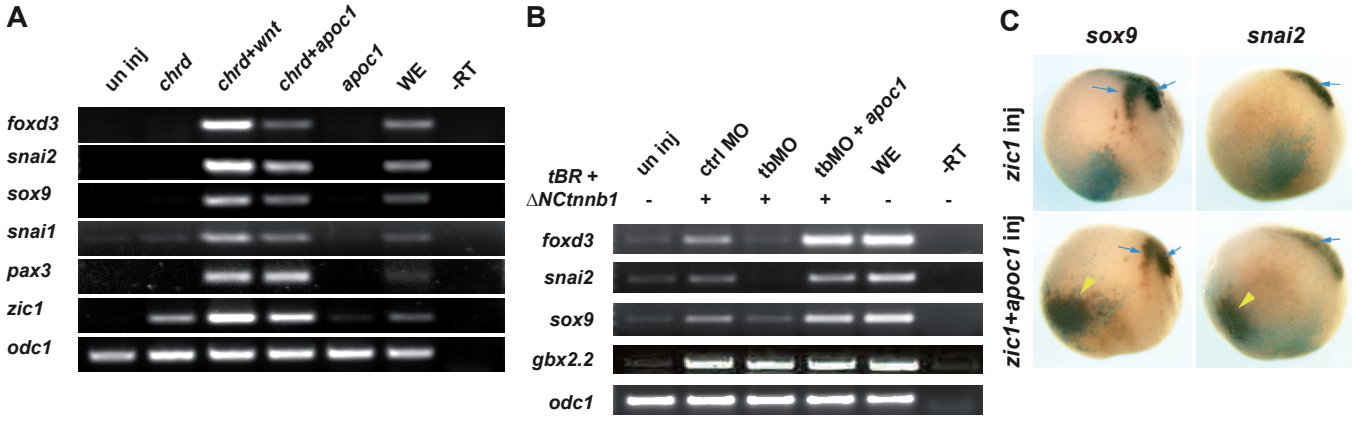

Fig. 5. Apoc1 can substitute for wnt in the animal cap neural crest induction assay. (A) *200 pg of* chrd*, 200 pg* wnt3a *and/or 300 pg of* apoc1 *was injected into two animal blastomeres of 4- or 8-cell stage embryos.* Animal caps *were dissected at stage 9 and cultured until stage 19. Gene expression was assayed by RT-PCR.* **(B)** *125 pg* tBR *(dominant negative form of* bmpr1a*) and 30 pg aN-HA-*Ctnnb1 *(constitutively active form of* ctnnb1*) mRNA was injected in two animal blastomeres at the 4- to 8-cell stage to induce neural crest cell marker expression. In addition, 40 ng of either control (ctrl) or translation blocking Apoc1 MO (tbMO) with or without 400 pg MO-resistant* apoc1 *mRNA was injected to determine if Apoc1 is required for neural crest induction.* Animal caps *were dissected at stage 9 and cultured until stage 16. Gene expression was assayed by RT-PCR.* **(C)** *Coinjection of* apoc1 *and* zic1 *into the ventral side of the embryo induced ectopic expression of* sox9 *and* snai2*. 50 pg of* zic1 *+/- 500 pg of* apoc1 *was injected into ventral marginal cells of 8 cell-stage embryos. The embryos were fixed at stage 14, and subjected to whole mount* in situ *hybridization. The blue arrows point to endogenous* sox9 *and* snai2 *expression. The yellow arrowheads show the area of ectopic* sox9 *and* snai2 *expression.*

mal ectoderm, is shifted laterally (Fig. 4C; supplementary material Table S2). Furthermore, expression of the border marker *msx1*, was depleted in Apoc1-depleted embryos (Fig. 4C; supplementary material Table S2). Interestingly, Msx1 morphants also exhibit an expanded *sox2* expression domain, and a lateral shift of the medial *xk81a1* expression boundary (Monsoro-Burq *et al.,* 2005).

Pax3 is a border specifier and required for neural crest induction. Furthermore, it is sufficient for neural crest induction *in vivo* and in animal caps when ectopically expressed together with *zic1* (Sato *et al.,* 2005). It has been shown that *pax3* is an immediate-early target of Wnt signaling in neural border induction (de Croze *et al.,* 2011). We then examined *pax3* expression in Apoc1 morphants and observed a dramatic downregulation in *pax3* expression in the neural folds (Fig. 4C; supplementary material Table S2). This result indicates that Wnt signaling directly induces initial expression of *pax3*, and *apoc1* maintains the expression of *pax3* in neural border. Together these results demonstrate that *apoc1* is required for border formation.

Apoc1 *together with BMP antagonists can induce neural crest markers in a neural crest induction assay*

We have demonstrated that *apoc1* is activated by Wnt/Ctnnb1 signaling both *in vivo* and in animal caps. The combined activity of Wnt ligands and BMP antagonists in animal caps results in a potent induction of neural crest cells (Saint-Jeannet *et al.,* 1997). Thus, we asked if *apoc1* could also induce neural crest markers with BMP antagonist in the animal cap neural crest induction assay. We injected embryos at the 4-8 cell stage with *apoc1*, the BMP antagonist *chordin* (*chrd*), *chrd*/*wnt3a*, and *chrd*/*apoc1*, dissected animal caps at stage 9 and cultured them until control embryos reached a late neurula stage (stages 19; Fig. 5A). As expected, RT-PCR analysis of the late neurula stage samples demonstrated that *chrd* alone can activate *zic1* expression, but does not result in neural crest induction. However, *chrd* in combination with *wnt3a* robustly induced the neural crest specifiers *foxd3*, *snai2*, *sox9*, *snai1*, and the border specifier *pax3*. Expression of *apoc1* alone did not activate any of the genes assayed, but in combination with *chrd*, *apoc1* robustly induced the expression of all markers. This result suggested that *apoc1* is a key mediator of Wnt/Ctnnb1 signaling in neural crest induction. To determine if *apoc1* is also required for Wnt-mediated neural crest induction in animal caps, we injected a dominant negative form of *bmpr1a* and a constitutively active form of *ctnnb1* with or without Apoc1 MO, dissected animal caps at stage 9, and performed RT-PCR at stage 16 (Fig. 5B). Apoc1 MO coinjection strongly suppressed the NC markers *foxd3, snai2* and *sox9*. Furthermore, the expression of these markers was restored by injecting morpholino resistant *apoc1* mRNA (Fig. 5B). Taken together, *apoc1* is required for Wnt-mediated neural crest induction in animal caps. As previous study showed (Li *et al.,* 2009), gbx2.2 was induced in the animal caps injected with dominant negative form of *bmpr1a* and a constitutively active form of *ctnnb1,* and its expression was not changed by injecting Apoc1 tbMO.

Chrd can be replaced both *in vitro* and *in vivo* with the *chrd*-induced gene *zic1* to, in combination with e.g. *pax3* or *gbx2.2*, induce neural crest markers (Sato *et al.,* 2005; Li *et al.,* 2009). As *apoc1* and *chrd* together could induce neural crest markers in vitro, we examined if *apoc1* together with *zic1* could induce ectopic neural crest markers *in vivo* (Fig. 5C). Injection of *zic1* alone in the ventral embryo could not induce *sox9* or *snai2*, but *apoc1* together with *zic1* induced robust ectopic *sox9* and *snai2* (Fig. 5C; supplementary material Table S3).

apoc1 *and* gbx2.2 *are both required for neural crest induction and are independently regulated by Wnt/Ctnnb1 signaling*

Li *et al.,* have suggested that *gbx2.2* is the earliest target of Wnt/Ctnnb1 signaling in neural crest induction (Li *et al.,* 2009). To determine the epistatic relationship between endogenous *apoc1* and *gbx2.2*, we analyzed *apoc1* expression in Gbx2.2 MO injected embryos and *gbx2.2* expression in Apoc1 MO injected embryos (Fig. 6A-D). Interestingly, neither gene appeared regulated by the other

(supplementary material Table S4), demonstrating that *apoc1* and *gbx2.2* are independently regulated during early *Xenopus* development. Fig. 5B results also indicated that *gbx2.2* expression in the neural crest induction assay is independent of Apoc1. In addition, the loss of the neural crest markers *sox9* and *snai2* in Apoc1 or Gbx2.2 MO injected embryos could not be rescued by coinjection of *gbx2.2* or *apoc1* mRNA, respectively (Fig. 6 E-H; supplementary material Table S4). Thus, *apoc1* and *gbx2.2* are both required for neural crest development, but independently regulated.

To determine whether both *apoc1* and *gbx2*.2 are required for Wnt/Ctnnb1 mediated neural crest induction *in vivo*, *GR-Ctnnb1* was targeted to the neural plate/neural border region and activated by DEX at an early gastrula stage. As expected, the *snai2* and *sox9* expression domains were both expanded anteriorly (Fig. 6I, J; supplementary material Table S4), and in some embryos, expression was even detected in the neural plate (Fig. 6I). Strong ectopic expression of both *apoc1* and *gbx2.2* was observed overlapping the entire area where *GR-Ctnnb1* was expressed (Fig. 6K, L; supplementary material Table S4). In addition, *msx1* and *pax3* were also found to be ectopically expressed (Fig. 6 M,N; supplementary material Table S4).

We then coinjected *GR-Ctnnb1* and Apoc1 tbMO or Gbx2.2 MO. The anterior expansion of *snail2* was rescued in both morphants (Fig. 6 O,Q; supplementary material Table S4) demonstrating that both *apoc1* and *gbx2.2* are important mediators of Wnt/Ctnnb1 signaling in neural crest induction *in vivo*. However, Apoc1 or Gbx2.2 depletion did not rescue the *GR-Ctnnb1*-induced ectopic expression of *gbx2.2* or *apoc1*, respectively (Fig. 6P, R; supplementary material Table S4). Taken together, the expression of *apoc1* and *gbx2.2* are regulated independently by Wnt/Ctnnb1 signaling, but both are required for neural crest induction *in vivo*.

Apoc1 *does not regulate regionalization of the border*

Our findings show that *apoc1* is a key mediator of Wnt/Ctnnb1 signaling in border formation, but Wnt/Ctnnb1 signaling has also been reported to posteriorize the border (Patthey *et al.,* 2008; Li *et al.,* 2009). Therefore, to investigate whether *apoc1* is sufficient for posteriorizing the neural border, *apoc1* mRNA was targeted to the neural border. Unlike after expression of *wnt* or *gbx2.2* in the anterior border (Li *et al.,* 2009), no anterior expansion of *pax3* or *snai2* was observed, and the preplacodal marker *foxi4.1* was maintained (Fig. 7 A-C; supplementary material Table S5). *Apoc1* is thus not sufficient to promote posteriorizaton of the anterior neural border.

In Gbx2.2 morphants, the posterior border is anteriorized. We therefore analyzed *foxi4.1* also in Apoc1 morphants (Fig. 7 D-F). Rather than a posterior expansion, we found that *foxi4.1* was lost in the most posterior part of the preplacodal domain (Fig. 7F; supplementary material Table S5). This result shows that *gbx2.2* and *apoc1* have opposite effects on preplacodal development; *gbx2.2* inhibits, but *apoc1* is required for expression of preplacodal markers. Thus, these results argue that while *apoc1* is required for mediating Wnt's border specification and neural crest induction activities, it is, unlike *gbx2.2*, not acting as a posteriorizing factor.

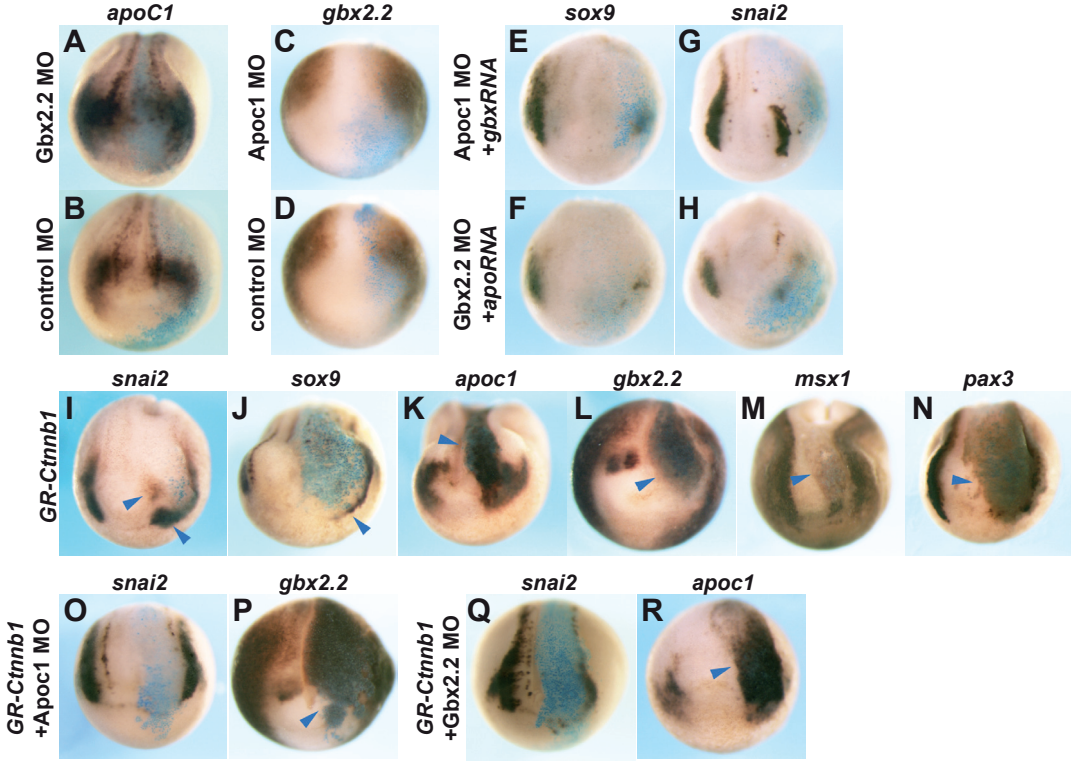

Fig. 6. Apoc1 and gbx2.2 are required for emergence of neural crest cells, but are independently regulated by Wnt signaling. (A-H) *The expression of* apoc1 *and* gbx2.*2 is independently regulated, but both genes are required for neural crest induction.* Apoc1 *expression in Gbx2.2* **(A)** *or control MO* **(B)** *injected embryos and* gbx2.2 *expression in Apoc1* **(C)** *or control MO* **(D)** *injected embryos. The reduction of* sox9 *and* snai2 *expression was not rescued by coinjection of 100 pg* gbx2.2 *mRNA in Apoc1* **(E,G)** *or 400 pg* apoc1 *mRNA in Gbx2.2* **(F**, **H)** *depleted embryos. 30 ng of MO and 100 pg* lacZ *mRNA was injected into one side of a single dorsoanimal blastomere of 8-cell stage embryos. The embryos were fixed at stage 15.* **(I-R)** Apoc1 *and* gbx2.*2 are both required for Wnt-mediated neural crest induction* in vivo. *200 pg of* GR-ctnnb1 *and 100 pg* lacZ *mRNA were injected into one side of a dorsoanimal blastomere of 8-cell stage embryos and the embryos were treated with DEX at stage 10.5. The embryos were fixed at stage 15 for whole mount* in situ *hybridization with* snail2 **(I)**, sox9 **(J)**, apoc1 **(K)**, gbx2.2 **(L)**, msx1 **(M)**, *and* pax3 **(N)**. *Ectopic expression of* gbx2.2 *in* GR-ctnnb1 *injected embryos was not reduced by coinjecting 30 ng Apoc1 tbMO* **(P)**, *but the anterior expansion of* snai2 *was rescued* **(O)**. *Ectopic expression of* apoc1 *in* GR-ctnnb1 *injected embryos was not reduced by coinjecting 30 ng Gbx2.2 MO* **(R)**, *but the anterior expansion of* snai2 *was rescued* **(Q)**. *Blue arrowhead indicates ectopic expression.*

To further support this claim, we turned to the animal cap assay and analyzed border and neural crest markers at stage 14 (early neurula stage; Fig. 7G). As was shown in late neurula stage animal explants (Fig. 5), *apoc1* (or *wnt*) together with *chrd* can induce *snai2* and *pax3* (Fig. 7G). The *zic1* level was even in all *chrd*-injected animal explants, and as expected, coinjection of *wnt* and *chrd* reduced expression of the preplacodal marker *six1* and the anterior neural and placodal marker *otx2* (Fig. 7G). These results confirm the posteriorizing effect of *wnt* expression. However, while coinjection of *chrd* and *apoc1* induced neural crest markers, it failed to repress *sox2*, *six1* and *otx2* (Fig. 7G). These three markers are all expressed in the anterior border or placodes. Our data thus suggests that *apoc1* is required to mediate the border specification, but not the border regionalization, activity of Wnt/Ctnnb1 signaling.

Discussion

We identified the apolipoprotein *apoc1* in a screen for Wnt/Ctnnb1-regulated genes in late blastula *Xenopus tropicalis* embryos. Using *Xenopus laevis*, we confirmed this finding and demonstrated that Apoc1-depleted tadpoles exhibited defects in the formation of neural crest- and placode-derived cell types. In vertebrates, both placodal and neural crest cells are generated from the border, albeit from distinct regions. While the anterior border produces placodal cells, the posterior border generates both neural crest (medial border) and placodal cells (lateral border). The emergence of neural crest and placodal cells are complex, multi-step processes (Meulemans and Bronner-Fraser 2004; Betancur *et al.,* 2010), which are initiated already in the late blastula/early gastrula stage embryo at which time cells of the border are induced (Patthey and Gunhaga 2011). Our study has revealed an absolute requirement for the Wnt/Ctnnb1 signaling target *apoc1* in border formation, suggesting that a Wnt signal is required in blastula stage embryos to promote border formation.

Wnt/Ctnnb1 signals are used reiteratively during neural crest development. Wnts have been clearly demonstrated to regulate antero-posterior border patterning and neural crest induction (Saint-Jeannet *et al.,* 1997; LaBonne and Bronner-Fraser 1998). Previous studies have provided conflicting data on whether Wnt activity is required for, or actually inhibits, border formation. While one study suggested that Wnt activity needs to be repressed for border induction, and then activated for neural crest induction (Steventon and Mayor 2012), another proposed that Wnt is required to initiate a BMP-dependent neural border induction mechanism (Patthey *et al.,* 2009). In addition, Wnt/Ctnnb1 has been shown to be required for inhibiting neural induction and promoting neural crest development (Heeg-Truesdell and LaBonne 2006). When Wnt activity was ablated using dominant negative *lef1* or *tcf3* constructs, or Ctnnb1 morpholinos, *c-myc* was repressed, the neural plate was expanded laterally and neural crest cells were not formed (Heeg-Truesdell and LaBonne 2006). Taken together with the observation that *c-myc* is required for border formation (Bellmeyer *et al.,* 2003), and our findings in this study: that *apoc1* functions upstream of *c-myc* in border formation and that *apoc1* is a Wnt/Ctnnb1 target, argues for an early Wnt/Ctnnb1 requirement for ensuring the formation of a proper border domain. Consistent with the idea that at least the neural crest producing border cells originate from the lateral neural tube, we showed that activating Wnt

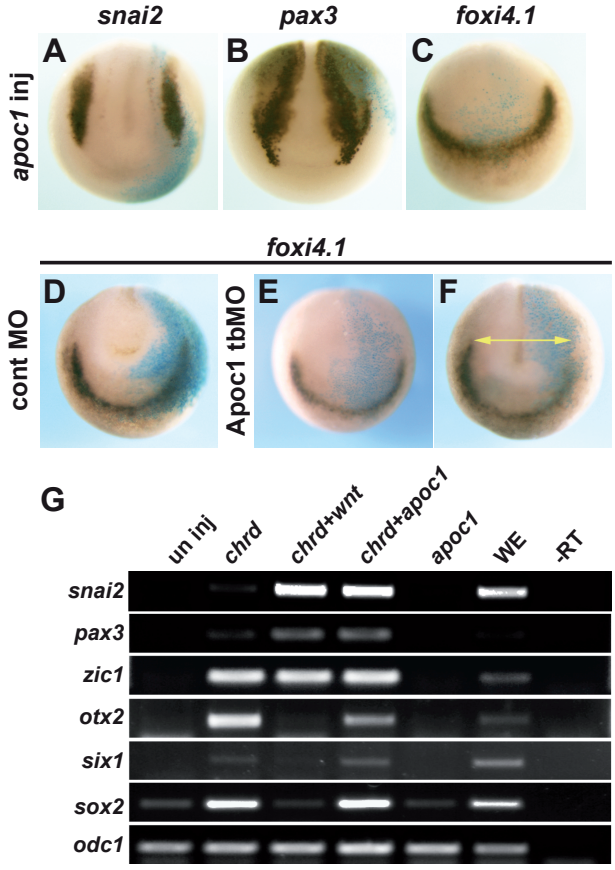

Fig. 7. Apoc1 does not posteriorize the border. (A-C) *Expression of* apoc1 *(400 pg) is not sufficient to posteriorize the anterior neural border as demonstrated by analysis of* snai2 **(A)**, pax3 **(B)** *and* foxi4.1 **(C)** *using* in situ *hybridization. One of the dorsoanimal blastomeres was injected with 400 pg* apoc1 *and 100 pg* lacZ *mRNAs at the 8-cell stage.* **(D-F)** Apoc1 *is required for expression of* foxi4.1 *in the posterior preplacodal domain. Whole mount* in situ *hybrydization of* foxi4.1 *in control MO* **(D)** *or Apoc1 tbMO* **(E,F)** *injected embryos. The yellow arrow shows the posterior limit of* foxi4.1 *expression on the uninjected side. 30 ng of MO was injected with 100 pg of* lacZ *mRNA into one side of a dorsoanimal blastomere of 8-cell stage embryos and fixed at stage 15.* **(G)** *200 pg of* chrd, *200 pg* wnt3a *and/or 300 pg of* apoc1 *was injected into two animal blastomeres at the 4- to 8-cell stage.* Animal caps *were dissected at stage 9 and cultured until stage 14. Gene expression was assayed by RT-PCR.* Odc1 *is a loading control.*

signaling in the early neural plate using DEX-inducible *GR-ctnnb1* results in strong ectopic expression of *apoc1, gbx2.2, msx1 and pax3* in the neural plate. In fact, in some embryos, expression of *snai2* was also detected in the neural domain, suggesting that a continuous Wnt/Ctnnb1 signal is sufficient to respecify some neural cells into neural crest-producing border cells.

Several studies have demonstrated that, at gastrula stages, Wnt/Ctnnb1 is required for patterning the border into separate anterior and posterior domains. Secreted or intracellular Wnt antagonists, such as *dkk1* or *kctd15*, are required for the border to acquire anterior character. Furthermore, the anterior border expresses posterior markers upon ectopic expression of *wnt* or *ctnnb1* and produces neural crest cells rather than placodal cells. *Gbx2.2* has been shown to be a direct target of Wnt/Ctnnb1 signaling and

to mediate its posteriorizing activity (Li *et al.,* 2009). In Gbx2.2-depleted embryos, *pax3* and *msx1* expression was reduced/lost and neural crest cells did not form. However, the border was formed and expressed the panplacodal marker *six1*. Conversely, ectopic expression of *gbx2.2* was sufficient for repressing *six1* expression and imposing a posterior character on the anterior border. *Gbx2.2* exerts this function by crossrepressing *otx2* (Steventon *et al.,* 2012), a mechanism that also patterns the central nervous system (Nordstrom *et al.,* 2002). In contrast, *apoc1* expression in the anterior border does not repress the placodal marker *foxi4.1*, and conversely, *foxi4.1* is not expanded posteriorly in Apoc1-depleted embryos. In fact, the posterior limit of the *foxi4.1* domain is shifter anteriorly, which is consistent with the observed loss of placodal fates in tadpole morphants and a requirement for *apoc1* in border formation.

As *pax3* and *msx1* is lost in Gbx2.2-depleted embryos, it had been suggested that *gbx2.2* should be placed at the top of the hierarchy of genes controlling neural crest development (Li *et al.,* 2009). However, unlike Apoc1-depleted embryos, Gbx2.2 morphants were not reported to exhibit an expanded neural plate and neural border formation defects. In fact, the border was formed but the patterning perturbed resulting in loss of neural crest fates. Given the spatiotemporal expression pattern of *apoc1*, first appearing in the dorsal region at the onset of gastrulation followed by expression in the border, except the most anterior part, *apoc1* is likely to be the immediate Wnt-regulated genes in border formation. This prompted us to examine the epistatic relationship between *apoc1* and *gbx2.2*. We found that *apoc1* and *gbx2.2* are independently regulated by Wnt signaling, both in the early embryo and upon targeting expression of *GR-ctnnb1* to the nervous system. Based on these observations, we propose a model in which Wnt regulates border formation and border patterning by two independent genetic programs. We suggest that *apoc1* mediates Wnts border formation activity and *gbx2.2* the anteroposterior patterning activity. Thus our study provides further insights into the genetic mechanism by which Wnt controls two distinct steps of the neural crest developmental program. Furthermore, *apoc1* remains expressed after border formation, in the neural folds and migrating neural crest, suggesting that it may be required also for neural crest induction, migration or differentiation.

The cellular and molecular mechanisms by which *apoc1* contributes to border formation remain to be elucidated. *Apoc1* is a member of the apolipoprotein family that binds lipids to form lipoprotein such as chylomicrons, IDL, LDL, VLDL, and HDL. These lipoproteins transport lipids through the lymphatic and circulatory systems. In addition, apolipoproteins have been demonstrated to act as ligands for cell surface receptors (Li *et al.,* 1988). There are four members of the *apoc* subfamily of low molecular weight apolipoproteins. The human *apoc1* gene is located in the *apoe/c1/c2* gene cluster on chromosome 19 (Jong *et al.,* 1999). Both *apoc1* and *apoe4* are known to be risk factors for Alzheimer's disease (AD), possibly by controlling the cellular cholesterol homeostasis in the brain (Leduc *et al.,* 2010), although a detailed mechanism has not been provided.

This study is one of the first to ascribe a function to *apoc1* during embryonic development. The demonstration that *apoc1* activity is required as early as during late blastula is interestingly since a circulatory system has yet to be established at such early stages of *Xenopus* development, possibly suggesting a novel molecular mechanism. In mice, both overexpression and knock out of *apoc1* results in impaired memory functions (Berbee *et al.,* 2011), but no embryonic defects have been reported. This suggests that, in mouse, there might be either redundancies, a requirement for a different apolipoprotein family member, or a different mechanism for establishing the neural border.

Several studies have shown that *apoe* is a physiologically relevant ligand for low-density lipopotein receptors (LDLRs) and LDLR related proteins (LRPs) (Zaiou *et al.,* 2000). *Apoc1* can also inhibit *apoe*-mediated β-VLVL binding to LRP (Weisgraber *et al.,* 1990) suggesting the possibility of direct binding of *apoc1* to LRPs. Therefore, we examined the possibility that *apoc1* modulates Wnt signaling by using a reporter construct (TOP-flash assay; Fig. S4). Since we showed that secretion of Apoc1 protein was required for its function (Fig. 4B), it might be possible that Apoc1 cooperates with extracellular Wnt components such as Wnt ligand or the Wnt antagonist Dkk1. We, therefore, examined TOP-FLASH reporter assays using *lrp6, apoc1* together with either low amount of *wnt3a* or *dkk1* in *Xenopus* animal caps (Fig. S4B), however, we did not observe any activation or repression of Wnt activity when *apoc1* was coexpressed (Fig. S4B). These data suggested that the molecular mechanism by which *apoc1* functions in early development is not by modulating wnt signaling. Further studies are required to fully understand the molecular function of *apoc1* in *Xenopus* embryos.

Materials and Methods

Xenopus *embryos and animal cap assays*

Animal care and research protocols were in accordance with institutional guidelines, and approved by the Etiska Nämnden on animal use. *Xenopus laevis* eggs were obtained by injecting frogs with 700 units human chorionic gonadotropin (Pregnyl®, Merck Sharp & Dohme). The eggs were fertilized using a sperm suspension. The embryos were dejellied with 1% thioglycolic acid, and cultured in 0.2x Marc's Modified Ringer's solution (MMR) at 18-20ºC. Staging was according to Nieuwkoop and Faber (Nieuwkoop and Faber 1994). Ectodermal explants (animal caps) were dissected at stage 9 in 0.2x MMR, and cultured in 0.4x MMR, 0.1% BSA until control embryos reached the desired stage. *Xenopus tropicalis* embryos were obtained by artificial insemination and cultured in 20% Steinberg's Solution (SS) at 23-25°C. Fertilized eggs were dejellied using 4% L-Cystein in 20% SS pH 8.0.

Microinjection and DNA constructs

Microinjections were performed in 4% Ficoll/0.3x MMR (*Xenopus laevis*) or 5% Ficoll/1x SS (*Xenopus tropicalis*). The maximum injection volume was 20 nl (*Xenopus laevis*) or 1 nl (*Xenopus tropicalis*) per embryo. The mMessage mMachine® sp6 Kit (Ambion) was used to synthesize *in vitro* capped mRNA. pCS2-super was generated by inserting an oligonucleotide fragment containing a polylinker sequence (EcoRI, PacI, SbfI, XmaI, XhoI, AscI, XbaI) into the EcoRI/XbaI sites of pCS2. pCS2-3xFLAG was generated by inserting a oligonucleotide fragment containing 3xFLAG followed by a stop codon into the XmaI/AscI sites of pCS2-super. To generate pCS2-*apoc1*, the *Xenopus laevis apoc1* open reading frame (ORF) was obtained by RT-PCR from embryonic cDNA and subcloned into the PacI/AscI sites of pCS2-super. pCS2-*apoc1-3xFLAG* was generated by PCR amplifying the *apoc1* ORF without the stop codon and subcloning into the PacI/XmaI sites of pCS2-3xFLAG. pCS2-*5'UTR-apoc1-3xFLAG* was generated by subcloning a PCR fragment containing 42 base pairs of the *apoc1* untranslated region directly upstream of the first methionine and the *apoc1* ORF (without the stop codon) into the PacI/XmaI sites of pCS2-3xFLAG. *Wnt3a* was isolated from a *Xenopus tropicalis* full-length clone library (Source BioScience). In addition, GR-Ctnnb1 (β-catenin-GR), dominant negative *bmpr1a* (tBR), and *chrd* was used for making mRNA for injections.

Morpholino antisense oligos

Morpholino antisense oligos were synthesized by Gene Tools, LLC (USA). To design the Apoc1 translation blocking morpholino (tbMO), sequences of more than 50 EST clones were compared to identify a suitable conserved sequence in the five prime untranslated region. To design the *apoc1* splice blocking MO (spMO), genomic DNA was amplified by PCR with the primer for exon 1 and 3, and several clones were sequenced, and the spMO was designed against the exon2-intron2 boundary. The sequences of the MOs are; tbMO: 5'-GGG CAC CTT CTT TAG AAA GCT CTG A-3', spMO: 5'-GTC AGG AAA AGA TAC TGT ACC TTG T-3', control MO: 5'-CCT CTT ACC TCA GTT ACA ATT TAT A-3'. The Gbx2.2 MO sequence has previously been reported (Li *et al.,* 2009). To test the efficiency of the tbMO, western blots were performed with a 5'UTR apoc1-flag construct described further below.

RNA isolation and RT-PCR assays

Total RNA extraction was performed using the TRIzol RNA isolation protocol (Life Technologies). The total RNA was treated with DNAseI (Roche), and cDNA was synthesized with the Maxima® First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific). The primers used were: *apoc1*-fwd CGC ACT GTC TGT GAT TCT GG, *apoc1*-rev CAG AGC TGG ACA CCT TTT CC, *nodal3.2* (Darras *et al.,* 1997), *odc1* (Heasman *et al.,* 2000), *foxd3, pax3* and *zic1* (sato *et al.,* 2005), *otx2* (Mizuseki *et al.,* 1998), *sox9* and *six1* (Li *et al.,* 2009), *snai1* and *snai2* (LaBonne and Bronner-Fraser 1998).

Blastocoel cavity fluid collection and western blot

The blastocoel fluid was collected by inserting a thin needle connected to a PL1-100 Deluxe Pico Injector (Harvard Apparatus, Holliston, Massachusetts, USA) into the blastocoel cavity of stage 10 embryos. The blastocoel fluid from 10 embryos (approximately 0.2 μl / embryo) per condition was collected. As a control, total protein from whole embryos (5-10 embryos) was extracted by homogenizing the tissue on ice in extraction buffer [Tris-HCl pH7.5, protease inhibitors (cOmplete, Mini Protease Inhibitor Cocktail Tablets, Roche)]. Homogenates were spun at 13,000 rpm for 2 minutes. The supernatant was prepared for Western blot by mixing with an equal volume of 2x reducing sample buffer and boiling. Approximately 1 embryo equivalent of protein extract was loaded on a 12% SDS-PAGE gel. The blastocoel fluid was diluted with extraction buffer, mixed with reducing sample buffer, and boiled. The entire blastocoel fluid sample was loaded on a 12% SDS-PAGE gel. The nitrocellulose membranes were blocked with 5% milk, incubated with anti-FLAG® M2 antibody (dilution 1:1125; F3165, Sigma Aldrich) overnight, and the signal was detected with SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific). Finally, the membranes were stripped and stained with a α-tubulin antibody (dilution 1:10000; T5168, Sigma Aldrich) as a loading control.

Whole-mount* in situ *hybridization

The embryos were fixed in MEMFA for 2 hours at room temperature. Digoxigenin *in situ* hybridization was performed as described in (Harland 1991) with some modifications. NBT/BCIP was used as substrate for alkaline phosphatase. The *Xenopus laevis apoc1* probe (pBKS-*apoc1*) was generated by ligating an EcoRI/XbaI fragment from pCS2-*apoc1* into pBluescript KS.

Cycloheximide treatment

For blocking protein synthesis, embryos injected with 200pg of *GR*-ctnnb1were treated with 100 μg/ml cycloheximide 30 minutes prior to the dexamethasone treatment at st11.5. Embryos were then fixed with MEMFA at st13 (*apc1, gbx2.2* and *pax3*) or st15 (*snail2*), and used for whole-mount *in situ* hybridization.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

J.M.S. and C.Y. conceived the project. J.M.S., C.Y., C.Å., S.T. designed and C.Y., C.Å., S.T. performed experiments. J.M.S., C.Y., C.Å., D.W.H. analyzed data and J.M.S., C.Y., C.Å. wrote the manuscript.

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