Maintenance of motor neuron progenitors in *Xenopus* requires a novel localized cyclin

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The ventral spinal cord contains a pool of motor neuron progenitors (pMNs), which sequentially generate motor neurons and oligodendrocytes in the embryo. The mechanisms responsible for the maintenance of pMNs are not clearly understood. We have identified a novel cyclin, *cyclin Dx* (*ccndx*), which is specifically expressed in pMNs in *Xenopus*. Here, we show that inhibition of *ccndx* causes paralysis in embryos. Furthermore, we show that maintenance of pMNs requires *ccndx* function. In addition, inhibition of *ccndx* results in the specific loss of differentiated motor neurons. However, the expression of interneuron or sensory neuron markers is unaffected in these embryos, suggesting that the role of *ccndx* is specifically to maintain pMNs. Thus, we have identified, for the first time, a tissue-specific cell-cycle regulator that is essential for the maintenance of a pool of neural progenitors in the vertebrate spinal cord.

Keywords: cell cycle; cyclin; *Xenopus tropicalis*; neurogenesis; motor neuron

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INTRODUCTION

The G1 phase of the cell cycle is important during neurogenesis and histogenesis; therefore, factors controlling this phase of the cycle have received much attention. Interestingly, the D-type cyclins, which are G1-phase regulators, are expressed in dynamic and tissue-specific patterns during embryonic development, often in mutually exclusive cell types in the CNS (Wianny *et al*, 1998). Knockout studies of the three murine D-type cyclins have shown that they have distinct roles in regulating the differentiation of specific neuronal cell types. *Cyclin D1*^{−/−} mice show a striking reduction in the thickness of all retinal layers owing to a decrease

in proliferation and specific apoptosis of photoreceptor cells (Sicinski *et al*, 1995; Ma *et al*, 1998). The cerebella of *cyclin D2*^{−/−} mice show a reduction in the number of granule cells and satellite interneurons, but not Golgi and basket interneurons. These neurons might arise from the same progenitors, suggesting that *Cyclin D2* is required not only for proliferation but also for correct differentiation of different neuronal cell types in the cerebellum (Huard *et al*, 1999). *Cyclin D3*^{−/−} mice are viable, but show lymphoid abnormalities (Sicinska *et al*, 2003). Mice lacking more than one of the D-type cyclins show phenotypes that represent the sum of the abnormalities observed in mice lacking individual cyclins (Ciemerych *et al*, 2002). Recent generation and characterization of mice lacking all the three D-cyclins unexpectedly exhibit relatively normal embryonic development until mid-gestation (Kozar *et al*, 2004). Interestingly, most cell types could proliferate in the absence of the D-type cyclins, with the exception of myocardial cells and haematopoietic stem cells, suggesting that only these cells critically require D-type cyclins for their proliferation (Kozar *et al*, 2004).

In *Xenopus*, a number of G1/S-phase cyclins have been identified including two D-type cyclins (Taieb & Jessup, 1996; Taieb *et al*, 1997), three E-type cyclins (Rempel *et al*, 1995; Chevalier *et al*, 1996) and two A-type cyclins (Minshull *et al*, 1990; Kobayashi *et al*, 1991). These G1 cyclins also show dynamic and tissue-restricted expression patterns during neurogenesis (Vernon & Philpott, 2003). However, a role for G1-phase cyclins during neurogenesis has not been addressed in *Xenopus*. We have isolated a novel D-type cyclin, which is specifically located within the ventral hindbrain and the ventral neural tube. This prompted us to investigate whether this novel cyclin, which we named Cyclin Dx (*Ccndx*), might regulate neurogenesis in the ventral neural tube.

RESULTS AND DISCUSSION

Cyclin Dx is a novel D-type cyclin

We recently performed an enhanced large-scale functional screen using a full-length *Xenopus tropicalis* clone set with the aim of identifying novel genes involved in early embryogenesis (Chen *et al*, 2005). One of the genes isolated in the screen, TGas111k06, led to a consistent phenotype with regards to apoptosis and showed a highly restricted pattern of expression. Sequence

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Fig 1 | Cyclin Dx is a novel D-type cyclin in *Xenopus*. (A) Protein sequence alignment of *X. tropicalis* Ccnd1 and Ccndx. Predicted retinoblastoma (pRb)-binding domain (green line) and conserved cyclin-box domain (red line) are indicated. Residues that match the consensus exactly are shaded black. (B) Ccndx binds to Cdk4. *Xenopus* embryos were injected with the indicated messenger RNAs and HEK 293T cells were cotransfected with the indicated constructs and lysed for co-immunoprecipitation (IP) using the Myc antibody. The immunoblot was then probed using the antibodies, as indicated. (C–E) Comparison of the expression patterns of *ccndx* (purple) and *ccnd1* (blue) shown by whole-mount double *in situ* hybridization at the neurula stage (St 18). The expression of *ccndx* is localized to the ventral spinal cord (vs), ventral retina (vr) and olfactory placode (op) at the tailbud stage (St 28). Transverse section in the eye (blue dash line) and spinal cord (orange dash line) shows that *ccndx* is localized to the ventral retina (D) and ventral spinal cord in a domain that includes the progenitors of motor neurons incorporating BrdU⁺ cells (E). The neural tube edge is demarcated with pink.

analysis showed that clone TGas111k06 is a novel gene encoding a predicted protein of 290 amino acids with 55% sequence identity to *Xenopus* Cyclin D1 (Fig 1A). In addition, TGas111k06 contains a retinoblastoma (pRb)-binding domain (Dowdy *et al*, 1993; Ewen *et al*, 1993) and a cyclin box domain (Zwicker *et al*, 1999), which indicate that TGas111k06 might be a new member of D-type cyclins (Fig 1A).

To confirm whether TGas111k06 encoded a novel D-type cyclin, we investigated whether it could bind to Cyclin-dependent kinase 4 (Cdk4) and phosphorylate pRb *in vivo*. Indeed, co-immunoprecipitation (co-IP) experiments showed that this novel cyclin protein could bind to Cdk4 in *Xenopus* embryos and HEK 293T cells (Fig 1B), resulting in hyperphosphorylation of pRb at serine 780, a Cdk4-specific site (Kitagawa *et al*, 1996; supplementary Fig S1 online). On the basis of these results, as well as the fact that this novel cyclin was more similar to mammalian Cyclin D1 than Cyclin D3, and the apparent lack of a clear *cyclin D3*

orthologue in the *X. tropicalis* genome, we have named this novel gene *cyclin Dx* (*ccndx*).

To test whether the expression pattern of *ccndx* was different from *ccnd1*, we performed whole-mount double *in situ* hybridization assays with both genes in *X. tropicalis* embryos (Fig 1C, left panel; supplementary Fig S2 online). At the late neural plate stage (St 18–20), *ccndx* expression was localized to the hindbrain and the ventral neural tube. By contrast, *ccnd1* transcripts were highly enriched in the midbrain–hindbrain boundary (MHB) and anterior neural plate (Fig 1C, left panel). No obvious overlapping domain between *ccnd1* and *ccndx* was shown by these assays, suggesting that each D-type cyclin might have specific functions in regulating local proliferation and/or differentiation, allowing them to function in a highly optimized manner (Ciemerych *et al*, 2002).

In tailbud-stage embryos (St 28), *ccndx* expression remained localized to the ventral hindbrain and spinal cord, with further domains apparent in the ventral retina and olfactory region (right

Fig 2 | Phenotype of *ccndx*-deficient embryos. (A) Diagram depicting the target location of the different MOs. (B) Reverse transcription (RT)-PCRs of MO-injected embryos. MOs targeting splice junctions result in the shift of bands (truncated, arrowheads) or absence of transcripts relative to the controls. Ui, uninjected embryos; Ct, 5-mismatch e1i1 control MO. (C,D) Embryos injected with *ccndx* (Dx) MOs have defects in the formation of the ventral retina (D, arrowhead), relative to control MO-injected embryos (C). Ct MO, 5-mismatched e1i1 control MO; Dx MO, e1i1 + e2i2 combined MOs; MO, morpholino antisense oligonucleotides; St 40, stage 40.

panel in Fig 1C and St 28, Fig 1D). Interestingly, *ccndx* is expressed in the apical ciliary margin zone (CMZ) of ventral retina at the tadpole stage (St 42, Fig 1D). Further investigation of the expression of *ccndx* in the ventral spinal cord showed that it is highly localized to the domain containing the progenitors of motor neurons (pMNs; Fig 1E, left panel) and in proliferating cells as shown by bromodeoxyuridine (BrdU) incorporation (Fig 1E, right panel). The highly localized expression pattern of *ccndx* prompted us to examine whether it is crucial for the maintenance of pMNs.

Loss of *ccndx* function leads to paralysis

We designed morpholino antisense oligonucleotides (MOs) to block either *ccndx* protein translation or mRNA splicing (Fig 2A). Each MO was designated by its target location: for example, ATG for the initiation codon; e1i1 for the exon 1–intron 1 boundary. To assess whether MOs targeting the splice junctions could interfere with the endogenous *ccndx* transcripts *in vivo*, we injected experimental and control MOs into one-cell stage embryos, allowed them to develop until the late neurula stage (St 20), and performed reverse transcription–PCR (RT–PCR) using primers located in exon1 and exon3 of the *ccndx* gene. As a control, a MO containing five mismatches to the e1i1 sequence control MO (Ct MO) was used. Control MO-injected embryos contained correctly spliced *ccndx* mRNA, whereas embryos injected with MOs targeting the splice junctions resulted in incorrectly spliced products or complete loss of amplified products (Fig 2B). *Ccndx* morphants appeared to be morphologically normal at the tailbud stage (St 25), apart from a small delay in development and a slight anterior/posterior truncation (supplementary movie online). This result was consistent with a reduction in body size observed in D-type cyclin-deficient mice (Ciemerych et al, 2002; Kozar et al, 2004). Further inspection showed that the

Fig 3 | *Ccndx* function is required to maintain motor neuron progenitors. (A) Experimental design. Embryos were injected at the one-cell stage, fixed at the early tailbud stage and analysed with the markers indicated. (B,C) The maintenance of pMNs is lost when *ccndx* function is inhibited, as assayed by *olig2* expression. However, neither the patterning of dorsal progenitors (*irx3*) nor ventral progenitors (*nkx6*) is affected by the loss of *ccndx* function. Ct MO, 5-mismatched e1i1 control MO; Dx MO, e1i1 + e2i2 combined MOs; MO, morpholino antisense oligonucleotides; pMNs, progenitors of motor neurons.

main defect in *ccndx* morphants was a lack of hatching response or to touch stimulation even after St 35 (early tadpole stage), when primary neurons readily mediate an escape response in Ct MO-injected embryos or uninjected control embryos (see supplementary movie online). At the tadpole stage (St 40), *ccndx* MO-injected embryos showed a defect in the formation of the ventral retina (Fig 2D), which was consistent with *ccndx* expression in this region (Fig 1C). All MOs designed to inhibit *ccndx* function gave rise to the same paralysed phenotype, suggesting that the phenotype was specific (supplementary Fig S3 online). As *ccndx* is maternally expressed (supplementary Fig S2 online), and we were particularly interested in the function of the zygotic transcripts in the CNS, we performed most of our subsequent knockdown experiments using combined splice MOs (e1i1 + e2i2; Dx MO).

ccndx is required to maintain pMNs in the spinal cord

To investigate whether *ccndx* affects dorso–ventral (D–V) patterning of the neural tube, we cloned the *X. tropicalis* orthologues of *irx3* and *nkx6* and used them as markers for the patterning of neuronal progenitors in the spinal cord (Briscoe et al, 2000). Transverse sections of the spinal cord showed that these genes exhibited a similar pattern of expression along the D–V axis in the neural tube in *X. tropicalis*, as compared with amniotic embryos (see Ct MO in Fig 3B). Compared with Ct MO-injected embryos,

Fig 4 | *Ccndx* function is required for motor neuron differentiation. (A) Experimental design. Embryos were injected at the one-cell stage, fixed at the late tailbud or tadpole stages and analysed with the markers or antibody indicated. INs, interneurons; MNs, motor neurons; pDNs: pan-differentiated neurons. (B) Inhibition of *ccndx* results in the specific loss of differentiated motor neurons (*isl1* in MN domain and *n-tubulin* at the ventral spinal cord), but interneurons are not affected (*evx1*). (C) Cell proliferation is reduced in the ventral spinal cord of *ccndx* morphants, as assayed by BrdU incorporation. d, dorsal spinal cord; v, ventral spinal cord. (D) Inhibition of *ccndx* causes the loss of differentiated motor neurons, assayed by *isl1*, and failure of the embryos to extend motor neuron axons, as assayed by immunofluorescence of acetylated tubulin. Ct MO, 5-mismatched e1i1 control MO; Dx MO, e1i1 + e2i2 combined MOs; MO, morpholino antisense oligonucleotides; RB, Rohon-Beard sensory neurons.

neither the distribution of the domain of dorsal neuronal progenitors (*irx3*; 77%, $n = 52$) nor the domain of ventral neuronal progenitors (*nkx6*; 79%, $n = 82$) were affected in the *ccndx* morphants (Fig 3B). However, the expression of *olig2*, a marker of pMNs (Novitsch et al, 2001), was absent in *ccndx* morphant embryos. These results indicate that the function of *ccndx* was to maintain pMNs, and not the patterning of neuronal progenitors in the spinal cord (Fig 3C; whole-mount *in situ*s are shown in supplementary Fig S4 online).

The expression domain of *ccndx* is broader than pMN marker *olig2* (Figs 1C, 3B); therefore, we investigated whether *ccndx* is required for ventral interneuron differentiation, in addition to motor neuron differentiation. We cloned the differentiated motor

neuron marker *isl1* (Pfaff et al, 1996) and the interneuron marker *evx1* (Moran-Rivard et al, 2001) from *X. tropicalis*. *Isl1* was expressed in the domain of differentiated motor neurons in the ventral neural tube and the domain of the Rohon-Beard (RB) sensory neurons in the dorsal spinal cord, whereas the expression of *evx1* was highly restricted in the ventral interneuron domain (see Ct MO in Fig 4B).

Embryos lacking *ccndx* function were analysed for the expression of *isl1*, *evx1* and *n-tubulin*, a general marker for differentiated neurons (Fig 4A; whole-mount *in situ* results are shown in the supplementary Fig S4 online). The expression of *isl1* was largely reduced in the domain of differentiated motor neurons, whereas *isl1* expression in the Rohon-Beard sensory

neuron domain was unaffected (64%, $n=55$; Fig 4B,D). This finding provided a good internal control for the specificity of the effect of knocking down *ccndx* function in the ventral spinal cord. Furthermore, all MOs targeting *ccndx* gave rise to this phenotype, confirming the specificity of the result (data not shown). In addition, *n-tubulin* was specifically eliminated in the ventral, but not the dorsal, neural tube in the *ccndx*-deficient embryos (65%, $n=48$; Fig 4B). Finally, the expression of the interneuron marker, *evx1*, was not affected by inhibiting *ccndx* (Fig 4B), thus providing strong evidence that the function of *ccndx* is very specific for motor neuron differentiation in the spinal cord.

To determine whether the loss of pMNs was caused by a failure of these cells to proliferate, we counted BrdU⁺ nuclei in consecutive sections along the spinal cord, covering a distance of 100 μ m (average of five sections per embryo). We found that although the number of BrdU⁺ nuclei in the dorsal spinal cord was unaffected (Ct: 32 ± 5.5 nuclei, $n=5$ embryos; Dx: 29 ± 6.6 nuclei, $n=9$), there was a significant reduction in BrdU⁺ nuclei in the ventral domain (Fig 4C; Ct: 30 ± 3.8 nuclei, $n=5$; Dx: 15 ± 5.4 nuclei, $n=9$; representative sections are shown in Fig 4B). The decrease of about 15 BrdU⁺ nuclei in 100 μ m corresponds to the number of BrdU⁺ cells that normally express *ccndx* in the spinal cord over 100 μ m (15 ± 5 cells, $n=3$; representative section shown in Fig 1E). Thus, we conclude that *ccndx* is required for the continued proliferation of the pMNs in the ventral spinal cord.

To examine whether motor axon formation was affected by inhibiting *ccndx*, we stained tadpoles with an antibody for acetylated tubulin. In control tadpoles, the tracts of motor neuron axons travelled from the spinal cord down the intermyotomal cleft. By contrast, axon projections were severely disrupted in the *ccndx* morphant tadpoles (Fig 4D). This result confirmed that the phenotype of paralysis in *ccndx* morphants was due to the defect in motor neuron formation.

Summary

Here, we describe a novel D-type cyclin, *ccndx*, which is specifically expressed in pMNs in *Xenopus* and that is essential for the maintenance of these cells (Fig 5). Maintenance of a pool of pMNs is likely to be essential for the generation of motor neuron subtypes and oligodendrocytes as the tadpole grows. The identification of a pMN-specific cyclin supports a model in which specific cyclins have essential roles in the maintenance of the specific progenitor pools of neural cells in the CNS (Kowalczyk *et al*, 2004; Berger *et al*, 2005). It will be interesting to determine whether similar mechanisms are involved in regulating the maintenance of different pools of stem cells and/or progenitor cells in the spinal cord in other vertebrates.

METHODS

Isolation of *ccndx* and plasmid constructs. *Ccndx* (TGas111k06 or GenBank accession code DQ508541) was identified in a gain-of-function screen (Chen *et al*, 2005). Other *X. tropicalis* clones used here were identified by searching the *X. tropicalis* Full-Length Project database (Gilchrist *et al*, 2004). These include *ccnd1* (TGas026a07), *evx1* (TTbA025j08), *irx3* (TNeu131p22), *isl1* (TTPA069e10) and *nkx6* (TNeu010j02). The *olig2* was cloned from embryos by RT-PCR and subcloned into pCR-BluntII-TOPO. Tagged constructs used here include *X. laevis* Myc-tagged *cdk4*

Fig 5 | Summary of Cyclin Dx function. In the spinal cord, *ccndx* is specifically expressed in pMNs and ensures that adequate numbers of differentiated motor neurons are generated, which are needed for the establishment of the constitution of functional motor circuits, and proper swimming and escape responses. In the absence of *ccndx*, tadpoles are paralysed owing to the loss of pMNs. However, the expression of interneuron (*evx1*) or sensory neurons (*isl1* and the *n-tubulin* in dorsal sensory neuron domain) is not affected when *ccndx* is inhibited. Thus, *ccndx* is a novel localized neuronal regulator that is essential for the maintenance of a pool of neural progenitor cells in the vertebrate spinal cord. MN, motor neurons; pMNs, progenitors of motor neurons.

in FTX5 (a gift from Angel R. Nebreda; Dinarina *et al*, 2005), *X. tropicalis* HA-tagged *ccnd1* and *ccndx* in pcHA (*Bam*HI-*Xho*I). **Whole-mount *in situ* hybridization, BrdU detection, immunofluorescence and histology.** Whole-mount *in situ* hybridization and double *in situ* hybridization of *X. tropicalis* embryos were performed as described (Chen *et al*, 2005). BrdU incorporation was then detected by using the substrate diaminobenzidine (Sigma-Aldrich, Dorset, UK) as previously described (Carruthers *et al*, 2003). Some specimens were sectioned after staining and these were post-fixed overnight in MEMFA (0.1 M MOPS pH 7.5, 2 mM EGTA, 1 mM MgSO₄, 3.7% formaldehyde) and then embedded in gelatin/albumin mixture and solidified with glutaraldehyde. Sections (20–30 μ m) were cut on a Leica VT1000M vibrotome, mounted in 90% glycerol and photographed with Nomarski optic. Embryos for immunofluorescence staining were fixed in Dent's solution and incubated with the primary antibody (acetylated tubulin, 1/300; Sigma), followed by the secondary antibody, goat anti-mouse Alexa488 (1/200; Invitrogen, Paisley, UK) staining.

Immunoblotting, co-immunoprecipitations and pRb phosphorylation analysis. Co-IP in *Xenopus* was performed as described previously (Dinarina *et al*, 2005). *Xenopus* embryos were injected at the two-cell stage and were analysed at stage 10. For immunoblotting, Myc-HRP and HA-HRP-conjugated antibodies (1/2000; Roche, Lewes, UK) were used. HEK 293T cells were cultured, co-transfected and lysed as described previously (Voigt

& Papalopulu, 2006). Western analysis using phosphoRb S780 (Abcam; 1/1000) and Rb (Sigma; 1/2500) antibodies was performed on H293T-transfected cell lysates.

Morpholino design and reverse transcription-PCR. Splice junctions were identified by aligning complementary DNA sequences with genomic sequences. *X. tropicalis* embryos were injected with 10–20 ng total MOs before first cleavage. MO sequences are listed as follows. Control MO: 5-mis *ccndx* e1i1, 5'-ACcTACgTCCAcCATC CACcAGcTG-3' (mismatch bases are lowercases), ATG MO: *Ccndx* ATG, 5'-CACAGCATAACAGCCTTCCCTCCAT-3', splice MOs (intron sequences are lowercase): e1i1, 5'-acgtacCTCCAGCATCCAG CAGGTG-3'; and e2i2, 5'-ggacacttaccAGCAGTCTTTGTC-3'.

Total RNA was isolated from MO-injected embryos by using the TRIzol reagent according to the manufacturer's instructions (Invitrogen). cDNA was synthesized using Avian Myeloblastosis Virus (AMV) reverse transcriptase (Roche) and PCR was performed using *Taq* polymerase (Roche) according to established protocols. Primer sequences are written 5'–3' as follows: Dx forward, 5'-TGGAGGGAAGGCTGTTATGCTGTG; reverse, 5'-GCCCCGCTG CACTGGAT.

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

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