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ORIGINAL PAPER

Comprehensive expression analysis for the core cell cycle regulators in the chicken embryo reveals novel tissue-specific synexpression groups and similarities and differences with expression in mouse, frog and zebrafish

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Abstract

The core cell cycle machinery is conserved from yeast to humans, and hence it is assumed that all vertebrates share the same set of players. Yet during vertebrate evolution, the genome was duplicated twice, followed by a further genome duplication in teleost fish. Thereafter, distinct genes were retained in different vertebrate lineages; some individual gene duplications also occurred. To which extent these diversifying tendencies were compensated by retaining the same expression patterns across homologous genes is not known. This study for the first time undertook a comprehensive expression analysis for the core cell cycle regulators in the chicken, focusing in on early neurula and pharyngula stages of development, with the latter representing the vertebrate phylotypic stage. We also compared our data with published data for the mouse, Xenopus and zebrafish, the other established vertebrate models. Our work shows that, while many genes are expressed widely, some are upregulated or specifically expressed in defined tissues of the chicken embryo, forming novel synexpression groups with markers for distinct developmental pathways. Moreover, we found that in the neural tube and in the somite, mRNAs of some of the genes investigated accumulate in a specific subcellular localisation, pointing at a novel link between the site of mRNA translation, cell cycle control and interkinetic nuclear movements. Finally, we show that expression patterns of orthologous genes may differ in the four vertebrate models. Thus, for any study investigating cell proliferation, cell differentiation, tissue regeneration, stem cell behaviour and cancer/cancer therapy, it has to be carefully examined which of the observed effects are due to the specific model organism used, and which can be generalised.

KEYWORDS
Cdc25 genes, *Cdk* genes, *Cdkn* genes, cell differentiation, cell division, Chicken embryo, comparison with mouse, frog, zebrafish expression data, *Cyclin* (*Ccn*) genes, in situ hybridisation, myogenesis, neurogenesis, neurula, pharyngula, subcellular mRNA localisation

1 | INTRODUCTION

The cell cycle is one of the fundamental processes in any living organism because it allows the repeated generation of new cells while maintaining genetic and metabolic stability. The cell cycle underpins the ability of unicellular organisms to populate an ecological niche. It was also the prerequisite for the evolution of multicellularity and the numerous cell types we know today. The cell cycle is crucial for embryonic development, tissue homoeostasis, and the various forms of tissue and organ regeneration. An aberrant cell cycle is incompatible with normal development and tissue function, and is the cause of cancer.

A typical animal cell cycle consists of four phases. At the start of the first gap phase (G1), cells decide whether to pursue a cell cycle, whether to adopt a quiescent stage (G0) or whether to exit cell cycle altogether. If a cell continues the cycle, it will replicate its DNA in the synthesis phase (S), prepare for cell division in the second gap phase (G2) and then execute the cell division in the mytosis phase (M). The transition between the distinct phases of the cell cycle is controlled by the type I subfamily of cyclin (Ccn) proteins that bind, trigger conformational changes and thus allow activation of a subfamily of cyclin-dependent kinases (Cdks), the key facilitators of the cycle (reviewed in (Malumbres, 2014; Ruijtenberg and van den Heuvel, 2016). Entry into the cell cycle occurs when in response to mitogens, CcnD genes are upregulated; the proteins bind to Cdk4/6-type proteins, which in turn partially phosphorylate members of the retinoblastoma (Rb) family. This weakens the interaction between the heterodimeric transcription factor E2F/DP (together referred to as E2F), releasing E2F to promote the expression of genes required for the G1-S transition and S phase progression, including CcnE and CcnA. In late G1 phase, CcnE proteins interact with Cdk2-type proteins and complete Rb phosphorylation. As a result, cells reach the restriction point, whereby cells become irreversibly committed to cell division. In a complex with CcnA proteins, Cdk2 proteins then control the S-phase; CcnO is a further, less well-characterised binding partner of Cdk2 (Kim et al., 2014). Cdk1 is the key factor that ensures cell cycle completion: partnered with CcnA proteins, it sees cells through the G2 phase, switching to CcnB proteins, it controls progression through mitosis.

The activity of Ccn–Cdk complexes in their allocated phases of the cell cycle and hence continued, but coordinated proliferation is controlled at four levels (Malumbres, 2014; Molina and Pituello, 2017; Ruijtenberg and van den Heuvel, 2016). First, Ccn proteins contain motifs that permit rapid proteolysis. Thus, the life time of these proteins is short, with CcnDs rapidly disappearing upon mitogen withdrawal, CcnEs peaking at the G1/S transition, CcnAs accumulating during S and G2 phases, and CcnBs peaking at the G2/M transition and persisting during M phase. Second, CcnH together with its Cdk7 partner promotes cell cycle, acting as general Cdk-activating kinase (CAK) by phosphorylating the cell cycle Cdks at sites made available upon interaction with their cognate Ccn. Third, the activity of Cdk proteins is repressed by phosphorylation at specific, conserved threonine and serine residues which prevents cell cycle progression for example in the case of DNA damage. Cdc25 phosphatases promote cell cycle by removing this phosphorylation. Fourth, Cdk activity and hence cell cycle is suppressed by three families of cyclin-dependent kinase inhibitors, the Cdkn1 (Cip/Kip) family, the Cdkn2 (Ink4) family and Cdkn3 (Kap), the product of a single, highly conserved gene (Hannon et al., 1994; Nalepa et al., 2013). These inhibitors act at different stages of the cell cycle, either targeting Cdk proteins or Cdk–Ccn complexes (reviewed in (Malumbres, 2014; Ruijtenberg and van den Heuvel, 2016).

Under physiological conditions, typically proliferating cells are being prevented from differentiation while differentiating cells permanently exit cell cycle. This is controlled by generic mechanisms including Ccn–Cdk-mediated phosphorylation activating pro-mitotic and blocking anti-mitotic transcription factors; Ccn–Cdks interacting with chromatin modifiers, thereby indirectly regulating the expression of pro- or anti-mitotic transcription factors; and CcnD acting as a transcription factor itself (Hydbring et al., 2016; Ruijtenberg and van den Heuvel, 2016). However, cell cycle regulators also play specific roles in cell differentiation. This is particularly well understood for the processes of neuronal development (neurogenesis) in the spinal cord (reviewed in (Hindley and Philpott, 2012; Molina and Pituello, 2017) and skeletal muscle formation (myogenesis) from the segmented paraxial mesoderm, the somites, in trunk (reviewed in (Singh and Dilworth, 2013). Both neurogenesis and myogenesis are regulated by families of basic helix–loop–helix (bHLH) transcription factors, the Atoh/NeuroG/NeuroD subfamily and the Ascl (Mash) subfamily for neurogenesis (Baker and Brown, 2018), and the MyoD family for myogenesis (Buckingham and Rigby, 2014). A common, direct link between cell cycle regulators and differentiation in both processes is the phosphorylation of bHLH proteins by Cdk1 and Cdk2. This results in reduced protein stability and reduced activation of target genes, thus inhibiting cell differentiation (Ali et al., 2011; Hindley et al., 2012; Ali et al., 2014; Kitzmann et al., 1999; Song et al., 1998; Tintignac et al., 2000). Conversely, when bHLH protein levels increase, they upregulate downstream differentiations genes as well as Cdkns to promote simultaneous terminal differentiation and cell cycle exit. Surprisingly, while CcnD proteins generally have pro-mitotic and anti-differentiation properties, these proteins may stimulate both neuronal and skeletal muscle differentiation. This has been shown for CcnD1 in the neural tube (Lukaszewicz and Anderson, 2011) and CcnD3 during the transition from myoblasts to myocytes (Athar and Parnaik, 2015; Cenciarelli et al., 1999; Gurung and Parnaik, 2012).

In pseudostratified epithelia, where nuclei are staggered across an epithelial monolayer, both cell cycle control and differentiation are linked to the curious phenomenon of nuclear shuttling, also known as interkinetic nuclear migration (Norden, 2017). This is well-studied in the vertebrate neural tube and in the neocortex (Lee and Norden, 2013; Molina and Pituello, 2017), but also applies to the somite (Ben-Yair et al., 2011; Venters and Ordahl, 2005) or the Drosophila wing disc (Kirkland et al., 2020). In the neural tube, cells take the decision to enter the G1 phase of the cell cycle when their nuclei are at the apical, luminal side of the tissue. The nuclei

then move to the basal, outer side of the neural tube, reaching it in S-phase. During G2, nuclei return to the apical side. This coincides with cellular rounding, which allows mitotic spindle assembly and chromosome separation, followed by a planar-oriented, symmetric cell division. Conversely, cells that have completed their last division and are ready for differentiation sever their connection to the apical surface, accumulate on the basal surface and leave the epithelium. The somitic dermomyotome first delivers the differentiating cells for the myotome from its four edges and later from its deepithelialising centre (Buckingham and Rigby, 2014). Thus, the release mechanisms for differentiating cells are distinct from that in the neural tube. However, also here, while the tissue is epithelial, nuclei are staggered and symmetric cell divisions take place apically (Ben-Yair et al., 2011; Venters and Ordahl, 2005). In pseudostratified epithelia, microtubules emanate from the apically positioned centrosome with all minus-ends pointing towards the apical side and plus-ends towards the basal side. In tall, several nuclei-diameter thick epithelia such as the cortex, nuclear movements depend on these microtubules, while in shorter epithelia, microtubules are less important and the process is actomyosin dependent. Yet blockage of nuclear migration interferes with cell division, and, where non-apical cell divisions occur, this leads to cell delamination or a disturbed layering of tissues (Hu et al., 2013; Strzyz et al., 2015). Moreover, at least in tall tissues, Cdk1 controls the recruitment of the microtubule motor protein dynein to the nuclear envelope during G2 (Baffet et al., 2015), indicating an intricate molecular link between cell cycle regulators and nuclear shuttling.

It is generally assumed that the same genes that control the cell cycle in humans would also feature in the established vertebrate model organisms, mouse, chicken, Xenopus and the zebrafish, because the basic machinery for cell cycle control is conserved across eukaryotes (Malumbres, 2014). Jawed vertebrates, however, have undergone two early rounds of whole genome duplications twice (Dehal and Boore, 2005; Holland et al., 1994; Kuraku et al., 2009) with further duplications in teleost fish, including zebrafish. This resulted in an expansion of cell cycle regulator genes. Novel players like zebrafish ccnd4/x (Lien et al., 2016), frog and zebrafish cdc25d (Dalle Nogare et al., 2007; Nakajo et al., 2011) and frog cdkn1x/xic1 (Vernon and Philpott, 2003a) have occasionally been described. We have found evidence that different vertebrate clades retained distinct paralogues of the duplicated cell cycle regulators (Schubert et al, manuscript in preparation). Moreover, we found evidence that in discrete vertebrate lineages individual gene duplication events occurred. Thus, the complement of cell cycle regulators presents in distinct vertebrate clades, and hence the details of their cell cycle control, might be more divergent than generally anticipated.

Knock-out or knock-down of cell cycle regulators, in particular of CcnDs and their cognate Cdk4/6-type partners, showed that with the exception of the indispensable Cdk1, there is a high degree of redundancy (reviewed in (Sherr and Roberts, 2004). This would infer that, whatever complement of cell cycle regulators different vertebrates may have, homologous genes may be expressed and function in the same spatiotemporal fashion. Yet specific expression patterns and functions have been reported for example for cdkn1x/xic1 (Vernon and Philpott, 2003a), a gene that has been retained in chondrichthyans, Latimeria and most amphibians, but is absent in teleosts and amniotes (Schubert et al, manuscript in preparation). Thus, the similarity as well as the possible divergence of vertebrate cell cycle regulators remains an unresolved issue.

To address this problem, we for the first time evaluated the expression of all core cell cycle regulators, that is all cell cycle promoting Cdc25, cyclin, Cdk genes and all cell cycle inhibiting Cdkn genes, in the chicken embryo, an easily accessible vertebrate model with a standard diploid genome. Genes with restricted expression pattern were then further analysed in comparison to specific differentiation markers. Finally, we compared the expression of the chicken genes with the published expression pattern of cell cycle regulators in mouse, Xenopus and zebrafish. Our study shows that many of the chicken cell cycle regulators are expressed near-ubiquitously, as expected for young, fast growing embryos. However, some genes show upregulated or even specific expression in some tissues, with *Pax6*, *Cdc25b*, *CcnD1*, *Cdk6* and *Cdkn2b* forming a synexpression group in the central spinal cord, *Pax3/7* and *CcnA2* forming a synexpression group in the dorsal spinal cord and in the somitic dermomyotome, and *Cdkn1b* being expressed together with *Myf5* and earlier than *MyoD*. Most notable is that orthologous genes present in several or all vertebrate models do not necessarily show the same expression pattern. Moreover, in the chicken, we were unable to detect any expression of the canonical partners for CcnD proteins besides *Cdk6*. Thus, we have to assume that in tissues with high *CcnD* but no *Cdk6* expression, other Cdk proteins serve to initiate the G1 phase. Finally, we report a novel finding, namely the accumulation of specific transcripts on the apical sides of both the neural tube and the somite/dermomyotome, correlating with the time the gene products are required during cell cycle and the apical positioning of cell nuclei.

Our work indicates that even for a process as fundamental as cell cycle control, vertebrate models differ. Thus, care has to be taken when extrapolating from one model to the next: any study investigating cell proliferation, cell differentiation, tissue regeneration, stem cell behaviour and cancer/cancer therapy has to carefully examine which of the observed effects are due to the specific model used, and which can be generalised.

2 | MATERIALS AND METHODS

2.1 | Culture and staging of chicken embryos

Fertilised chicken eggs from a mixed flock (Henry Stewart Ltd, Norfolk) were incubated in a humidified atmosphere at 38.5°C and staged according to (Hamburger and Hamilton, 1951). Embryos were harvested in 4% PFA. At HH13-14, the telencephalon was opened from the left side, the midbrain was opened dorsomedially. This was

to avoid poor exchange of solutions during the in situ hybridisation that can cause a coloured precipitate to form on the inside of vesicular structures.

2.2 | *In situ* hybridisation (ISH)

Whole mount ISH was carried out as described by (Dietrich et al., 1997). Probes are detailed in the table provided as Supplementary Material 1. Staining reactions were carried out either until robust staining was obtained or until the negative control began to show blue shading. Each ISH was repeated at least three times.

2.3 | Vibratome sectioning

For detailed analyses, embryos subjected to whole mount staining were embedded in 20% gelatine and cross-sectioned to 30–50 μm on a Pelco 1000 Vibratome as described in (Dietrich et al., 1997).

2.4 | Photomicroscopy

Embryos and sections were photographed, using Nomarski optics on a Zeiss Axioskop. Images were acquired using the Axiocam/Axiovision system, and processed using Adobe Photoshop.

2.5 | Bioinformatics

To predict the specificity of probes derived from non-chicken sequences and the possibility of these probes hybridising with mRNAs from paralogous genes, cDNA sequences were extracted from the NCBI and Ensembl databases, aligned in Bioedit using ClustalW, trimmed to the length of the probe, and then the DNADist algorithm was used to generate a distance matrix. This matrix was then introduced into Excel to colour-code sequences with high similarity (= small distance values) or lower similarity (= larger distance values). The data are shown as Supplementary Material 2.

2.6 | Comparison of our data with published expression data

The results obtained with our experiments were compared with the published data for chicken embryos as deposited in http://geisha.arizona.edu/, using the gene names as search terms. In Geisha, the automated links to http://www.informatics.jax.org/gxd for the mouse, http://www.xenbase.org/ for Xenopus and http://zfin.org/ for the zebrafish were used to find the expression pattern of orthologous genes. These databases as well as https://www.embrys.jp/ as additional source for the mouse were also interrogated to find expression patterns of paralogous genes. The comparison of expression data is shown in Table 1.

3 | RESULTS

The aim of this study was to comparatively analyse the embryonic mRNA expression of all chicken core cell cycle regulators, both in cells undertaking mitosis or withdrawing from it. We therefore focused on early neurula stages at HH8–HH10 to early somite stage/pharyngula embryos at HH13-14, because (i) these stages still have actively dividing, immature cells in the primitive streak and tailbud, (ii) many tissues contain committed, but still mitotically active precursors, (iii) yet at many sites, differentiating cells begin to build the first organs including the central nervous system, the blood and cardiovascular system, and the skeletal musculature. Moreover (iv), the pharyngula stage is seen as the vertebrate phylotypic stage when species are the most similar, and thus, this facilitates cross-species comparison of expression patterns (Irie and Sehara-Fujisawa, 2007). Whole embryos including annotated schematic representations are shown in Figures 1–4, whole embryos and close-ups are also shown in Figure 6, and cross sections for markers with more specific expression patterns are shown in Figure 5; for the ease of navigation, paralogues appear in the same alphabetical, then numerical, order throughout. A comparison of the chicken, mouse, frog and zebrafish expression data is presented in Table 1; unless further specified, the publicly available data are direct entries to the http://geisha.arizona.edu/, http://www.informatics.jax.org/gxd, https://www.embrys.jp/, http://www.xenbase.org/ and http://zfin.org/ databases.

Given that at the chosen stages many tissues contain dividing cells, we expected that many of the positive regulators of the cell cycle would show widespread or near-ubiquitous expression. To ensure that positive results we not due to unspecific background staining, we in included a *CcnB2* sense probe as negative control (Figure 1bi–iii). As positive controls we used (i) *Pax6* as marker for the early diencephalon, caudal telencephalon, eye and the dorsoventrally central area of the spinal cord that delivers specific subtypes of interneurons and motor neurons (Figures 1Ci-iii, 5b; [Bel-Vialar et al., 2007]), (ii) *NeuroD4* (=*NeuroM*) as marker for post-mitotic, differentiating neurons (Figure 1di–iii, 5c; [Roztocil et al., 1997]), (iii) *Pax7* as marker for the dorsal neural tube that delivers subtypes of dorsal interneurons, and as a marker for developing somites, somitic dermomyotomes/embryonic muscle stem cells and craniofacial neural crest cells (Figures 1ei–iii, 5d), (iv) *Myf5* as marker for cells committed to skeletal muscle formation (Figiures 1fi–iii, 5e) and (v) *MyoD* as marker for cells beginning muscle differentiation (Figures 1gi–iii, 5f; [Berti et al., 2015] and references therein). All controls delivered the expected expression patterns.

TABLE 1 Summary: Expression of cell cycle regulators in vertebrate model organisms from early neurula to pharyngula stages of development

Genes	Comment	Expression in chicken; this study and http://geisha.arizona.edu/	Expression in mouse; http://www.informatics.jax.org/gxd and https://www.embrys.jp/	Expression in Xenopus; http://www.xenbase.org/	Expression in zebrafish; http://zfin.org/
Cdc25 phosphatases					
Cdc25a	Tetrapod-specific Cdc25b-duplicate	Widespread, upregulated in the neural tube and gut endoderm, later somitic dermomyotomes (Bénazéraf 2006)	Ubiquitous	Cleavage and early blastula stages, later: neural plate (Nakajo 2011)	–
Cdc25b	Not found in parrots, divergent genomic environment in passeriformes	Widespread, upregulated in the central neural tube, somite, lateral mesoderm, gut endoderm (Bénazéraf 2006)	(No data)	Neural plate, optic placode (Nakajo 2011)	Widespread during epiboly, then upregulated in the neural plate, neural tube, retina, pharynx, somites, tailbud (Dalle Nogare 2007)
Cdc25c	Divergent phosphatase domain with 221 aa in Xt, 34 aa in Hs, absent in Mm	Gene not found in neognath birds	(No data)	Similar to cdc25a: cleavage and early blastula stages, later: neural plate (Nakajo 2011)	Gene not found in ostariophysii and in neoteleosts
Cdc25d	possibly no phosphatase domain, divergent C-terminus	Gene not found in crocodiles and birds	Gene not found in placental mammals	Late neurula: epidermal ectoderm, liver diverticulum (Nakajo 2011)	Limited expression in neural plate and ventral myotome (Dalle Nogare 2007)
Group I cyclins					
CcnA1		No or low level expression, later: mesonephros	(no data, later stages: developing teeth)	Maternally-supplied, largely degraded at the MBT, remaining expression widespread (Vernon and Philpott 2003)	After 36 hpf, low level expression in head and pectoral fins
CcnA2		Widespread, upregulated in the dorsal neural tube, surface ectoderm, somitic dermomyotome, pharyngeal arches, later limb fields	Widespread, upregulated in the neural plate/ neural tube, eyes, allantois	Neural plate, neural tube, eyes, cranial neural crest cells, pronephros, pre-somitic mesoderm, somites (Vernon and Philpott 2003)	Widespread during epiboly, then neural plate, neural tube, eyes/retina, somites, pharyngeal arches, pectoral fin, tailbud
CcnB1		Not found in galloansers and passeriformes	Limited data, ventricular zone of neural tube (Zhao 1995)	Initially widespread, then neural plate/ neural tube, eyes, otic vesicle, nasal pits, cranial neural crest cells, cement gland, tailbud (Vernon and Philpott 2003)	Widespread during epiboly, then neural tube-brain, eyes, otic vesicles, pharyngeal arches, pectoral fin
CcnB2		Widespread, upregulated in the primitive streak, neural plate/ tube, notochord, somite, lateral mesoderm, gut endoderm	Limited data, ventricular zone of neural tube (Zhao 1995)	Strong and widespread, some upregulation in the neural tube, notochord, somitic dermomyotome, surface ectoderm	Widespread during epiboly, then neural tube-brain, retina, pharyngeal arches
CcnB5	Xenopus-specific duplication of Ccnb2	–	–	(No data)	–

TABLE 1 (Continued)

CcnB3	CcbB3 is not a CcnB1/CcnB2 ohnologue	Strong, widespread throughout the embryo	(No data)	Strong and widespread, some upregulation in the neural tube, eye, surface ectoderm	testis (Ozaki 2011)
CcnD1		Strong expression in the primitive streak/ tail bud, neural tube, pharyngeal arches, somites, later: limb fields; (Lobjois 2004)	Initially widespread, then upregulation in the primitive streak, neural plate, head mesoderm, somites, pharyngeal endoderm, later ventricular zone of neural tube (Zhao 1995, Wianny 1998)	After MBT, initially widespread then upregulation in prechordal plate, rostral neural plate, neural tube, cranial neural crest cells, eyes, tail bud, transient in the lateral edge of somites (Vernon and Philpott 2003, Zhang 2017)	Widespread during epiboly, then notochord, neural tube-brain, retina, pharyngeal arches, somites, tailbud
CcnD2		Widespread; (Lobjois 2004)	Initially widespread, then upregulation in the primitive streak, neural plate/ neural tube, later marginal zone of neural tube (Zhao 1995, Wianny 1998)	After MBT, weaker than ccnd1, cranial neural crest cells, brain/neural tube, nasal pits, otic vesicles, pronephric duct, ventral blood islands (Vernon and Philpott 2003)	ccnd2a blood vessels, pharyngeal arches (Covassin 2006) ccnd2b (No data)
CcnD3	lost in amphibians	Widespread, upregulated in blood islands	Initially widespread, then upregulation in the foregut and blood islands (Wianny 1998)	–	Mid-somitogenesis stages: head, neural tube, pectoral fins
CcnD4/x	Lost in amniotes	–	–	motor neuron progenitors in hindbrain and spinal cord (Chen 2005)	Mid-somitogenesis stages: motor neuron progenitors in hindbrain/spinal cord (Lien 2016)
CcnE1		Widespread, upregulated in blood islands and somitic sclerotome	(Limited data; widespread)	Low level and widespread, upregulated in rostral neural plate/ neural tube, cranial neural crest cells, eyes (Vernon and Philpott 2003)	Widespread during epiboly, then neural tube, lateral line system
CcnE2		Low Level expression, widespread at HH13/14, upregulated somitic sclerotome and lateral mesoderm	(Limited data, expressed in mesonephros/ urogenital system; GUDMAP Consortium)	(No data)	neural tube, retina, blood
CcnO	About 30 copies in Xt	Extraembryonic	(No data)	(No data)	(No data)
Group II cyclins					
CcnH		Strong, widespread	(Limited data, limb mesenchyme)	(No data)	Ubiquitous during cleavage and epiboly, then upregulated in rostral neural tube, brain, eyes, ovary, testis, liver and heart (Liu 2007)

TABLE 1 (Continued)

Cdk interacting with group I Cyclins					
Cdk1		Strong, widespread (Bénazéraf 2006)	(Limited data, ventricular zone of neural tube; Zhao 1995)	From st15 onwards, widespread, upregulated in neural tube and eyes, lower in epidermis (Vernon and Philpott 2003, Zhang 2017)	(No data)
Cdk2	Cdk2 sequences in birds often with frame shifts or incomplete	Low level, enriched in early neural plate/neural tube	Widespread, low level expression	Early animal hemisphere, then downregulation, then re-emergence of transcripts in rostral neural plate/neural tube, eyes, otic vesicles, cranial neural crest cells (Vernon and Philpott 2003)	Widespread during epiboly, then neural tube-brain, pharyngeal arches, retina
Cdk3 (=Cdk2-like)	'Cdk2-like' gene in Latimeria and chondrichthyans is the orthologue of amniote Cdk3	Early neural plate/neural tube, later more widespread incl. heart and somitic sclerotome	Widespread	–	–
Cdk4	Gene predicted for quails, but absent in chicken or turkeys	–	Ubiquitous	Initially widespread, then upregulated in neural plate, eyes, cranial neural crest cells, newly formed caudal mesoderm, downregulated by late tailbud stages (Vernon and Philpott 2003)	(No data)
Cdk6		Widespread, upregulated in the neural tube, lateral mesoderm, moderately in the sclerotome	Widespread, upregulated in the neural tube, pharyngeal arches, limb buds (Lewandowski 2015)	(No data)	(No data)
Cdk21	Ohnologue of Cdk4 and Cdk6, lost in tetrapods	–	–	–	Ovary, testis
Cdk interacting with group II Cyclins					
Cdk7		Widespread, particular in early embryos	Ubiquitous	(No data)	Widespread (Liu 2007)
Cyclin dependent kinase inhibitors					
Cdkn1a		=CIP1 Low level, widespread	=p21 Widespread, upregulated in neural tube and somitic myotomes (Magdaleno 2006)	=xic2; cement gland, pre-somitic mesoderm, somites, lens, otic vesicle, tail bud (Daniels 2004, Zhang 2017)	Low or no expression (Osborn 2011)

TABLE 1 (Continued)

Cdkn1b	In sauria and the elephant shark: linked Cdkn1b and 1d genes, transcribed in opposite direction	=Kip1 Neural plate/ neural tube, very prominent in somitic myotome	= p27 (Limited information, lens epithelial cells; Ho 2009)	=xic3; unspecific staining in the neural plate and st33 head (Daniels 2004, Zhang 2017)	cdkn1ba somitic myotomes, otic vesicle; (Osborn 2011, Radosevic 2011) cdkn1bb widespread, but upregulated in otic vesicle, lens, later brain, retina, pharyngeal arches, somitic myotomes, pectoral fins; (Osborn 2011)
Cdkn1c		=Kip2 Widespread, upregulated in neural plate/neural tube, myotome, cardiogenic mesoderm	= p57 (Limited information, heart, skeletal muscle, cerebellum, pancreas primordium (Georgia 2006, Andrews 2007, Seto 2014)	present in many amphibians, possibly absent in Xenopus	cdkn1ca notochord, neural tube-primary neurons, adaxial cells, somitic myotomes, then brain, spinal cord, trigeminal ganglion, otic vesicle, lateral line precursor (Osborn 2011, Radosevic 2011) cdkn1cb (No data)
Cdkn1d	Lost in mammals and some bird, amphibian and actinopterygian clades	No or low level expression	–	–	(No data)
Cdkn1x	Lost in amniotes and actinopterygians	–	–	=xic1, cdkn1x, cdknx; initially widespread, then lens, nasal, otic and neurogenic cranial placodes, lateral line placode, brain, retina, pre-somitic mesoderm, somitic myotome, craniofacial and abdominal muscle anlagen	–
Cdkn2a-ARF (p19 ARF)	Located between Cdkn2b and 2a; delivers alternative 1st exon for Cdkn2a; found in placental and marsupial mammals and some neognath birds, possibly independent evolution in birds and mammals	No or low level expression	(No data)	–	–

(Continues)

TABLE 1 (Continued)

Cdkn2a (p16, Ink4a)	The distinct amniote Cdkn2a and 2b genes arose from a tandem gene duplication, with a secondary establishment of long splices, cutting out the 2nd exon from 2b and the 1st exon from 2a.	No or low level expression	Widespread low level expression, upregulated in neural tube (Magdaleno 2006)	Low level, with upregulation in the craniofacial region, heart, pre-somitic mesoderm, somites (Zhang 2017)	(No data)
Cdkn2b (p15, Ink4b)		Widespread, upregulated in neural plate and blood islands, at HH13/14 upregulated in neural tube, sclerotome, lateral mesoderm	Widespread low level expression, upregulated in neural tube (Magdaleno 2006)		
Cdkn2c (p18, Ink4c)		Low level, widespread, upregulated in neural tube	Widespread low level expression, upregulated in neural tube and oral ectoderm (Magdaleno 2006)	(No data)	(No data)
Cdkn2d (p19, Ink4d)	Lost in birds	(gene shown as Cdkn2d in Geisha is Cdkn2c)	Widespread low level expression, upregulated in neural tube and oral ectoderm (Magdaleno 2006)	Low level, enriched in brain, eye, somites (Doherty 2014)	(No data)
Cdkn3 (KAP)		Early: closing neural tube, later: widespread	(No data)	Present in Nanorana parkeri, but absent in Xenopus	somitic myotomes, low level in brain

Note: Published expression data have been extracted from the databases indicated at the top of the panel; further specific resources are also indicated. The presence of a gene in the genome is indicated by the green shading of the respective table cell; upregulated or specific gene expression is indicated by the red type face.

Abbreviations: Hs, homo sapiens, Mm, mus musculus, Gg, Gallus gallus, Xt, Xenopus tropicalis, Xl, Xenopus laevis; MBT, midblastula transition.

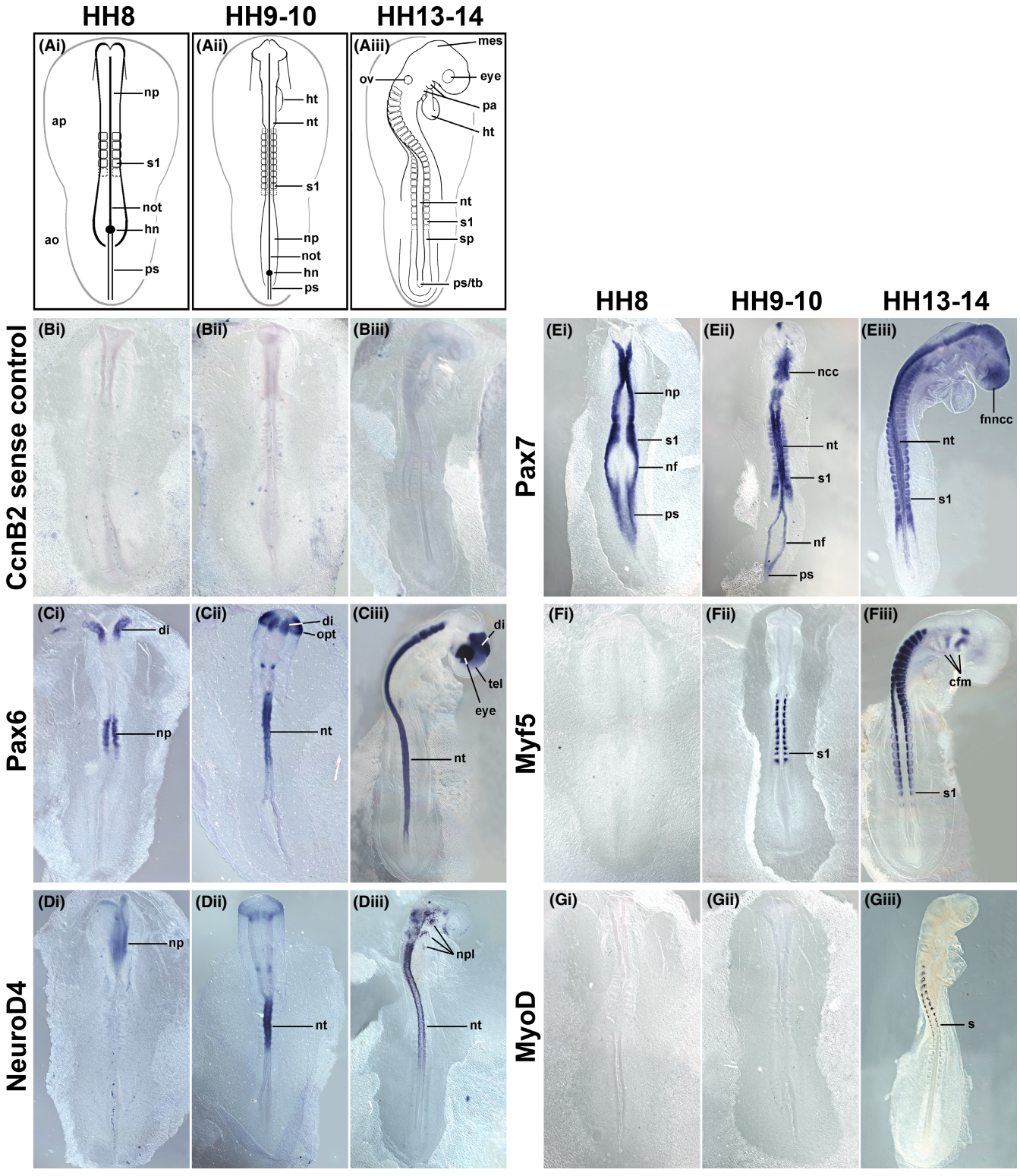


FIGURE 1 Expression of control markers. (Ai–Aiii) Schematic representation of whole chicken embryos at stages HH8, HH10 and HH14 of development, dorsal views, rostral is up. (B–G, i–iii) Whole embryos at stages HH8, HH9-10, HH13-14 subjected to in situ hybridisation; probes and marker genes are indicated on the left of each series. The probes reproduce the known, specific marker gene expression patterns; the sense probe does not produce a staining. Abbreviations: ao, area opaca; ap, are pellucida; cfm, craniofacial muscle anlagen; di, diencephalon; eye, eye; fnncc, frontonasal neural crest cells; hn, Hensen's node; ht, heart; mes, mesencephalon; ncc, neural crest cells; nf, neural folds; not, notochord; np, neural plate; npl, neurogenic placodes; nt, neural tube; opt, optic placode; ov, otic vesicle; pa, pharyngeal arches; ps, primitive streak; s, somite; s1, youngest somite; sp, segmental plate; tb, tail bud; tel, telencephalon

FIGURE 2 Expression of Cdc25 and cell cycle Ccn genes. Stages, views and annotations as in Figure 1. Abbreviations as in Figure 1 and bi, blood islands; lm, lateral mesoderm. Arrows indicate the onset of gene expression in the early neural plate, arrowheads indicate markers expressed in the more mature neural plate/neural tube. Cdc25 and most Ccn genes are expressed widely, in tune with the high mitotic activity in most tissues. Note, however, the restricted expression pattern for CcnD1 and the strong expression in blood islands for CcnD3

3.1 | Expression of Cdc25 phosphatase genes

Many clades of jawed vertebrates have *Cdc25b,c,d* genes, with *Cdc25c* encoding a reduced, *Cdc25d* possibly no phosphatase domain. In tetrapods, *Cdc25b* was duplicated, creating a novel *Cdc25a* gene, yet *Cdc25a* and *b* are the only *Cdc25* genes retained in the chicken (Schubert et al., manuscript in preparation). Previous studies suggested *Cdc25a* expression in the early neural plate, with *Cdc25b* expression commencing in the more mature neural tube (Bénazéraf et al., 2006). Our analysis suggests a rather widespread expression of the genes: at HH8-10, *Cdc25a* was expressed in the neural plate/neural tube, and in the rostral primitive streak at the junction to the neural plate/neural tube (Figure 2bi–iii, arrow). At the early pharyngula stage at HH13-14, prominent expression encompassed the neural tissues, the somites and the lateral mesoderm bordering the segmental plate. Cross sections through the neck confirmed this widespread expression, with a strong signal in the endoderm and a curious accumulation of transcripts both along the apical surface of the neural tube and the apical surface of the somitic dermomyotome (Figure 5g). Prominent *Cdc25b* expression was first detected in the rostral neural plate/neural tube, spreading widely at HH13/14 (Figure 2ci–iii). Notable is the expression in the more rostral lateral mesoderm compared to the more caudal expression of *Cdc25a* (compare Figure 2biii, ciii), and the strong upregulation in the central region of the spinal cord (Figure 5h) that also expresses *Pax6* (Figure 5b). Similar to the chicken, Xenopus *Cdc25a* but also *cdc25c* were reported to be expressed before *cdc25b*, with all genes being upregulated in the neural plate; the divergent *cdc25d* gene in contrast was expressed in the epidermal ectoderm and the liver anlage of the late neurula (Table 1; (Nakajo et al., 2011). The zebrafish lacks the tetrapod-specific *Cdc25a* duplicate, and the expression of its *cdc25b* including the expression during epiboly is more akin to that of tetrapod *Cdc25a*. Notably, the zebrafish seems to have lost the *cdc25c* gene; its *cdc25d* gene was reported to have limited expression in the neural plate and ventral myotome only (Table 1; (Dalle Nogare et al., 2007). Expression data for the mouse unfortunately are insufficient.

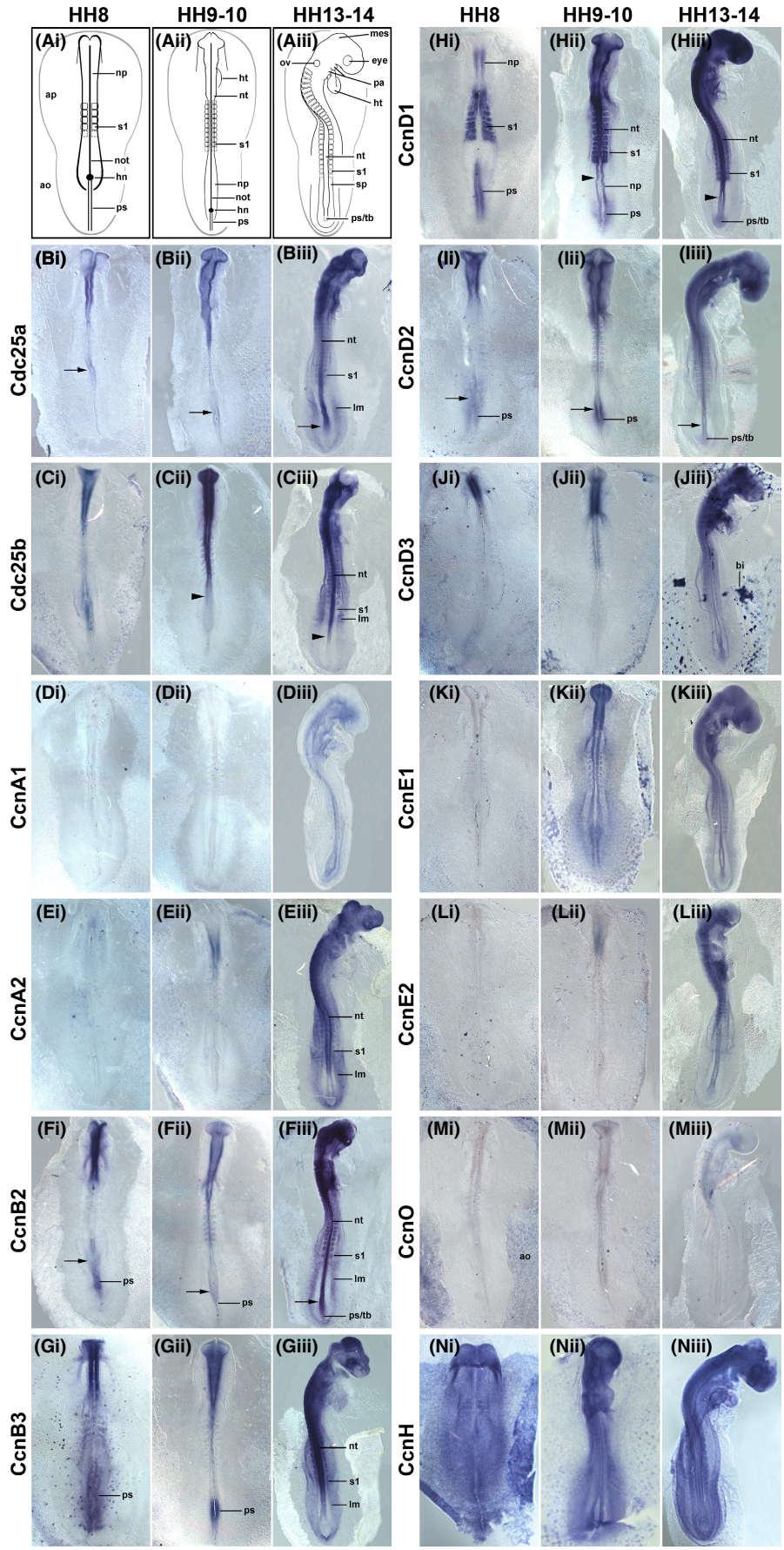
3.2 | Expression of cell cycle controlling cyclins

All jawed vertebrates have *CcnA1,2* genes, most also have *CcnB1,2* genes, with *CcnB1* being absent in galloanserae, and Xenopus carrying a tandem duplicate of *ccnb2* currently named *ccnb5* (Schubert et al., manuscript in preparation). All vertebrates also have a *CcnB3* gene, but it has to be noted that a distinct *ccnb3* gene exists already in invertebrates and hence the vertebrate gene is not an ohnologue (=orthologue created by the two vertebrate whole genome duplications) of *CcnB1,2* (Lozano et al., 2012). Jawed vertebrates consistently have *CcnD1,2,3*, (note: two *ccnd2* genes in the zebrafish), with a *ccnd4/x* gene being present in all but amniotes. Jawed vertebrates also share *CcnE1,2* genes, one *CcnO* gene (with a curious amplification of this gene in *Xenopus tropicalis*) and a single *CcnH* gene (Schubert et al., manuscript in preparation).

We were unable to detect expression for chicken *CcnA1* at stages HH8-10, and signals for HH13-14 were weak (Figure 2di–iii). However, data deposited in the chicken expression database suggest a later expression in the metanephros (http://geisha.arizona.edu/). Expression data for mouse, *Xenopus* and zebrafish are limited; in the frog the transcript seems mainly maternally supplied (Table 1; (Vernon and Philpott, 2003b). *CcnA2* was initially barely detectable, but at HH13-14 robust expression was found throughout the embryo, with particularly strong expression in the dorsal neural tube and somitic dermomyotome, coinciding with the expression of *Pax7* (Figure 2ei–iii, Figure 5i, d). Widespread expression, upregulated in neural tube and somites and pharyngeal arches has also been described for *CcnA2* in the other vertebrate models, suggesting that *CcnA2* is the main S-G2-driver in the embryo (Table 1; (Vernon and Philpott, 2003b).

Chicken *CcnB2* showed strong expression in the primitive streak and neural plate at HH8-10, with robust and widespread expression at HH13-14, particularly strong in the notochord, neural tube, the endoderm and the adjacent splanchnic lateral mesoderm (Figures 2fi–iii, 5j). Transcripts accumulated along the apical surface of the neural tube and the somitic dermomyotome, which correlates with CcnB proteins acting during late G2 and M phase and symmetric cell divisions in pseudostratified epithelia taking place apically (Lee and Norden, 2013). Data for the mouse are limited, but both *CcnB1* and *B2* have been found in the ventricular layer of the neural tube that contains mitotically active cells (Table 1, [Zhao et al., 1995]). In the frog and the fish, *ccnb1* was reported to initially be expressed widely; expression then becomes more restricted with the central nervous system, eye, ear and nasal vesicles and the neural crest cells filling the pharyngeal arches being the most prominent sites. Frog *ccnb2* expression resembled that of the chicken, zebrafish *ccnb2* expression was more restricted (Table 1; (Vernon and Philpott, 2003b); expression data for frog *ccnb5* are currently not available. Together, these data suggest similar expression patterns for vertebrate *CcnB1/2* genes with *CcnB2* being the sole driver of the G2/M cell cycle transition in the chicken.

Chicken *CcnB3* expression was strong and widespread throughout the embryo, with particularly high expression in the neural tube; the postmitotic myotome is negative (Figures 2gi–iii, 5k). No data are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HH9-10
HH13-14
HH8
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HH13-14
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s1
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(Aiii)
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pa
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s1
sp
ps/tb
CcnD1
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(Hii)
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Cdc25a
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(Miii)
CcnB3
(Gi)
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s1
lm
CcnH
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available for the mouse, the frog gene is expressed widely with some upregulation in the neural tube and surface ectoderm, expression for the zebrafish gene has only been reported for the testis (Table 1, [Ozaki et al., 2011]).

Chicken *CcnD1* has a remarkably strong and specific expression in the primitive streak, the developing somites and the neural plate/neural tube, at HH13/14 accompanied by expression in the neural crest cells populating the pharyngeal arches and the pharyngeal endoderm. Cross sections through the neck of HH13/14 embryos revealed that the expression in the spinal cord was particularly prominent in the *Pax6*-positive central region; signals in the somite were elevated along the apical surface of the dermomyotome (Figures 2hi–iii, 5l; [Lobjois et al., 2004]). Mouse, frog and zebrafish *CcnD1* expression in contrast was reported to initially be widespread, later showing upregulated expression in the neural tube, cranial neural crest cells, eyes, somites and tailbud, similar to the chicken gene (Vernon and Philpott, 2003b; Wianny et al., 1998; Zhang et al., 2017; Zhao et al., 1995). Yet zebrafish *ccnd1* is also expressed in the notochord, a structure that did not express the gene in the chicken (see http://zfin.org/). Chicken *CcnD2* expression was strongest in the rostral neural plate/tube and the primitive streak before becoming widespread (Figure 2li–iii; [Lobjois et al., 2004]); expression in the mouse was reported as widespread, with later upregulation in the neural tube (Wianny et al., 1998; Zhao et al., 1995). Expression of frog *ccnd2* occurs after zygotic genome activation and seems weaker than that for *ccnd1*, encompassing the neural tube, otic and nasal vesicles, the pronephric duct but also blood islands (Vernon and Philpott, 2003b); expression data for the two zebrafish *ccnd2* genes are limited, but *ccnd2a* was reported to be expressed in blood islands and the pharyngeal arches (Covassin et al., 2006). Chicken *CcnD3* was expressed in the rostral neural plate and blood islands, eventually being expressed widely (Figures 2ji–iii, 5m), expression of the mouse gene was reported as initially widespread, becoming upregulated in the foregut and blood islands (Wianny et al., 1998); the zebrafish gene, however, is expressed late and seems to have a more restricted expression in the nervous system and the pectoral fins. Finally, both Xenopus and zebrafish *ccnd4/x* show restricted expression in motor neuron progenitors in the hindbrain and spinal cord (Chen et al., 2005; Lien et al., 2016). Thus, vertebrate *CcnD1-3* expression is similar but not identical; ccnd4/x seems to have acquired a specific role at least in the osteichthyans that have kept it.

Chicken *CcnE1* was found to be expressed widely, with elevated expression in blood islands and the somitic sclerotome (Figures 2ki–iii; 5n). *CcnE2* expression was weak but also widespread, yet somewhat upregulated in the sclerotome and lateral mesoderm (Figures 2li–iii; 5o). Data for the mouse are limited, but expression has been reported for *CcnE2* in the urogenital system. Expression of frog *ccne1* has been described as low-level, upregulated in the rostral neural tube, neural crest cells and eyes (Vernon and Philpott, 2003b), no data are available for *ccne2*. Zebrafish *ccne1* expression was reported as widespread during epiboly, becoming restricted to the lateral line system and neural tube; *ccne2* was expressed in the neural tube, eye and blood (Table 1).

Finally, chicken *CcnO* was expressed extra-embryonically (Figure 2mi–iii) whereas *CcnH* expression was ubiquitous (Figure 2ni–iii). Data for the other vertebrate models are limited; zebrafish *ccnh* expression has been reported as ubiquitous during cleavage stages and epiboly, later being upregulated in the rostral neural tube, reproductive organs, liver and heart (Liu et al., 2007); (Table 1).

3.3 | Expression of cell cycle controlling Cdk genes

Despite their various whole genome duplications, vertebrates have a single *Cdk1* gene only (Schubert et al, manuscript in preparation), explaining why the loss of this gene is detrimental to development (reviewed in (Sherr and Roberts, 2004). All vertebrates have a *Cdk2* gene, yet chondrichthyans as well as Latimeria and amniotes have also kept the *Cdk2-like/Cdk3* ohnologue. Most vertebrates have a *Cdk4* and a *Cdk6* gene, in holocephali, neopterygians and Latimeria accompanied by an ohnologue named *cdk6-like/cdk21*. Remarkably, in many birds including chicken as well as in marsupials, no *Cdk4* was found, leaving these animals with Cdk6 alone to partner CcnD proteins. All vertebrates harbour a single *Cdk7* gene (Schubert et al, manuscript in preparation).

Chicken *Cdk1* showed a strong, ubiquitous expression, with transcripts accumulating along the apical surfaces of the neural tube and somitic dermomyotomes (Figures 3bi–iii, 5p; (Bénazéraf et al., 2006)). *Cdk2* was expressed at low levels, but somewhat upregulated in the neural plate/neural tube (Figure 3ci–iii). *Cdk3* was initially expressed in the primitive streak and rostral neural plate/neural tube before becoming more widespread (Figure 3di–iii). Expression in the somite was most prominent in the sclerotome (Figure 5q).

To accommodate for the possibility that the absence of *Cdk4* in the chicken genome was due to poor sequencing of the gene locus, we designed probes based on the sequence information available for the Tibetan ground tit (*Pseudopodoces humilis*, Phum), a passeriform bird and the American alligator (*Alligator mississippiensis*, Amis). The probes are directed against the 5′ portion of the open reading frames that encodes the conserved ATP binding and catalytic domain (Material S1). To determine the likelihood of the probes hybridising with *Cdk4* sequences, we constructed a distance matrix for the *Cdk4* probes, currently available bird *Cdk4* sequences, *Cdk6* and other closely related *Cdk* sequences (Material S2A). This analysis indicated that the probes will preferably detect *Cdk4*, if present, before detecting *Cdk6* or *Cdk2*. The Phum *Cdk4* probe did not produce a signal in chicken embryos (Figure 3ei–iii); with the Amis probe, we after 3 weeks of staining obtained a pattern that appeared like a combination of the patterns for *Cdk6* and *Cdk2*, the two genes to which the Amis probe has a slightly higher affinity than the Phum probe (Figure 3fi–iii; Material S2). We tested the probes also on embryos of the zebra finch (Taeniopygia guttata), a passeriform bird lacking *Cdk4*, and could not detect any reasonable expression either (not shown), suggesting that indeed, in many birds *Cdk4* is not available. The *Cdk6* probe, however, delivered a strong signal in the central neural tube and the

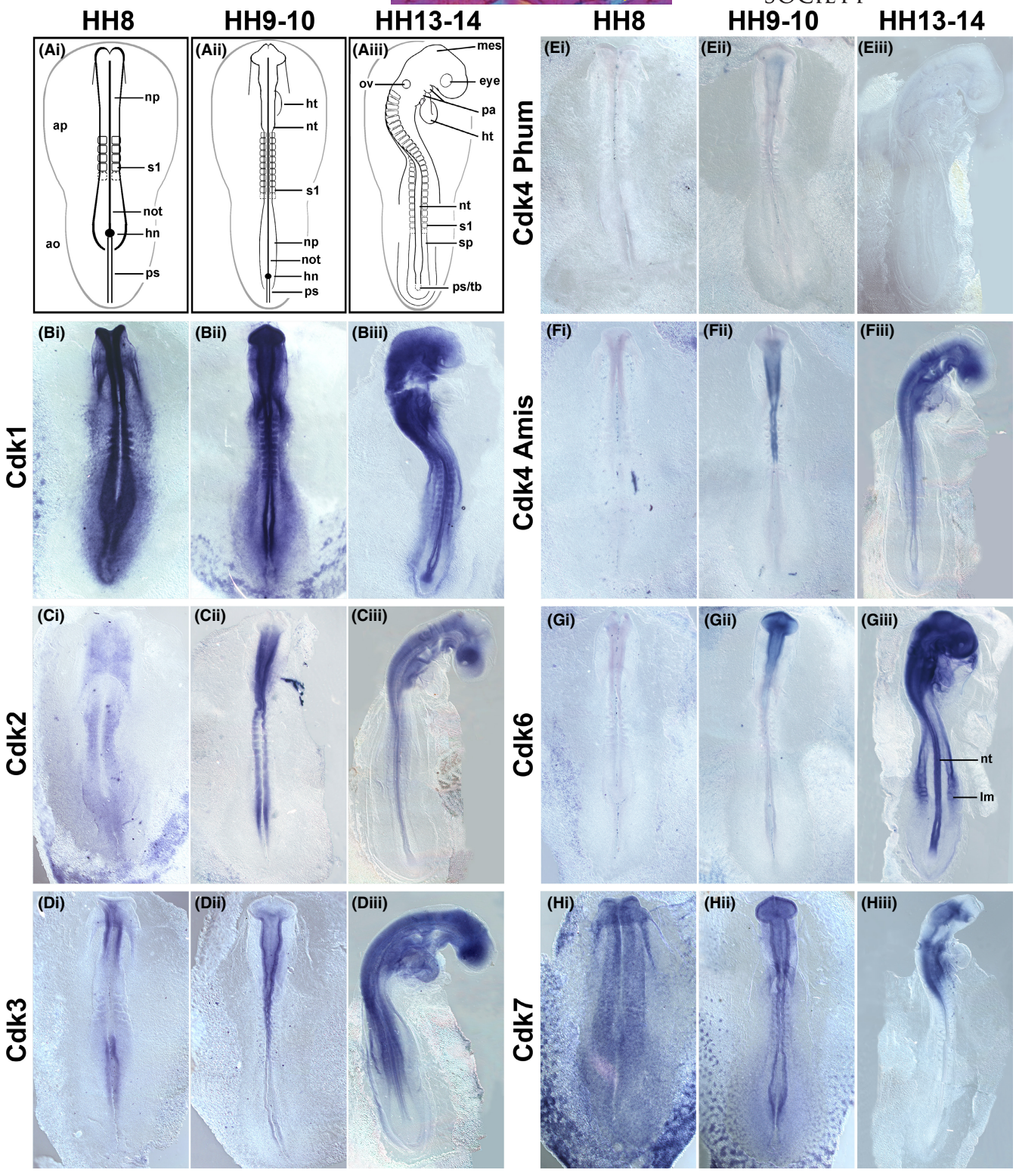


FIGURE 3 Expression of cell cycle Cdk genes. Stages, views, annotations and abbreviations as in Figures 1, 2. Note the strong, widespread expression of Cdk1 and the weaker but also widespread expression of Cdk2, Cdk3/Cdk2-like and Cdk7. No reliable expression was detected with the Cdk4 probe from the Tibetan ground tit Pseudopodoces humilis (Phum). The Cdk4 probe from the American alligator (Alligator mississippiensis, Amis) produced a signal after prolonged staining, possibly because this probe has slightly more similarity to chicken Cdk2 and 6 than the Phum Cdk4 probe. The Cdk6 probe delivered a robust staining particularly in the neural tube and lateral mesoderm

FIGURE 4 Expression of Cdkn genes. Stages, views and annotations as in Figure 1. Abbreviations as in Figures 1, 2 and cm, cardiac mesoderm. Note the relatively restricted expression of Cdkn1b and Cdkn2b. Also note: the Cdkn2a–ARF probe and the conventional Cdkn2a probe derived from the zebra finch (Taeniopygia guttata, Tg) only produced a low-level signal after prolonged staining

lateral mesoderm, with weaker signals in the somitic sclerotome (Figure 3gi–iii, Figure 5r). Yet other tissues showed no or only low-level expression, suggesting that here, distantly related Cdk proteins may functionally replace Cdk6 to partner CcnDs. In contrast to chicken, the mouse has ubiquitous *Cdk4* expression and widespread expression for *Cdk6* with some upregulation in the neural tube, pharyngeal arches and limb buds (Lewandowski et al., 2015); for the frog, initially widespread expression for *cdk6* followed by temporary upregulation in the neural plate, eyes, cranial neural crest cells, newly formed caudal mesoderm was reported (Vernon and Philpott, 2003b). No data are available for frog *cdk6* and zebrafish *cdk4* and *cdk6*, but the *cdk4/6* ohnologue *cdk21* is expressed in reproductive organs (Table 1).

Finally, *Cdk7* showed strong, widespread expression in particular at early stages of development, including prominent expression in blood islands (Figure 3hi–iii); likewise widespread expression has been reported for the mouse and the zebrafish (no data for the frog; Table 1, (Liu et al., 2007).

3.4 | EXPRESSION OF CYCLIN-DEPENDENT KINASE INHIBITORS

3.4.1 | Cdkn1/Cip-Kip genes

Cdkn1 genes are ancient to the animal kingdom as they can be found in cnidarians, protostomes (Drosophila gene: *dacapo*) and deuterostomes (Schubert et al., manuscript in preparation). Distinct *Cdkn1a,b,c* genes have been reported for mammals, but originally, vertebrates had five genes with *Cdkn1a,d* forming one, *Cdkn1b,c,x* forming another subgroup. Notably, in many animals, *Cdkn1b* and *d* are linked. Chicken have retained *Cdkn1a,b,c,d*, Xenopus has retained *cdkn1a* (*xic2*), *cdkn1b* (*xic3*), *cdkn1x* (*xic1*; *cdkn1c,d* are present in other amphibian clades), zebrafish has retained *cdkn1a,ba,bb,ca,cb,d*.

Chicken *Cdkn1a* was barely detectable at HH8-10, and showed low-level, widespread expression at HH13/14 (Figures 4bi–iii, 5S; Table 1). Mouse *Cdkn1a* has been reported to be expressed widely, with upregulated expression in the neural tube and somitic myotome (Magdaleno et al., 2006). The frog ohnologue is expressed specifically in the cement gland, pre-somitic mesoderm, somites, lens, otic vesicle, tail bud (Daniels et al., 2004; Zhang et al., 2017); whereas the fish gene shows little if any expression (Osborn et al., 2011).

Cdkn1b was expressed in the early primitive streak, the neural plate/neural tube and the cardiac crescent; the transcript was also very prominent in the somitic myotome (Figures 4Ci–iii, 5t; Table 1). To better determine the onset of the gene in comparison to *Myf5* indicating the commitment to myogenesis and *MyoD* indicating the start of myogenic differentiation, we analysed expression earlier at stages HH4-7 and later at HH15-16 and HH20. We found that somitic *Cdkn1b* expression began at HH7+ (Figure 6b), somewhat earlier than the expression of *Myf5* at HH10 (Figure 6i). From HH10 onwards, *Cdkn1b* expression was concomitant with that of *Myf5* (compare Figure 6c–f, i–l), but always earlier than that of *MyoD* (Figure 6o–r; for myogenic gene expression, see also Berti et al., 2015). For the mouse *Cdkn1b* gene, little information besides expression in the lens is available (Ho et al., 2009); expression of the frog gene in the neural plate and stage 33 head was described as unspecific (Daniels et al., 2004; Zhang et al., 2017). Zebrafish *cdkn1ba* is expressed specifically somitic myotomes and the otic vesicle (Osborn et al., 2011; Radosevic et al., 2011), *cdkn1bb* is expressed widely, with some upregulation in the otic vesicle and lens, later in the brain, retina, pharyngeal arches, somitic myotomes and pectoral fins (Osborn et al., 2011).

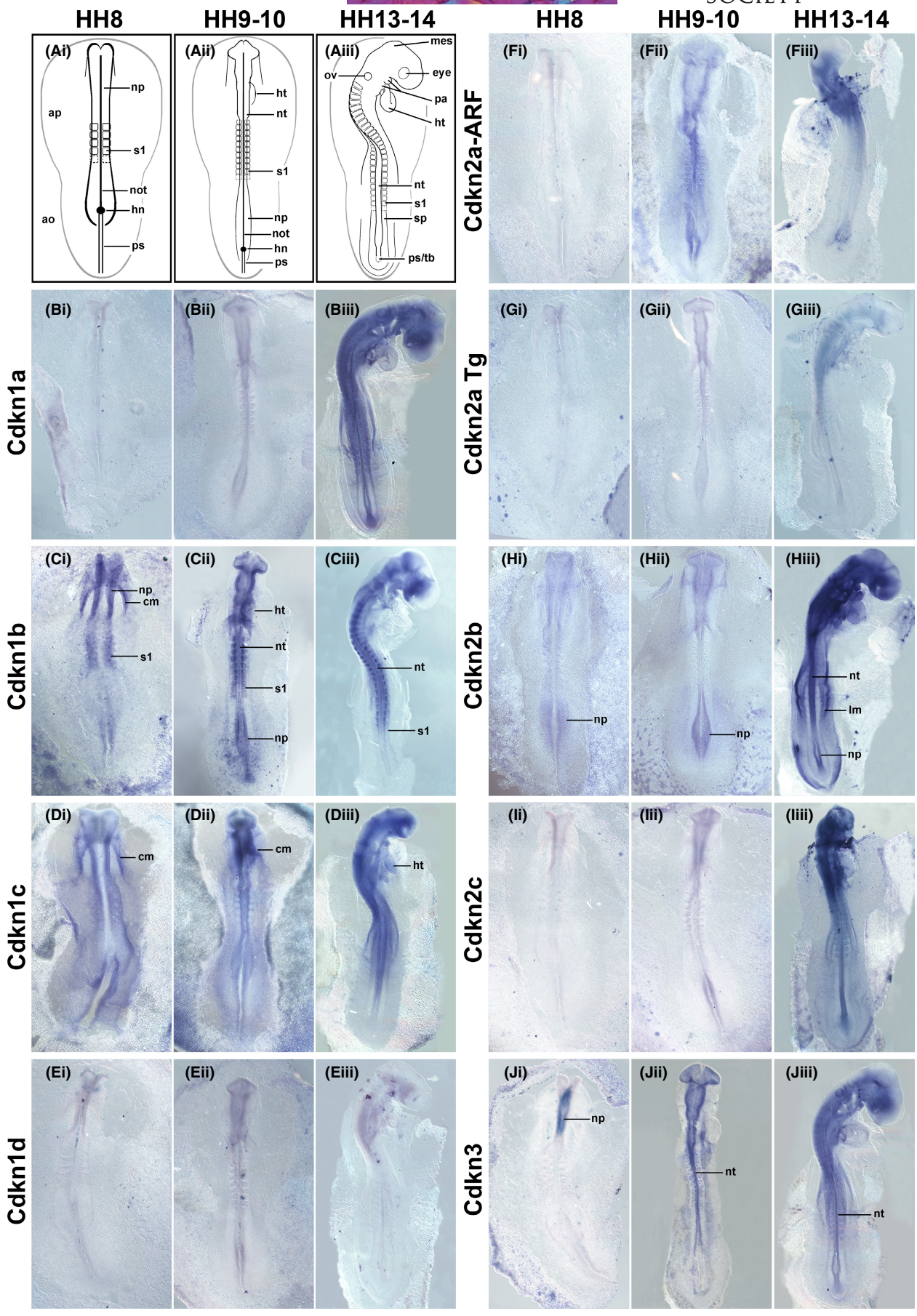
Chicken *Cdkn1c* expression was widespread, with some upregulation in the neural plate/neural tube, myotome, cardiogenic mesoderm (Figures 4di–iii, 5u; Table 1). For mouse *Cdkn1c*, limited information is available, but the gene seems expressed in the heart, skeletal muscle, cerebellum and pancreas primordium (Andrews et al., 2007; Georgia et al., 2006; Seto et al., 2014). Zebrafish *cdkn1ca* is specifically expressed in the notochord, neural tube-primary neurons, adaxial cells, somitic myotomes, later the brain, spinal cord, trigeminal ganglion, otic vesicle and lateral line precursor (Osborn et al., 2011; Radosevic et al., 2011); no data are available for the *cdkn1cb* gene.

Finally, chicken *Cdkn1d* showed no or low-level expression (Figures 4ei–iii; Table 1), with no information available for fish *cdkn1d*; frog *cdkn1x/cdknx/xic1* is expressed specifically in the lens, nasal, otic and neurogenic cranial placodes, the lateral line placode, the brain, retina, pre-somitic mesoderm, somitic myotome, craniofacial and abdominal muscle anlagen (Table 1).

Taken together, while all vertebrates have one or more *Cdkn1* genes showing restricted expression, different paralogues have taken on these specific roles, suggesting distinct sub-functionalisation events.

3.4.2 | Cdkn2/Ink4 family genes

Cdkn2 genes typically consist of two main exons delivering an ankyrin repeat protein. Genes can be traced to a single ancestor that is present already in cnidarians (Schubert et al., manuscript in preparation). In mammals, four family members, *Cdkn2a,b,c,d*, have been identified. *Cdkn2a* and *2b* arose from a tandem gene duplication thought to have occurred before the divergence of amniotes ([Gilley and Fried, 2001] and references therein), but more likely before the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Cdkn2a Tg
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(Iiii)
Cdkn1d
(Ei)
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(Eiii)
Cdkn3
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FIGURE 5 Cervical cross sections of select HH13/14 embryos. (a) Schematic cross section, (b–w) cross sections of embryos stained for the expression of the gene indicated on the left of each image. Abbreviations as in Figures 1, 2 and da, dorsal aorta; dm, somitic dermomyotome; ect, ectoderm; end, endoderm; nd, nephric duct; nm/t, nephric mesoderm/tubules; m, somitic myotome, scl, somitic sclerotome. The asterisks indicate the apical sides of the pseudostratified neural and dermomyotomal epithelia. The coloured frames link similar expression patterns. Note that Cdk25b, CcnD1, Cdk6, but also Cdkn2b and NeuroD4 are expressed within the central, Pax6-positive neural tube (red frames). Pax7 and CcnA2 are both expressed in the dorsal neural tube and the dermomyotome (green frames). Cdkn1b expression coincides with that of Myf5 and MyoD in the myotome (turquoise frames). The mRNAs of Cdc25a,b; CcnB2, D1, E1, E2; Cdk1, Cdk3/2-like, Cdk6 and Cdkn2b are enriched on along the apical side of the pseudostratified epithelia that express the genes

divergence of tetrapods (Schubert et al., manuscript in preparation). However, Xenopus and also zebrafish have a single *cdkn2a/b* gene only, accompanied by *cdkn2c* and *cdkn2d*. In birds the *Cdkn2d* gene was lost, and there is a tendency to also disable or lose one of the *Cdkn2b/a* duplicates, with the downstream located *Cdkn2a* being affected more frequently. The earlier Galgal4 version of the chicken genome suggested that a cryptic exon1 for *Cdkn2a* might exist, but the current Galgal5 version of the genome indicates that only the second *Cdkn2a* exon was retained. Thus, *Cdkn2c* is the best preserved avian *Cdkn2* paralogue (Schubert et al., manuscript in preparation).

In amniotes, a sequence stretch located between the upstream *Cdkn2b* and the downstream *2a* gene has been made expressible, delivering an alternative first exon for *Cdkn2a* (Kim et al., 2003; Szklarczyk et al., 2007). This exon, however, sets a distinct reading frame and thus creates a protein unrelated to the canonical Cdkn2 proteins, often referred to as Cdkn2a–ARF (alternative reading frame). ARF is an upstream suppressor of the cell cycle, suppressing the activity of Mdm2 (Kim et al., 2003; Szklarczyk et al., 2007). To distinguish the expression of the ARF mRNA from that of *Cdkn2a*, we designed a probe specific for its unique first exon (Material S1). We detected no or low-level expression, in line with (Kim et al., 2006). Yet this may be partly due to the probe being short (Figure 4ei–iii). Unfortunately, no data are available for the mouse embryo.

Since a cryptic exon1 had been initially been proposed for *Cdkn2a*, we designed a *Cdkn2a* probe based on the well preserved first exon of the zebra finch (*Taeniopygia guttata*, Tg) gene (Material S1, 2B). This probe neither detected significant expression in the chicken (Figure 4gi–iii) nor in zebra finch embryos corresponding to chicken stages HH14-20 (not shown). The *Cdkn2b* probe, however, detected a signal in the chicken embryo which was widespread, but upregulated in neural plate and blood islands at HH8-10 (Figure 4hi,ii), and upregulated in the *Pax6*-positive, central region of the neural tube, the lateral mesoderm and the somitic sclerotome at HH13/14 (Figures 4hiii; 5v). For the mouse, low-level expression with some upregulation in the neural tube has been reported, both for *Cdkn2a* and *2b* (Magdaleno et al., 2006). The Xenopus *cdkn2a/b* gene is expressed at low levels, upregulated in the craniofacial region, the heart, the pre-somitic and somitic mesoderm (Zhang et al., 2017); no data are available for the zebrafish *cdkn2a/b* gene. Expression of chicken *Cdkn2c* was low level and widespread, with some enrichment in the central nervous system (Figure 4li–iii), mouse *Cdkn2c* has been reported to be expressed at low levels, but upregulated in the neural tube and the oral ectoderm (Magdaleno et al., 2006); no data are available for frog and zebrafish. Finally, mouse *Cdkn2d* is expressed in a similar fashion as *2c* (Magdaleno et al., 2006), frog *cdkn2d* expression is low level with some upregulation in the brain, eyes and somites (Doher, 2014), no data are available for the fish.

3.4.3 | Cdkn3

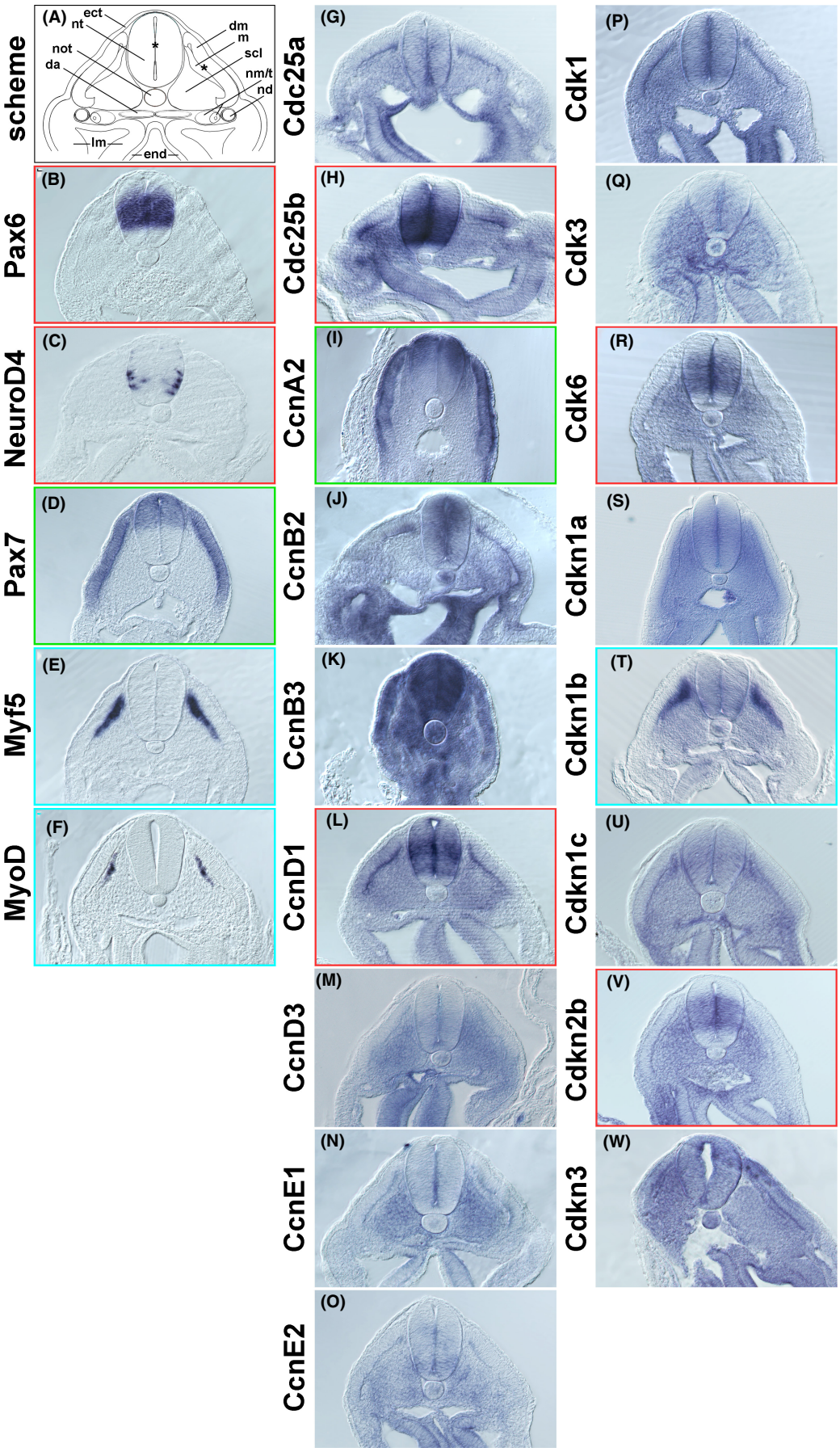
A single *Cdkn3* gene is present in cnidarians, protostomes and deuterostomes, but seems to have been shed in Xenopus (Schubert et al., manuscript in preparation). The chicken gene was initially expressed in the closing, rostral neural plate (Figure 4ji); eventually, expression became more widespread (Figures 4jii–iii, 5w). In contrast, specific *cdkn3* expression in the somitic myotomes and, at lower levels, in the brain had been reported for the zebrafish gene; no data are available for the mouse (Table 1).

4 | DISCUSSION

The aim of this study was to provide a comprehensive expression analysis of all core cell cycle regulators in the chicken embryo, focusing on early neurula to early somite stage/pharyngula stages of development. The second aim was to compare the data obtained for the chicken with those available for mouse, Xenopus and zebrafish, the other commonly used vertebrate models. Our study is the first to attempt this comprehensive analysis as data in particular for the mouse are incomplete. Where the comparison of expression patterns was possible, we found similarities but also remarkable differences between vertebrates. Moreover, we found novel synexpression groups and a curious association of mRNA localisation, cell polarity and nuclear shutting in the pseudostratified epithelia of the neural tube and the somite.

4.1 | Similar but not identical expression of vertebrate cell cycle promoting genes in mitotically active tissues

At neurula and pharyngula stages of development, most developing organs contain a large proportion of actively dividing cells. Thus, we expected widespread expression for genes associated with the promotion of cell cycle. This indeed was true for *Cdc25b*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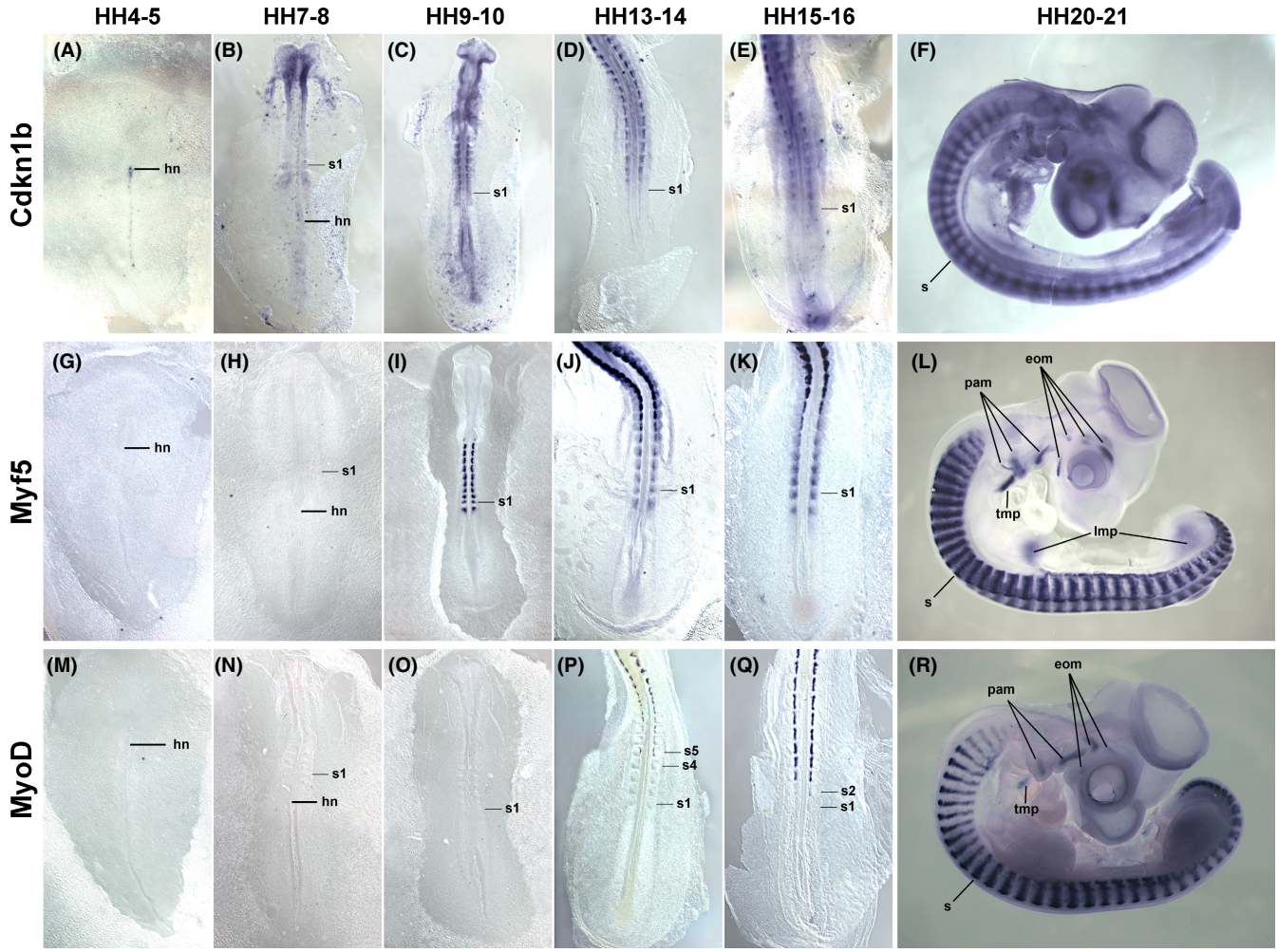

FIGURE 6 Time course for Cdkn1b, Myf5 and MyoD expression. (a–c, g–i, m–o) Dorsal views of embryos at stages HH4-5, 7–8 and 9–10; note the embryo shown in (b) is HH7+, younger than the HH8+ embryo shown in Figure 4Ci. (d, e, j, k, p, q) Dorsal views of the caudal region of embryos at HH13-14 and 15–16. (f, l, r) Lateral view of whole embryos at HH20-21. The markers are indicated on the left, abbreviations are as in Figure 1 and eom, extraocular muscle anlagen; lmp, limb muscle anlagen; pam, pharyngeal arch muscle anlagen; tmp, tongue muscle/hypobranchial muscle anlagen. Note that at HH7-10, somitic expression of Cdkn1b commences before that of Myf5, later both markers appear simultaneously, but always before the onset of MyoD. No specific Cdkn1b expression was detected in craniofacial muscle anlagen which do express first Myf5 and then MyoD

and its tetrapod-specific duplicate *Cdc25a*, the two *Cdc25* genes retained in the chicken (Schubert et al, manuscript in preparation). It was also true for *CcnB3*, *CcnH*, *Cdk1* and *Cdk7*, and at lower levels for *CcnA2*, *B2*, *D2*, *D3*, *E1*, *E2* and *Cdk2*, *Cdk3*/*Cdk2-like* and *Cdk6*. No or very low-level expression was found for *CcnA1*; expression of *CcnO* was extraembryonic. Expression for the lowly expressed genes was barely detectable at the early stages investigated here, and was often confined to the neural plate/neural tube and the primitive streak. This may be linked to specific cell cycle dynamics in these tissues, may reflect that cell density is higher as these tissues are epithelially organised, or may indicate that mRNA levels are not in tune with protein abundance. Nonetheless, our data suggest that in principle, the activating CcnH–Cdk7 complex, activating Cdc25 proteins and CcnD, CcnE, CcnA, CcnB cyclins, able to interact with Cdk6, Cdk1 and Cdk2-type kinases, are available to dividing cells.

There are, however, deviations from the expected: First, entry into the cell cycle is driven by CcnD cyclins and Cdk4/6/21 kinases (Malumbres, 2014; Ruijtenberg and van den Heuvel, 2016). Tetrapods lack the *Cdk21* paralogue, and in addition, the status of the *Cdk4* gene is unclear in marsupials and birds (Schubert et al, manuscript in preparation). To nonetheless try and reveal any *Cdk4* expression, we designed *Cdk4* probes based on the sequences of two archosauromorphs (=crocodiles and birds) that may carry a *Cdk4* gene, the American alligator and the Tibetan ground tit (a passeriform bird). Yet we could not detect any reliable expression, neither in the chicken nor in the zebra finch (also a passeriform), even though the probes were predicted to bind Cdk4 sequences. This suggests that the chicken and many other birds may only have *Cdk6* to trigger cell cycle entry. Notably, *Cdk6*, while at stage HH13-14 strongly expressed in the neural tube and lateral mesoderm, showed no expression in the somites. The somites, however, had strong and specific

CcnD1 expression from stage HH8 onwards, with expression being maintained in the mitotically active dermomyotome and sclerotome. This suggests that in the somites, CcnDs may not cooperate with a Cdk4/6/21 family members, but instead cooperate with Cdk1, which is strongly expressed here, or with Cdk3/2-like proteins, that all can compensate for the absence of Cdk4/6/21 (Malumbres et al., 2004) reviewed in (Sherr and Roberts, 2004).

The second deviation from the expected are species-specific differences in expression. As summarised in Table 1, a number of cell cycle promoting genes have been reported to have upregulated or specific expression in distinct mouse, frog or zebrafish tissues, and we observed the same for the chicken. This expression pattern was at times shared between vertebrates, but at times also deviated. For example, the widespread expression found for chicken *Cdc25a*, frog *Cdc25a,c* and zebrafish *cdc25b* was very similar and may reflect the original pattern of an ancestral *Cdc25* gene. Chicken *Cdc25b* expression was a more restricted version of the *Cdc25a* pattern (Bénazéraf et al., 2006), this study), and where the divergent *Cdc25d* genes are present, they seem to be expressed in unrelated patterns (Dalle Nogare et al., 2007; Nakajo et al., 2011). Another example is *CcnA2* whose expression is upregulated in the neural tube in all vertebrate models (http://www.informatics.jax.org/gxd and https://www.embrys.jp/, http://www.xenbase.org/, http://zfin.org/, (Vernon and Philpott, 2003b). Yet in the chicken neural tube, we found *CcnA2* expression confined to the dorsal territory only (this study). In a similar vein, chicken, mouse and frog *CcnB2* genes are all expressed widely with local upregulation in the notochord, neural tube and somites, while zebrafish *ccnb2* expression was reported to be more restricted (http://www.informatics.jax.org/gxd and https://www.embrys.jp/, http://www.xenbase.org/, http://zfin.org/, [Zhao et al., 1995], this study). Likewise, chicken *CcnD1* has a remarkable restricted expression pattern (this study) while mouse, frog and zebrafish *CcnD1* expression has been reported to at least initially be widespread (Vernon and Philpott, 2003b; Wianny et al., 1998; Zhang et al., 2017; Zhao et al., 1995). Chicken and mouse lack a *CcnD4/x* paralogue, yet the gene is present in Xenopus and zebrafish and is specifically expressed in motor neuron progenitors in the hindbrain and spinal cord (Chen et al., 2005; Lien et al., 2016). Chicken and mouse have two *Cdk2* paralogues that are expressed widely; frog and zebrafish only harbour a single *cdk2* gene, reported to have distinct expression patterns (http://www.informatics.jax.org/gxd and https://www.embrys.jp/, http://www.xenbase.org/, http://zfin.org/, (Vernon and Philpott, 2003b), this study). Expression data for *Cdk6*-type genes are incomplete, hampering comparisons, yet among the four vertebrate models, different family members have been retained, with *cdk21* only being present in the fish and expressed in ovary and testis (http://zfin.org/). It is well established that during vertebrate evolution, the genome was duplicated twice, with further gene and genome duplication events having occurred in different taxa (Dehal and Boore, 2005; Evans, 2008; Glasauer and Neuhauss, 2014; Holland et al., 1994; Kuraku et al., 2009; Taylor et al., 2001). Gene loss, sub- and neo-functionalisation also occurred (Huminiecki and Heldin, 2010), leaving different species with similar but not identical sets of cell cycle regulators (Schubert, manuscript in preparation). Thus, perhaps it is less surprising then to find that gene expression patterns of cell cycle promoting genes are similar, but not identical.

4.2 | Similar but not identical expression of cell cycle inhibitors: a specific role for chicken *Cdkn1b* in the myotome

Cell cycle inhibitors facilitate the exit from cell cycle that is associated with, and in many cases required for, terminal differentiation (Hydbring et al., 2016; Ruijtenberg and van den Heuvel, 2016). Past studies implicated the Cdkn1b protein in mouse neurogenesis ((Nguyen et al., 2006); reviewed in (Hindley and Philpott, 2012)), and Cdkn1a,c and Cdkn2a and the alternative protein generated from the *Cdkn2a* locus, Cdkn2a–ARF, in mouse myogenesis, with mouse Cdkn1b playing a role in the maintenance of adult muscle stem cells (Chakkalakal et al., 2014; Pajcini et al., 2010; Wang and Walsh, 1996; Wei and Paterson, 2001; Zhang et al., 1999); (reviewed in (Kitzmann and Fernandez, 2001; Sherr and Roberts, 1995; Singh and Dilworth, 2013). Moreover, cdkn1c has been shown to cooperate with myod during zebrafish myogenesis (Osborn et al., 2011). Yet as for the cell cycle promoting genes, the cell cycle inhibitors retained in vertebrate genomes are not the same (Schubert et al., manuscript in preparation), and for those that are shared, expression patterns often differ.

For example, a single *Cdkn1a* gene exists in all four vertebrate models. We found low-level, widespread expression for the chicken gene, suggesting an involvement in cell cycle withdrawal and differentiation at many sites and in many organs (this study). Widespread, but upregulated expression in the neural tube and the postmitotic somitic myotomes was reported for mouse *Cdkn1a*; specific expression in a number of tissues was reported for the frog; and low or no expression for the zebrafish (Daniels et al., 2004; Magdaleno et al., 2006; Osborn et al., 2011; Zhang et al., 2017). *Cdkn2* genes present an even more complex picture: a single *cdkn2b/a* gene exists in actinopterygians such as the zebrafish and lower sarcopterygians such as *Latimeria*; yet tetrapods experienced a tandem gene duplication that created a *Cdkn2b–Cdkn2a* pair ([Gilley and Fried, 2001; Kim et al., 2003], Schubert et al., manuscript in preparation). Both genes are active in mammals; *Xenopus* only retained one gene, and in the chicken and many other birds, *Cdkn2a* was disabled. Yet an upstream alternative first exon for *Cdkn2a* delivering Cdkn2a–ARF exists not only in mammals, but also throughout amniotes ([Kim et al., 2003], Schubert et al., manuscript in preparation). We have designed probes to separately detect genuine *Cdkn2a* and *ARF* mRNAs, but at the stages analysed, we have not found evidence for their expression, in line with (Kim et al., 2006). Chicken *Cdkn2b* expression, however, was widespread with upregulation in the neural tube, sclerotome, lateral mesoderm and blood islands (this study). There are no expression data for mouse *ARF*, but both *Cdkn2b* and *2a* were reported to show low-level embryonic expression, with

some upregulation in the neural tube (Magdaleno et al., 2006); in the frog *cdkn2b* was expressed at low levels, with some upregulation in the craniofacial region, heart, pre-somitic mesoderm and somites (Zhang et al., 2017), no data are currently available for the zebrafish. Finally, the highly conserved *Cdkn3* gene showed restricted expression in the closing neural tube of the chicken, with expression later becoming widespread. In the zebrafish, the gene was reported to be expressed in the somitic myotomes and, at low levels, in the brain (http://zfin.org/), there are no expression data for the mouse, and Xenopus seems to have lost the *cdkn3* gene.

A remarkable association between *Cdkn* gene expression and differentiation was found for chicken *Cdkn1b*. The gene was highly expressed in the postmitotic cells of the somitic myotomes. Yet mRNA expression commenced at the time *Myf5* expression began, always before the onset of *MyoD* and *MyoG*. This was unexpected since cell cycle withdrawal and terminal differentiation is thought to be controlled by MyoD and MyoG (reviewed in (Singh and Dilworth, 2013)). It is possible that *Cdkn1b* RNA is transcribed and stored for subsequent translation. Yet this would suggest a novel control mechanism involving RNA-binding proteins that warrants further investigation. Notably, among the other vertebrate model organisms, specific myotomal expression has only been established for zebrafish *cdkn1ba* (Osborn et al., 2011; Radosevic et al., 2011). Moreover, in the mouse *Cdkn1a,c* seem to be the main *Cdkn1* paralogues associated with myogenesis (Mademtzoglou et al., 2018; Wang and Walsh, 1996). In zebrafish this role falls to *cdkn1c* (Osborn et al., 2011), whereas in the chicken, *Cdkn1c* is expressed in the myotome at low levels only (this study). Furthermore, the frog has retained the *cdkn1x/xic1* gene, and this gene is strongly expressed in the myotomes (Vernon and Philpott, 2003a). Thus, while a role in myogenesis may be a basic function of the original *cdkn1* gene prior to the vertebrate genome and gene duplications, sub-functionalisation seems to have assigned this role to different paralogues in different vertebrate taxa. Thus, *Cdkn* cell cycle inhibitors are expressed at many sites of cell differentiation; different vertebrates use different paralogues for that purpose.

4.3 | *Pax6, CcnD1, Cdk6, Cdc25b and Cdkn2b* form a synexpression group in the central spinal cord, which also encompasses *NeuroD4*-expressing differentiating neurons

Previous studies established that *CcnD2*, *CcnB2*, *Cdk1* and *Cdc25a* are expressed in the immature chicken trunk neural plate/neural tube, the precursor of the spinal cord. When under the influence of retinoic acid the tissue matures, *Pax6* is turned on in the central domain, and cell cycle gene expression shifts to *CcnD1* and *Cdc25b*, facilitating a lengthening of cell cycle (reviewed in (Hindley and Philpott, 2012; Molina and Pituello, 2017). With the exception of the proposed differential expression of *CcnE* genes, our expression analysis was able to confirm and further add to previous studies: We found that in the *Pax6* expressing central neural tube, *CcnD1*, *Cdk6* and *Cdc25b*, but also the cell cycle inhibitor *Cdkn2b* are co-expressed. Thus, these genes form a synexpression group. Moreover, expression overlaps with the expression of *NeuroD4* in cells that are completing, or have completed, their last cell cycle and prepare for neuronal differentiation, suggesting that the genes are part of a gene regulatory network that specifically controls proliferation and differentiation of central neuronal subtypes. Moreover, our data suggest a specific role of *Cdkn2b* in this process.

4.4 | *Pax3/7* and *CcnA2* form a synexpression group in the dorsal spinal cord and in the somitic dermomyotome

In contrast to *Pax6*, *Pax3* and *Pax7*, two *Pax* ohnologues that arose during the vertebrate specific genome duplications, are already expressed in the immature neural plate, labelling the neural folds from which neural crest cells will emerge. However, at the time that *Pax6* expression commences in the central neural tube, *Pax3/7* adopt a specific expression domain in the dorsal neural tube (Buckingham and Relaix, 2015; Holland et al., 1999; Maczkowiak et al., 2010; Seo et al., 1998). *Pax3/7* also begin to be expressed in the developing somites, with expression being retained in the dermomyotome (Berti et al., 2015; Buckingham and Relaix, 2015). In both tissues, *Pax3/7* positive cells are mitotically active and harbour a number of the widely distributed cell cycle regulators (this study). Remarkably, we also found that both in the neural tube and in the dermomyotome, *CcnA2* is expressed in the same pattern as *Pax3/7*. *CcnA2* has further sites of expression in the surface ectoderm and cranial neural crest cells, suggesting a role in a number of gene regulatory networks. In the dorsal neural tube and somite, however, chicken *CcnA2* is in a synexpression group with *Pax3/7*. For mouse, frog and zebrafish *CcnA2*, upregulated expression has been reported for the neural tube, but expression seems to commence in the neural plate, that is, earlier than in the chicken, and a link to the *Pax3/7* expression domains has not been established. Likewise, mouse, frog and zebrafish *CcnA2* expression has been reported for the somites, but a link to the dermomyotome has not been made (http://www.informatics.jax.org/gxd and https://www.embrys.jp/, http://www.xenbase.org/, http://zfin.org/, (Vernon and Philpott, 2003b). Thus, more detailed analyses in mouse, frog and zebrafish will be required to establish whether the *Pax3/7-CcnA2* link is evolutionarily conserved.

4.5 | *Transcripts* of many cell cycle promoting genes accumulate along the apical surface of both the spinal cord and the somitic dermomyotome

Both the vertebrate neural tube and newly formed somites are pseudostratified epithelia, each harbouring a well-defined outer, basal side and an apical side that faces the lumen of the tissue; this tissue organisation is maintained in the somitic dermomyotome until it deepithelialises to release the dermal progenitors and the embryonic muscle stem cells (Buckingham and Rigby, 2014; Kim et al., 2006). In the polarised

cells of epithelia, many proteins have a distinct apical or basal localisation, in tune with their localised function. Localised mRNA accumulation in subcellular domains has also been demonstrated, for example for the intestinal epithelium, and was associated with translation efficiency (Moor et al., 2017). Yet this phenomenon has not been reported for the neural tube or the somites. We found, however, that in both tissues, *Cdc25a,b*, *CcnB2,D1,E1,E2* and *Cdk1* transcripts accumulated on the apical side of the cells. In the neural tube, further apical accumulation was observed for *Cdk3/2-like*, *Cdk6* and *Cdkn2b*.

In the neural tube, cell nuclei shuttle between the apical and basal surface during cell cycle: when cells enter the G1 phase of the cell cycle, nuclei are located at the apical side. The nuclei move to the basal side, reaching it in S-phase. During G2 nuclei return to the apical side, and this is also where cell division takes place (Lee and Norden, 2013; Molina and Pituello, 2017). Likewise, for the dermomyotome, symmetric cell divisions that drive the growth of the tissue take place apically (Ben-Yair et al., 2011; Venters and Ordahl, 2005), and localised cell division and nuclear shuttling has been proposed to be a phenomenon typical for all pseudostratified epithelia (Lee and Norden, 2013; Norden, 2017). At least in tall tissues, interference with the dynein/microtubule motor system not only blocked the interkinetic nuclear migration, but it also interfered with cell cycle entry, indicating that nuclear shuttling is required for cell cycle progression (Hu et al., 2013). Moreover, Cdk1 controls the recruitment of dynein to the nuclear envelope during G2 (Baffet et al., 2015), indicating that the core cell cycle regulators and nuclear shuttling are molecularly intertwined.

Ccns have been reported to show cyclic expression, with CcnE proteins peaking at the G1/S transition, CcnAs accumulating during S and G2 phases, and CcnBs peaking at the G2/M transition and persisting during M phase (Malumbres, 2014; Ruijtenberg and van den Heuvel, 2016). Thus, the observed accumulation of *CcnB2,D1,E1,E2* mRNA on the apical side of the neural tube and the somite correlates with the time when the genes are transcribed, with the time and site of action for the protein and also with the apical position of the nucleus. Likewise, given that the decision to enter or withdraw from cell cycle as well as the actual cell division are carried out when the cell nucleus is on the apical side, also the apical mRNA localisation for *Cdc25a,b*, *Cdk6*, *Cdk1* and *Cdkn2b*, possibly linked to high translation efficiency and protein localisation, is also plausible. However, the possible purpose of apical localisation of *Cdk3/2-like* mRNA which according to the classical model would be needed in late G1 and during S-phase (Malumbres, 2014; Ruijtenberg and van den Heuvel, 2016) is less clear.

Nuclear shuttling in proliferative epithelia has been shown to predominantly depend on actomyosin II activity, in particular for the basal to apical movement. In tall tissues, dynein or kinesin motors acting on microtubules have also been implicated (Lee and Norden, 2013; Molina and Pituello, 2017). Actomyosin as well as dynein and kinesin motors have been shown to influence mRNA transport, with RNA-binding proteins (RBPs) serving as mediators of the transport and as anchors at the site of destination. Moreover, RBP may suppress translation during mRNA transport, ensuring localised protein production (Buxbaum et al., 2015). Our data suggest the exciting prospect that cell cycle, nuclear movement and mRNA localisation for cell cycle genes may be part of a to-be-explored regulatory system.

4.6 | Summary

In summary, we found novel synexpression groups that hint at unexplored aspects of gene regulatory networks controlling cell proliferation and differentiation. Moreover, for the pseudostratified epithelia of the neural tube and the somite we discovered a remarkable association of cell cycle regulator mRNA localisation with cell polarity and nuclear shuttling, which opens new avenues for investigation. Finally, our study revealed similarities but also differences in the expression of cell cycle regulators in the four established vertebrate model organisms which is linked to divergent gene retention and sub- and neofunctionalisation of paralogues. The consequence of this finding is that in the future, studies investigating tissue regeneration, stem cell behaviour and cancer have to be interpreted in a species/model specific context, and the applications of findings to humans have to be done with utmost care.

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AUTHOR CONTRIBUTIONS

Susanne Dietrich designed the work, performed the bioinformatics work to derive the novel probes as indicated in Supplementary Materials 1–3, wrote the manuscript and carried out the revisions. Marta Alaiz Noya and Federica Berti carried out the majority of in situ hybridisations and the photomicroscopy. All authors contributed to the discussion of the work.

DATA AVAILABILITY STATEMENT

Data available in article supplementary material.

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