Notch targets the Cdk inhibitor Xic1 to regulate differentiation but not the cell cycle in neurons

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The proneural protein neurogenin (XNGNR1) drives differentiation of primary neurons in combination with the cyclin-dependent kinase (Cdk) inhibitor Xic1. Differentiation is inhibited by Notch signalling, resulting in a scattered neuronal distribution. Here we show that Notch signalling regulates the level of Xic1 transcription, yet this does not correlate with Notch's ability to perturb the cell cycle. Instead, Notch may regulate Xic1 levels to control its differentiation function directly, which is required in parallel with XNGNR1 to promote primary neurogenesis. Indeed, Notch-mediated repression of both XNGNR1 and Xic1 must be relieved for neuronal differentiation to occur. Interestingly, although Xic1 is required for XNGNR1-mediated neurogenesis, it is not required for XNGNR1-mediated upregulation of Delta, allowing establishment of the negative feedback loop involved in lateral inhibition. Therefore, Notch targets Cdk inhibitor expression to regulate differentiation of primary neurons, and its effects on the cell cycle may be of secondary importance.

Keywords: Notch; cdk; neuron; cell cycle; neurogenin

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INTRODUCTION

During early development of the toad *Xenopus laevis*, inductive interactions result in specification of the neural plate. In the neural plate, all cells seem to be competent to undergo neurogenesis, but initially only a small subset of scattered cells actually exit from the cell cycle and differentiate into the so-called primary neurons, found in three distinct domains on either side of the midline. Over recent years, an understanding of the molecular events underlying primary neurogenesis has begun to emerge. First, differentiation is thought to be initiated by expression of the basic-helix–loop–helix (bHLH) transcription factor neurogenin (XNGNR1; Ma *et al*, 1996), which in turn upregulates the transcription factors XMyT1

(Bellefroid *et al*, 1996) and NeuroD (Lee *et al*, 1995). This eventually results in cell-cycle exit and differentiation. In addition, high levels of XNGNR1 are also able to activate the expression of Delta, a transmembrane ligand. Delta signals to the Notch receptor on adjacent cells, through Suppressor of Hairless (Su(H)) homologues, to downregulate XNGNR1 transcription and activity in these cells in a negative feedback loop, thus suppressing neurogenesis (Coffman *et al*, 1993; Chitnis *et al*, 1995). Reinforcing the difference between cells expressing high and low levels of XNGNR1, those cells with the highest level of XNGNR1 and Delta expression also turn on XMyT1 expression, which further confers resistance to Notch-mediated inhibition of XNGNR1 in the cells that are destined to form neurons (Bellefroid *et al*, 1996). Hence, an initial fairly uniform population of precursors showing stochastic variations in XNGNR1 levels between cells is sorted into a scattered 'salt and pepper' distribution, interspersing differentiated neurons and neural progenitors. This process is known as lateral inhibition. In this scheme, cell-cycle exit of neurons must be coordinated with differentiation, although how this is achieved is poorly understood.

One candidate for a pivotal coordinator of cell-cycle exit and differentiation is Xic1, the principal *Xenopus* cyclin-dependent kinase (Cdk) inhibitor, which shows homology to mammalian p21^{Cip1}, p27^{Kip1} and p57^{Kip2}, and is expressed very early on in primary neurons (Su *et al*, 1995; Vernon *et al*, 2003). We have previously shown that Xic1 is essential for primary neurogenesis and its overexpression results in the formation of extra neurons. Surprisingly, however, Xic1 acts very early on in neurogenesis, in parallel with XNGNR1 and before cell-cycle exit. Moreover, Xic1's function in promoting neural differentiation resides in its amino terminus and is distinct from its ability to inhibit the cell cycle or overall Cdk kinase activity (Vernon *et al*, 2003).

In this paper, we have investigated whether Notch signalling acts in part to generate scattered neurons by regulating Xic1 levels and whether Notch may use such control as a method of coordinating cell-cycle exit and differentiation.

RESULTS AND DISCUSSION

Notch-induced cell-cycle arrest and neural differentiation

In most tissues, it is thought that cell-cycle exit must precede differentiation. In some contexts, such as the mouse nervous system, Notch signalling keeps cells in the cell cycle and has

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been proposed as a mechanism whereby cells maintain an undifferentiated state (Solecki *et al*, 2001). However, in other tissues such as the epidermis, Notch has been shown to drive cell-cycle exit, possibly by upregulation of the Cdk inhibitor p21^{Cip1}, and at the same time to potentiate differentiation (Coffman *et al*, 1993; Rangarajan *et al*, 2001). Additionally, Notch may also be able to influence cdk levels post-transcriptionally (Sarmiento *et al*, 2005). These observations, among others, indicate that the effect of Notch is highly dependent on cellular context and that interactions with the cell-cycle machinery may have crucial roles in transducing and regulating Notch function. However, whether effects on the cell cycle *per se* are directly influencing the Notch-mediated regulation of differentiation remains unclear. We set out to address this question in the *Xenopus* neural plate.

To determine whether Notch signalling could be regulating the cell cycle, messenger RNAs encoding modified Notch pathway members were injected into embryos and their effect on cell proliferation in the neural plate was determined by staining for phosphohistone H3 (pH3), a marker of mitosis (Saka & Smith, 2001). Notch-ICD is a truncated form of the Notch receptor (Chitnis *et al*, 1995), and Suppressor of Hairless-Ankyrin (XSu(H)-ANK) is a key downstream component of the Notch signal transduction pathway that has been rendered constitutively active by the addition of ankyrin repeats (Wettstein *et al*, 1997). Overexpression of both these constructs results in the loss of primary neurons (Chitnis *et al*, 1995; Wettstein *et al*, 1997; data not shown). In contrast, expression of X-Delta^{STU}, a dominant-negative form of the Delta ligand, can block Notch signalling and promote primary neuron formation (Chitnis *et al*, 1995), although effects on other pathways cannot be ruled out.

Interestingly, overexpression of Notch-ICD and XSu(H)-ANK, while maintaining neural cells in an undifferentiated state, resulted in a significant decrease in the number of cycling cells in the neural plate on the injected side of the embryo (Fig 1A–B'). Thus, Notch-ICD and XSu(H)-ANK keep neural cells in an undifferentiated state, yet still inhibit the cell cycle. Unexpectedly, although X-Delta^{STU} inhibits Notch signalling and promotes neural differentiation, its overexpression also results in cell-cycle inhibition (Fig 1C,C'). See the supplementary information online for additional quantitative analysis.

Hence, both activation and inhibition of Notch signalling result in cell-cycle inhibition, indicating that, at least in this context, Notch signalling does not promote proliferation as a means of inhibiting differentiation.

Notch does not regulate the cell cycle via Xic1 modulation

The Cdk1 Xic1 is expressed during early *Xenopus* development in differentiating primary neurons and is therefore a potential mediator of cell-cycle arrest in the neural plate (Vernon *et al*, 2003). To determine whether Notch signalling is controlling cell-cycle exit by modulating Xic1 levels, we injected the Notch signalling activators Notch-ICD and XSu(H)-ANK, or the Notch inhibitor X-Delta^{STU}, into one cell of two-cell stage embryos and determined the effect on Xic1 expression on the injected side by *in situ* hybridization. Embryos expressing Notch-ICD or XSu(H)-ANK show a substantial decrease in Xic1 expression (Fig 2A–C, 90%, *n*=24; 80%, *n*=30, respectively). This loss of expression is apparent not only in the primary neurons (Fig 2A,B, black arrows

Fig 1 | Notch activation suppresses cell proliferation. Embryos were injected with (A,A') Notch-ICD, (B,B') XSu(H)-ANK or (C,C') X-Delta^{STU} along with β -gal (light blue), fixed at stage 15 and stained for phosphorylated histone H3 (pH3; purple). Enlargements of the boxed areas in the neural plate (A–C), midline as indicated (injected side to left).

on the injected side) but also in the underlying myotome in the injected region (Fig 2A–C, m), where Xic1 is also strongly expressed. Xic1 is expressed very early on from stage 11 in the precursors of primary neurons (Fig 2A, black arrow, injected side). This very early Xic1 expression, which occurs well in advance of neural differentiation, is also clearly downregulated by Notch signalling (52%, *n*=23). In contrast to the loss of Xic1 expression observed after constitutive activation of Notch signalling, blocking the pathway by X-Delta^{STU} mRNA injection results in upregulated expression of Xic1, primarily in more densely arrayed scattered cells of a modestly expanded stripe in the lateral neural plate (Fig 2D, black arrow; 68%, *n*=68), similar to the pattern of more dense neurons seen after X-Delta^{STU} overexpression (Chitnis *et al*, 1995). Indeed, expansion of Xic1 staining is clearly seen in deep layer cells of the neural plate on transverse sections (Fig 2E, arrow).

Castella *et al* (2000) have demonstrated that mammalian Hes-1, a hairy/E(Spl) homologue and component of the Notch signalling pathway, can repress p21^{Cip1} expression in PC12 cells, in addition to inhibiting nerve-growth-factor-induced differentiation, whereas Rangarajan *et al* (2001) have shown that Notch1 induces p21^{Cip1} and promotes differentiation in the epidermis.

Fig 2 | Notch-ICD downregulates Xic1 expression. Embryos were injected with (A,B) Notch-ICD, (C) XSu(H)-ANK or (D) Delta^{STU} along with β -gal (light blue), fixed at stage 11 (A) or stage 15 (B–D) and assayed for Xic1 expression (purple) by *in situ* hybridization (injected side to left). (E) A section through an X-Delta^{STU}-injected embryo stained for Xic1 expression in purple (arrow). (Myotome, m, stains light blue owing to poor penetrance of BM Purple stain in whole mount.) (F) Western blot to detect Xic1 in embryos injected into two of two cells with (lane 1) Notch-ICD, (lane 2) XSu(H)-ANK or (lane 3) X-Delta^{STU}. (Lane 4) Uninjected embryo. Sections of embryos injected as in Fig 1A,B are shown in (G,H) with arrows to cells in the deep layer of the neural plate staining for pH3, uninjected side.

Notch signalling has been shown to regulate the expression of Skp2, an E3 ligase, which can in turn degrade p27^{Kip1} and p21^{Cip1}, mammalian homologues of Xic1 (Sarmiento *et al*, 2005). Therefore, to determine whether Notch regulates Xic1 at the level of protein stability, Notch pathway modulators were injected and the levels of Xic1 protein at neurula stage were determined by western blotting. Although Notch signalling clearly down-regulated Xic1 transcription (Fig 2A,B), it has no obvious effect on the overall Xic1 protein level (Fig 2F). Although this may seem puzzling, we note that Xic1 expression appears transiently in scattered cells in the epidermis just before this embryonic stage (Vernon *et al*, 2003), and that Xic1 protein accumulates in this tissue (data not shown). Interestingly, Notch signalling regulates scattered ciliated cells in the epidermis (Deblandre *et al*, 1999) but, in contrast to neurogenesis, our preliminary data (I.H. and A.P.) indicate that Xic1 levels do not have a role in this process, and we have no evidence that Notch regulates Xic1 levels in this tissue. Hence, Xic1 protein accumulated in the skin is likely to obscure changes in Xic1 protein levels in primary neurons and myotome.

We have shown that Notch activation results in the transcriptional downregulation of Xic1 (Fig 2), as well as inhibition of primary neuron formation (Chitnis *et al*, 1995), even though it also promotes cell-cycle arrest (Fig 1). Primary neurons arise from deep layer cells of the neural plate, and Xic1 is expressed only in these deep layer cells in the neural plate (Fig 2E; Vernon *et al*, 2003). On sectioning pH3-stained embryos, we observed that Notch-ICD and Su(H)-ANK inhibited mitosis in both the superficial and deep layers of the neural plate (Fig 2G,H), again indicating that Notch signalling does not target Xic1 expression as a means to regulate cell proliferation in the neural plate.

We have previously shown that Xic1 is absolutely required for primary neurogenesis in parallel with XNGNR1, and that this requirement is for an N-terminal function, which is distinct from Xic1's ability to arrest the cell cycle (Vernon *et al*, 2003). Given the data above, we wanted to explore further the possibility that Xic1 levels are regulated to maintain cells in an undifferentiated state irrespective of Xic1's effect on the cell cycle.

Notch regulates Xic1 to control neural differentiation

For XNGNR1-expressing cells to completely escape lateral inhibition by Notch signalling, they must coexpress the XNGNR1 target gene XMyT1. Notch-ICD suppresses XNGNR1's ability to induce neurons unless XMyT1 is co-injected (Bellefroid *et al*, 1996). Overexpression of XNGNR1, with or without XMyT1, does not induce ectopic Xic1 expression (Vernon *et al*, 2003; data not shown). Therefore, we postulated that Xic1 and XNGNR1 must be repressed in parallel by Notch signalling and therefore the repression of both should be overcome by coexpression of XMyT1 to allow resistance to lateral inhibition. To test this, embryos were co-injected with Notch-ICD, XNGNR1 and XMyT1 and analysed for Xic1 expression at neural plate stages. Notch-ICD inhibits Xic1 expression in both the myotome and the lateral stripe of primary neurons (Fig 3A",B", uninjected sides, black arrows), even in the presence of XNGNR1 (91% of embryos have reduced Xic1, $n=79$; Fig 3A, compare injected side A' and uninjected side A"). However, coexpression of XMyT1 with XNGNR1 substantially alleviates the inhibition of Xic1 by Notch-ICD (only 40% of embryos have reduced Xic1, $n=120$; Fig 3B, compare injected side B' and uninjected side B"), as it does with Notch-mediated inhibition of XNGNR1 expression (Bellefroid *et al*, 1996). Therefore, Xic1 expression can be maintained in the presence of active Notch signalling when both XNGNR1 and XMyT1 are present, and maintenance of Xic1 may be a crucial downstream event that allows XMyT1 to cooperate with XNGNR1 in overcoming lateral inhibition.

Therefore, we investigated whether co-overexpression of X-NGNR1 and Xic1 alone might be sufficient to confer insensitivity to Notch-mediated lateral inhibition, by co-injection of Notch-ICD, XNGNR1 and Xic1. Notch-ICD inhibits endogenous neurogenesis (Fig 3C, 91%, $n=23$). Overexpression of Xic1 results in a modest upregulation in the number of primary neurons in the neural plate (Fig 3D, 53%, $n=36$), whereas XNGNR1 can induce ectopic neurons both in the neural plate and in the non-neural ectoderm (Fig 3E, 100%, $n=34$; Fig 3F, 100% $n=31$). As expected, Notch-ICD can repress neurogenesis even in the presence of ectopic Xic1 (Fig 3G, 88%, $n=25$). However, when all three are co-injected, neurons are still largely absent on the injected side (Fig 3H, 100%, $n=29$), indicating that co-injection

Fig 3 | XNGNR1 and XMyT1 synergize to overcome repression of Xic1 by Notch-ICD. Embryos were injected with (A) Notch-ICD + XNGNR1 or (B) Notch-ICD + XNGNR1 + XMyT1 with β -gal as a tracer (light blue), fixed at stage 15 and assayed for Xic1 by *in situ* hybridization (purple; injected side to left). (A',A'',B',B'') Enlargements of the boxed areas in (A,B); black arrows indicate the lateral stripe of primary neurons (injected side to left). XNGNR1 and Xic1 alone cannot overcome Notch-mediated lateral inhibition. Embryos were injected with RNA encoding (C) Notch-ICD, (D) Xic1, (E) XNGNR1, (F) Xic1 and XNGNR1, (G) Xic1 and Notch-ICD or (H) XNGNR1, Xic1 and Notch-ICD, along with β -gal (light blue, injected side to left), and assayed for neural β -tubulin expression at stage 15 by *in situ* hybridization as labelled. Notch-ICD can still inhibit primary neurogenesis even when XNGNR1 and Xic1 are co-injected.

of XNGNR1 and Xic1 RNAs alone is not enough to overcome lateral inhibition. This observation may be explained by the fact that Notch-ICD can directly inhibit the function of XNGNR1 protein by another as yet unidentified mechanism (Bellefroid *et al*, 1996; Ma *et al*, 1996), inhibition that is also alleviated by XMyT1. Notch signalling has been shown to potentiate protein turnover of the related proneural protein hASH1 (Sriuranpong *et al*, 2002) and to upregulate the bHLH factor Hes1, which can antagonize the function of other proneural proteins (Kageyama *et al*, 2005). Moreover, it is clear that other molecules must be present for XNGNR1 to drive differentiation of primary neurons, as overexpression of XNGNR1 in ectodermal explants (animal caps) cannot drive expression of the terminal differentiation marker neural β -tubulin (β tub) at the same stage as it occurs in whole embryos (Papalopulu & Kintner, 1996), even when coexpressed with Xic1 (data not shown). However, Xic1 protein is still absolutely required for primary neurogenesis: primary neurons do not form when XNGNR1, XMyT1, Notch-ICD and

Fig 4 | Xic1 is not required for XNGNR1-mediated activation of Delta. Embryos were injected with Xic1 Mo (A,C) or Con Mo (B), without (A) or with XNGNR1 (B,C) and analysed by whole-mount *in situ* hybridization for expression of XDelta1 (β -gal is light blue, injected side to left). (D) Model illustrating the interactions between the Notch pathway, XNGNR1 and Xic1.

Xic1 Mo are all co-injected (data not shown). Moreover, when Notch signalling is blocked with Delta^{STU}, neurons are unable to form in the absence of Xic1 (see supplementary Fig 1 online).

XNGNR1 upregulation of Delta does not require Xic1

To suppress neurogenesis in cells adjacent to those with the highest levels of XNGNR1, and thus to generate scattered neurons, XNGNR1 establishes a negative feedback loop by upregulating Delta expression on the cell surface (Ma *et al*, 1996). Delta activates Notch signalling in adjacent cells, which represses both the expression and activity of XNGNR1 as well as Xic1 (Fig 2) and hence suppresses primary neurogenesis, resulting in a 'salt and pepper' distribution of neurons. We have previously shown that Xic1 is absolutely required for XNGNR1-mediated expression of NeuroD (Vernon *et al*, 2003), but we wished to determine whether Xic1 is required for XNGNR1-mediated upregulation of Delta, by injection of Xic1 Mo. Unlike the expression of N β tub or NeuroD (Vernon *et al*, 2003), endogenous Delta expression is unaffected by injection of Xic1 Mo (Fig 4A, 100%, $n=38$). Moreover, Xic1 Mo injection does not prevent XNGNR1-mediated upregulation of Delta (Fig 4B,C, 52% upregulated, $n=48$; 59% upregulated, $n=44$, respectively), even though it is very efficient at blocking Xic1 protein translation (Vernon *et al*, 2003). Thus, although Xic1 is required for XNGNR1 to activate downstream effectors of neurogenesis such as XMyT1 and NeuroD, there is a lesser requirement for Xic1 in XNGNR1-mediated expression of Delta, which promotes lateral inhibition.

Notch regulates Xic1 and XNGNR1 to control neurogenesis

The data presented above allow us to draw the following model (Fig 4D). In the early neural plate, cells stochastically express variable levels of XNGNR1 and Xic1, with threshold levels of both being required for primary neurogenesis. XNGNR1 can upregulate Delta transcription independently of Xic1 levels.

Thus, in cells with higher XNGNR1, Delta signals to and maintains Notch activation in adjacent cells, repressing both XNGNR1 and Xic1 and thus keeping these surrounding cells in an undifferentiated state. In contrast, in cells with a higher initial level of XNGNR1 and which are destined to overcome lateral inhibition and form primary neurons, XNGNR1 accumulates and upregulates XMyT1. This combination overcomes any Notch-mediated repression of both XNGNR1 and Xic1 from adjacent cells, allowing these proteins to accumulate in parallel. Xic1 then acts with XNGNR1, possibly by stabilizing the XNGNR1 protein (Vernon *et al*, 2003), to upregulate NeuroD and thus promote neuronal differentiation. Moreover, it is possible that further targets are coordinately repressed by Notch signalling to keep neurons in an undifferentiated state, or that Notch signalling has a more direct role in regulating the transcriptional activity of XNGNR1.

Although Xic1 can simultaneously inhibit the action of cyclin/Cdk complexes in neuronal cells promoting cell-cycle exit required for differentiation, this activity may be secondary to the Cdk function required to promote differentiation directly. In support of this theory is the finding that NeuroD overexpression can promote neural differentiation in the absence of Xic1 (Vernon *et al*, 2003). Moreover, the evidence presented here indicates that Notch signalling does not regulate cell-cycle exit through changes in Xic1 levels. How, then, does Notch signalling influence the cell cycle in differentiating neuronal tissue? We see that modulation of Notch signalling alters the expression of cyclin A2 and Cdk2 in anterior neural structures where they are strongly expressed (supplementary Fig 2 online). However, the effects of Notch signalling on the cell cycle and cell-cycle regulators do not correlate with its effects on differentiation if one considers a simple model where cell-cycle exit directly promotes differentiation. Thus, other mechanisms to link the two processes must be considered.

Speculation

Much has been made of Notch targeting of cell-cycle regulators, including Cdkis, as a potential means of maintaining an undifferentiated state, altering the balance between the stem cell and progenitor compartments and also influencing tumour formation (Sarmiento *et al*, 2005, and references therein). However, it is clear that cell-cycle regulators in general, and Cdkis in particular, have other and separable roles in the processes of differentiation, as well as in cell signalling and movement (Coqueret, 2003). Indeed, it has recently been demonstrated that Cdkis may not even exert their ability to block cell-cycle progression by inhibiting Cdk2 as had long been thought (Bashir & Pagano, 2005), illustrating that we still have a lot to learn about their functions. Although the cell cycle may be coordinately, or even coincidentally, regulated, non-cell-cycle or Cdk inhibitory functions of Cdkis may be equally important; these deserve much more attention if we are to understand the complex regulatory networks linking cell-cycle regulation, differentiation and Notch signalling.

METHODS

Xenopus embryos, fixation and β -galactosidase staining. *X. laevis* embryos were obtained, staged and stained for β -galactosidase by standard methods (Vernon *et al*, 2003). Embryo extracts were

prepared and western blotted to detect Xic1, as described by Vernon *et al* (2003).

mRNA injection and morpholino antisense oligo. Capped RNAs were synthesized *in vitro* using the SP6 Message Machine kit (Ambion, Huntingdon, Cambridgeshire, UK). Injected RNAs were nuc- β -gal (250 pg), Notch-ICD (1–2 ng), X-Delta^{STU} (1.5–3 ng; Chitnis *et al*, 1995), XNGNR1 (50 pg; Ma *et al*, 1996), XMyT1 (250 pg; Bellefroid *et al*, 1996), XSu(H)-ANK (1 ng), XSu(H)-DBM (1 ng; Wettstein *et al*, 1997) and Xic1 (30–50 pg; Su *et al*, 1995). Morpholinos used (20 ng) have been described previously (Vernon *et al*, 2003).

Whole-mount *in situ* hybridization and antibody staining. Whole-mount *in situ* hybridization was performed as described (Shimamura *et al*, 1994). Linearized Bluescript plasmid from N β tub (*Bam*H1/T3), cyclin A2 (*Eco*R1/T7), Cdk2 (*Eco*R1/T7) and X-Delta-1 (*Not*I/T7) was used to generate digoxigenin-11-UTP-labelled (Boehringer Mannheim, Nutley, NJ, USA) antisense RNA probes. Xic1 (*Bam*H1) probes were from linearized pCS2⁺ and used the T7 promoter. BM Purple (Boehringer Mannheim) was used as a substrate. Whole-mount antibody staining was performed as described (Sive *et al*, 2000) using an anti-phosphohistone H3 (TCS Biologicals, Buckingham, UK) at 1:500, an alkaline phosphatase-conjugated secondary antibody and nitroblue tetrazolium/BCIP (5-bromo-4-chloro-3-indolylphosphate) as colour substrates. Embryos were sectioned after embedding in gelatin and albumin using a vibratome.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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