Int. J. Dev. Biol. 46: 255-258 (2002)

Short Communication

Notch is required for outgrowth of the *Xenopus* tail bud

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ABSTRACT It has previously been shown that *Notch, Delta* and *Lunatic Fringe* are expressed together in the leading edge of the tail bud of the *Xenopus* embryo prior to outgrowth (Beck and Slack, 1998). It has also been shown that ectopic expression of a constitutive form of Notch, Notch-ICD, will provoke ectopic tail formation (Beck and Slack, 1999). Here we show that inhibition of Notch activity *in vivo* prevents outgrowth of the tail bud. This is achieved using inhibitors of the protease that carries out the ligand-induced intramembranous cleavage of Notch. Other protease inhibitors that do not inhibit Notch cleavage do not affect tail outgrowth.

KEY WORDS: *Notch, tail bud, protease inhibitors, outgrowth, Xenopus*

In order to prove the involvement of a molecule in a developmental process it is necessary to show that the molecule is expressed at the appropriate time and place, that it has the appropriate biological activity, and that its specific removal causes the process to fail (Slack, 1994). Outgrowth of the tail bud in *Xenopus* commences at stage 27. It is associated with the onset of expression of *lunatic fringe*, a gene not expressed at the earlier stages during which the tail forming region becomes established (Tucker and Slack, 1995a; Tucker and Slack, 1995b; Beck and Slack, 1998). The ventral part of the tail-forming region expresses genes encoding the cell surface receptor Notch and its ligand Delta from the end of gastrulation. At around stage 27, a small area of overlap between *lunatic fringe* and *Notch/Delta* becomes established in the region destined to become the distal tail bud. This expression data suggests that Notch signalling may be active in the leading edge of the tail bud during outgrowth, and led us to investigate the biological activity of the Notch system in tail formation. In a previous study, we demonstrated that ectopic tails are formed from grafts of animal cap expressing a constitutively active form of Notch and inserted into the posterior neural plate (Beck and Slack, 1999). These experiments have satisfied two of the three conditions for proving that Notch signalling is required for tail bud outgrowth: those of appropriate expression and biological activity. In this paper we complete the proof by showing that inhibition of Notch activity prevents tail bud outgrowth in intact embryos.

The Notch protein is a transmembrane receptor. Following binding of the ligand it undergoes proteolytic cleavage within the plasma membrane to release the Notch intracellular cytoplasmic domain (N-ICD), which activates transcription factors of the RBP Jκ/Suppressor of Hairless group (Brown *et al*., 2000; Mumm *et al*., 2000). This proteolytic cleavage of Notch can be inhibited by reagents that interfere with the γ-secretase activity, which cleaves the Alzheimer Precursor Protein and is involved in the pathology of Alzheimer's disease (De Strooper *et al*., 1998; De Strooper *et al*., 1999; Song *et al*., 1999; Steiner *et al*., 1999; Struhl and Greenwald, 1999). It has been proposed that the γ-secretase activity is encoded by the *presenilin* genes, elimination of which result in developmental defects due to severe reduction in Notch processing (Shen *et al*., 1997; Wong *et al*., 1997; Donoviel *et al*., 1999). Several peptide aldehyde protease inhibitors, including MG-132 and MDL28170, can inhibit the cleavage of Notch *in vitro*, while specific proteasome inhibitors, such as lactacystin, do not (De Strooper *et al*., 1999). This suggested that it might be possible to investigate the requirement for Notch through the use of suitable protease inhibitors applied to whole embryos. In this paper we show that application of Notch cleavage inhibitors to *Xenopus* embryos can prevent tail bud outgrowth, completing the proof for the need for Notch signalling in this process.

Previous embryological experiments have shown that only the distal half of the tail arises from the tail bud. The proximal part of the tail does not come from the tail bud but is formed during gastrulation and becomes displaced posterior to the proctodeum by later morphogenetic movements (Tucker and Slack, 1995b). Consistent with this, surgical removal of the tail bud at stage 30 prevents formation of just the distal half of the tail at stage 40. Complete inhibition of tail bud outgrowth should not therefore result in the total absence of the tail but in the formation of a shortened tail, corresponding to the loss of the tail bud contribution.

Although other studies (De Strooper *et al*., 1999) have shown which protease inhibitors do, and do not, inhibit Notch cleavage, to confirm these results for *Xenopus* we examined their effects on neurogenesis. It is known that primary neurogenesis is controlled by Notch signalling such that developing neurons suppress neurogenesis in surrounding cells (Chitnis *et al*., 1995). Inhibition of Notch signalling

Abbreviations used in this paper: N-ICD, Notch intracellular cytoplasmic domain.

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0214-6282/2002/$25.00

Printed in Spain
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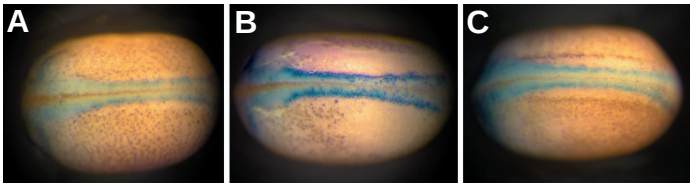


Fig. 1. Effect of protease inhibitors on primary neuron formation. *Embryos cultured in protease inhibitors from 2-cell stage and stained for expression of* N-tubulin *(blue staining in midline), a marker of primary neurons, at stage 16.* **(A)** *Control, no inhibitor.* **(B)** *MG132 (25 μM).* **(C)** *Lactacystin (25 μM). Anterior is to left, dorsal uppermost.*

should therefore result in a larger number of primary neurons being formed. *Xenopus* embryos were incubated in protease inhibitors from the 2-cell stage onwards. At stage 16 they were fixed and the primary neurons were stained by *in situ* hybridisation for *N-tubulin* expression (Fig. 1). Embryos treated with the Notch inhibitor MG132 showed more primary neurons (Fig. 1B) than either untreated controls (Fig. 1A) or embryos treated with the serine protease inhibitor lactacystin (Fig. 1C). We conclude that the effects of inhibitors on Notch signalling are similar in *Xenopus* embryos to the mammalian systems examined previously. The demonstration of an effect on neurogenesis also shows that inhibitors such as MG132 are able to penetrate the embryos effectively.

To study the effects on tail development, we applied various protease inhibitors to early neurula embryos, starting the treatment at stage 15. The Notch cleavage inhibitors MG-132, MDL28170, MG-115 and calpeptin all resulted in formation of a shorter tail, with the average length of tail formed being similar to that resulting from surgical removal of the tail bud (Figs. 2, 3). MG-132 and MG-115 are fairly broad inhibitors of both serine and cysteine proteases, which can inhibit the proteasome as well as Notch processing. However, proteasome inhibitor I and lactacystin, specific inhibitors of the proteasome which do not affect Notch cleavage, had no effect on tail length, suggesting that a cysteine protease rather than a serine protease is required for tail outgrowth. Also AEBSF, a specific serine protease inhibitor, had no effect on tail formation, nor did pepstatin A nor DFK-167, both inhibitors of aspartyl proteases (data not shown).

The *Xenopus* tail bud, if isolated, can be grown in culture and will autonomously produce myotomes, notochord, neural tube

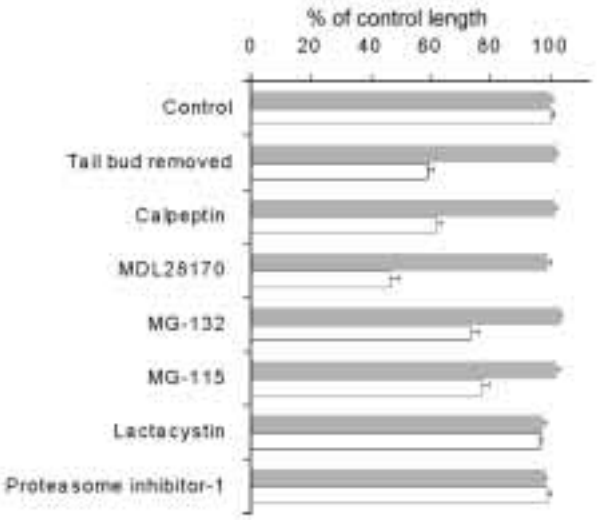
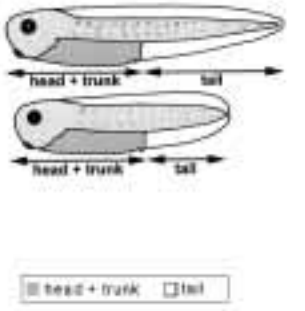


Fig. 2. Effect of protease inhibitors on tadpole head, trunk and tail length. *Tadpoles were cultured in 25 μM of the various protease inhibitors and then fixed at 3 days of development. Treatments were compared both to controls and to embryos which had the tail bud removed at stage 30 (2 days old). For each treatment the distance from head to proctodeum (head and trunk) and from proctodeum to tail tip (tail) was recorded for 20 randomly chosen tadpoles and expressed as a percentage of control (vehicle-treated) tadpole. Results are shown as mean ± SD, n = 20.*



and fin (Tucker and Slack, 1995a). Tail buds excised at stage 30 were treated with Notch cleavage inhibitors for 24 hours and the results observed reproduced the effects seen in the whole embryo with the buds failing to elongate or differentiate (Fig. 4). This shows that the effects of the inhibitors are local to the tail bud and are not an indirect consequence of action elsewhere in the developing embryo.

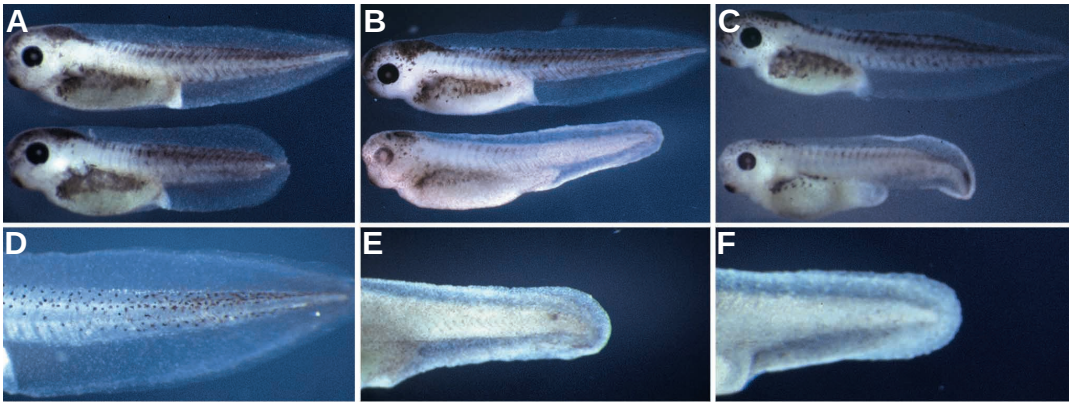


Fig. 3. Notch inhibitors reduce tail bud outgrowth, fin and pigment cell development. *(A–C) Whole embryos at stage 40 (3 days old) following vehicle treatment (upper) or inhibitor treatment (lower) from stage 16 (24 hours).* **(A)** *The lower embryo has had the tail bud surgically removed at stage 30 (2 days old). Note that the proximal tail still forms as a result of the posterior displacement of trunk tissue relative to the proctodeum.* **(B)** *The lower embryo has been treated with calpeptin (25 μM) and shows reduction of the tail equivalent to loss of the tail bud.* **(C)** *The lower embryo has been incubated in MG-132 (25 μM) and the majority of the tail bud-derived tail is missing. Anterior is to the left. (D-F) Higher power views to show effects on fin and pigmentation.* **(D)** *Vehicle-treated embryo, note length, well developed fin and pigmentation.* **(E)** *Following calpeptin (25 μM) treatment. The tail is much shorter, lacks pigmentation and the fin is poorly developed with thickened epidermis.* **(F)** *MG-132 (25 μM). The phenotype is similar to that seen with calpeptin.*

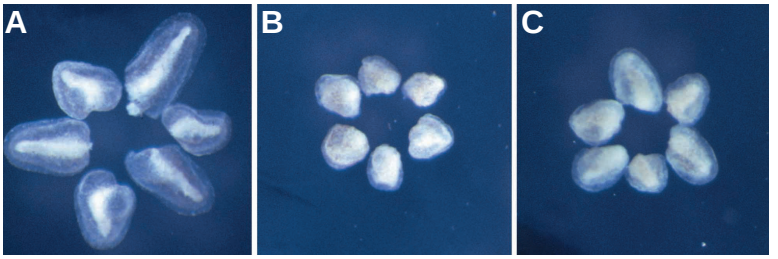


Fig. 4. Effects on isolated tail buds. *Tail buds were excised at stage 30 (2 days old) and cultured alone or in the presence of protease inhibitors until stage 40 (3 days old).* **(A)** *Control, no inhibitor.* **(B)** *Calpeptin (25 μM).* **(C)** *MDL28170 (25 μM).*

We have previously shown that Notch signalling in the growing tail tip activates transcription of *Xhox3*, an *even-skipped* homeobox gene, and that blocking *Xhox3* function at the stage of outgrowth results in the specific loss of tail bud-derived structures (Beck and Slack, 1999). In the current experiments, expression of *Xhox3* was examined by *in situ* hybridisation and it was found that it is much reduced or absent following treatment with the Notch cleavage inhibitors (Fig. 5). We have also reported elsewhere the effect of one Notch inhibitor, calpeptin, on the formation of ectopic tails from animal cap grafts containing tail-promoting mRNAs (Beck *et al.*, 2001). These studies showed that calpeptin would inhibit tail formation promoted by mNotchΔE, a Notch mutant which undergoes ligand-independent proteolytic cleavage, but had no effect on tail formation promoted by Notch ICD, which does not require cleavage at all. This further validates the specificity of the results reported here.

The Notch gene is required for many different functions during development (see Simpson, 1998, and accompanying articles, for review). It is not known, however, whether proteolytic cleavage and N-ICD formation is required for all of these. In terms of gross effects on body level formation, the effects of the Notch cleavage inhibitors was specific to the tail. However, the treated embryos also fewer melanocytes and had poorly formed, thickened, fins and thicker epidermis than controls (Fig. 3 E,F). Surprisingly however, we found the myotomes formed normally in treated embryos, although they were reduced in number owing to a lack of contribution from the tail bud (Fig. 6). This may indicate that somitogenesis, although dependent on Notch, (Jiang *et al.*, 1998) may operate through a different signalling pathway not requiring cleavage and release of the N-ICD.

The present results show that inhibitors of Notch cleavage will prevent the outgrowth of the tail bud and prevent the expression of *Xhox3*, which encodes a transcription factor required for tail development. They are complementary to our previous results showing that *Notch, Delta* and *Lunatic Fringe* are expressed in the prospective tail bud, and that tail outgrowth can be provoked by ectopic expression of constitutively active forms of Notch. Notch is expressed in the tail bud of several vertebrate species (Reaume *et al.*, 1992; Bettenhausen *et al.*, 1995; Dunwoodie *et al.*, 1997; Westin and Lardelli, 1997; Forsberg, Crozet and Brown, 1998; Smithers *et al.*, 2000). Although overexpression data is lacking for other species, a similar tail shortening phenotype has been described in knockout mice lacking *Presenilin-1*, or both *Presenilin-1* and *-2* (Shen *et al.*, 1997; Wong *et al.*, 1997; Donoviel *et al.*, 1999). These comparative data make it likely that this role of the Notch pathway is not just confined to *Xenopus* but that it is required for tail bud outgrowth in all vertebrates.

Experimental Procedures

Embryo Culture and Analysis

Xenopus embryos were cultured in $^{1}/_{10}$ NAM (Beck and Slack, 1999) supplemented with 25 μM protease inhibitors (from 400x stock in DMSO) from stage 16 (Nieuwkoop and Faber, 1967) (1 day), to stage 40 (3 days). Control siblings were incubated in $^{1}/_{10}$ NAM containing 0.05% DMSO. At 3 days they were fixed in batches of 20 and measured using a graduated eyepiece on a Wild dissecting microscope set to 25x. Each tadpole was measured from head to proctodeum (head and trunk) and from proctodeum to tail tip (tail). Tail buds were extirpated at stage 30 and cultured for 24 hours as described previously (Tucker and Slack, 1995b). Myotomes were detected by staining with monoclonal antibody 12/101(Kintner and Brockes, 1984), as described elsewhere (Tucker and Slack, 1995b). *In situ* hybridisation for *Xhox3* has been previously described (Beck and Slack, 1998). *N-tubulin* was a kind gift of Nancy Papalopulu, and was linearised with BamHI and transcribed using T3 polymerase.

Protease Inhibitors

Calpeptin (z-Leu-Nle-CHO), (N-acetyl-Leu-Leu-Nle-CHO), MDL28170 (z-Val-Phe-CHO), MG-115 (z-Leu-Leu-Nva-CHO), MG-132 (z-Leu-Leu-Leu-CHO), proteasome inhibitor I (z-Ile-Glu(OtBu)-Ala-Leu-CHO) and lactacystin were purchased from CN Biosciences, (Nottingham, U.K.). AEBSF (p-Aminoethylbenzenesulfonyl Fluoride, HCl) and Pepstatin A (Isovaleryl-Val-Val-4-amino-3-hydroxy-6-methyl-heptanoyl-Ala-4-amino-3-hydroxy-6-methylhepatanoic acid) were purchased from Calbiochem. DFK-167 was purchased from Enzyme Systems Products. All inhibitors were initially prepared at 10 mM in 100% DMSO.

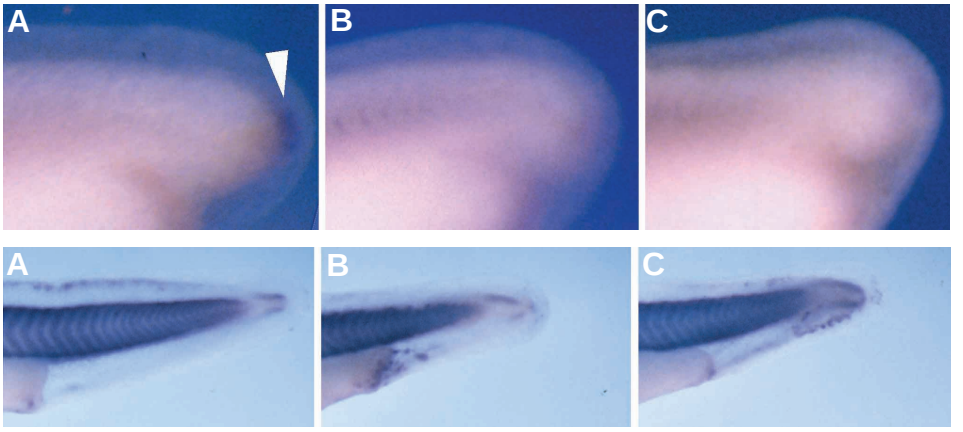


Fig. 5. (Upper panel) Effects of inhibitors on *Xhox3* expression. In situ *hybridisation of* Xhox3 *in the tail bud at stage 30. View of tail bud from side with anterior to the left.* **(A)** *Vehicle-treated control expresses* Xhox3 *(dark blue staining) in the distal tail bud (white arrow).* **(B)** *Embryo treated with 25 μM calpeptin does not express* Xhox3 *at detectable levels.* **(C)** *Embryo treated with 25 μM MG-132 expresses* Xhox3 *at barely detectable levels.*

Fig. 6. (Lower panel) Myotome structure in embryos treated with protease inhibitors. *Myotomes are visualised using the antibody 12/101 on 3 day-old embryo tails (dark blue staining).* **(A)** *Control (number of myotomes 42±1, n=19).* **(B)** *Embyros treated with MDL28170 have fewer myotomes, but they appear to be normally formed (number of myotomes 30±1, n=17).* **(C)** *Embryos treated with MG-132 also have fewer, normally formed, myotomes (number of myotomes32±1, n=13). Anterior to the left.*

Acknowledgements

The authors would particularly like to thank Dr. Mark Shearman of Merck, Sharpe and Dohme Research Labs (Harlow, UK) both for initiating our interest in protease inhibitors as a means to inhibit Notch signalling and for the kind provision of many of the reagents used. We would also like to thank Nadine Black for N-tubulin in situ hybridisations, and Nancy Papalopulu for N-tubulin.

This work was supported by the Wellcome Trust and the Medical Research Council.

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Received: August 2001
Reviewed by Referees: October 2001
Modified by Authors and Accepted for Publication: February 2002