Reiterative AP2a activity controls sequential steps in the neural crest gene regulatory network

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The neural crest (NC) emerges from combinatorial inductive events occurring within its progenitor domain, the neural border (NB). Several transcription factors act early at the NB, but the initiating molecular events remain elusive. Recent data from basal vertebrates suggest that *ap2* might have been critical for NC emergence; however, the role of AP2 factors at the NB remains unclear. We show here that AP2a initiates NB patterning and is sufficient to elicit a NB-like pattern in neuralized ectoderm. In contrast, the other early regulators do not participate in *ap2a* initiation at the NB, but cooperate to further establish a robust NB pattern. The NC regulatory network uses a multistep cascade of secreted inducers and transcription factors, first at the NB and then within the NC progenitors. Here we report that AP2a acts at two distinct steps of this cascade. As the earliest known NB specifier, AP2a mediates Wnt signals to initiate the NB and activate *pax3*; as a NC specifier, AP2a regulates further NC development independent of and downstream of NB patterning. Our findings reconcile conflicting observations from various vertebrate organisms. AP2a provides a paradigm for the reiterated use of multifunctional molecules, thereby facilitating emergence of the NC in vertebrates.

Pax3 | Zic1 | Hairy2 | Msx1 | *Xenopus* embryo

The neural crest (NC), a vertebrate embryo multipotent population, gives rise notably to peripheral nervous system, melanocytes and craniofacial structures (1). Combined Wnt, FGF, and BMP signals emanating from the paraxial mesoderm, neural plate, and nonneural ectoderm activate NC specifiers (e.g., *snail2*, *foxd3*, *sox10*) that are expressed in the premigratory NC and essential for further NC development (2–5). NC induction starts during gastrulation with the establishment of a broad competence domain at the neural border (NB) (6–8). The NB specifiers (e.g., *pax3*, *msx1*, *zic1*, *hairy2*) are essential for NC induction, but usually are not maintained in the NC cells after induction (5, 6, 9). They integrate FGF, Wnt, and BMP signals into a coherent induction of NC specifiers by diverse actions (6, 9–11). The hierarchical organization of this network is conserved across vertebrate evolution (12). Our understanding of this complex network remains preliminary, however; defining the roles of early actors is essential to understanding how this network might have emerged in vertebrates. Comparative analyses in amphioxus and lamprey have highlighted *ap2* transcription factor (tfap2) up-regulation at the NB in vertebrates (13). AP2 transcription factors are well conserved in vertebrates and are essential for both nonneural ectoderm development and NC induction (14–16). Expression in the ectoderm is shared among chordates, whereas the up-regulation at the NB is a hallmark of vertebrates (13). AP2a depletion or mutation in mice or zebrafish results in a variety of NC defects (16–18). Recent data in a basal vertebrate (lamprey) suggest its role in NB formation, whereas results in zebrafish embryos identified a function during NC postspecification steps (8, 19, 20). To reconcile these different observations, we analyzed the relationships between AP2a and the NB specifiers Hairy2, Msx1, Pax3, and Zic1, as well as with the secreted inducers FGF and Wnt and the NC specifiers Snail2 and Foxd3. We found that AP2a activates NB formation and maintenance and is critical for later NC specification.

Results

AP2a Is Required Upstream of NB Induction. We compared the expression of *ap2a* to that of the earliest known NB specifiers, *hairy2, msx1*, *pax3*, and *zic1*, from gastrulation to NC emigration (Fig. 1*A* and Fig. S1*A*; *snail2* marks the NC). *Ap2a* was detected in the ectoderm from the onset of gastrulation (stage 10) to neurula stage 18. At stage 11–12, *ap2a, hairy2*, and *msx1* expression were simultaneously up-regulated at the NB and colocalized with *pax3* as it first appeared (Fig. 1*A*; ref. 6). During neurulation (stage 14–18), all four transcripts marked the NB (Fig. 1*A*). In parallel, the neural plate marker *zic1,* which initially overlaps with *sox2* expression (21), was progressively restricted around the neural plate, including the non–NC-forming anterior neural fold (Fig. 1*A*; ref. 10). Finally, only *ap2a* and *snail2* were maintained as the NC migrated (stage 20; Fig. 1*A*). These patterns suggest that AP2a could play several roles from early NB formation stage to NC migration stage.

Although both *ap2a* and *ap2b* are expressed in premigratory NC, AP2a morpholino (AP2aMO)-mediated depletion blocks NC induction in *Xenopus laevis* efficiently (15, 22). AP2a depletion caused the loss of the three NB specifiers *hairy2*, *msx1*, and *pax3* in vivo at neurula stage 17, accompanied by lack of *snail2* induction on the injected side, as would be expected after the loss of either NB specifier (Fig. 1*B*, Fig. S1*B*, and Table S1; refs. 6, 9, 15). This phenotype was efficiently rescued by *ap2* gain of function (GOF) (Fig. 1*D*). In sharp contrast, *zic1* expression was either unchanged or expanded, as was observed for *sox2* (Fig. 1*B* and Fig. S1*B*; ref. 15). This suggests that AP2a morphants either do not form or do not maintain an NB, despite proper neural induction (*sox2*) and activation of *zic1*. Indeed, we found that *pax3*, *hairy2*, and *msx1* expression was not initiated at the edge of the early neural plate (stage 10.5–11) (Fig. 1*B* and Fig. S2). In contrast, *zic1* NB expression was increased only moderately at this early stage (Fig. 1*B*). All control MO- and AP2a-mismatch MO–injected embryos were normal (Table S1; ref. 15). We conclude that when AP2a is depleted, the earliest activities essential for further NC development are not switched on; thus, AP2a is essential for NB initiation.

Reciprocally, we analyzed the effect of Pax3, Zic1, Msx1, and Hairy2 depletion on *ap2a* expression (Table S1). Initial *ap2a* expression at the NB was not affected by depletion of Pax3, Hairy2, Msx1 or Zic1 (stage 10.5–12; Fig. 1*C*), whereas *ap2a* expression declined at mid-neurula stage 14 in injected siblings. At stage 17, Pax3 and Hairy2 depletion strongly decreased *ap2a* expression at the NB, similar to the *snail2* phenotype observed in siblings (Fig. 1*C*). These phenotypes were efficiently rescued (Pax3 or Hairy2 GOF; Fig. 1*D*). In contrast, Zic1 and Msx1 depletion moderately affected *ap2a* expression, whereas injected siblings demonstrated strongly decreased *snail2* expression, suggesting indirect regulation (Fig. 1*C*).

Finally, we compared the GOF for each NB specifier to the depletion experiments. Increased AP2a activity expands all of the other NB transcripts analyzed, including *zic1*, whereas *sox2* was decreased (Fig. S1 and Table S1). This effect is similar to the mutual activation by GOF of the other NB specifiers Pax3, Zic1,

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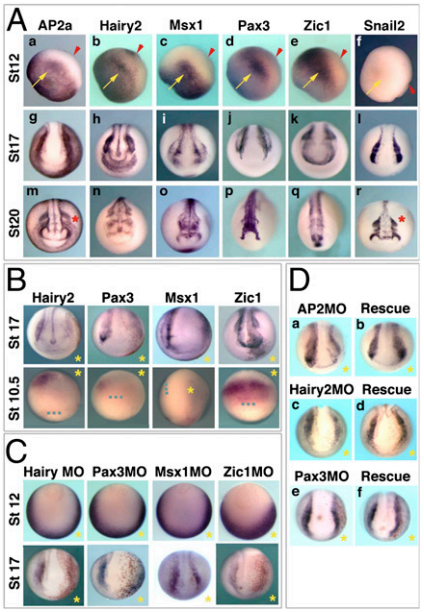

Fig. 1. AP2a controls NB formation. (*A*) NB specifier patterns from the late gastrula stage (stage 12) to late neurula stage (stage 20). ISH results for *ap2a*, *hairy2*, *msx1*, *pax3*, *zic1*, and *snail2*. (a–f) Gastrulas, side views. Arrowheads indicate the dorsal blastopore lip; arrows indicate the NB. (g–r) Anterior views of mid (stage 17; g–l) and late (stage 22; m-r) neurulae. (*B*) AP2a controls NB formation. NB expression of *hairy2*, *msx1*, and *pax3* depends on AP2a, whereas AP2a down-regulates *zic1* expression. AP2a morphants were analyzed at stage 10.5–17 for *hairy2*, *msx1*, *pax3*, and *zic1* ISH [stage 17, anterior views; stage 10.5, blastopore (dots) views]. (*C*) Up-regulation, but not initiation, of *ap2a* at the NB depends on the other NB specifiers. *ap2a* ISH in Hairy2, Pax3, Msx1, or Zic1 morphants (stage 12, dorsal views, blastopore on top; stage 17, anterior views). (*D*) MO specificity is validated by rescue analysis: AP2 (a and b, ISH for *pax3*), Hairy2, and Pax3 (c–f, ISH for *ap2*). The star indicates the injected side.

Hairy2, and Msx1 (6, 9, 10). Reciprocally, when Pax3, Msx1, or Zic1 was activated, expression of both *ap2a* and *snail2* increased (Fig. S1 and Table S1). These data suggest that all NB specifiers reinforce one another's expression and create a competence area for further NC induction.

AP2a Is Sufficient for NB Specification. We reasoned that AP2a might be sufficient to transform neuralized ectoderm into NB. To test this hypothesis, we used blastula ectoderm (animal caps) treated with Noggin (Fig. 2*A*; ref. 23). Neuralized explants do not express NC markers unless additional inducers (e.g., Wnt, FGF) are present (3). *Hairy2* was expressed in both uninjected and noggin-injected ectoderm (Fig. S2), likely reflecting its expression in the nonneural ectoderm, at the neural midline, and all around the NB (Fig. 1), and so was not diagnostic for NB formation. As expected for potent neural induction, Noggin-injected animal caps expressed *sox2*, *zic1*, and loose *epidermal keratin* (*epk*) (Fig. 2*A* and Fig. S2; ref. 21). In contrast to caps treated with Noggin alone, caps injected with both Noggin and AP2a demonstrated activation of *snail2*, *sox9*, *sox10*, *pax3*, and *msx1*, along with maintenance of *sox2*, *zic1*, and *hairy2* and loss of *epk* (Fig. 2*A* and Fig. S2). This indicates that AP2a is sufficient to induce the full complement of NB and NC specifiers in neural ectoderm explants. Together, these results demonstrate that AP2a is both necessary and sufficient to initiate typical NB development. Once induced, all NB specifiers cooperate and establish a feed-forward maintenance loop that stabilizes and expands the NB domain.

AP2a Is Essential for NB and NC Induction by FGF and Wnt Signaling. Several NB specifiers integrate the FGF and Wnt inductive signals secreted from the surrounding ectoderm, neural plate, or mesoderm (6, 9). Both Wnt (15) and FGF were found to be necessary for the induction and expansion of *ap2a* expression (Fig. S3 and Table S2). In addition, FGF could induce *ap2a* expression in Noggin-neuralized ectoderm, but this required de novo protein synthesis and thus was indirect (Fig. 2*B*). Finally, FGF required β-catenin activity to induce *ap2a* and *snail2* expression (Fig. 2*C* and Table S2; ref. 24). These findings suggest that FGF participates in *ap2a* pattern regulation indirectly via the Wnt–β-catenin pathway.

NB and NC can be induced and expanded by FGF and Wnt. Epistasis tests have shown that NC induction (*snail2* and *foxd3*) by FGF requires active Pax3, Hairy2, and Msx1, whereas NC induction by Wnt requires Pax3 but not Hairy2 or Msx1 (6, 11). To analyze the epistatic relationships among FGF, Wnt, and AP2a, we first tested whether NB (*hairy2* and *msx1*) and NC (*snail2*) induction by FGF and Wnt pathways required AP2a activity. Activation of FGF signaling by caFGFR4 and activation of Wnt signaling by stabilized β-catenin (Δβcat) (25) resulted in robustly increased *snail2*, *hairy2*, and *msx1* expression, whereas AP2a depletion decreased *snail2*, *hairy2*, and *msx1* expression in both contexts (Fig. 2*E*, Fig. S3, and Table S2). This indicates that AP2a is essential for NB and NC induction by either pathway. Thus, AP2a activity is required both when endogenous FGF and Wnt signals are acting (Fig. 1) and in the context of FGF and Wnt GOF (Fig. 2).

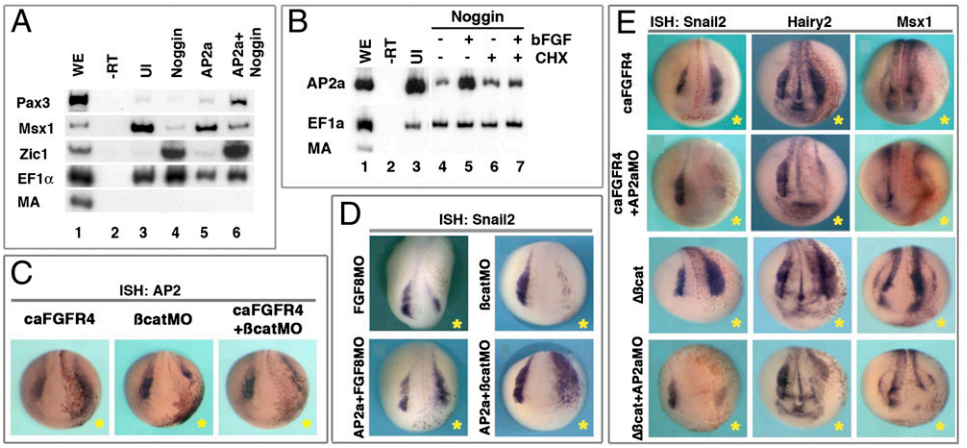

Fig. 2. AP2a induces NB genes in neuralized ectoderm explants and is essential for NC induction by FGF and Wnt. (*A*) Although explants neuralized by Noggin express *zic1*, AP2 activates coexpression of *pax3* and *msx1*. RT-PCR for NB markers *pax3*, *msx1*, and *zic1* after injection of Noggin (lane 4), *ap2a* (lane 5), or *ap2a* combined with Noggin (lane 6). Controls are shown in lanes 1–3 (*SI Materials and Methods*). (*B*) FGF controls *ap2a* at the NB in a β-catenin–dependent manner. RT-PCR analysis of *ap2a* in neuralized explants (lanes 4–7) and treated with bFGF (lane 5), CHX (lane 6), or CHX followed by bFGF (lane 7). Controls are shown in lanes 1–3 (*SI Materials and Methods*). (*C*) Expansion of *ap2a* by FGF is abolished by β-catenin depletion. Injections of a constitutively active FGFR4 (caFGFR4), β-catenin MO, or both at stage 17, anterior views. (*D*) AP2a restores *snail2* expression in both FGF and β-catenin morphants. Injections of *ap2a,* FGF8MO, and βcatMO alone and combined. (*E*) AP2a mediates NB induction by FGF and Wnt. AP2a morphants lack *snail2, hairy2*, and *msx1* expression even with FGF or Wnt GOF. Injections of caFGFR4, AP2aMO, and activated β-catenin (Δβcat) alone or combined at stage 17, anterior views. The star indicates the injected side.

Because AP2a is sufficient to induce the other NB markers, we tested whether increasing AP2a activity also could be sufficient to compensate for the loss of either FGF or Wnt signaling pathways in NC induction. Whereas FGF8MO decreased *snail2* expression, coinjection with *ap2a* mRNA resulted in normal or expanded expression (Fig. 2*D*, Fig. S3, and Table S2). Similarly, whereas β-catenin MO injections resulted in a loss of *snail2* expression, coinjection of β-catenin MO with *ap2a* mRNA produced normal or expanded expression (Fig. 2*D*, Fig. S3, and Table S2). These findings indicate that AP2a GOF is sufficient to rescue the loss of *snail2* induction observed when either FGF or Wnt β-catenin signaling is blocked.

AP2 Cooperates with Wnt Signaling to Directly Activate NB Marker *pax3*. We investigated whether AP2a is a target of Wnt signals at the NB and in turn activates the other NB markers. First, to identify immediate-early targets of Wnt at the NB, we activated Wnt signaling [using an inducible T cell factor (TCF)–VP16GR fusion] in the presence of cycloheximide (CHX) in animal caps, and noted activation of *ap2a*, *pax3*, and *msx1* (Fig. 3*A*). We next tested whether NB markers are activated by distinct levels of TCF, using increasing doses (20–120 pg), and found that *ap2a* is the most sensitive to Wnt signals (Fig. 3*B*). We hypothesized that AP2a might cooperate with Wnt to favor NB induction. Indeed, in the presence of AP2a MO, TCF activation no longer induced *pax3* or *msx1* (Fig. 3*C*). Moreover, AP2a was necessary for Wnt to activate *pax3* in vivo, and AP2a GOF overrided the loss of Wnt signaling (using β-catenin MO; Fig. S4). Finally, we examined AP2a binding to *pax3* gene regulatory elements. First, using CHX and an inducible AP2a construct, we found that *pax3* is an immediate-early AP2a target (Fig. 3*D*). We then identified two putative AP2-binding elements in *X. tropicalis pax3* proximal promotor (Fig. 3*E*). When fused to GFP, this 0.5-kb region was found to drive expression in the neural plate and the NB, reminiscent of *pax3* expression (Fig. 3*F* and Fig. S4). Moreover, AP2a morphants showed a dramatic loss of GFP expression compared with WT embryos (Fig. 3*E*). Finally, EMSA demonstrated direct binding of AP2a to these two elements. Binding specificity is further supported by the finding that an AP2-specific antibody allowed supershifting of the complex, whereas mutated sequences did not bind AP2a (Fig. 3*G* and Fig. S4). Together, this series of experiments demonstrates that AP2a cooperates with Wnt and directly activates the most specific NB specifier, *pax3*.

AP2a Acts Downstream of Pax3 and Zic1 to Specify Early NC. NC specifier induction has been shown to involve the combined activity of Pax3 and Zic1 downstream of FGF and Wnt signaling (6, 10). This combination of two transcription factors is sufficient to activate *snail2* or *foxd3* in ectopic ventral locations as well as in non-neural ectoderm explants. *Ap2a* is expressed in those two situations, suggesting that AP2a function might be important in both cases.

We examined the epistatic relationships among AP2a, Pax3 and Zic1 at the NB in vivo to establish their potential hierarchical activities upstream of *snail2* and *foxd3* induction (Fig. 4 and Table S3). We found that *snail2* and *foxd3* expression was decreased when either Pax3, Zic1, or AP2a was depleted separately. In contrast, *snail2* expression was normal or increased in the majority of embryos when *ap2a* mRNA was coinjected with Pax3MO, while it was decreased with the injection of Pax3MO alone (Fig. 4*A*). *Foxd3* expression was normal or expanded in the majority of embryos but nonetheless decreased in many embryos, representing a modest rescue (Fig. 4*A*). Similarly, *ap2a* mRNA coinjection with Zic1MO restored both *snail2* and *foxd3* expression (Fig. 4*A*). These findings suggest that AP2a GOF compensates for the depletion of Pax3 or Zic1 (i.e., low-level Pax3 and Zic1 achieved by knockdown), and that AP2a either acts downstream of Pax3 and Zic1 or exhibits redundant activities with these factors at the NB. In the former case, depletion of AP2a would block an increase in *snail2* or *foxd3* expression by GOF of Pax3 or Zic1; in the latter case, increasing Pax3 or Zic1 would compensate for the loss of AP2a activity.

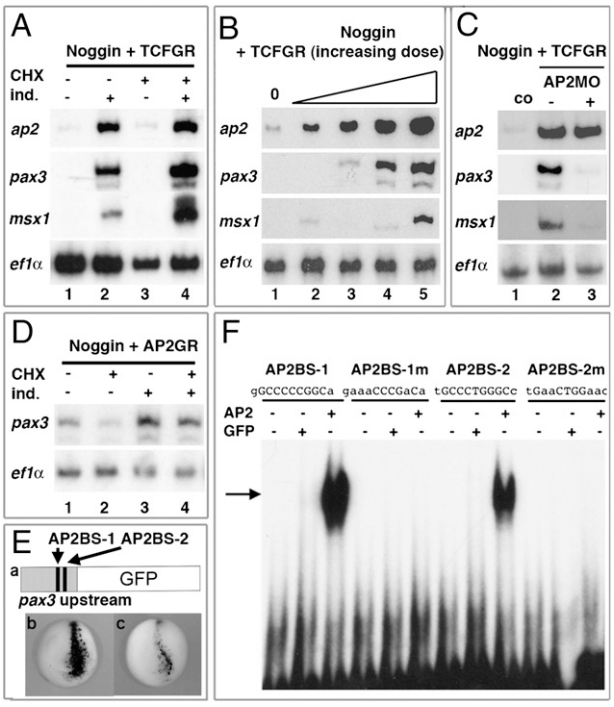

Fig. 3. AP2a mediates Wnt signals and directly triggers *pax3* at the NB. (*A*) All three NB genes *ap2a*, *pax3*, *msx1* are immediate-early targets of Wnt signaling in NB induction (inducible constitutively active TCF–VP16GR with CHX). (*B*) *ap2a* activation is the most sensitive to increasing amounts of TCF (0, 20, 40, 80, and 125 pg). (*C*) AP2a is required for Wnt signaling (125 pg of TCF–VP16GR) to trigger *pax3* and *msx1*. (*D*) *Pax3* is an immediate-early target of AP2a. (*E*) Two putative AP2-binding sites are seen in the *pax3* upstream gene sequence (a) that drives robust GFP expression at the NB, roughly similar to the *pax3* pattern seen in WT embryos (b), but very weakly in AP2a morphants (c). (*F*) EMSA analysis of each putative AP2-binding site (upper-case letters) and the corresponding mutants (lower-case letters) shows specific AP2a binding to these two elements.

To test whether AP2a is required downstream of Pax3 and Zic1, we used AP2a depletion in the context of either Pax3 or Zic1 GOF (Table S3). Although increased Pax3 activity resulted in increased *snail2* and *foxd3* expression, depleting AP2a in this context resulted in normal or decreased expression of both genes (Fig. 4*A*). When Zic1 was increased, expression of both *snail2* and *foxd3* was expanded. In embryos coinjected with AP2aMO and *zic1* mRNA, expression of *snail2* and *foxd3* was normal or decreased (Fig. 4*A*). AP2a depletion was seen to counteract the expansion phenotype observed after Pax3 or Zic1 GOF. Thus, we conclude that AP2a acts downstream of both Pax3 and Zic1 at the NB.

We also tested whether the ectopic induction of these two early NC specifiers by Pax3 and Zic1 in a ventral location in vivo was dependent on AP2a activity. Although neither Pax3 nor Zic1 is known to induce *snail2* or *foxd3* when injected alone ventrally (6), the combined injection activates *snail2* robustly and *foxd3* modestly. When AP2a was depleted, Pax3 and Zic1 coinjection failed to activate *snail2* in the majority of cases, but, surprisingly, expression of *foxd3* was robust in this scenario (Fig. 4*B* and Table S3).

The findings of our three sets of experiments suggest an essential function of AP2a downstream of Pax3 and Zic1 in vivo, with a difference in *foxd3* regulation between the NB and the ventral location. To further examine these relationships, we analyzed ectoderm explants in vitro. We induced *snail2* and *foxd3* in animal caps by combining Pax3 and Zic1 injections. Combining AP2aMO with these two factors resulted in severely impaired induction of both *snail2* and *foxd3* (Fig. 4*C*). Although the findings of our ectopic induction assay suggest some differences in the fine regulation of these two NC genes, we conclude that AP2a

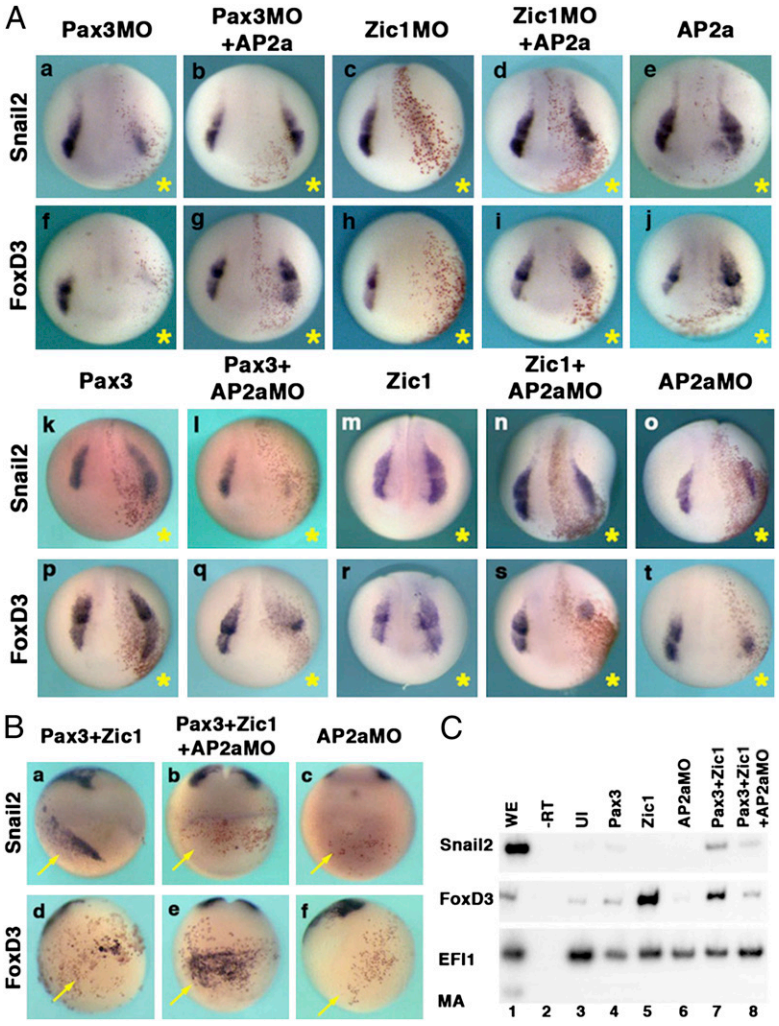

Fig. 4. AP2a induces NC downstream of Pax3 and Zic1. (*A*) AP2a restores *snail2* and *foxd3* expression in Pax3 or Zic1 morphants. Injections of Pax3MO, Zic1MO, and *ap2a* alone or in combination. In contrast, Pax3 and Zic1 do not maintain/increase the NC domain in AP2a morphants. Injections of Pax3, Zic1, or AP2aMO alone or in combination at stage 17, anterior views. (*B*) Embryos were injected ventrally with *pax3* or *zic1* mRNAs, AP2aMO, or all three. Ectopic *snail2*, but not *foxd3*, induction by Pax3/Zic1 is lost in AP2a morphants. Side (a, d, and f) and ventral (b, c, and e) views are shown; stars and arrows indicate injected areas. (*C*) The *Pax3/Zic1* combination does not induce *snail2* and *foxd3* in AP2a-depleted explants. RT-PCR analysis of *snail2* and *foxd3* in animal caps injected with *pax3* (lane 4), *zic1* (lane 5), or AP2aMO (lane 6) or coinjected with *pax3* and *zic1* (lane 7) or with all three (lane 8). Controls are shown in lanes 1–3 (*SI Materials and Methods*).

activity is essential for induction of the NC specifiers *snail2* and *foxd3* downstream of Pax3 and Zic1 both at the NB and in ectoderm explants.

AP2a Is a Typical NC Specifier. Because AP2a plays a role downstream of Pax3 and Zic1, we reasoned that it also might act as a bona fide NC specifier. In this case, AP2a would reinforce the expression of other NC specifiers and promote further NC development independent of its action at the NB. To investigate this, we used dexamethasone-activated forms of AP2a to trigger AP2a GOF at specific stages. Using unilateral injections of an AP2a/glucocorticoid receptor fusion, AP2aGR (14), we compared the effects of AP2a activation before gastrulation (stage 8) and at early and mid-neurulation stages (stage 12–14) on NB and NC development. In siblings injected with AP2aGR with the addition of only carrier (ethanol), no effect was observed (Fig. S5). Although activation at stage 8 mimicked the previously described *ap2a* GOF phenotypes (i.e., increased *snail2*, *hairy2*, *msx1*, and *zic1* expression and decreased *sox2* expression), later activation (at stage 12 and 14) failed to expand NB markers or to alter *sox2* expression (Fig. 5*A*). In contrast, *snail2* expression was increased at those two stages (Fig. 5*A* and Fig. S5), indicating that AP2a plays roles at NC specification stages independent of its action in NB development.

We also tested stage dependence in ectoderm explants. When we combined *Noggin* mRNA injections with AP2aGR, activated at the equivalent of stage 12, we found robust *snail2* (and *sox8*) induction (Fig. 5*B* and Fig. S5, lane 3). When we blocked protein synthesis with CHX before induction (lanes 4 and 5), *snail2* was still induced efficiently but *sox8* was not (Fig. S5), indicating that *snail2* is an immediate–early AP2a target (lanes 3 and 5). This finding, in concert with the action of AP2aGR on *snail2* at mid-neurulation, suggests that AP2a regulates this early NC specifier independent of NB function.

Finally, we analyzed whether late AP2a activation at mid-neurulation would affect NC maturation using *sox10* expression at tailbud stages. Although ethanol-treated embryos displayed normal *sox10* expression, the induced siblings showed a dramatic expansion of the migrating NC cells, accompanied by ectopic expression along the dorsal neural tube (Fig. 5C). We confirmed this late effect of AP2a by using an inducible dominant interfering construct (Fig. S5). Activation of this construct at mid-neurula stage 14 resulted in a significant loss of *sox10*-positive migratory NC cells at both head and trunk levels, where specified NCs were seen above the neural tube but the migrating cells were either much fewer in number (head) or absent (trunk) (Fig. 5C and Fig. S5). In addition, *snail2*- and *sox9*-migrating NCs were strongly depleted, and *sox2*-positive trigeminal ganglion was lost, whereas *sox9*-positive otic cup and *sox2*-positive CNS remained intact (Fig. S5).

Finally, to examine the interactions with other NC specifiers (12, 26, 27), we tested the effects of depleting either Sox8 or c-Myc on late *ap2a* expression. As expected, we found that both depletions resulted in defective NC migration (26, 27). Whereas *ap2a* was robustly expressed in the NC cells that remained lateral to the neural tube of Sox8 morphants, c-Myc depletion was accompanied by decreased *ap2a* expression (Fig. 5*D*).

Together, these findings indicate that AP2a can be classified as an NC specifier, acting during neurulation, in addition to its

essential role as an NB initiator during gastrulation. Thus, we propose a new position for AP2a, a multifunctional actor of NC development, in the current model of NC early specification (Fig. 5*E*).

Discussion

We have analyzed the role of the transcription factor AP2a within the vertebrate NC gene regulatory network (NC-GRN) by systematically addressing its epistatic and regulatory relationships with the four early transcriptional regulators acting at the top of the cascade: Hairy2, Msx1, Pax3, and Zic1. We have shown that AP2a displays a unique multistep role in early NC development, first in positioning of the NB and directly activating *pax3* in cooperation with Wnt signals, followed by specification of the NC. The present analysis thus reconciles different results obtained in the two model organisms, zebrafish and lamprey, in which this question was addressed. Whereas chordate and vertebrate embryos express *ap2* at low levels in the nonneural ectoderm, greater *ap2* expression at the NB in basal vertebrates (lamprey) coincides with the appearance of NC in vertebrate evolution (8, 13). Whether this increased expression was a cause or a consequence of the establishment of the NC-GRN remained unclear so far. Our data indicate that AP2a is both necessary and sufficient for establishing an NB domain in vivo as well as in neuralized ectodermal explants, and provide clear evidence for the initiation of the NC-GRN by AP2a. These findings suggest that the refinement of *ap2* expression at the NB might have provided the impetus for NC development in basal vertebrates.

AP2a Triggers NB Patterning. Gene expression pattern dynamics at the prospective NB from gastrulation to early tailbud stages indicate that *ap2a* is the first NB specifier to appear in the gastrula ectoderm, and that expression of *ap2a*, *hairy2*, *msx1*, and *pax3* is up-regulated simultaneously during early neurulation (Fig. 1*A*). These observations and previous findings reported in lamprey embryos (8) do not indicate which gene is responsible for activating the others at the NB, however. Thus, we analyzed the relationships among all NB specifiers—AP2a, Hairy2, Msx1, Pax3, and Zic1—during initiation and maintenance of the NB. Using AP2a knockdown, we found that AP2a is essential for the induction of other early NB specifiers (Fig. 1B and Fig. S1). In sharp contrast, both the pan-neural plate marker *sox2* and the NB specifier *zic1* are expanded, indicating that the impaired NB is now specified as a neural plate. This early patterning defect results in the absence of NC induction (i.e., lack of NC specifier *snail2*, *sox9*, or *foxd3*) (15). In contrast, depletion of the other NB specifiers does not affect initial *ap2a* expression at the NB (Fig. 1*C*). Moreover, naive ectoderm neuralized by the BMP antagonist Noggin is converted into a neural and NB-like tissue (simultaneously expressing *sox2*, *msx1*, *pax3*, and *zic1*) on AP2a activation (Fig. 2), and *pax3* is directly activated by AP2a (Fig. 3). These findings underscore the importance of AP2a up-regulation at the NB for activation of the NC-GRN at the end of gastrulation (Fig. 5).

In a second step, at the newly induced NB, AP2a, Hairy2, Msx1, Zic1, and Pax3 activate the expression of one another in a positive and reciprocal manner. Whether these interactions are direct or indirect remains to be determined. Such feed-forward loops also exist among Msx1, Pax3, Zic1, and Hairy2 in the later *Xenopus* neurula (6, 9, 10, 12). Disruption of this loop by depletion of any single factor results in defective NB development and lack of NC induction (Fig. 1 *B* and *C*). In addition, this mechanism seems to be largely conserved between lamprey and *Xenopus ap2*, *msx1*, and *pax3/7* genes (8). The only apparent difference is that *Xenopus* Zic1 depletion has no significant affect on *ap2* expression. This might be due to redundant Zic activities in higher vertebrates compared with basal vertebrates (28). Similarly, redundant activities might explain the apparent lack of early AP2a roles in zebrafish (19).

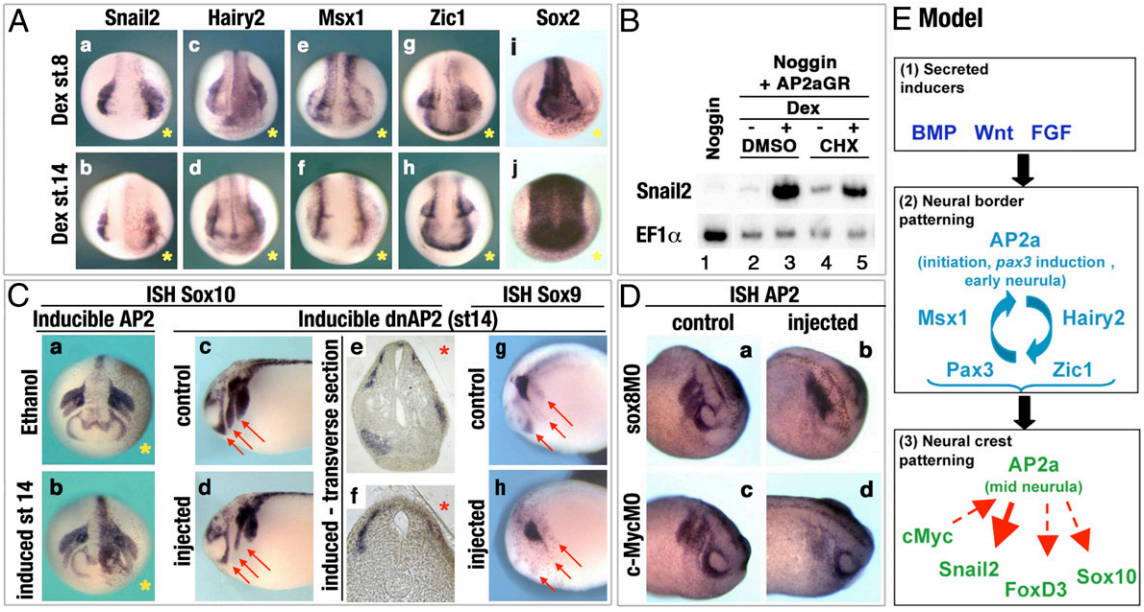

Fig. 5. AP2a specifies NC independent of its NB specifier role. (*A*) Early APaGR induction (stage 8–17) enlarges both the NB and the NC, whereas late induction (stage 14–17) expands *snail2* (b) without affecting NB markers (d, f, h, and j). Anterior views, ISH *snail2*, *hairy2*, *msx1*, *zic1*, and *sox2*. (*B*) *Snail2* is an immediate-early target of AP2a. RT-PCR for *snail2* in neuralized explants: Noggin (lane 1) and Noggin/AP2aGR (lanes 2–5) (Fig. S5). AP2aGR was induced (Dex+) from stage 11–13 in DMSO- or CHX-treated explants. (*C*) AP2aGR activation at mid-neurulation (stage 14) increases *sox10* expression in the neural tube and NC (stage 22, anterior views, with a star indicating the injected side), whereas an inducible dominant interfering AP2 mutant prevents NC emigration on the injected side (stage 22 embryos, *sox9* and sox*10* ISH). (*D*) Late (stage 22) *ap2a* expression is regulated by other NC specifiers. c-Myc morphants exhibit decreased *ap2a* expression, whereas Sox8 depletion affects NC migration but not *ap2a* levels (a and c, uninjected side; b and d, injected side). (*E*) Model of AP2a dual function during NC development in vertebrates. Interactions among the neural plate, ectoderm, and paraxial mesoderm induce NB (lane 1). At a low dose, Wnt increases *ap2a* expression, which initiates NB formation (notably by direct activation of *pax3*) and triggers the NB specifiers gene cassette (lane 2). Once a robust NB pattern is established, AP2a also acts as a downstream NC specifier, with direct activation of some NC specifiers (*snail2*, solid arrow) but not others (*foxd3,* interrupted arrow) and regulation by other NC specifiers (i.e., cMyc) (lane 3).

Finally, NB specifiers are regulated by FGF and Wnt from the surroundings and mediate their NC induction. We have shown that FGF regulates *ap2a* in a β-catenin–dependent fashion; that *ap2a*, *pax3*, and *msx1* are immediate-early targets of Wnt signaling; and that *ap2a* is the most sensitive to Wnt signaling. In turn, AP2a activity is required for Wnt activation of *pax3* and NC both in explants and in vivo, in contrast to Hairy2 and Msx1 activity, which is not required for NC induction by Wnt (Figs. 2 and 3; refs. 6 and 9). Moreover, AP2a is sufficient to rescue *snail2* induction when the FGF or Wnt pathway is blocked, similar to the Msx1 rescue of *snail2* after FGF knockdown (Fig. 2; ref. 6).

In conclusion, our findings strongly suggest that AP2a upregulation at the NB of the vertebrate ancestor could have recruited and triggered the coordinate expression of a group of transcription factors that now cooperate to define and strengthen the NC progenitor domain.

AP2a Acts as an NC Specifier After NB Specification. NB activity is executed by Pax3 and Zic1 cooperation, which is sufficient to elicit *snail2* and *foxd3* ectopic induction in two contexts with *ap2a* basal expression: the ventral ectoderm in vivo and ectoderm explants (6). We have shown that AP2a activity is necessary in both ectopic situations (Fig. 4). Further epistatic analysis among AP2a, Pax3, and Zic1 demonstrated that AP2a is also required downstream of the NB network (Fig. 4). Using inducible AP2a to activate AP2a after establishment of the NB, at the mid-neurula stage, we found that AP2a GOF still expanded the *snail2* NC domain without modifying the neural plate or NB (Fig. 5*A*; refs. 1 and 5). In addition, this mid-neurula stage activation of AP2a resulted in robust activation of the NC specifier *sox10* in both the migrating NC and the neural tube. In contrast, an inducible dominant interfering mutant activated at the mid-neurula stage depleted *sox10*-, *snail2*-, and *sox9*-positive migrating NC cells at the head and trunk levels, as well as *sox2*-positive trigeminal ganglia (Fig. 5*C* and Fig. S5). Cross-regulations between NC specifiers are typical, illustrated here by the induction of *snail2* by AP2a as an immediate-early target (Fig. 5*B*) and *ap2* regulation by c-Myc (but not by Sox8) in migratory NCs at the tailbud stage (Fig. 5*D*). Together, these results demonstrate that AP2a acts at the NB of mid-neurula stage *Xenopus* embryos as a bona fide NC specifier. This second level of action of AP2a supports previous results in zebrafish embryos suggesting that AP2 plays a role in NC fate diversification downstream of NB induction and NC specification (19).

Conclusion

We propose an updated model for the NC-GRN that accounts for the establishment of this network in vertebrates (Fig. 5*E*). Our data suggest that early refinement of AP2a is necessary and sufficient for establishing the neural plate border. AP2a might be an ancestral gene that was co-opted for a regulatory role in the NC-GRN. We found that AP2a has unsuspected sequential and particularly complex roles, first in initiating the NB at the onset of neurulation, then in inducing NC cells within this border by cooperating with other NB specifiers, and finally in acting as an NC specifier during NC development. Our findings demonstrate an additional level of complexity in the previous linear and stepwise model of NC induction and suggest how an evolutionary innovation such as the NC might have arisen through the recruitment of a few multifunctional regulators.

Materials and Methods

See *SI Materials and Methods* for more detailed information. In brief, *X. laevis* embryos were obtained, staged, and injected following standard procedures, and animal caps were cut at stage 9 (29, 30). ISH was optimized for NC structures (31). mRNAs were synthesized with the mMESSAGE mMACHINE Kit (Ambion). We have used previously validated MOs against AP2a, Pax3, Msx1, Zic1, Hairy2, β-catenin, and FGF8. Inducible constructs were activated by dexamethasone (32). Treatment with basic FGF was performed as described previously (33). CHX protein synthesis inhibition was done before the addition of dexamethasone or bFGF. Explants were analyzed by semiquantitative RT-PCR (30). AP2 binding to *pax3* regulatory sequences was validated by standard EMSA procedures.

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