Development 139, 1718-1723 (2012) doi:10.1242/dev.077552

Post-translational modification of Ngn2 differentially affects transcription of distinct targets to regulate the balance between progenitor maintenance and differentiation

Christopher Hindley[1,*], Fahad Ali[1,*], Gary McDowell[1], Kevin Cheng[1], Alison Jones[1], Francois Guillemot[2] and Anna Philpott[1,‡]

SUMMARY

Neurogenin 2 (Ngn2) controls neuronal differentiation cell-autonomously by transcriptional activation of targets such as *NeuroD*, while simultaneously controlling progenitor maintenance non-cell-autonomously by upregulating *Delta* expression and Notch signalling. Reduction in Cdk-dependent multisite phosphorylation of Ngn2 enhances its promoter binding affinity. This leads specifically to an increase in neuronal differentiation without an apparent increase in progenitor maintenance via Delta-Notch signalling, although the mechanism underlying this imbalance remains unclear. Here we show in *Xenopus* embryos and mouse P19 cells that the *NeuroD* promoter is substantially more sensitive to the phosphorylation status of Ngn2 than the *Delta* promoter, and that this can be attributed to differences in the ease of promoter activation. In addition, we also show that the phosphorylation status of Ngn2 regulates sensitivity to Notch signalling. These observations explain how Ngn2 post-translational modification in response to changes in the cell cycle kinase environment results in enhanced neuronal differentiation upon cell cycle lengthening.

KEY WORDS: Ngn2, Cell cycle, Delta, NeuroD, Neuronal differentiation, Phosphorylation

INTRODUCTION

Transcription factors undergo very dynamic binding cycles on promoters (Hager et al., 2009; Michel, 2009). 'Slow' promoters are thought to require more extensive chromatin modification for activation than 'fast' promoters and hence require greater promoter residence time for activation. Consistent with this, increased dwell time on promoters is associated with more active transcription (Stavreva et al., 2004; Hager et al., 2009; Voss et al., 2011). Could differential requirements for transcription factor occupancy be used to differentially control distinct targets of a master regulatory transcription factor in response to a changing cellular environment? We have explored whether such a mechanism might coordinate cell cycle length and differentiation during nervous system development.

The proneural basic helix-loop-helix (bHLH) transcription factor neurogenin 2 (Ngn2) is required for generation of glutamatergic neurons in many regions of the central nervous system (Bertrand et al., 2002) and has multiple downstream functional effectors (Seo et al., 2007). However, as well as controlling neuronal differentiation cell-autonomously, Ngn2 also controls progenitor maintenance, largely non-cell-autonomously, by direct transcriptional activation of the Notch ligand *Delta* (Ma et al., 1996). Classically, Ngn2-driven Delta expression in cells destined to become neurons activates the Notch pathway in adjacent cells. This results in expression of Hes proteins, which inhibit *Ngn2* transcription and activity in these adjacent cells, maintaining their progenitor status (Chitnis et al., 1995), a process known as lateral inhibition. Recent evidence demonstrates an oscillatory relationship between Ngn2 and Notch signalling as a requirement for maintenance of progenitor status (Kageyama et al., 2008b; Shimojo et al., 2008), further defining the molecular basis of lateral inhibition. Escape from lateral inhibition-driven progenitor maintenance requires expression and activity of the bHLH transcription factor NeuroD, which is also a direct target of Ngn2 (Lee, 1997; Seo et al., 2007).

Thus, Ngn2 is required non-cell-autonomously for progenitor maintenance via Delta upregulation and, conversely, drives neuronal differentiation cell-autonomously via upregulation of NeuroD. The *Delta* promoter responds rapidly to Ngn2 expression and requires minimal chromatin modification, whereas upregulation of *NeuroD* transcription is considerably slower and requires chromatin modifiers such as p300 and Brg1 (Koyano-Nakagawa et al., 1999; Seo et al., 2005). This difference in promoter timing has been postulated to allow progenitor maintenance to be established before differentiation occurs (Koyano-Nakagawa et al., 1999), but how a difference in transcription factor binding requirements for promoter activation can act mechanistically as a developmental switch in response to the cellular environment has remained unclear.

Progenitor maintenance predominates at early stages of cortical development, whereas neuronal differentiation increases as the embryo develops, and this transition is coordinated by an average cell cycle lengthening (Lange and Calegari, 2010). We have previously shown that preventing Cdk-mediated phosphorylation of Ngn2, by mutating its serine-proline (SP) target sites to alanine-proline (9S-A Ngn2), significantly enhances the ability of Ngn2 to drive neuronal differentiation by enhancing its binding to downstream target promoters such as *NeuroD*, without apparently increasing non-cell-autonomous progenitor maintenance (Ali et al., 2011). However, un(der)phosphorylated Ngn2 also binds more

[1]Department of Oncology, University of Cambridge, Hutchison/Medical Research Council (MRC) Research Centre, Cambridge CB2 0XZ, UK. [2]Division of Molecular Neurobiology, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK.

*These authors contributed equally to this work
‡Author for correspondence (ap113@cam.ac.uk)

Accepted 13 March 2012

tightly to the *Delta* promoter (Ali et al., 2011), which might be expected to simultaneously promote non-cell-autonomous progenitor maintenance. In practice, mutation of Cdk phosphorylation sites substantially enhances the ability of Ngn2 to drive neuronal differentiation in the cells in which it is expressed, both in vitro and in vivo (Ali et al., 2011). If un(der)phosphorylation of Ngn2 enhances occupancy of both the *NeuroD* and *Delta* promoters (Ali et al., 2011), why does it promote neuronal differentiation cell-autonomously preferentially over non-cell-autonomous Delta/Notch-driven progenitor maintenance?

We find that phosphorylation of Ngn2 differentially controls the activation of the *NeuroD* and *Delta* promoters by exploiting differences in the kinetics of activation in response to promoter occupancy by Ngn2. Hence, post-translational modification of Ngn2 in response to cellular conditions can directly control the balance between progenitor maintenance and differentiation by exerting different effects on the control of individual downstream targets.

MATERIALS AND METHODS

Xenopus laevis extracts and embryos

Acquisition of *Xenopus laevis* embryos, preparation and injection of synthetic mRNA, staging of embryos and in situ hybridisation were as described previously (Vernon et al., 2003; Vosper et al., 2007; Vosper et al., 2009; Ali et al., 2011). Degradation assays were performed as described (Ali et al., 2011).

Cell culture and transfections

Cell culture and transfections were conducted as described previously (Ali et al., 2011).

Quantitative real-time PCR (qPCR)

cDNA was generated from mouse P19 cells or stage 15 *Xenopus* embryos and 50 ng used per qPCR reaction, which was performed in the LightCycler 480 PCR system (Roche) using SYBR Green mix (Roche), with β-actin and ornithine decarboxylase (*ODC*) as housekeeping genes for normalisation. Thermal cycling conditions were: 95°C for 5 minutes, then 45 cycles of 95°C for 10 seconds, 60°C for 10 seconds and 72°C for 10 seconds. Primer sequences (5′ to 3′): β-actin Fwd, CAGGGTGTGATGGTGGGAATG; β-actin rev, ATGGCTGGGGTGTTGAAGGTC; ODC Fwd, GAATCACCCGAATGCAAAGC; ODC rev, CCACTGCCAACATGGAAACTC.

Statistical analysis

Statistical analysis used a two-tailed Student's *t*-test ($P<0.05$). Error bars indicate s.e.m.

RESULTS AND DISCUSSION

Phosphorylation of Ngn2 has similar effects on its binding to the *NeuroD* and *Delta* promoters, as assayed by chromatin immunoprecipitation (ChIP), but dephosphorylated Ngn2 preferentially drives neuronal differentiation over progenitor maintenance (Ali et al., 2011). We hypothesised that the *Delta*

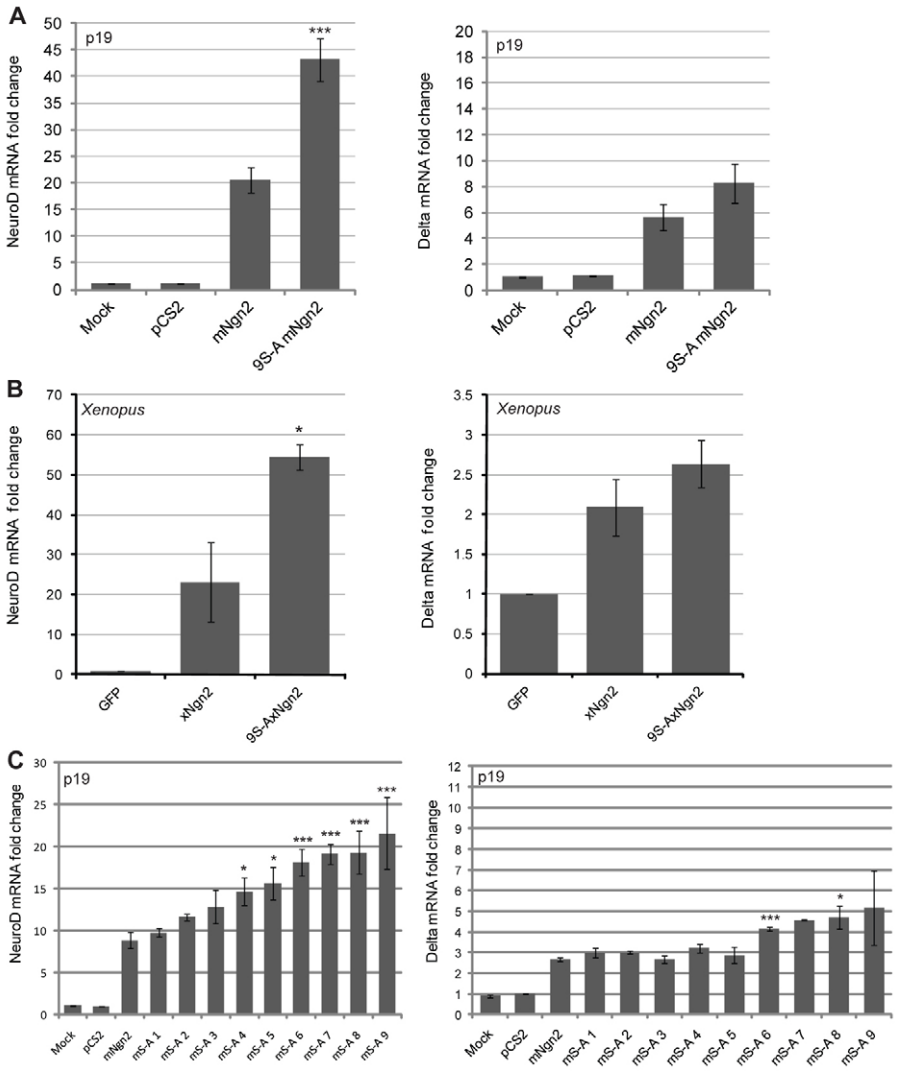

Fig. 1. Phosphorylation on multiple serine-proline sites regulates *NeuroD* and *Delta* expression differentially. qPCR analysis of *NeuroD* and *Delta* in mouse P19 cells and *Xenopus* embryos. (**A**) P19 cells were transiently transfected with mNgn2 or 9S-A mNgn2 for 24 hours. (**B**) *Xenopus* embryos were injected with 20 pg xNgn2 or 9S-A xNgn2 mRNA and harvested at stage 15. (**C**) P19 cells were transiently transfected with mNgn2 and cumulative phosphorylation site mutants thereof (see supplementary material Fig. S1) for 24 hours. Average fold increase in mRNA expression is shown normalised to β-actin (P19) or to GFP-injected control embryos (*Xenopus*), with mean normalised values calculated together with the s.e.m. *, $P\leq0.05$; ***, $P\leq0.005$.

and *NeuroD* promoters might respond differentially to changes in Ngn2 un(der)phosphorylation; a differential response of the *Delta* and *NeuroD* promoters to increased promoter occupancy, resulting from Ngn2 post-translational modification changes occurring on cell cycle lengthening, could result in an alteration in the balance between progenitor maintenance and differentiation within a population of neural progenitors (Vosper et al., 2009; Ali et al., 2011). To test this hypothesis, we compared the response of *NeuroD* and *Delta* (*Neurod1* and *Dll1* in mouse) expression to overexpression of mouse Ngn2 (mNgn2) and a mutant version thereof, 9S-A mNgn2, in which the nine SP sites that can be phosphorylated by Cdks have all been mutated to alanine-proline (Ali et al., 2011) (Fig. 1A; supplementary material Fig. S1). *Delta* expression was upregulated to a similar extent in response to Ngn2 or 9S-A Ngn2 in mouse P19 cells and *Xenopus* embryos. By contrast, un(der)phosphorylated 9S-A Ngn2 induced approximately twice as much *NeuroD* expression as wild-type Ngn2 (Fig. 1A,B). Thus, the *Delta* and *NeuroD* promoters respond differently to the phosphorylation status of Ngn2.

We have previously shown that mutation of an increasing number of Ngn2 phosphorylation sites progressively increases its affinity for E-box DNA in an environment with high Cdk kinase activity. This translates to an enhanced association of 9S-A Ngn2 with both the *Delta* and *NeuroD* promoters (Ali et al., 2011); ChIP assays demonstrate that 9S-A mNgn2 binding to the *NeuroD1* and *Delta* promoters is increased ~2- and 3-fold, respectively, over binding of wild-type Ngn2. Since the *NeuroD* promoter requires greater promoter remodelling for activation than the *Delta* promoter (Koyano-Nakagawa et al., 1999; Seo et al., 2005), we reasoned that the cumulative effect of individual phosphorylation events, each one having a small effect on DNA binding and, hence, promoter occupancy by Ngn2, would result in a quantitative shift in the ratio of *NeuroD* to *Delta* expression in response to progressively reduced Ngn2 phosphorylation. To test this, a panel of S-A mutant versions of mNgn2, in which mutation of SP sites was undertaken cumulatively from the N-terminus (i.e. mS-A1 has the most N-terminal SP site mutated; see supplementary material Fig. S1), was assayed for their ability to upregulate *NeuroD* and *Delta* expression.

In this assay (Fig. 1C), 9S-A mNgn2 drove *NeuroD* expression to twice the level of that achieved by the same amount of mNgn2. By contrast, expression of *Delta* driven by mNgn2 and 9S-A mNgn2 was similar, consistent with results presented in Fig. 1A,B. When assaying our cumulative mutant series, we observed that *NeuroD* expression showed an essentially linear increase for each additional SP site of Ngn2 that was mutated. However, *Delta* expression increased very little with progressive SP mutations, confirming that the *Delta* promoter is markedly less sensitive to Ngn2 phosphorylation status than the *NeuroD* promoter (Fig. 1C). We have previously shown that expression of the neuronal marker βIII-tubulin also gradually increases as additional SP sites are mutated (Ali et al., 2011).

Our results are consistent with a model whereby *Delta* transactivation requires little chromatin remodelling and can occur with both phosphorylated and un(der)phosphorylated Ngn2, whereas only enhanced promoter dwell time achieved by un(der)phosphorylated Ngn2 is sufficient to remodel the *NeuroD* promoter (Koyano-Nakagawa et al., 1999; Seo et al., 2005). To confirm this, we investigated the kinetics of activation of the *Delta* and *NeuroD* promoters in response to expression of Ngn2 in P19 cells, normalising to the maximum expression of each individual transcript after 10 hours. Both *Delta* and *NeuroD* expression showed a lag post-transfection, which was at least partly due to the time taken for Ngn2 proteins to accumulate from transfected plasmids. However, *Delta* expression was significantly elevated within 3-4 hours post-transfection and continued to rise (Fig. 2A), whereas the *NeuroD* promoter took 5-6 hours to respond to Ngn2 expression.

We next compared promoter responsiveness to mNgn2 and 9S-A mNgn2 over a longer timecourse. 9S-A mNgn2 induced *Delta* transcription at a somewhat higher rate than the wild-type protein, although this difference was small (see also Fig. 1); 9S-AmNgn2 induced on average 20-30% more *Delta* transcripts than mNgn2.

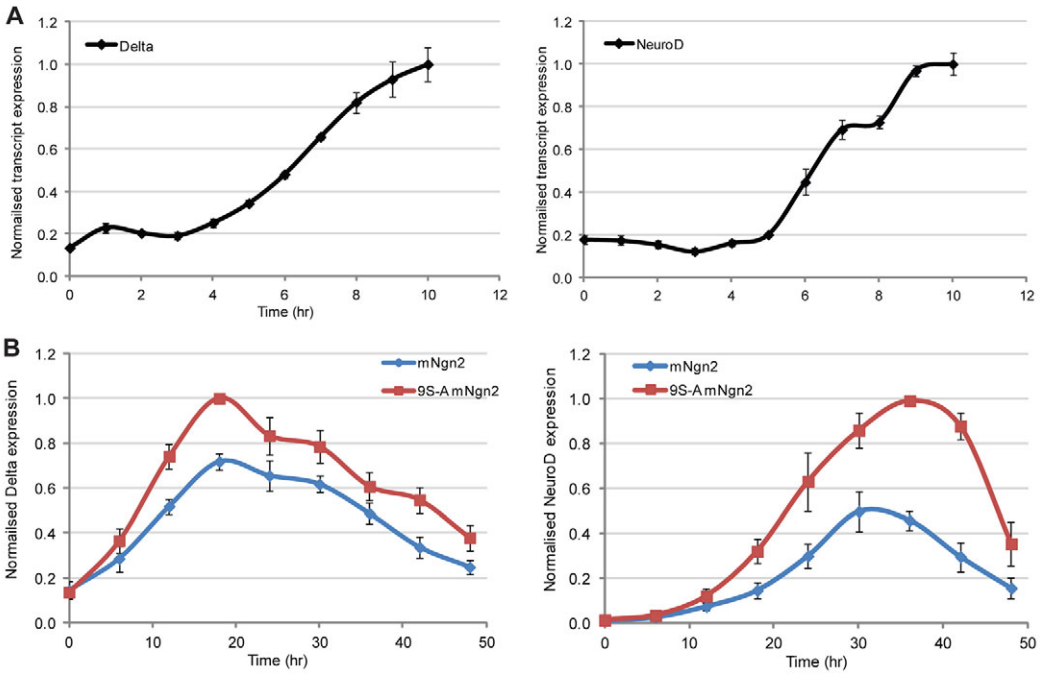

Fig. 2. Ngn2 phosphorylation differentially regulates timing of *NeuroD* and *Delta* transcription. qPCR analysis of *NeuroD* and *Delta* expression in P19 cells (**A**) over 10 hours after mNgn2 transfection or (**B**) over 48 hours after transfection with mNgn2 or 9S-A mNgn2. Average fold increase in mRNA expression is shown normalised to the maximum expression of each transcript, with mean normalised values calculated together with s.e.m.

After peaking at ~18 hours, *Delta* expression in response to mNgn2 and 9S-A mNgn2 declined at similar rates. As previously seen, the onset of *NeuroD* transcription showed a longer lag than that of *Delta* after transfection of mNgn2 or 9S-A mNgn2. However, once *NeuroD* transcription had commenced, 9S-ANgn2 expression resulted in a more rapid accumulation of *NeuroD* transcripts than wild-type Ngn2, resulting in a 2-fold enhancement of expression by 18 hours. Moreover, whereas expression of *NeuroD* in response to Ngn2 peaked at ~30 hours and then underwent slow decline, expression in response to 9S-A Ngn2 continued to rise until ~35 hours before declining more rapidly (Fig. 2B).

Taken together, it is clear that 9S-A Ngn2 induces *NeuroD* expression more rapidly and for longer when compared with induction by the wild-type protein, whereas *Delta* expression profiles are similar after Ngn2 or 9S-A Ngn2 transfection. These data provide further confirmation that the *Delta* and *NeuroD* promoters have differential responses to distinct phosphorylated forms of Ngn2 protein. This is consistent with a model in which these differences are a result of the differing affinity of phosphorylated forms of Ngn2 for E-box-containing promoters (Ali et al., 2011), resulting in differential gene expression depending on the extent of chromatin remodelling required for promoter activation.

To test more directly whether manipulating the stability of Ngn2, and hence its ability to bind DNA, has different effects on the *NeuroD* and *Delta* promoters, we investigated whether artificially destabilised N-terminally ubiquitin-fused forms of *Xenopus* Ngn2 (UbxNgn2 and Ub9S-A xNgn2), which would target Ngn2 for rapid destruction via the ubiquitin fusion/degradation (UFD) pathway (Johnson et al., 1995), would differentially affect *Delta* and *NeuroD* transcription. First, we investigated the half-life of these proteins in in vitro degradation assays in *Xenopus* egg extracts (Vosper et al., 2007; Vosper et al., 2009).

As expected, in *Xenopus* egg extract, N-terminal ubiquitin fusion decreased the half-life of UbxNgn2 and Ub9S-A xNgn2, resulting in proteins that were approximately half as stable as the non-ubiquitylated forms (Fig. 3A). E protein addition has been shown to stabilise Ngn2 protein (Vosper et al., 2007) (Fig. 3A). Addition

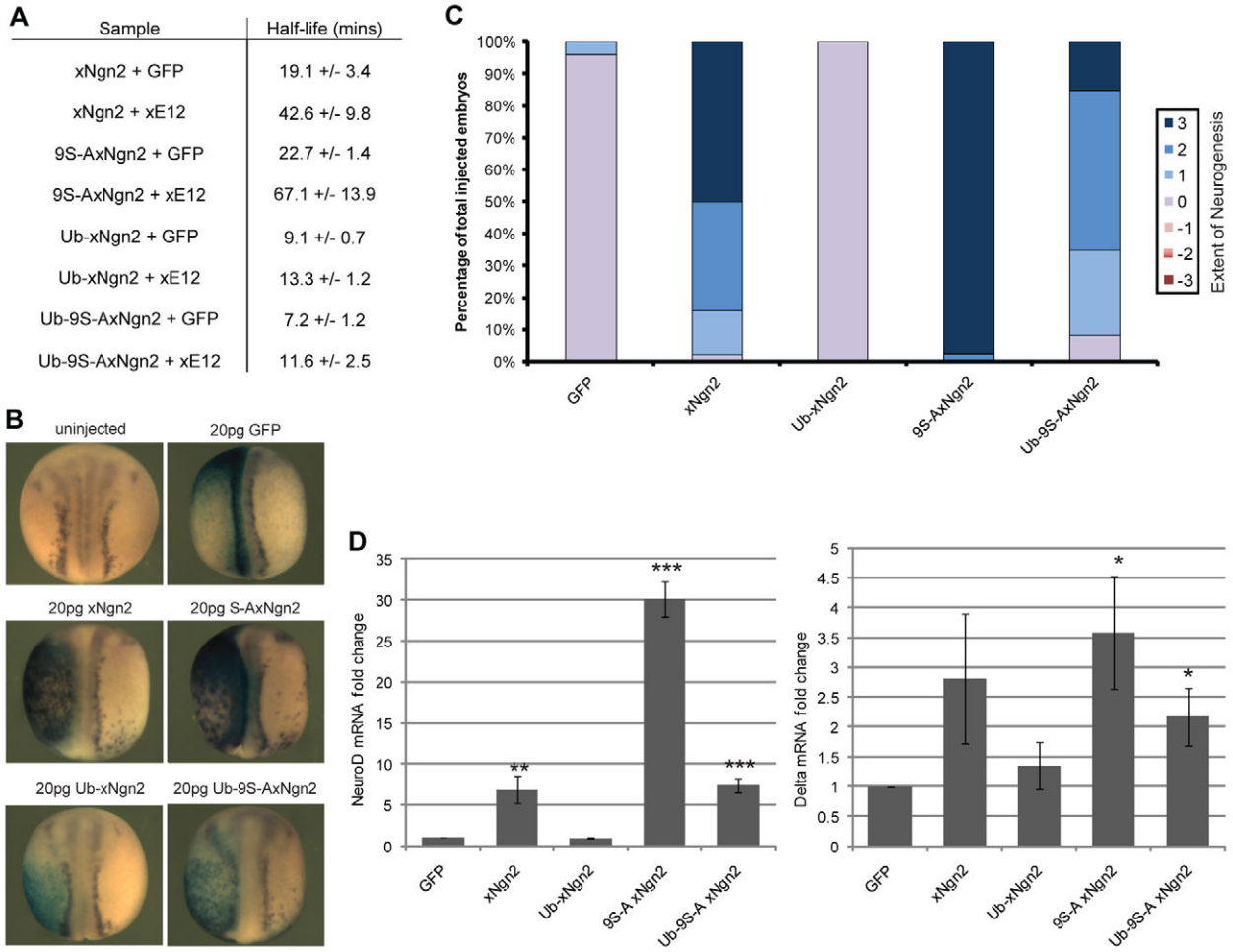

Fig. 3. The *NeuroD* promoter requires greater Ngn2 availability for activation than the *Delta* promoter. (**A**) The half-life of Ngn2 proteins, calculated using first-order rate kinetics (errors calculated using s.e.m.). In vitro translated ^{35}S-methionine-labelled xNgn2, UbxNgn2, 9S-A xNgn2 or Ub9S-AxNgn2 were incubated in *Xenopus* mitotic egg extracts with unlabelled in vitro translated GFP or E12, and the rate of degradation was determined. (**B**) *Xenopus* embryos were injected in one of two cells with 20 pg xNgn2, UbxNgn2, 9S-A xNgn2 or Ub9S-A xNgn2 mRNA and subject to in situ hybridisation at stage 15 for neural β-tubulin expression ($n \geq 76$) (injected side, left). (**C**) Semi-quantitative analysis of in situ hybridisation data ($n \geq 76$). (**D**) qPCR analysis of *NeuroD* and *Delta* in *Xenopus* embryos. Embryos were injected at the one-cell stage with 20 pg xNgn2, UbxNgn2, 9S-A xNgn2 or Ub9S-A xNgn2 mRNA and harvested at stage 15. Average fold increase in mRNA expression compared with the GFP control, normalised to house keeping gene expression (*ODC*), with mean normalised values calculated together with the s.e.m. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

of E12 led to only a small increase in the UbxNgn2 and Ub9S-A xNgn2 half-lives, which were still considerably shorter than those of their non-ubiquitylated counterparts with or without E12 (Fig. 3A).

We then tested the ability of these Ngn2 derivatives to drive neuronal differentiation in developing *Xenopus* embryos. Whereas injected xNgn2 mRNA induced moderate ectopic neurogenesis, the same amount of UbxNgn2 mRNA was completely inactive in this assay, indicating that the Ngn2 protein half-life regulates its ability to drive neuronal differentiation. However, both 9S-A xNgn2 and Ub9S-A xNgn2 could induce ectopic neurogenesis (Fig. 3B,C), with 9S-A xNgn2 showing the greatest activity. This indicates that mutating phosphorylation sites, and hence promoting Ngn2-E protein complex stability and enhancing DNA binding, can at least partially compensate for constitutive Ngn2 ubiquitylation and reduced half-life when assaying the ability to induce neuronal differentiation.

Ubiquitylation of xNgn2 resulted in reduced *Delta* expression (Fig. 3D), which was not significantly rescued by mutation of xNgn2 phosphorylation sites, confirming that phosphorylation of xNgn2 does not play a major role in the regulation of *Delta* expression. By contrast, whereas UbxNgn2 was completely unable to upregulate *NeuroD*, Ub9S-A xNgn2 elevated *NeuroD* transcription ~7-fold (Fig. 3D). Thus, mutation of phosphorylation sites in Ngn2 that enhance promoter binding can partially compensate for a shorter protein half-life when driving *NeuroD* expression but not when driving *Delta* transcription. This is consistent with the stability of promoter association playing a key role in regulating the level of *NeuroD* transcription but not that of *Delta*.

We find that un(der)phosphorylation of Ngn2 promotes transcription of *NeuroD*, driving cell-autonomous differentiation, while having little effect on the transcription of *Delta* that activates Notch signalling in adjacent cells, promoting progenitor maintenance. Mechanistically, as well as effecting *Ngn2* transcriptional repression, Notch-induced Hes1 protein inhibits Ngn2 post-translationally by binding and sequestering E proteins, inhibiting proneural protein-mediated transcriptional activation directly (Kageyama et al., 2008a). Un(der)phosphorylated Ngn2 is stabilised by E protein binding to a greater extent than the wild-type protein (Ali et al., 2011) (Fig. 3A), and also binds to downstream target promoters more

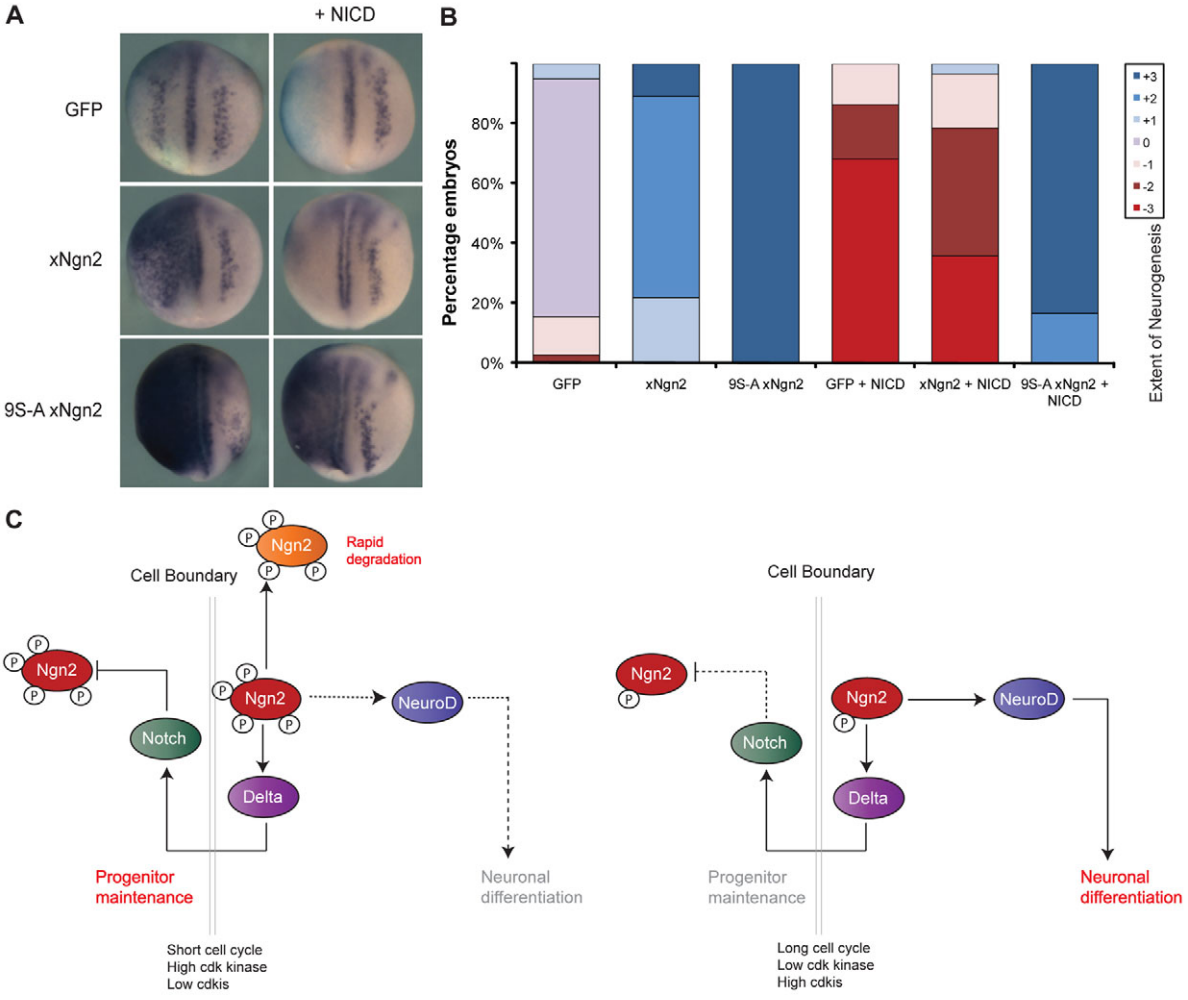

Fig. 4. 9S-A Ngn2 is less sensitive than Ngn2 to Notch signalling. (**A**) *Xenopus* embryos were injected in one of two cells with 20 pg mRNA for GFP, xNgn2 or 9S-A xNgn2 and with 1 ng mRNA for GFP or Notch intracellular domain (NICD) as indicated (injected side, left). Stage 15 embryos were subject to in situ hybridisation for neural β-tubulin expression. (**B**) Semi-quantitative analysis of in situ hybridisation data (see supplementary material Fig. S2 for scoring schematic); *n*=23-33. (**C**) Model to explain why phosphorylated Ngn2 favours progenitor maintenance, whereas un(der)phosphorylated Ngn2 favours neuronal differentiation. See text for description. cdkis, cyclin-dependent kinase inhibitors.

efficiently (Ali et al., 2011), indicating more stable E protein association. We speculated that the ability of Notch signalling to regulate Ngn2 protein function might depend upon the phosphorylation status of Ngn2, and a feed-forward loop might be set up when Ngn2 phosphorylation drops whereby the reduction of Cdk kinase activity results in both a Ngn2-driven relative enhancement of NeuroD expression over Delta expression and an increase in the resistance of Ngn2-dependent transcription to Notch-mediated inhibition.

To test this, we injected mRNA encoding the constitutively active Notch intracellular domain (NICD) (Chitnis et al., 1995) along with xNgn2, 9S-A xNgn2 or GFP (as a control), then investigated subsequent neuronal differentiation. As expected, NICD inhibited differentiation of endogenous primary neurons on the injected side (Fig. 4A,B). In addition, NICD inhibited the ability of xNgn2 mRNA to induce ectopic primary neurons, demonstrating post-transcriptional inhibition of xNgn2 function. However, 9S-A xNgn2 was essentially completely resistant to the inhibitory activity of Notch signalling (Fig. 4A,B). This might be a consequence of more stable Ngn2-E protein binding to DNA, resulting in resistance to the E protein sequestration by the Hes1 protein that is induced by Notch signalling.

Taken together, our results suggest a model (Fig. 4C) whereby phospho-Ngn2 would predominate in rapidly cycling cells, resulting in Ngn2-E protein dimers with weak promoter affinity. This is insufficient to activate the *NeuroD* promoter (Figs 1, 2), an activation that is required for cell-autonomous neuronal differentiation. However, phospho-Ngn2 is still able to activate the *Delta* promoter, resulting in non-cell-autonomous Notch-mediated progenitor maintenance.

By contrast, within individual cells, cell cycle lengthening favours un(der)phosphorylated Ngn2-E protein dimers that exhibit greater promoter occupancy (Ali et al., 2011), which would be permissive for both *NeuroD* and *Delta* transcription. This un(der)phosphorylated Ngn2 is resistant to Notch-mediated inhibition (Fig. 4), which further potentiates expression of downstream targets, including NeuroD (Figs 1, 2). In addition, the accumulating NeuroD protein thus produced is relatively insensitive to Delta-driven Notch-mediated inhibition (Chitnis and Kintner, 1996). Rising NeuroD expression against a backdrop of constant Delta-mediated Notch signalling (Fig. 1), coupled with a growing resistance of un(der)phosphorylated Ngn2 to Notch-mediated lateral inhibition (Fig. 4), favour differentiation over progenitor maintenance in response to cell cycle lengthening.

In reality, within a population of neural progenitors the cell cycle length may be variable while showing an average increase. This variation in cell cycle length and hence variation in Ngn2 protein activity might contribute to the 'salt and pepper' heterogeneity of neuronal differentiation seen during CNS development. Moreover, SP pairs can act as substrates for a variety of kinases, such as GSK3β [which have already been shown to target SP sites in Ngn2 (Ma et al., 2008)] and MAP kinases, so phosphoregulation of Ngn2 might act to integrate a number of cellular signalling events in addition to cell cycle length.

Acknowledgements
We thank Helen Wise, Christelle Fiore-Heriche, Romana Kucerova and Jon Vosper for important preliminary observations; Chris Hurley and Venkat Pisupati for technical assistance; and Luke Wylie, Gert Veenstra, Adele Murrell, Jeremy Gunewardena, Hanno Steen, Marc Kirschner and Bill Harris for helpful discussions.

Funding
This work was supported by a Medical Research Council (MRC) Research Grant [G0700758 to A.P. and F.A.]; MRC DTA Studentship (G.M.); Cancer Research UK Studentship (C.H.); and MRC Grant-in-Aid [U117570528 to F.G.]. Deposited in PMC for release after 6 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.077552/-/DC1

References

Ali, F., Hindley, C., McDowell, G., Deibler, R., Jones, A., Kirschner, M., Guillemot, F. and Philpott, A. (2011). Cell cycle-regulated multi-site phosphorylation of Neurogenin 2 coordinates cell cycling with differentiation during neurogenesis. *Development* **138**, 4267-4277.
Bertrand, N., Castro, D. S. and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**, 517-530.
Chitnis, A. and Kintner, C. (1996). Sensitivity of proneural genes to lateral inhibition affects the pattern of primary neurons in Xenopus embryos. *Development* **122**, 2295-2301.
Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D. and Kintner, C. (1995). Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. *Nature* **375**, 761-766.
Hager, G. L., McNally, J. G. and Misteli, T. (2009). Transcription dynamics. *Mol. Cell* **35**, 741-753.
Johnson, E. S., Ma, P. C., Ota, I. M. and Varshavsky, A. (1995). A proteolytic pathway that recognizes ubiquitin as a degradation signal. *J. Biol. Chem.* **270**, 17442-17456.
Kageyama, R., Ohtsuka, T. and Kobayashi, T. (2008a). Roles of Hes genes in neural development. *Dev. Growth Differ.* **50 Suppl. 1**, S97-S103.
Kageyama, R., Ohtsuka, T., Shimojo, H. and Imayoshi, I. (2008b). Dynamic Notch signaling in neural progenitor cells and a revised view of lateral inhibition. *Nat. Neurosci.* **11**, 1247-1251.
Koyano-Nakagawa, N., Wettstein, D. and Kintner, C. (1999). Activation of Xenopus genes required for lateral inhibition and neuronal differentiation during primary neurogenesis. *Mol. Cell. Neurosci.* **14**, 327-339.
Lange, C. and Calegari, F. (2010). Cdks and cyclins link G(1) length and differentiation of embryonic, neural and hematopoietic stem cells. *Cell Cycle* **9**, 1893-1900.
Lee, J. E. (1997). Basic helix-loop-helix genes in neural development. *Curr. Opin. Neurobiol.* **7**, 13-20.
Ma, Q., Kintner, C. and Anderson, D. J. (1996). Identification of neurogenin, a vertebrate neuronal determination gene. *Cell* **87**, 43-52.
Ma, Y. C., Song, M. R., Park, J. P., Henry Ho, H. Y., Hu, L., Kurtev, M. V., Zieg, J., Ma, Q., Pfaff, S. L. and Greenberg, M. E. (2008). Regulation of motor neuron specification by phosphorylation of neurogenin 2. *Neuron* **58**, 65-77.
Michel, D. (2009). Fine tuning gene expression through short DNA-protein binding cycles. *Biochimie* **91**, 933-941.
Seo, S., Richardson, G. A. and Kroll, K. L. (2005). The SWI/SNF chromatin remodeling protein Brg1 is required for vertebrate neurogenesis and mediates transactivation of Ngn and NeuroD. *Development* **132**, 105-115.
Seo, S., Lim, J. W., Yellajoshyula, D., Chang, L. W. and Kroll, K. L. (2007). Neurogenin and NeuroD direct transcriptional targets and their regulatory enhancers. *EMBO J.* **26**, 5093-5108.
Shimojo, H., Ohtsuka, T. and Kageyama, R. (2008). Oscillations in notch signaling regulate maintenance of neural progenitors. *Neuron* **58**, 52-64.
Stavreva, D. A., Muller, W. G., Hager, G. L., Smith, C. L. and McNally, J. G. (2004). Rapid glucocorticoid receptor exchange at a promoter is coupled to transcription and regulated by chaperones and proteasomes. *Mol. Cell. Biol.* **24**, 2682-2697.
Vernon, A. E., Devine, C. and Philpott, A. (2003). The cdk inhibitor p27Xic1 is required for differentiation of primary neurones in Xenopus. *Development* **130**, 85-92.
Vosper, J. M., Fiore-Heriche, C. S., Horan, I., Wilson, K., Wise, H. and Philpott, A. (2007). Regulation of neurogenin stability by ubiquitin-mediated proteolysis. *Biochem. J.* **407**, 277-284.
Vosper, J. M., McDowell, G. S., Hindley, C. J., Fiore-Heriche, C. S., Kucerova, R., Horan, I. and Philpott, A. (2009). Ubiquitylation on canonical and non-canonical sites targets the transcription factor neurogenin for ubiquitin-mediated proteolysis. *J. Biol. Chem.* **284**, 15458-15468.
Voss, T. C., Schiltz, R. L., Sung, M. H., Yen, P. M., Stamatoyannopoulos, J. A., Biddie, S. C., Johnson, T. A., Miranda, T. B., John, S. and Hager, G. L. (2011). Dynamic exchange at regulatory elements during chromatin remodeling underlies assisted loading mechanism. *Cell* **146**, 544-554.