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A developmental pathway controlling outgrowth of the *Xenopus* tail bud

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SUMMARY

We have developed a new assay to identify factors promoting formation and outgrowth of the tail bud. A piece of animal cap filled with the test mRNAs is grafted into the posterior region of the neural plate of a host embryo. With this assay we show that expression of a constitutively active *Notch* (*Notch ICD*) in the posterior neural plate is sufficient to produce an ectopic tail consisting of neural tube and fin. The ectopic tails express the *evenskipped* homologue *Xhox3*, a marker for the distal tail tip. *Xhox3* will also induce formation of an ectopic tail in our assay. We show that an antimorphic version of *Xhox3*, *Xhox3VP16*, will prevent tail formation by *Notch ICD*, showing that *Xhox3* is downstream of Notch signalling. An inducible version of this reagent, *Xhox3VP16GR*, specifically blocks tail formation when induced in tailbud stage embryos, comfirming the importance of *Xhox3* for tail bud outgrowth in normal development.

Grafts containing *Notch ICD* will only form tails if placed in the posterior part of the neural plate. However, if *Xwnt3a* is also present in the grafts they can form tails at any anteroposterior level. Since *Xwnt3a* expression is localised appropriately in the posterior at the time of tail bud formation it is likely to be responsible for restricting tail forming competence to the posterior neural plate in our assay. Combined expression of *Xwnt3a* and active *Notch* in animal cap explants is sufficient to induce *Xhox3*, provoke elongation and form neural tubes. Conservation of gene expression in the tail bud of other vertebrates suggests that this pathway may describe a general mechanism controlling tail outgrowth and secondary neurulation.

Key words: *Xenopus laevis*, Tail bud outgrowth, Secondary neurulation, *Notch*, *Delta*, *lunatic fringe*, *Xhox3*, *Wnt3a*

INTRODUCTION

The cells of the *Xenopus* tail bud give rise to the neural tube, somites and fin of the posterior part of the tadpole tail (Tucker and Slack, 1995a). As the tail grows, the structures derived from the tail bud become continuous with, and are indistinguishable from, those of the trunk-derived mid-axis. Despite some detailed mapping of the tail bud anatomy on the basis of gene expression domains (Gont et al., 1993; Beck and Slack, 1998; Gawantka et al., 1998), to date nothing is known about the molecular mechanisms involved in tail bud outgrowth and differentiation.

We have previously shown that tail bud determination is dependent on the interactions between three regions that are brought into apposition at the completion of gastrulation (Tucker and Slack, 1995b). These are the main neural plate (N), the posterior 100 μm of the neural plate (M), which is actually mesodermal in both fate and commitment, and the underlying dorsal mesoderm (C). A tail bud will only form when the junction of the N and M regions directly overlies C. We believe that the action of C is exerted over the period of the early neurula, stages 13-16, as it is over this stage range that the capacity to form double tails following duplication of the NM junction by neural plate rotation becomes well established (Tucker, 1995). However, outgrowth of the tail bud does not start until about stage 27, well after the neural tube has closed and the primary body axis of the embryo is complete. We have previously shown that there are two phases of gene expression in the *Xenopus* tail bud (Beck and Slack, 1998). An early group of genes is expressed from gastrulation onwards and continues to be expressed in the tail bud until the end of tail outgrowth. These genes include *X-Notch-1* and *X-delta-1*. The second phase of gene expression occurs just before tail bud outgrowth, at around stage 26, and includes *lunatic fringe (lfng)*, *Xwnt3a* and *Xhox3*. Our results showed that the early and late gene domains together defined seven distinct regions of the tail bud, and this has now been increased to ten regions following the study of additional genes (Gawantka et al., 1998). The particular feature of interest for the present study is the small region of overlap between *X-Notch-1* and *X-delta-1*, expressed together in the posterior wall, and *lunatic fringe (lfng)*, expressed in the dorsal roof of the neural tube. The region of overlap corresponds to the junction between N and M territories in the posterior of the embryo, which defines the direction of tail outgrowth. At around the same time, *Xhox3* (a *Xenopus* homologue of *Drosophila evenskipped*), is expressed in a subset of cells of the overlap region corresponding to the caudal tip, or most distal cells, of the future tail bud. We reasoned that, by analogy with other systems undergoing outgrowth such as the *Drosophila* wing disc and the chick limb (Fleming et al., 1997; Laufer

et al., 1997; Panin et al., 1997; Rodruigez-Esteban et al., 1997), Notch signalling may be restricted by lfng to the overlap region, and this could be the molecular basis of the N-M interaction in the posterior wall that leads to outgrowth of the tail bud.

In the present study we have investigated the mechanisms underlying tail bud outgrowth in *Xenopus* using a simple grafting assay to study the effects of ectopic expression and inhibition of late tail bud genes. We show that ectopic tail-like projections can be formed by posterior grafts expressing a constitutively active form of *X-Notch-1*, *Notch ICD*. The resulting buds express *Xhox3* as well as many other tail bud markers. Furthermore, grafts expressing *Xhox3* form identical ectopic tails, suggesting that *Xhox3* is a downstream target of Notch signalling. Evidence that *Xhox3* is actually a critical step in bud outgrowth is provided by the use of an antimorphic form of *Xhox3*, which prevents ectopic tail formation by *Notch ICD*, and also prevents tail development in intact embryos.

These results provide evidence that the N-M interaction initiates a new molecular pathway controlling tail bud outgrowth, involving activation of Notch signalling at the future tail tip and consequent activation of *Xhox3*. We also show that expression of *Xwnt3a* is required in addition to Notch signalling and that tail outgrowth may restricted to the posterior as a result of this additional requirement. We conclude that extension of the caudal neural tube and tail outgrowth in *Xenopus* occurs by mechanisms which show some similarity to those involved in appendage outgrowth in other organisms. Given the similar expression patterns for *Notch*, *Wnt3a* and *evenskipped*-related genes in mouse (Takada et al., 1994; Gofflot et al., 1997) and zebrafish (Joly et al., 1993; Westin and Lardelli, 1997), this may provide a general mechanism for formation of the tail in all vertebrates.

MATERIALS AND METHODS

Culture of embryos

Xenopus laevis embryo collection was as described in Godsave et al. (1988). Embryos were cultured in 1× NAM salts (110 mM NaCl, 2 mM KCl, 1 mM $Ca(NO_3)_2.4H_2O$, 1 mM $MgSO_4.7H_2O$, 0.1 mM Na_2EDTA) with 1 mM $NaHCO_3$, 5 mM Hepes, pH 7.5, and 2.5 μg/ml gentamycin sulphate (Sigma) initially and then transferred to NAM/10 before gastrulation (0.1× NAM salts with 5 mM Hepes, pH 7.5, and 2.5 μg/ml gentamycin sulphate). Embryos were staged according to Nieuwkoop and Faber (1967).

Antimorphic and inducible *Xhox3* fusion constructs

Full-length *Xhox3* cDNA was truncated just 3′ to the homeobox (Pro 233) and fused in-frame to the engrailed repressor domain (encoding amino acids (aas) 1-298) in CS2+ vector to create *Xhox3EnR*, or to the VP16 activator domain (aas 410-490), also in CS2+, to create *Xhox3VP16* (Fig. 1). An inducible version of the antimorphic construct *Xhox3VP16* was subsequently made by in-frame fusion of the glucocorticoid receptor ligand-binding domain (aas 512-777; Kolm and Sive, 1995) 3′ to the VP16 moiety. The sizes of the fusion constructs were tested by in vitro translation using a rabbit reticulocyte lysate system (Promega). Synthesised proteins were labelled with [^{35}S]methionine and analysed by SDS-PAGE and autoradiography.

mRNA synthesis

Capped synthetic mRNA was made using an SP6 MEGAscript in vitro transcription kit (Ambion) with the addition of 5 mM cap analogue (Ambion) and reduction of GTP to 0.5 mM in the reaction. *Notch ICD* cDNA in CS2+ was cut with *Not*I and transcribed with SP6 (Coffman et al., 1993). *eFGF* and *Xwnt3a* cDNAs in pSP64T were cut with *Bam*HI and transcribed with SP6 (Isaacs et al., 1995; Wolda et al., 1993). *Lfng* and *Xhox3* cDNAs were subcloned into CS2+, cut with *Not*I and transcribed with SP6 (Wu et al., 1996; Ruiz-i-Altaba and Melton, 1989a). *Xhox3VP16* and *Xhox3VP16GR* were cut with *Not*I and transcribed with SP6, and *Xhox3EnR* was cut with *Sac*II, filled using Klenow DNA polymerase and transcribed with SP6.

Assay for tail formation

Embryos were injected with mRNA into all four blastomeres at the 4-cell stage and cultured to stage 9. Animal caps were removed in NAM/2 (0.5× NAM salts with 1 mM $NaHCO_3$ and 5 mM Hepes, pH 7.5) and cut into 600×100 μm strips for grafting (Fig. 2A). Host embryos at stage 14 were placed in NAM/2 containing 10 μg/ml type IX trypsin (Sigma). The host neural plates were slit with a tungsten needle perpendicular to the anterior-posterior axis and an opening of 600×100 μm was provided for the graft. As the slit widened automatically, no tissue needed to be removed from the host, therefore the host tail bud region is unaffected. Both graft and host were then transferred to NAM/2 containing 20 μg/ml soybean trypsin inhibitor (Sigma), and hosts were placed in agar wells. The grafts were positioned so that the open face of the graft contacted the underlying notochord and presomitic mesoderm of the host, then held in place using small glass bridges and left to heal for 30 minutes. The bridges were then removed and the host embryos cultured individually in NAM/2 overnight at 18°C, then transferred to NAM/10 and cultured for 24 hours at 18°C before fixation.

Tail scoring

Ectopic projections formed by the grafts were scored for tail characteristics using the index of Tucker and Slack (1995a). One point is awarded each for the presence of notochord, neural tube and paired somites, two points for a complete fin, one point for tapering and one point for elongation. A total of seven points is given for a normal tail.

LacZ staining and FDA labelling

Many of the grafts were labelled with nuclear β-galactosidase by incorporating a total of 50 pg lacZ mRNA into the injections (Pownall et al., 1996). X-gal staining could then be used to trace the final position of the graft. Embryos were fixed in MEMFA (4% formaldehyde, 100 mM MOPS, 1 mM $MgSO_4{\cdot}7H_2O$, 1 mM EGTA, pH 7.4) for 30 minutes, washed twice for 10 minutes in PBSA, then once in LacZ buffer (10 mM $K_3Fe(CN)_6$, 10 mM $K_4Fe(CN)_6$, 2.2 mM citric acid, 156 mM $NaHPO_4$) for 10 minutes, before staining in LacZ buffer containing 2.7 mg/ml X-gal (Boehringer Mannheim) at 37°C for 15 minutes. Embryos were then washed twice for 10 minutes with PBS containing 0.1% Tween 20 (Sigma) and re-fixed for 30 minutes in MEMFA. These embryos could subsequently be used for in situ hybridisations.

For FDA (fluorescein dextran amine) labelling, host embryos were injected bilaterally at the 2-cell stage with a total of 9.6 nl of dialysed FDA at 50 mg/ml as in Tucker and Slack (1995a).

In situ hybridisations

Whole-mount in situ hybridisations used the method of Harland (1991), with modifications as detailed in Pownall et al. (1996). Digoxygenin-labelled antisense probes for *Xbra*, *Xhox3*, *Xcad3*, *lfng*, *X-shh* and *X-delta-1* were made as described in Beck and Slack

(1998). Probe for *Xenopus FGF-8* was made as described in Christen and Slack (1997) and probe for *Xwnt3a* was made by cutting XP-2 with *Cla*I and transcribing with T7 as in Wolda et al. (1993).

Histology

Histology of embryos and animal caps was as detailed in Godsave et al. (1988). Samples were block-stained overnight in Borax Carmine in 35% ethanol, washed for 4 hours in 70% ethanol, 1% HCl, dehydrated and paraffin-embedded. 8 μm serial sections were cut, rehydrated and counterstained with Picroblueblack, dehydrated and mounted in Depex (BDH). Sections were photographed using a colour CCD camera (Hamamatsu) as in Chalmers and Slack (1998). Images of FDA-labelled sections were captured using a cooled monochrome CCD camera (Hamamatsu).

RNAse protection assays

RNA was made from groups of 30 animal caps by homogenising in 250 μl of 0.1 M NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA and 0.5% SDS, extracting with phenol-chloroform and precipitating with ethanol. RNAse protection assays were performed on 5 μg of RNA (5-10 caps) using the method of Isaacs et al. (1992). Probes were cut and transcribed as follows: *Xhox3* was cut with *Dde*I and transcribed with T7 (230 bp protected; Saha and Grainger, 1992). *NCAM* was cut with *Eco*RI and transcribed with SP6 (200 bp protected; Balak et al., 1987). The ubiquitously expressed control probe *ODC* was made by cutting with *Bgl*II and transcribed with T7 polymerase (91-bp protected; Isaacs et al., 1992).

RESULTS

Formation of ectopic tails by activated Notch

We have previously shown that tail bud outgrowth occurs at the position where there is an overlap in the expression of *lfng* with *X-Notch-1* and *X-delta-1* (Beck and Slack, 1998). By analogy with the situation in the *Drosophila* wing disc, this suggested to us that fringe might be needed for the activation of Notch by Delta and that activation of Notch might be a necessary condition for outgrowth.

We therefore grafted small strips of animal cap tissue expressing a constitutively active form of *Notch*, *Notch ICD* (Coffman et al., 1993), into the posterior neural plate of stage-13 host embryos (Fig. 2A). Of 143 cases, 81% of the grafts formed tail-like projections (Fig. 2D, see also Table 1). By contrast, grafts of pieces of animal cap tissue taken from uninjected embryos or embryos injected with *β-galactosidase* mRNA formed ectopic projections in only 2% of cases (*n*=94, Fig. 2B, Table 1). Other mRNAs, including *eFGF* and *lfng* (Table 1) also gave negative results, showing that the *Notch* effect is specific.

Table 1. Summary of ectopic tail forming ability in posterior graft assays

mRNAs injected (pg/embryo)	% ectopic tails	Number of grafts
Uninjected	4	46
β-*galactosidase* (200)	0	48
Notch ICD (500)	81	143
Xhox3 (500)	62	42
Xhox3EnR (500)	85	20
Xhox3VP16 (500)	0	12
Xwnt3a (500)	19	27
lunatic fringe (500)	0	6
eFGF (10)	0	11
Xhox3VP16 (500) + *Notch ICD* (500)	0	12
eFGF (10) + *Notch ICD* (500)	92	12

β-galactosidase labelling of the grafts showed that *Notch ICD* ectopic tails are formed from the part of the graft which comes to lie more posteriorly (Fig. 2E). By contrast, control grafts were simply incorporated into the host neural tube (Fig. 2C). *Notch ICD* ectopic tails score 5/7 points on the tail index of Tucker and Slack (1995a) as they contain neural tube, fin and loose mesenchyme, and are elongated and somewhat tapered. However the notochord and somites found in a normal tail are always missing (Fig. 2F,G). Graft-derived tails form in the host midline and the ectopic neural tube branches directly from that of the host. Participation of host tissue in the ectopic tails was examined by making *Notch ICD* grafts to FDA-labelled hosts. This showed that the ectopic tail neural tube and mesenchyme are primarily graft-derived while the epidermis is mainly host-derived (Fig. 2H,I).

Grafts were positioned between 100 and 200 μm anterior of the blastopore slit at stage 13 to avoid physically splitting the M region (the posteriormost neural plate fated to become posterior tail somites; see Tucker and Slack, 1995b). Grafts placed more posteriorly were often seen to generate a split tail, where the host forms two tails with contributions from the graft. As these arise equally from *Notch ICD* or control animal caps we believe they arise simply by mechanical splitting of the host tail bud forming region.

We have previously shown that *Xhox3* is expressed in the distal tip of the tail bud, occupying a region of the posterior wall centered on the overlap of *lfng* and *X-delta-1* expression (Beck and Slack, 1998). *Xhox3* is therefore a potential downstream target of Notch activation. Ectopic tails formed by grafts of *Notch ICD*-expressing tissue also switch on expression of *Xhox3* at the distal tip, resembling the expression

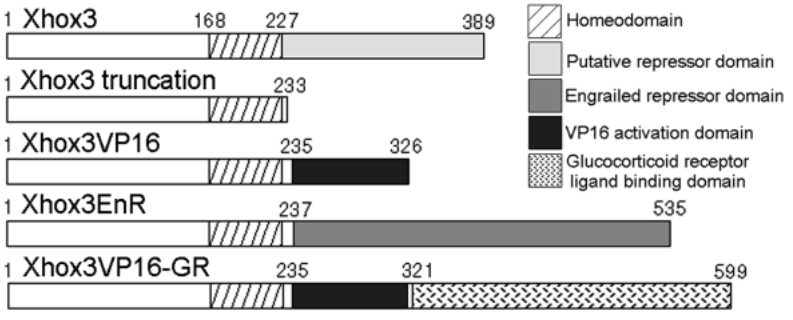

Fig. 1. Constructs used in this study. VP16 or EnR effector domains are substituted for the putative repressor domain of the wild-type *Xhox3*. In the inducible version, a glucocorticoid receptor ligand-binding domain is added at the 3′ end.

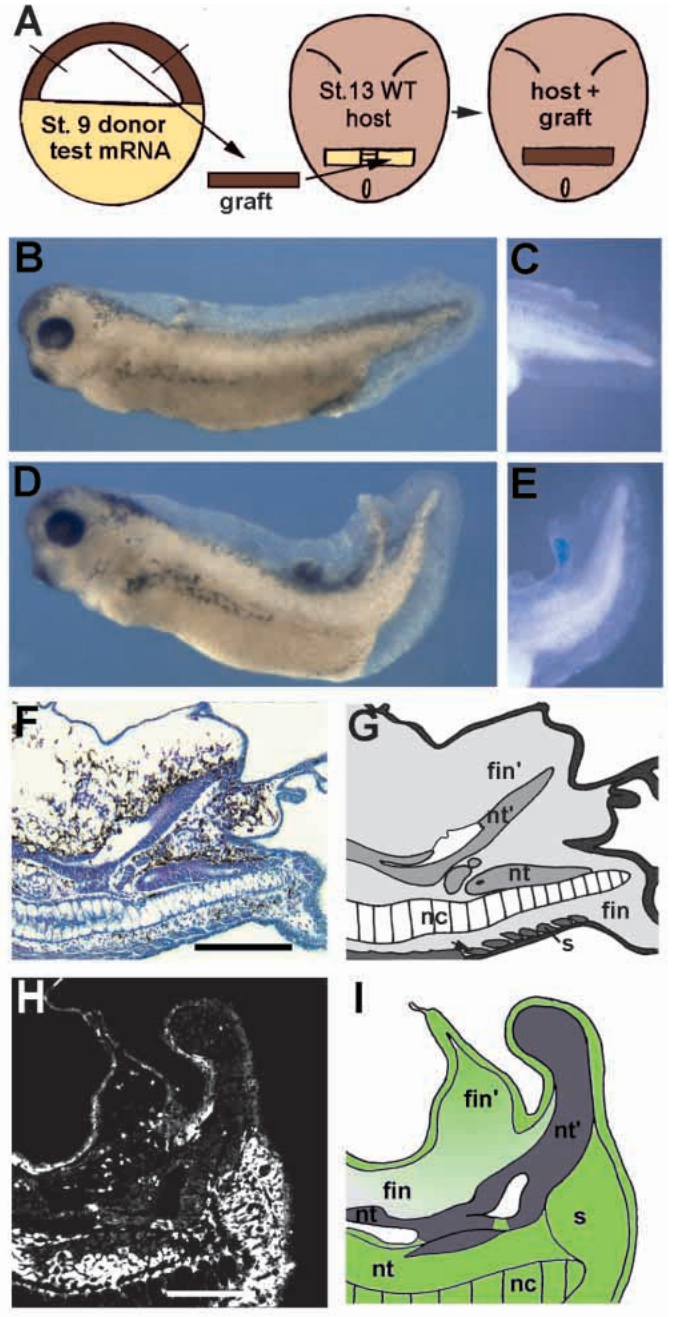

Fig. 2. Ectopic tails formed by grafts expressing *Notch ICD*. (A) Assay for outgrowth-forming ability. Test mRNA was injected quadrilaterally into 4-cell-stage embryos, which were then allowed to develop to stage 9. Animal caps were removed and trimmed into strips of 100×600 μm. A slit 600 μm long was made in the host neural plate 100-200 μm anterior to the slit blastopore, and the neural plate pushed forward to create an opening for the graft. Grafted embryos were analysed for ectopic tail formation at stage 34-36. (B,C) Embryos grafted with control animal cap. There is no pattern disruption, and visualisation of the graft by X-gal staining (cyan) shows incorporation of the graft into the host neural tube. (D,E) Embryos grafted with *Notch ICD* expressing animal cap develop an ectopic tail. The X-gal stain shows that grafted tissue which ends up more anteriorly is incorporated into the host neural tube, while the more posterior graft tissue is incorporated into the ectopic tail bud. (F,G) Section though a *Notch ICD* grafted embryo at stage 35 to show the lack of somites and notochord in the ectopic tail formed from the graft. (H,I) Section through a *Notch ICD* graft onto a host labelled with FDA to show that the ectopic tail epidermis is composed of host cells (fluorescent in H, green in I), whereas the neural tube of the ectopic tail is composed primarily of non-fluorescent, graft-derived cells. The composition of the fin mesenchyme is mixed. fin, host fin; fin′, ectopic fin; nt, neural tube; nt′, ectopic neural tube; nc, notochord; s, somites. Bars, 250 μm (F), 100 μm (H).

in normal tails (Fig. 3B). Despite the lack of notochord and somites in the ectopic tails, the *Notch ICD* tail buds resemble real tail buds in the expression and localisation of the tail bud markers: *Xbra, Xwnt3a, X-delta-1, lfng, Xshh, FGF-8* and *Xcad3* (Fig. 3). Recent identification of a functional Suppressor of hairless binding site in the ascidian *Brachyury* promotor suggests that *Xbra* may also be a direct target of Notch signalling (Corbo et al., 1998). While *Xbra* is always expressed in ectopic tails, eFGF, which has been shown to activate *Xbra* during gastrulation (Isaacs et al., 1994), it fails to form an ectopic tail in the grafting assay (Table 1).

Xhox3 generates ectopic tails and acts downstream of *Notch* activation

The late onset of *Xhox3* expression in the distal tip of the tail bud suggests that it may be a result of Notch signalling (Beck and Slack, 1998), and might therefore be an important component of the outgrowth mechanism. We tested this by grafting pieces of animal cap tissue expressing *Xhox3* into the posterior neural plate of stage 13 hosts. These grafts generated

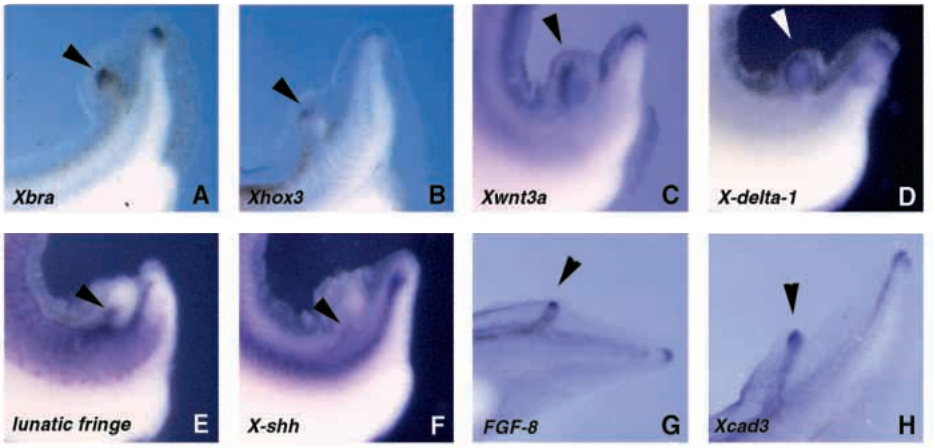

Fig. 3. Expression of tail bud genes in *Notch ICD* ectopic tails. (A) *Xbra* in tail bud, (B) *Xhox3* in the distal tail bud, (C) *Xwnt3a* localised to the tail bud and dorsal side of the tail neural tube, (D) *X-delta-1* is not restricted ventrally as in the host tail bud, but is expressed throughout the ectopic tail, (E) *lfng* expression is dorsal in the proximal tail, (F) *X-shh* expression in ectopic tails is much reduced compared to the host tail and may represent floor plate expression. (G) *FGF-8* and (H) *Xcad3* expression in ectopic tails resembles that of wild-type tail buds. Arrowheads indicate staining in ectopic tails formed from the grafts. All embryos are aged between stages 33 and 36. Posterior (host tail bud) is to the right and dorsal uppermost.

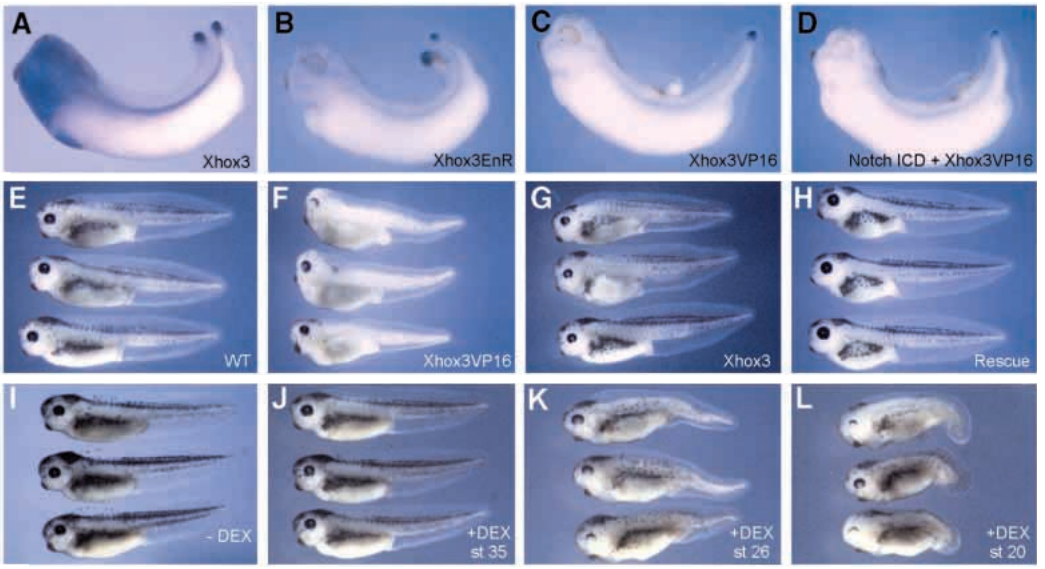

Fig. 4. *Xhox3* can induce ectopic tails and functions downstream of *Notch ICD*. (A-D) Animal cap grafts were made into the posterior neural plate of stage 13 hosts and assayed for formation of an ectopic tail bud showing expression of *Xbra* at stage 35. (A) *Xhox3* grafts can generate an ectopic tail identical to those formed by *Notch ICD*. (B) Grafts expressing *Xhox3EnR*, a strong repressing version of *Xhox3*, also form ectopic tails. (C) Grafts expressing an activating (antimorphic) form of *Xhox3*, *Xhox3VP16*, do not generate ectopic tails. (D) Co-expression of the antimorphic *Xhox3VP16* with *Notch ICD* prevents ectopic tail formation. (E-H) Expression of *Xhox3VP16* in embryos causes tail, head and pigmentation defects and can be rescued by coinjection of wild-type *Xhox3* mRNA. (E) Wild-type embryos at stage 40. Tails contain 42 somites on average. (F) Embryos injected with 100 pg of *Xhox3VP16* have a reduced tail with 28-34 somites at stage 40, pigment cells are lacking and the head is slightly reduced. (G) Embryos injected with 50 pg *Xhox3* mRNA have slightly reduced heads but normal tails, with 42 somites on average. (H) Co-injection of 50 pg of *Xhox3* mRNA with 100 pg *Xhox3VP16* completely rescues all the phenotypic effects of *Xhox3VP16* and tails contain 42 somites. (I-L) Effect of inducing *Xhox3VP16GR* with dexamethasone at different developmental stages. (I) Injection of 400 pg of *Xhox3VP16GR* has no phenotypic effect if embryos are cultured in the absence of dexamethasone, or (J) treated with dexamethasone at stage 35. (K) Embryos injected with 400 pg *Xhox3VP16GR* and treated with dexamethasone at stage 26 have reduced tails and slight eye defects. (L) Embryos treated at stage 20 have vestigal tails and more severe eye defects.

ectopic tails at high frequency (Fig. 4A, 62%, *n*=42, Table 1), with identical histology to the *Notch ICD* tails (not shown), suggesting that expression of *Xhox3* can substitute for Notch signalling to enable tail outgrowth.

To further investigate *Xhox3* function, two domain-swapped constructs were made. The wild-type *Xhox3* was truncated just C-terminal to the homeobox and fused to the activation domain of VP16 to create *XhoxVP16* and *Xhox3EnR* (Fig. 1). The replaced region of *Xhox3* maps to the repressor domains in the homologous mammalian *Evx* and *Drosophila evenskipped*

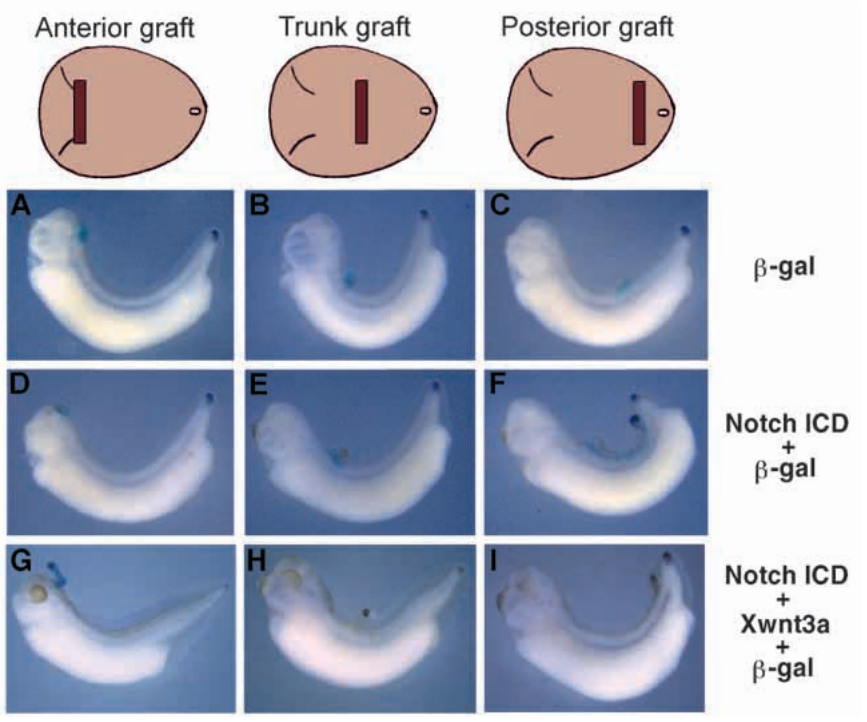

Fig. 5. Posterior restriction of the tail-forming ability of *Notch ICD* grafts can be overcome by coexpression of *Xwnt3a*. Animal cap pieces 100×600 μm are grafted into one of three positions along the anterior posterior axis of stage 13 hosts as shown. The final position of the graft is marked by Xgal staining (cyan, absent in H,I) and tail buds (host and ectopic) are marked by *Xbra* expression (dark blue). (A-C) Control animal cap pieces expressing β-*galactosidase* do not form tails when grafted into anterior (A), mid-trunk (B) or posterior (C) positions. (D-F) *Notch ICD* animal caps do not form ectopic tails if grafted into the anterior (D) or trunk (E) positions, but ectopic tails are formed from 81% of posterior grafts (F). (G-I) Grafts expressing both *Notch ICD* and *Xwnt3a* can form ectopic tails in anterior (G), trunk (H) or posterior (I) positions.

Table 2. Summary of anterior and trunk grafting data

mRNAs injected (pg/embryo)	Position	% ectopic tails	Number of grafts
β-*galactosidase* (200)	Anterior	0	12
β-*galactosidase* (200)	Trunk	0	11
Notch ICD (500)	Anterior	0	9
Notch ICD (500)	Trunk	0	8
Notch ICD (500) + *Xwnt3a* (500)	Anterior	62	29
Notch ICD (500) + *Xwnt3a* (500)	Trunk	57	51
Notch ICD(500) + *Xhox3* (500) + *Xwnt3a* (500)	Trunk	88	8
Xwnt3a (500)	Trunk	0	16
XhoxEnR (500)	Trunk	0	8

proteins (Biggin and Tijan, 1989; Johnson and Krasnow, 1992; Han and Manley, 1993; Tenharmsel et al., 1993; Briata et al., 1995, 1997).

The effects of the fusion proteins on tail outgrowth were tested using the same grafting procedure. As expected, *Xhox3EnR* grafts into the posterior neural plate generated ectopic tails even more effectively than *Xhox3* itself (85%, *n*=20, Table 1), confirming that Xhox3, like other evenskipped family members, functions as a repressor (Fig. 4B). In contrast, *Xhox3VP16* grafts did not produce any ectopic tails (*n*=12, Fig. 4C, Table 1), and *Xhox3VP16* also prevented ectopic tail formation when co-injected with *Notch ICD* (*n*=12, Fig. 4D, Table 1). These results show that Xhox3 functions downstream of Notch signalling in tail outgrowth. Overexpression of *Xhox3* in embryos has previously been shown to reduce anterior structures (Ruiz-i-Altaba and Melton, 1989b). We observe similar effects after injection of more than 100 pg/embryo of either wild-type *Xhox3* or *Xhox3EnR* mRNA (data not shown). Conversely, expression of *Xhox3VP16* results in truncations of the posterior, most obvious as a reduction in the number of tail somites from 42 to an average of 31 at stage 40 (Fig. 4F). Loss of eye pigmentation and a lack of neural crest-derived pigment cells are also seen in these embryos. Posterior defects were previously observed by Ruiz-i-Altaba and colleagues, who injected anti-Xhox3 antibodies into embryos, although eye and pigment defects were not noted (Ruiz-i-Altaba et al., 1991). Although these results are consistent with a role for endogenous *Xhox3* in tail outgrowth, the tail is not the only region affected, suggesting the additional existence of an earlier role for Xhox3.

In order to confirm the specificity of *Xhox3VP16*, rescue experiments were conducted by co-injecting different levels of wild-type *Xhox3* into whole embryos. Co-expression of very low levels of *Xhox3* (Fig. 4G) can completely rescue the phenotype of *Xhox3VP16*-injected embryos, resulting in normal development of the tadpoles (Fig. 4H). Co-injection of higher doses of *Xhox3* mRNA results in progressive loss of anterior structures, as might be expected when it is in excess (not shown). These results make it very likely that Xhox3VP16 acts as a specific activator of target genes that are normally repressed by Xhox3, and can therefore specifically antagonise its action in vivo.

Inhibition of Xhox3 at the time of tail bud outgrowth selectively truncates the tail

The endogenous *Xhox3* expression in the future tail bud becomes visible at about stage 26. However, the phenotype of embryos expressing *Xhox3VP16* is suggestive of an additional earlier developmental role. We therefore constructed an inducible form of Xhox3VP16, by the addition of the glucocorticoid receptor DNA binding domain (GR) at the C terminus (Fig. 1). Transcription factors containing the GR are nonfunctional, since after translation they are sequestered in a complex by heat shock proteins (Hollenberg et al., 1993). When the synthetic steroid hormone dexamethasone is added, the transcription factor is released from the complex and can enter the nucleus and act on its target genes.

We have used this inducible system to study the effect of interfering with Xhox3 function during development of the tail. Embryos were injected with 400 pg of *Xhox3VP16GR* mRNA and then treated with 10 μM dexamethasone at different stages. Addition of dexamethasone to uninjected embryos at stage 14 did not affect development (not shown). Embryos injected with *Xhox3VP16GR* but not treated with dexamethasone also developed normally (Fig. 4I), as did those treated at stage 35 (Fig. 4J). Addition of dexamethasone at stage 26 resulted in reduced tail development (Fig. 4K), whereas addition at stage 20 resulted in embryos with vestigal tails (Fig. 4L). Eye development is delayed in more severe cases. Earlier treatment with dexamethasone at stage 14 resulted in severe head and tail defects and treatment from the time of injection was lethal (not shown). Taken together with the ability of Xhox3VP16 to block tail formation by *Notch ICD*, these results show that Xhox3 is indeed required for tail outgrowth, and that it performs this function by acting downstream of Notch signalling.

Formation of ectopic tails by *Notch ICD* requires Wnt signalling

Generation of an ectopic tail by animal caps filled with *Notch ICD* only works if the grafts are placed near the late dorsal blastopore lip, but still outside the M region. This was found by placing the grafts at different anteroposterior levels along the neural plate. Posterior grafts were as described previously, anterior grafts were placed just posterior to the head neural folds and trunk grafts were placed midway along the anterior-posterior axis (Fig. 5). The grafts were co-injected with *β-galactosidase* mRNA to enable visualisation of the final position. Control grafts expressing this marker did not form ectopic tails regardless of position (Fig. 5A-C, Table 2). *Notch ICD* grafts were only able to form ectopic tails in the posterior position (Fig. 5D-F, Table 2). Together with our

observation that it is tissue at the posterior end of the *Notch ICD* grafts which undergoes outgrowth, this suggests a requirement for a second signal, defining competence for tail outgrowth, which is tightly localised to the posterior neural plate.

We have previously reported that *Xwnt3a* is expressed in the extreme posterior midline just prior to tail bud outgrowth (Beck and Slack, 1998). Posterior grafts of animal cap tissue expressing very high levels of *Xwnt3a* only generated ectopic tails in 19% of cases (n=27, Table 1), and never did so if grafted further towards the anterior (n=16, Table 2). As *Xwnt3a* had only this rather limited ability to provoke tail outgrowth on its own, we considered that it might be a candidate for the factor limiting competence for Notch-induced tail outgrowth to the posterior of the embryo.

To test this, pieces of animal cap expressing both *Notch ICD* and *Xwnt3a* were grafted to the three positions along the dorsal midline, and proved to be capable of forming ectopic tails at any point along the anterior posterior axis of the host (Fig. 5G-I, Table 2). In contrast to the *Notch ICD* tails, which always form from the posterior region of the graft, the cores of these tails were formed by the entire graft, leaving the host's neural tube unlabelled (Fig. 5G). As with the *Notch ICD* tails, the core is graft-derived and the epidermis is derived from the host (not shown). Xwnt3a is therefore a strong candidate for the posteriorly localised competence factor for tail outgrowth.

Formation of neural tube-like structures in animal caps

In order to evaluate the activity in isolation of *Notch ICD* and *Xwnt3a*, whole animal caps were injected as before and simply cultured to stage 38 for analysis. Uninjected caps or caps injected with *β-galactosidase* mRNA (50 pg) or *Xwnt3a* alone (250 pg) develop into atypical epidermis (Fig. 6A,C,F). Caps injected with *Notch ICD* alone (500 pg) look like atypical epidermis externally (Fig. 6B), but contain smaller, more densely staining cells of unknown identity (Fig. 6G). Animal caps containing both *Notch ICD* and *Xwnt3a* are strikingly different; elongation takes place during control neurula stages, and around stage 27 the caps swell and become surrounded by fin, acquiring a tail-like appearance (Fig. 6D). When these were sectioned they were found to contain multiple structures resembling neural tubes (compare Fig. 6I to the transverse section of normal tail at the same stage, Fig. 6E). In about half of the caps the neural tube-like tissue forms a core running along the centre of the long axis of the cap and is surrounded by fin-like mesenchyme and an epidermis (Fig. 6H). In the remaining cases multiple neural tubes are surrounded by condensed neural tissue and mesenchyme (Fig. 6I).

To confirm that the tubes formed by the *Notch ICD/Xwnt3a* caps were really neural in character, we carried out in situ hybridization for the pan-neural marker *NCAM* (Fig. 6J-O), and RNAse protection for *NCAM* and *Xhox3*. The protections showed that uninjected animal caps or caps injected with either *Xwnt3a* or *Notch ICD* alone contained no detectable *NCAM* or *Xhox3* message. In contrast, RNA from caps expressing both contained high levels of *NCAM* and *Xhox3* (Fig. 7).

DISCUSSION

A model for tail bud outgrowth

The results presented here provide evidence for the existence of a molecular pathway controlling tail bud outgrowth in *Xenopus* (Fig. 8). Previous work has shown that three regions of the dorsal-posterior of the late gastrula must come into alignment at the end of gastrulation in order that a tail bud shall be formed. These regions are known as N (neural plate), M (neural plate posterior to N with a mesodermal fate) and C (caudal dorsal mesoderm). To form a tail bud, N and M must overlie and contact the C region (Tucker and Slack, 1995b; Fig. 8A). We believe that the C region exerts its effect in the early neurula period (Tucker, 1995). Later in development, the junction of N and M forms the tip of the tail bud as it begins to extend.

We have found that *X-Notch-1* and *X-delta-1* are expressed in the M region from gastrulation onwards. After fusion of the neural folds over the chordoneural hinge these genes are expressed in the lateral blastopore lips, which become the posterior wall, while the N region becomes the dorsal roof. At around stage 26 the N region begins to express *lfng* (Fig. 8B) and *Xwnt3a* is expressed in the posterior dorsal part of N. As outgrowth of the tail bud begins, expression of *lfng* slightly overlaps that of *X-Notch-1* in the distal tip of the tail bud. Boundaries between *fng*+ and *fng*− cells have been implicated in many mechanisms involving signalling through the Notch protein, but it is the role of these boundaries in appendage outgrowth that has been best studied. In the *Drosophila* wing disc, *fng* modulates the interaction of Notch with its ligands to establish a local Notch signal along the boundary of dorsal and ventral compartments, which promotes outgrowth of the wing (Panin et al., 1997). Based on observation of *lfng, X-Notch-1 and X-delta-1* expression, the *Xenopus* tail bud fulfils the basic requirements for the establishment of localised Notch activation in the posterior wall of the tail bud at the time outgrowth begins (Beck and Slack, 1998; Fig. 8D). In this report, we have shown that a small graft expressing the constitutively active cytoplasmic domain of *Xenopus* Notch and placed in the posterior neural plate can induce ectopic tail outgrowth. The resulting tails contain fin and neural tube but lack the mesodermal components, suggesting that caudal notochord and somites are not formed under the control of Notch signalling. It is likely that these structures are formed from the tail bud chordoneural hinge, as is the case in amniotes (Le Douarin et al., 1998).

The *evenskipped* homologue *Xhox3* satisfies three criteria for being an essential component of the outgrowth mechanism: it is expressed in the forming tail tip; it will provoke outgrowth of a new tail bud in our grafting assay; and a specific antagonist will block formation of the tail in intact embryos. As expected, the domain swap experiments show that Xhox3 functions as a transcriptional repressor, like other eve-related proteins. We have shown that *Xhox3* is activated by Notch signalling and that *Xhox3VP16* will block the formation of tails provoked by *Notch ICD*. These experiments show that Notch exerts its activity by induction of *Xhox3*, and that Xhox3 acts downstream of Notch. Like *Notch*, evenskipped-type homeobox gene expression is also

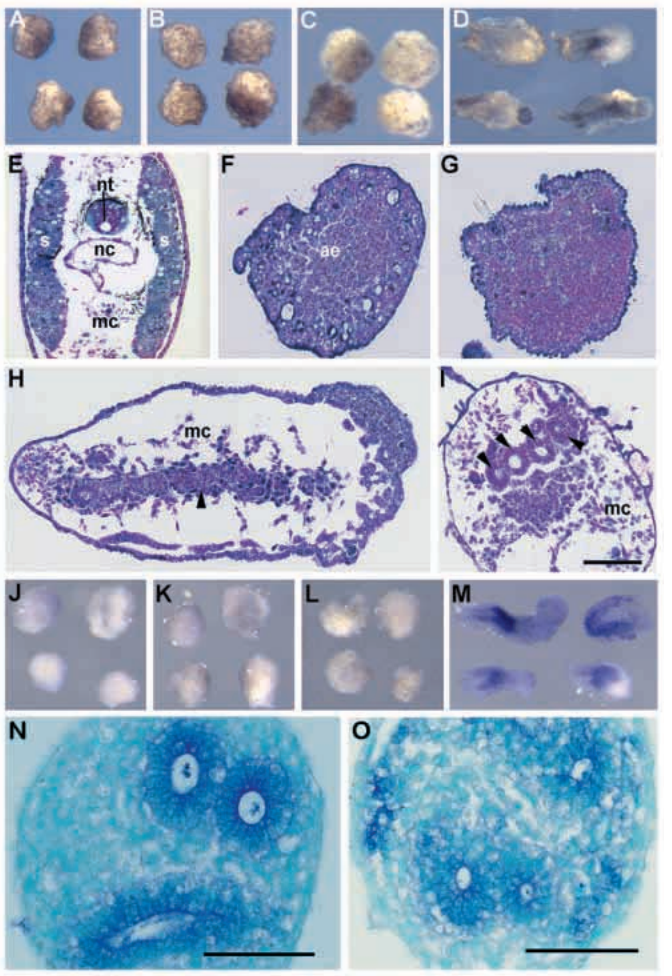

Fig. 6. Combined Notch and Wnt signalling induces formation of neural tube-like structures in animal caps. (A-D) Animal caps at stage 38. (A) Control caps expressing β-*galactosidase*; (B) caps expressing *Notch ICD* alone resemble controls. (C) Caps expressing *Xwnt3a* alone resemble controls. (D) Caps co-expressing *Xwnt3a* and *Notch ICD* elongate and swell. (E-I) Histology of injected caps at stage 38 compared to embryo of the same stage. (E) Transverse section through a *Xenopus* late embryo tail showing arrangement of tissues. (F) Section through uninjected animal cap showing atypical epidermis is formed. (G) Section through cap expressing *Notch ICD* showing poorly differentiated dense tissue. (H,I) Sections through animal caps co-expressing *Notch ICD* and *Xwnt3a* to show presence of fin-like mesenchyme (mc) and neural tube-like structures (arrows). (J-M) In situ hybridisation to *NCAM*, a marker of neural tissue (dark blue stain); (J) uninjected caps, (K) *Notch ICD* caps, (L) *Xwnt3a* caps, (M) caps injected with *Notch ICD* and *Xwnt3a*. (N-O) Sections of caps from M counterstained with methyl green (pale green) as in Beck and Slack (1998), to show that NCAM staining (dark blue) is restricted to the neural tube structures. ae, atypical epidermis; mc, mesenchyme; nc, notochord; nt, neural tube; s, somite. Bars, 100 μm (I), 50 μm (N,O).

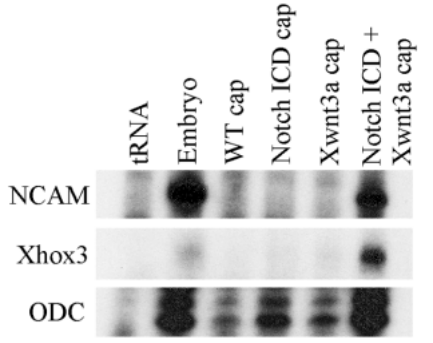

Fig. 7. Combined Notch and wnt signalling induces neural tissue in animal caps. RNAse protections showing *Xhox3* and *NCAM* are strongly induced in the presence of both *Notch ICD* and *Xwnt3a* but absent from uninjected caps, or caps injected with either RNA alone. Whole stage-38 control embryonic RNA is included for comparison; tRNA lane is a negative control.

implicated in outgrowth formation in other organisms. *Evx-2* is expresssed in the distal limb bud mesenchyme of mice and the fin of zebrafish and loss of Evx-2 function in mice leads to limb defects (Sordino et al., 1996; Herault et al., 1996), whereas *Evx-1* is expressed in the tail bud in both mice and zebrafish as well as the mouse limb bud (Dush and Martin, 1992; Joly et al., 1993; Gofflot et al., 1998). Mice lacking Evx-1 function fail to gastrulate, indicating an early requirement for this gene in development (Spyropoulos and Capecchi, 1994). Our results suggest that localised *Xhox3* expression in these areas of outgrowth may also be a result of Notch signalling.

We have shown that Notch ICD can only provoke tail bud outgrowth if the grafts are placed near the posterior end of the neural plate, suggesting a requirement for a posteriorly localised competence factor. *Xwnt3a* expression is tightly localised in this region from stage 26 and is therefore a good candidate for this factor. Animal cap grafts co-expressing *Xwnt3a* with *Notch ICD* can form ectopic tails if grafted anywhere along the anterior-posterior axis, demonstrating that Xwnt3a can act as the posterior competence factor in tail morphogenesis. It is therefore probable that it also fulfils this role in normal development. In support of this, mouse *Wnt3a* expression is also localised to the tail bud (Takada et al., 1994) and the apical ectodermal ridge of the limb bud (Kengaku et al., 1998). Interactions between Notch and wnt signalling have been observed in other systems and may be antagonistic (Axelrod et al., 1996; Uyttendaele et al., 1996) or synergistic (Neumann and Cohen, 1996). We have shown here that active Notch requires Xwnt3a to turn on *Xhox3*. However, Xhox3 may also require continued wnt signalling to promote outgrowth of the tail and caudal neural tube (Table 2).

A mechanism for secondary neurulation?

Although the trunk- and tail bud-derived components of the tail merge seamlessly, one difference between the the mode of formation of the axial body structures in the trunk and tail of the embryo concerns the neural tube. In the anterior and trunk, the neural tube of the *Xenopus* embryo, like that of other vertebrates, is formed by rolling up of the neural plate, which in turn is formed during gastrulation from the ectoderm overlying the dorsal mesoderm. In contrast, the tail bud-derived neural tube is formed directly by cavitation in avian and mammalian embryos, a process termed secondary neurulation (Nievelstein et al., 1993; Catala et al., 1995; Le Dourain et al., 1998). However, amphibians are often said to

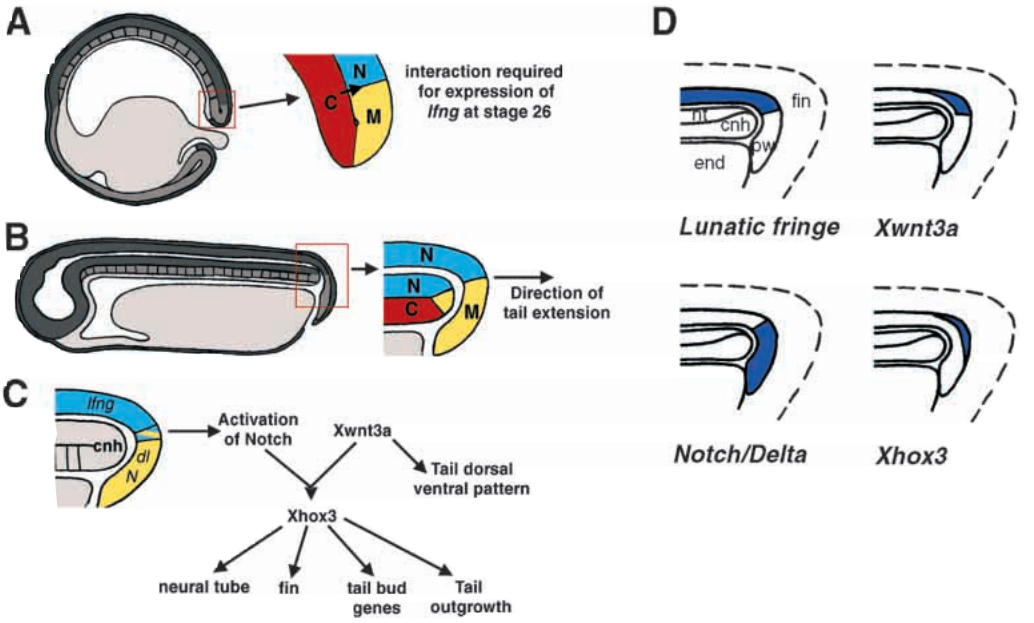

Fig. 8. A model for tail outgrowth in *Xenopus*. (A) The NMC model for tail bud initiation. At stage 13 three regions, N, M and C, shown here in a section through the dorsal midline, come into alignment so that the junction of N and M overlies C. This interaction is essential for tail formation and is also required for onset of expression of *lfng* and *Xwnt3a* at stage 26. (B) Rearrangement of N, M and C occurs by the time of outgrowth. By stage 26, closure and extension of the neural tube results in formation of the posterior wall of the neural tube from the lateral blastopore lips, and movement of the N-M junction to the tip of the tail. (C) A mechanism for tail bud outgrowth mediated by Notch signalling. At around stage 26, *lfng* expression begins in the dorsal roof of the neural tube. Expression terminates at the posterior of the embryo in a sharp boundary, which slightly overlaps expression of the early genes *X-Notch-1* and *X-delta-1* in the posterior wall. This arrangement results in localised activation of Notch signalling at the leading edge of the tail bud. In the presence of Xwnt3a, also turned on at stage 26 in the extreme posterior dorsal roof and distal tip of the tail bud, this results in *Xhox3* expression in the distal tip of the tail bud. Xhox3 is required at least for outgrowth of the tail, and formation of the tail bud-derived neural tube. In addition, Xwnt3a may be required for dorsal-ventral patterning of the tail bud-derived neural tube. (D) Summary of the expression of key genes at the time of tail outgrowth. Tailbud expression of *lfng*, *Xwnt3a*, *Notch*, *Delta* and *Xhox3* is shown in blue for comparison. Drawings are based on sections of stage-30 embryos shown in Beck and Slack (1998). cnh, chordoneural hinge; end, endoderm; nt, neural tube; pw, posterior wall.

be excluded from this since the condensation and cavitation occur simultaneously and the tail neural tube appears to arise as an extension of the trunk neural tube (e.g. Gilbert, 1997). The results presented here show that secondary neurulation can occur in amphibians, since direct formation of neural tubes in caps expressing *Xwnt3a* and *Notch ICD* occurs in the absence of any existing neural tube or dorsal mesoderm. These animal caps may therefore represent a simple model of the secondary neurulation process in vertebrates. In support of this, loss of wnt3a, lfng or Notch-1 function in mice has been shown to lead to posterior defects (Takada et al., 1994; Conlon et al., 1995; Evrard et al., 1998; Zhang and Gridley, 1998). This model system may be of particular interest if it turns out that some defects of the posterior spinal cord in higher vertebrates are due to faults in the Notch signalling pathway.

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REFERENCES

Axelrod, J. D., Matsuno, K., Artavanis Tsakonas, S. and Perrimon, N. (1996). Interaction between wingless and Notch signaling pathways mediated by dishevelled. *Science* **271**, 1826-1832.

Balak, K., Jacobson, M., Sunshine, J. and Rutishauser, U. (1987). Neural cell-adhesion molecule expression in *Xenopus* embryos. *Dev. Biol*. **119**, 540-550.

Beck, C. W. and Slack, J. M. W. (1998). Analysis of the developing *Xenopus* tail bud reveals separate phases of gene expression during determination and outgrowth. *Mech. Dev.* **72**, 41-52.

Briata, P., Vandewerken, B., Airoldi, I., Ilengo, C., Diblas, E., Boncinelli, E. and Corte, G. (1995). Transcriptional repression by the human homeobox protein Evx1 in transfected mammalian-cells. *J. Biol. Chem.* **270**, 27695-27701.

Briata, P., Ilengo, C., Van de Werken, R. and Corte, G. (1997). Mapping of a potent transcriptional repression region of the human homeodomain protein Evx1. *FEBS Lett.* **402**, 131-135.

Biggin, M. D. and Tjian, R. (1989). A purified *Drosophila* homeodomain protein represses transcription in vitro. *Cell* **58,** 433-440.

Catala, M., Teillet, M. A. and Le Douarin, N. M. (1995). Organization and development of the tail bud analyzed with the quail-chick chimera system. *Mech. Dev.* **51,** 51-65.

Chalmers, A. D. and Slack J. M. W. (1998). Development of the gut in *Xenopus laevis*. *Dev. Dynam*. **212,** 509-521.

Christen, B. and Slack, J. M. W. (1997). FGF-8 is associated with anteroposterior patterning and limb regeneration in *Xenopus*. *Dev. Biol*. **192**, 455-466.

Coffman, C.R., Skoglund P., Harris, W. A. and Kintner, C. R. (1993). Expression of an extracellular deletion of Xotch diverts cell fate in *Xenopus*-embryos. *Cell* **73**, 659-671.

Conlon, R. A., Reaume, A. G. and Rossant, J. (1995). Notch1 is required for the coordinate segmentation of somites. *Development* **121**, 1533-1545.

Corbo, J. C., Fujiwara, S., Levine, M. and DiGregorio, A. (1998). Suppressor of Hairless activates *Brachyury* expression in the Ciona embryo. *Dev. Biol.* **203**, 358-368.

Dush, M. K. and Martin, G. R. (1992). Analysis of mouse *Evx* genes: *Evx-1* displays graded expression in the primative streak. *Dev. Biol*. **151**, 273-287.

Evrard, Y. A., Lun, Y., Aulehala, A., Gan, L. and Johnson, R. L. (1998).

Lunatic fringe is an essential medaitor of somite segmentation and patterning. *Nature* **394**, 377-380.

Fleming, R. J., Gu, Y. and Hukriede, N. A. (1997). Serrate-mediated activation of Notch is specifically blocked by the product of the gene *fringe* in the dorsal compartment of the *Drosophila* wing imaginal disc. *Development* **124,** 2973-2981.

Gawantka, V., Pollet, N., Delius, M., Vingron, R., Pfister, R., Nitsch, C., Blumenstock, C. and Niehrs, C. (1998). Gene expression screening in *Xenopus* identifies molecular pathways, predicts gene function and provides a global view of embryonic patterning. *Mech. Dev.* **77**, 95-143.

Gilbert, S. F. (1997). *Developmental Biology*, 5th edition. Sinauer associates Inc., USA.

Godsave, S. F., Isaacs, H. V. and Slack, J. M. W. (1988). Mesoderm-inducing factors – a small class of molecules. *Development* **102,** 555-566.

Gofflot, F., Hall, M. and Morriss-Kay, G. M. (1997). Genetic patterning of the developing mouse tail at the time of posterior neuropore closure. *Dev. Dynam.* **210**, 431-445.

Gont, L. K., Steinbeisser, H., Blumberg, B. and DeRobertis, E. M. (1993). Tail formation as a continuation of gastrulation – the multiple cell-populations of the *Xenopus* tailbud derive from the late blastopore lip. *Development* **119,** 991-1004.

Han, K. Y. and Manley, J. L. (1993). Transcriptional repression by the *Drosophila* evenskipped protein – definition of a minimal repression domain. *Genes Dev.* **7**, 491-503.

Harland, R. M. (1991). In situ hybridisation: an improved whole mount method for *Xenopus* embryos. *Meth. Cell Biol.* **36,** 685-695.

Herault, Y., Hraba Renevey, S., Van der Hoeven, F. and Duboule, D. (1996). Function of the *Evx-2* gene in the morphogenesis of vertebrate limbs. *EMBO J.* **15**, 6727-6738.

Hollenberg, S. M, Cheng, P. F. and Weintraub, H. (1993). Use of a conditional MyoD transcription factor in studies of MyoD transactivation and muscle determination. *Proc. Natl. Acad. Sci. USA* **90**, 8028-8032.

Isaacs, H. V., Tannahill, D. and Slack, J. M. W. (1992). Expression of a novel FGF in the *Xenopus* embryo – a new candidate inducing factor for mesoderm formation and anteroposterior specification. *Development* **114**, 711-720.

Isaacs, H. V., Pownall, M. E. and Slack, J. M. W. (1994). eFGF regulates *Xbra* expression during *Xenopus* gastrulation. *EMBO J.* **19**, 4469-4481.

Isaacs, H. V., Pownall, M. E. and Slack, J. M. W. (1995). *eFGF* is expressed in the dorsal midline of *Xenopus-laevis*. *Int. J. Dev. Biol.* **39,** 575-579.

Johnson, F. B. and Krasnow, M. A. (1992). Differential regulation of transcription preinitiation complex assembly by activator and repressor homeo domain proteins. *Genes Dev.* **6**, 2177-2189.

Joly, J. S., Joly, C., Schulte-Merker, S., Boulekbache, H. and Condamine, H. (1993). The ventral and posterior expression of the zebrafish homeobox gene *eve1* is perturbed in dorsalized and mutant embryos. *Development* **119**, 1261-1275.

Kengaku, M., Capdevila, J., Rodriguez-Esteban, C., DeLaPena, J., Johnson, R. L., Belmonte, J. C. I. and Tabin, C. J. (1998). Distinct WNT pathways regulating AER formation and dorsoventral polarity in the chick limb bud. *Science* **280**, 1274-1277.

Kolm, P. J. and Sive, H. L. (1995). Efficient hormone-inducible protein function in *Xenopus-laevis*. *Dev. Biol.* **171**, 267-272.

Laufer, E., Dahn, R., Orozco, O. E., Yeo, C. Y., Pisenti, J., Henrique, D., Abbott, U. K., Fallon, J. F. and Tabin, C. (1997). Expression of *radical fringe* in limb-bud ectoderm regulates apical ectodermal ridge formation. *Nature* **386,** 366-373.

LeDouarin, N. M., Teillet, M. A. and Catala, M. J. N. (1998). Neurulation in amniote vertebrates: a novel view deduced from the use of quail-chick chimeras. *Int. J. Dev. Biol*. **42**, 909-916.

Neumann, C. J. and Cohen, S. M. (1996). A hierarchy of cross-regulation involving Notch, wingless, vestigial and cut organizes the dorsal/ventral axis of the *Drosophila* wing. *Development* **122**, 3477-3485.

Nieuwkoop, P. D. and Faber, J. (1967). *Normal Table of Xenopus laevis* (Daudin). Reprinted Garland (1994).

Nievelstein, R. A. J., Hartwig, N. G., Vermeijkeers, C. and Valk, J. (1994). Embryonic development of the mammalian caudal neural-tube. *Teratology* **48**, 21-31.

Panin, V. M., Papayannopoulos, V., Wilson, R. and Irvine, K. D. (1997). Fringe modulates Notch ligand interactions. *Nature* **387,** 908-912.

Pownall, M. E., Tucker, A. S., Slack, J. M. W. and Isaacs, H. V. (1996). *eFGF, Xcad3* and Hox genes form a molecular pathway that establishes the anteroposterior axis in *Xenopus*. *Development* **122,** 3881-3892.

Rodriguez-Esteban, C., Schwabe, J. W. R., DeLaPena, J., Foys, B., Eshelman, B. and Belmonte, J. C. I. (1997). Radical fringe positions the apical ectodermal ridge at the dorsoventral boundary of the vertebrate limb. *Nature* **386,** 360-366.

Ruiz-i-Altaba, A. and Melton, D. A. (1989a). Bimodal and graded expression of the *Xenopus* homeobox gene *Xhox3* during embryonic-development. *Development* **106,** 173-183.

Ruiz-i-Altaba, A. and Melton, D. A. (1989b). Involvement of the *Xenopus* homeobox gene *Xhox3* in pattern-formation along the anterior posterior axis. *Cell* **57**, 317-326.

Ruiz-i-Altaba, A, Choi, T. and Melton, D. A. (1991). Expression of the Xhox3 homeobox protein in *Xenopus* embryos – blocking its early function suggests the requirement of Xhox3 for normal posterior development. *Dev. Growth Differ*. **33**, 651-669.

Saha, M. S. and Grainger R. M. (1992). A labile period in the determination of the anterior posterior axis during early neural development in *Xenopus*. *Neuron* **8,** 1003-1014.

Sordino, P., Duboule, D. and Kondo, T. (1996). Zebrafish *HoxA* and *Evx-2* genes: Cloning, developmental expression and implications for the functional evolution of posterior Hox genes. *Mech. Dev.* **59**, 165-175.

Spyropoulos, D. D. and Capecchi, M. R. (1994). Targeted disruption of the even-skipped gene, Evx-1, causes early postimplantation lethality of the mouse conceptus. *Genes Dev.* **8,** 1949-1961.

Takada, S., Stark, K. L., Shea, M. J., Vassileva, G., McMahon, J. A. and McMahon, A. P. (1994). Wnt3a regulates tailbud and somite formation in the mouse embryo. *Genes Dev.* **8**, 174-189.

Tenharmsel, A., Austin, R.J., Savenelli, N. and Biggin, M. D. (1993). Cooperative binding at a distance by even-skipped protein correlates with repression and suggests a mechanism of silencing. *Mol. Cell. Biol*. **13**, 2742-2752.

Tucker, A. S. (1995). Tail development in *Xenopus laevis*. DPhil thesis, University of Oxford.

Tucker, A. S. and Slack, J. M. W. (1995a). The *Xenopus-laevis* tail-forming region. *Development* **121,** 249-262.

Tucker, A. S. and Slack, J. M. W. (1995b). Tail bud determination in the vertebrate embryo. *Curr. Biol.* **5,** 807-813.

Uyttendaele, H., Soriano, J. V., Montesano, R. and Kitajewski, J. (1998). Notch4 and Wnt-1 proteins function to regulate branching morphogenesis of mammary epithelial cells in an opposing fashion. *Dev. Biol.* **196**, 204-217.

Westin J. and Lardelli, M. (1997). Three novel *Notch* genes in zebrafish: Implications for vertebrate *Notch* gene evolution and function. *Dev. Genes Evol.* **207**, 51-63.

Wolda, S. L., Moody, C. J. and Moon, R. T. (1993). Overlapping expression of *Xwnt-3a* and *Xwnt-1* in neural tissue of *Xenopus laevis* embryos. *Dev. Biol.* **155**, 46-57.

Wu, J. Y., Wen, L., Zhang, W. J. and Rao, Y. (1996). The secreted product of *Xenopus* gene *lunatic fringe*, a vertebrate signaling molecule. *Science* **273,** 355-358.

Zhang, N. and Gridley, T. (1998). Defects in somite formation in *lunatic fringe*-deficient mice. *Nature* **394,** 374-377.