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Cerebellar cortical lamination and foliation require *cyclin A2*

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ABSTRACT

The mammalian genome encodes two A-type cyclins, which are considered potentially redundant yet essential regulators of the cell cycle. Here, we tested requirements for *cyclin A1* and *cyclin A2* function in cerebellar development. Compound conditional loss of *cyclin A1/A2* in neural progenitors resulted in severe cerebellar hypoplasia, decreased proliferation of cerebellar granule neuron progenitors (CGNP), and Purkinje (PC) neuron dyslamination. Deletion of *cyclin A2* alone showed an identical phenotype, demonstrating that *cyclin A1* does not compensate for *cyclin A2* loss in neural progenitors. *Cyclin A2* loss lead to increased apoptosis at early embryonic time points but not at post-natal time points. In contrast, neural progenitors of the VZ/SVZ did not undergo increased apoptosis, indicating that VZ/SVZ-derived and rhombic lip-derived progenitor cells show differential requirements to *cyclin A2*. Conditional knockout of *cyclin A2* or the SHH proliferative target *Nmyc* in CGNP also resulted in PC neuron dyslamination. Although *cyclin E1* has been reported to compensate for *cyclin A2* function in fibroblasts and is upregulated in *cyclin A2* null cerebella, *cyclin E1* expression was unable to compensate for loss-of *cyclin A2* function.

Introduction

Normal central nervous system (CNS) development requires precise regulation of neural progenitor proliferation by extrinsic organizing signals, mitogens, and cell-intrinsic programs. Cell proliferation is driven by cyclins and their catalytic partners, the cyclin-dependent kinases (CDKs) (Bloom and Cross, 2007). Cyclin/CDK complexes, in concert with other proteins, control cell cycle through regulation of multiple cell cycle phases. In the classical model, particular cyclin/CDK complexes regulate transitions through G1, S, G2, and M cell cycle phases. This notion has been challenged by experiments demonstrating significant redundancy in cyclin-CDK binding and cell cycle progression (Aleem et al., 2005). Furthermore, cyclins have been shown to regulate other processes not traditionally associated with classical function, including CNS synapse development (Odajima et al., 2011) and DNA repair (Jirawatnotai et al., 2011).

The mammalian genome encodes two A-type cyclins, testis-specific *cyclin A1* and ubiquitously expressed *cyclin A2* (Sweeney et al., 1996; Yang et al., 1997a). Whereas male meiosis is dependent on *cyclin A1* (Liu et al., 1998), *cyclin A2* was originally shown to be required for the onset of DNA replication (Girard et al., 1991). Analyses of conventional *cyclin A2* null mice revealed that these animals fail to develop after 5.5 days postcoitum, underscoring a critical function for this cyclin in cell proliferation (Murphy, 1999). In addition, conditional ablation of the A-type cyclins revealed that *cyclin A2* function is essential for cell-cycle progression of hematopoietic and embryonic stem cells, yet is redundant with *cyclin E1* in mouse embryonic fibroblasts (Kalaszczynska et al., 2009). While *cyclin A2* expression can be induced by Sonic hedgehog (SHH) signaling in cerebellar granule neuron precursors (CGNP) (Zhao et al., 2002), *cyclin A2* function has not been investigated in cerebellar development.

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The cerebellar external granule layer (EGL) is populated by cerebellar granule neuron precursors (CGNP). SHH signaling is required for both cerebellar morphogenesis and CGNP proliferation (Dahmane and Ruiz i Altaba, 1999; Wechsler-Reya and Scott, 1999). CGNP cells emerge from the rhombic lip and then migrate to the EGL, while Purkinje (PC) neurons originate in the hindbrain ventricular/subventricular zone (Hatten and Heintz, 1995; ten Donkelaar et al., 2003) and develop to become the source of SHH proteins (Wallace, 1999). We tested the hypothesis that proper cerebellar development generally requires function of A-type cyclins through a conditional gene targeting approach. Deleting *cyclins A1* and *A2* in neural progenitor cells results in dysmorphic cerebella characterized by reduced growth of the CGNP population, abnormal foliation, and PC dyslamination. Similar findings were obtained in *nestin-cre, cyclin A2$^{fl/fl}$* animals showing that *cyclin A1* is unable to compensate for loss of *cyclin A2*. Increased programmed cell death was noted during early embryonic development of *cyclin* A2-null neural progenitors. PC dyslamination and abnormal proliferation was also noted in *Math1-cre, cyclin A2$^{fl/fl}$* mice, suggesting that these defects are intrinsic to the CGNP population.

Materials and methods

Transgenic mice and animal husbandry

The generation of *cyclin A1* and conditional *cyclin A2$^{fl/fl}$* knockout mice is described in Kalaszczynska et al. (2009) and references therein (Kalaszczynska et al., 2009). These animals were then bred to *cyclin A1* null mice (Liu et al., 1998), generating *cyclin A1$^{-/-}$A2$^{fl/fl}$* animals. *A1$^{-/-}$A2$^{fl/fl}$* and *A2$^{fl/fl}$* mice were bred to *nestin-cre* mice (Tronche et al., 1999). *Nestin-cre* mice show recombination in the CNS, including all cells of the cerebellum by E13.5 (Huang et al., 2010) and Purkinje cells of adults (Jennemann et al., 2005). Verification of *A2* loss was performed by western blot (Supplemental Fig. S2), *in situ* hybridization (Supplemental Fig. S1), and immunofluorescence (Fig. 4). *Math1-cre* (Schuller et al., 2008) was crossed to *A2$^{fl/fl}$* mice and *Nmyc$^{fl/fl}$* (Knoepfler et al., 2006) to generate *Math1-cre, A2$^{fl/fl}$* mutants and *Math1-cre, Nmyc$^{fl/fl}$* mutants. Verification of *Nmyc* loss was performed by *in situ* hybridization. Developmental analyses in wild-type mice were performed in the CD-1 strain (Charles River). In BrdU pulsing experiments, animals were injected with 1 mg BrdU intraperitoneally 2 h prior to sacrifice. All animal experimentation was performed in full compliance with the institutional review board requirements of Dana Farber Cancer Institute, University of California, San Francisco, and The Ohio State University.

Histology, immunofluorescent staining, in situ hybridization, and photomicroscopy

To evaluate brain morphology, experimental and control mice were sacrificed at P0, P7, and adult ages, perfused, and heads or brains were dissected and incubated in 4% paraformaldehyde. Evaluation of embryonic time points was performed by sacrificing the pregnant mouse and drop-fixing whole embryos into 4% paraformaldehyde. Post-fixation processing, immunoblotting, and ISH were performed as described previously by our group (Fancy et al., 2011). Briefly, animals were perfused with 4% PFA, and brains were then drop fixed in 4% PFA overnight. Tissue was incubated in 30% sucrose-1XPBS at 4 °C for 24–36 h, embedded in OCT, and cryosectioned at 12–14 μm. Cre-negative littermates represent negative controls.

The following primary antibodies were used for immunohistochemistry and western blotting: GFAP (Abcam, ab7777-500), Calbindin (Sigma, C8666), AP2β (Santa Cruz Biotechnology, SC-8976), Zic-1 (kind gift from R. Sega, Dana Farber Cancer Center), cleaved Caspase 3 (Cell Signaling, 9664 and 9661), Ki67 (Vector Labs, VP-RM04), phosphohistone H3 (Cell Signaling, 2650S), BrdU (GeneTex, gtx26326), Tuj1 (Covance, MMS-435 P-250), Pax6 (Santa Cruz Biotechnology, SC-7750), cyclin A (Santa Cruz, SC-53230 and SC-596), cyclin E1 (for IHC, antibody was provided by Dr. B. Clurman; for western blotting we used Santa Cruz, SC-481), cdk2 (Santa Cruz, sc-163 AC M2), cre (Millipore, MAB3120), GFP (Aves Lab, 1020), cdk1 (Abcam ab71939), synapsin-I (Millipore, AB1543). Appropriate secondary antibodies were purchased from Invitrogen/Molecular Probes. For brightfield and epifluorescence, a Zeiss Axioscope with an Axiocam HRC camera and a Nikon 80i microscope with Hamatasu Orca-R2 camera was used. Confocal microscopy instrumentation included Axioscope 2 mot plos with high-resolution color and monochrome camera and Axiovision 4.5 Image analysis software

In situ hybridization probe constructs for *Gli1, Math1* and *Shh* were generously provided by A.L. Joyner (Memorial Sloan Cancer Center, New York, New York, USA), J. Johnson (UT Southwestern, Dallas, USA) and A.P. McMahon (UCSF, USA), respectively. Probes for *cyclin D1* (Sicinski et al., 1995) and *Nmyc* (Charron et al., 2002) and patterning genes *Foxp4, Foxp2, Esrrb, Nrsf1* (Schuller et al., 2006) have been described and the hybridization protocols are specified therein. The *cyclin A2 in situ hybridization* probe was generated from cyclin A2 cDNA construct purchased from Open Biosystems (Clone BC052730 in pYX-Asc vector). This represents a 2770 bp fragment of cyclin A2 cDNA. To generate the antisense probe, the plasmid DNA was linearized with NheI (NEB) and transcribed with T3 polymease. Digoxigenin-labeled antisense RNA probes were made using plasmid DNA as the template (Roche). *In situ* hybridization with frozen sections was performed as described previously (Zhao et al., 2002). Western blotting was performed as described previously (Odajima et al., 2011). To evaluate protein levels across wells, band intensity was normalized to GAPDH band intensity determined by the densitometry function of Image J.

Unbiased stereology

Unbiased stereology was performed to determine estimates of volume and total number of cleaved-caspase 3 positive cells in the embryonic cerebellum (Stereoinvestigator™, MBF Biosciences, Fig. 6). Brains were cryosectioned at 50 μm (section cut thickness) and every 5th section (section evaluation interval) was immunostained with cleaved-caspase 3; Dako Envision™ kits developed the DAB reactions, nuclei were counterstained with hematoxylin (Electron Microscopy Solutions), and cover slips were mounted with Permount™ (Fisher). Note that peroxidase-DAB reactions using thick sections require increased incubation times in the final peroxidase reaction, and therefore it is common to see increased background when using a thick section. Cavalieiri estimation generated volume estimates with the following parameters: grid size = 30 μm, shape factor = 4. Cleaved caspase-3 total cell number estimates were obtained using optical fractionator probes with the following parameters: counting was performed under oil immersion with 100× objective, dissector height = 20 μm, dissector volume = 50,000 μm^3, counting frame height and width = 50 μm, sampling grid was 153.9 μm × 162.5 μm. We use the term ventricular zone/sub-ventricular zone (VZ/SVZ) to designate the 4th ventricular germinal neuroepithelium of the cerebellum, which for quantification purposes included the dense band of neural progenitor cells lining the 4th ventrical.