Homeodomain Transcription Factor Phox2a, via Cyclic AMP-Mediated Activation, Induces p27^{Kip1} Transcription, Coordinating Neural Progenitor Cell Cycle Exit and Differentiation^{∇†}

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Mechanisms coordinating neural progenitor cell cycle exit and differentiation are incompletely understood. The cyclin-dependent kinase inhibitor p27^{Kip1} is transcriptionally induced, switching specific neural progenitors from proliferation to differentiation. However, neuronal differentiation-specific transcription factors mediating p27^{Kip1} transcription have not been identified. We demonstrate the homeodomain transcription factor Phox2a, required for central nervous system (CNS)- and neural crest (NC)-derived noradrenergic neuron differentiation, coordinates cell cycle exit and differentiation by inducing p27^{Kip1} transcription. Phox2a transcription and activation in the CNS-derived CAD cell line and primary NC cells is mediated by combined cyclic AMP (cAMP) and bone morphogenetic protein 2 (BMP2) signaling. In the CAD cellular model, cAMP and BMP2 signaling initially induces proliferation of the undifferentiated precursors, followed by p27^{Kip1} transcription, G₁ arrest, and neuronal differentiation. Small interfering RNA silencing of either Phox2a or p27^{Kip1} suppresses p27^{Kip1} transcription and neuronal differentiation, suggesting a causal link between p27^{Kip1} expression and differentiation. Conversely, ectopic Phox2a expression via the Tet-off expression system promotes accelerated CAD cell neuronal differentiation and p27^{Kip1} transcription only in the presence of cAMP signaling. Importantly, endogenous or ectopically expressed Phox2a activated by cAMP signaling binds homeodomain *cis*-acting elements of the p27^{Kip1} promoter *in vivo* and mediates p27^{Kip1}-luciferase expression in CAD and NC cells. We conclude that developmental cues of cAMP signaling causally link Phox2a activation with p27^{Kip1} transcription, thereby coordinating neural progenitor cell cycle exit and differentiation.

Cyclin-dependent kinase (Cdk) inhibitors controlling G₁ progression and entry into S phase have been implicated as important regulators of cell cycle exit during development (15, 40, 76), displaying transcriptional induction when progenitor cells exit the cell cycle and differentiate (20, 22, 46, 55). Two families of Cdk inhibitors exist in mammalian cells: the inhibitors of Cdk4 (p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c}, and p19^{Ink4d}), which inactivate Cdk4 and Cdk6 (61), and the Cip/Kip family of inhibitors (p21^{cip1}, p27^{Kip1}, and p57^{Kip2}), which inactivate Cdk2 and Cdk1.

p27^{Kip1} inhibits Cdk2, which is required for entry into S phase, thus playing a central role as a negative regulator of cell cycle progression in a variety of tissues (63). p27^{Kip1} homozygous null mice exhibit seemingly normal prenatal development but display hyperplasia in many tissues that undergo postnatal growth (23, 34, 53). p27^{Kip1} also acts as a cell-intrinsic timer or switch regulating the transition from proliferation to differentiation of progenitors in various cell lineages (18, 21, 41, 70, 73), including neural progenitors (10, 17, 22, 28, 42). Despite the demonstrated involvement of p27^{Kip1} in regulating proliferation of various neural progenitors (10, 15, 22, 28), the transcription factors mediating p27^{Kip1} transcription during neuronal differentiation have not been identified. In this study, we

employed two cellular models of noradrenergic neuron differentiation and investigated whether developmental signals activating the homeodomain (HD) transcription factor Phox2a coordinate cell cycle exit and differentiation by inducing the transcription of p27^{Kip1}.

The proneural homeodomain transcription factor Phox2a (43, 50) is a key regulator in the differentiation of neural progenitors generating noradrenergic neurons of the central nervous system (CNS) and the peripheral nervous system (PNS). Noradrenergic neurons are characterized by synthesis and storage of catecholamines (2, 27) by expression of the regulatory biosynthetic enzymes tyrosine hydroxylase (TH) and dopamine-β-hydroxylase. Major CNS noradrenergic neurons are those of the locus ceruleus (36), derived from neural progenitors located in the ventricular zone (30). Noradrenergic neurons of the PNS include sympathetic ganglia and adrenal medullary cells (3, 25), which are derived from the trunk region of the neural crest (NC) during neural tube closure (38).

Although the progenitors giving rise to noradrenergic neurons of the CNS and PNS have different developmental origins (30), the mechanisms involved in their differentiation appear to be the same (12). Bone morphogenetic protein 2 (BMP2) and cyclic AMP (cAMP) signaling pathways synergistically induce catecholaminergic neuron differentiation via Phox2a transcription (5) and Phox2a activation (12). Specifically, *in vivo* loss- and gain-of-function studies have established that expression of Phox2a in committed progenitors requires BMPs inducing the lineage-determining transcription factor MASH1, which is necessary for Phox2a expression (60, 62, 64, 67). In addition to BMPs, moderate activation of the cAMP pathway

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is also required for Phox2a transcription (5), Phox2a activation (12), and development of the sympathoadrenal (SA) lineage originating from the NC (7, 12, 44). As with BMP2 (64), moderate activation of cAMP signaling is instructive (7) and is essential (12) in SA cell development. The cAMP pathway is also required for neuronal differentiation of the CAD cell line (8), a variant of the catecholaminergic CNS-derived Cath.a cell line (68). Cath.a cells originated from locus ceruleus brain tumors of transgenic mice expressing the simian virus 40 T antigen under the control of the TH promoter (68). Cath.a cells, similarly to noradrenergic SA cells, are characterized by TH and dopamine- β -hydroxylase expression, synthesis of catecholamines, and neurite development. CAD cell differentiation depends on cAMP-mediated Phox2a expression and activation (12). In agreement with similar observations by others (1, 69, 75), we reported that cAMP signaling regulates CAD and NC cell neuronal development by regulating Phox2a DNA binding and transcriptional activity, mediated by a protein phosphatase 2A (PP2A)-like phosphatase which dephosphorylates and activates Phox2a (12).

Despite this mechanistic understanding of Phox2a involvement in noradrenergic neuron differentiation, how activated Phox2a mediates neuronal differentiation via cross talk with cell cycle regulators is not yet understood. Phox2b, a proneural, Phox2a-related homeodomain transcription factor, coordinately regulates neuronal cell cycle exit and differentiation, but the mechanism has not been determined (19).

Since p27^{Kip1} switches various neural progenitors from proliferation to differentiation by promoting cell cycle exit (10, 17, 28, 42, 57) and since the cAMP pathway via p27^{Kip1} inhibits the Cdk-activating kinase from activating Cdk4 and cell cycle progression (33), we examined whether the cAMP pathway induces noradrenergic neuron differentiation by regulating cell cycle exit of neural progenitors in a p27^{Kip1}-dependent mechanism. Here, employing two cellular models of noradrenergic neuron differentiation, avian primary NC cultures (7) and the murine CAD cell line (68), we demonstrate that cAMP-mediated Phox2a activation directly induces p27^{Kip1} transcription. In turn, p27^{Kip1} mediates G₁ cell cycle arrest of neural progenitors, leading to differentiation of noradrenergic neurons of CNS and NC origin.

MATERIALS AND METHODS

NC cultures. NC cultures were prepared from 47.5-h Japanese quail embryos as previously described (7, 12).

CAD cells. CAD cells were grown as described previously (5, 59). CAD cell neuronal differentiation was induced by addition to serum-containing medium of 10 ng/ml BMP2 and 100 μ M IBMX (SBI). The presence of BMP2, IBMX, and 1 nM okadaic acid (SBI+OA) are conditions inhibiting neuronal differentiation. For CAD cell neuronal differentiation, 3×10^5 cells were plated per 6-cm dish, treated as described above, and maintained for 3 days in the same medium without refeding.

Tetracycline-regulated Phox2a-expressing CAD cell lines. Tetracycline-regulated Phox2a-expressing CAD cell lines were constructed as previously described (71), employing the Tet-off vector system described by Gossen and Bujard (26). Briefly, Phox2a was cloned in frame with three copies of the FLAG epitope in the PUHD10-3 vector (26), employing PCR amplification, standard cloning methodology, and DNA sequencing. Phox2a-FLAG was expressed in the CAD-Phox2a-FLAG cell line by tetracycline removal for 72 h and was purified by immunoaffinity chromatography using anti-FLAG M2 affinity gel (Sigma) and elution with FLAG octapeptide (Sigma). Phox2a-FLAG DNA binding to the HD binding site was analyzed by electrophoretic mobility shift assay (EMSA) (12) and Southwestern blot assays (4).

Immunocytochemistry. NC and CAD cell immunofluorescence analysis was performed as described previously (8). Modifications used for each antibody are provided in Table S1 in the supplemental material. CAD cells used for confocal microscopy were grown on Permanox plastic slides (Nalge Nunc International) and mounted using the SlowFade Light Antifade kit (Molecular Probes).

Real-time PCR. Total RNA from NC, CAD, or CAD-Phox2a-FLAG cells was extracted with TRIzol (Invitrogen). Real-time PCR was performed and quantified as described previously (39). The primers used are listed in Table S2 in the supplemental material.

Flow cytometry. CAD cells grown for 24 h or 48 h were fixed in ethanol at -20°C for 1 h, followed by 30 min of incubation with 1 μ l of SYBR green (Molecular Probes) per ml of cells, and analyzed with a Cytomics FC 500 instrument (Beckman-Coulter) at a flow rate of $<1,000$ cells/s. Analyses were performed using WinList 5.0 software (Verity Software House).

RNA interference. Phox2a and p27^{Kip1} silencing by small interfering RNA (siRNA) was performed by transfecting CAD cells with a pool of Phox2a- or p27^{Kip1}-specific siRNA (M-048526-00 or M-040178-00, respectively; SMART-pool kit from Dharmacon). Transfections were performed with Oligofectamine (Invitrogen) according to the manufacturer's instructions. The siControl basic kit (Dharmacon) employed was comprised of the siControl lamin A/C siRNA, Non-Targeting siRNA, Non-Targeting siRNA pool, and RISC-Free siRNA. The CAD cell transfection efficiency of siRNA duplexes was assessed with the siGLO RISC-Free siRNA (Dharmacon).

ChIP assays. Chromatin immunoprecipitation (ChIP) assays were performed as described previously (5), employing 2 μ g Phox2a antibody (Sigma) or 2 μ g immunoglobulin G (IgG), either with CAD cells grown to confluence in 10-cm dishes and treated for 24 h with SBI with or without OA (1 nM) or with CAD-Phox2a-FLAG cells grown with or without 5 μ g/ml tetracycline for 72 h and treated with or without forskolin (5 μ M) and OA (1 nM) for 4 h. Immunoprecipitated DNA corresponding to the p27^{Kip1} promoter was quantified by real-time PCR as described previously (39). Primer pairs used for sites 1, 2, and 3 of p27^{Kip1} promoter are listed in Table S3 in the supplemental material.

Transient-transfection assays. The mouse p27^{Kip1} promoter spanning nucleotides +50 to -1156 was amplified by PCR and cloned in a luciferase reporter. The p27^{Kip1}-luciferase plasmid (500 ng) was transfected by the Eugene 6 method (Roche Molecular Biochemicals) in CAD or NC cells treated with SBI with or without OA (1 nM). Transfected cells were harvested at 24 h and assayed for luciferase activity, which was normalized per μ g of protein extract.

RESULTS

The CAD cell line: model characterization for cell cycle exit and neuronal differentiation. In this study, the CNS-derived CAD cell line was used as a cellular model to investigate the mechanism by which Phox2a activation coordinates cell cycle exit of neural progenitors and differentiation. The CNS-derived CAD cells, similarly to NC cells, differentiate to noradrenergic neurons by combined treatment with BMP2 and the cAMP-elevating agent IBMX (5, 8, 12). cAMP signaling is necessary for Phox2a activation and differentiation, inducing a PP2A-like phosphatase which is inhibited by 1 to 5 nM OA (12).

We measured the growth and differentiation characteristics of CAD cell cultures (Fig. 1A) grown in the presence of serum, with BMP2 and IBMX treatment in serum-containing medium (SBI), or with SBI plus 1 nM OA. CAD cells treated for 24 h with SBI or SBI+OA grow at the same rate and display a statistically significant ($P < 0.05$) increase in total cell number in comparison to those grown with serum alone. Importantly, 1 nM OA in serum does not affect CAD cell growth, excluding effects of OA (1 nM) on the cell cycle, in agreement with similar observations by others (49). These results demonstrate that CAD cells treated with SBI or SBI+OA enter a proliferative phase within 24 h. Since 1 nM OA inhibits Phox2a activation (12), this proliferative phase of CAD cells induced by SBI is independent of Phox2a activation. Employing phosphohistone 3 immunostaining, a marker of cells in mitosis, we

FIG. 1. The CAD cell line as a model for cell cycle exit and neuronal differentiation. A. Growth curves of CAD cells grown for 48 h in indicated media. S, growth medium containing serum (5% calf serum and 10% fetal bovine serum in Dulbecco modified Eagle medium); SBI, neuronal differentiation medium comprised of growth medium (S), BMP2 (10 ng/ml), and IBMX (100 μ M); SBI+OA, medium that inhibits neuronal differentiation, comprised of growth medium (S), BMP2, IBMX, and okadaic acid (1 nM). The quantification of cell number in triplicates is from three independent experiments ($P < 0.05$). B. Quantification of CAD cell proliferation by phospho-histone 3 immunostaining. CAD cells were grown as indicated for panel A. Data represent averages from three independent experiments. C. Peripherin immunostaining of CAD cells at 24 h and 48 h of culture, grown as for panel A. D. Quantification of CAD cell apoptosis by cleaved caspase 3 immunostaining. CAD cells were grown as for panel A. Data represent averages from three independent experiments, quantifying an average of 9,000 cells. Error bars indicate standard errors.

quantified the relative number of proliferating cells at 24 h and 48 h following addition of SBI with or without OA (Fig. 1B). SBI+OA results in a continued increase in phospho-histone 3 immunostaining at 48 h, in comparison to control (serum) or SBI-treated cultures (Fig. 1B). Immunostaining for TH, an early noradrenergic cell lineage marker, and peripherin, a ter-

минаl neuronal differentiation marker delineating the neurites, was used to define the differentiation phase (Fig. 1C). Neuronal differentiation of CAD cells occurs after 24 h and before 48 h of SBI treatment. By contrast, OA treatment inhibits neuronal differentiation (Fig. 1C), increasing the number of proliferating cells at 48 h, as measured by phospho-histone 3

TABLE 1. Percentages of CAD cells in respective cell cycle phases

Medium	% of cells ^a at:					
	24 h			48 h		
	G ₁ /G ₀	S	G ₂ /M	G ₁ /G ₀	S	G ₂ /M
Serum	47 ± 4	21 ± 1	32 ± 7	49 ± 2	20 ± 3	31 ± 1
SBI	55 ± 4	14 ± 1	31 ± 3	67 ± 5	11 ± 0	22 ± 3
SBI+OA	48 ± 3	16 ± 0	36 ± 1	47 ± 2	12 ± 3	41 ± 7
Serum free	56 ± 2	15 ± 4	29 ± 3	69 ± 7	14 ± 2	17 ± 1

^a CAD cell cultures grown as described in Materials and Methods, treated as indicated, were analyzed by flow cytometry. Data represent the averages and standard errors from three independent experiments.

immunostaining (Fig. 1B). Since such an increase in proliferation is not observed with cultures induced to differentiate by SBI (Fig. 1B), the results suggest that neuronal differentiation is linked to cell cycle exit. Lastly, employing immunostaining for active caspase 3, we demonstrate an apoptotic phase occurring after 48 h treatment due to serum depletion (Fig. 1A and D).

In summary, CAD cells treated with SBI (differentiation medium) display proliferation lasting until 24 h, followed by differentiation occurring between 24 h and 48 h. By contrast, treatment with differentiation medium in the presence of OA (SBI+OA), which is known to inhibit Phox2a activation and neuronal differentiation (12), promotes proliferation extended to 48 h (Fig. 1A and B) and an absence of differentiation by 48 h (Fig. 1A to C). These observations suggest a link between activated Phox2a, cell cycle exit, and neuronal differentiation.

CAD cells accumulate in G₁/G₀ upon differentiation to catecholaminergic neurons. To directly demonstrate that exit from the cell cycle and CAD cell differentiation are linked, we quantified by flow cytometry the percentage of CAD cells in each phase of the cell cycle at 24 h and 48 h after treatment (Table 1 and Fig. 2A). Nearly 70% of the CAD cells grown with SBI for 48 h are in the G₁ phase. Likewise, serum-free medium, which also is known to induce CAD cell neuronal differentiation (8, 59), promotes nearly 70% of the cells into the G₁ phase. By contrast, in CAD cell cultures grown for 48 h with SBI+OA blocking differentiation, 40% of the cells are in G₁ and 40% are in the G₂/M phases (Fig. 2A and Table 1).

In our study, CAD cell cultures could not be synchronized by serum starvation because they underwent differentiation (8, 59). Accordingly, to confirm the flow cytometric results by another approach, CAD cell cultures were immunostained with cyclin D or A antibodies at 5 to 20 h following addition of SBI with or without OA (Fig. 2B and C). Cyclin D and A immunostaining is indicative of cells in the G₁ and S phases, respectively. CAD cell cultures treated with SBI+OA exhibit increased immunostaining for both cyclins D and A by 20 h of treatment, demonstrating that these cells are actively progressing through the G₁ and S phases of the cell cycle (Fig. 2B and C). Thus, the continued expression of cyclins D and A under conditions inhibiting neuronal differentiation (SBI+OA) demonstrates that the undifferentiated CAD cells continue cell cycle progression. By contrast, CAD cells induced to differentiate by SBI treatment display basal cyclin D expression at 20 h of treatment (Fig. 2B), leading to their accumulation in the G₁/G₀ phase of the cell cycle (Table 1).

FIG. 2. CAD cells accumulate in G₁ phase upon differentiation. A. Flow cytometric quantification of CAD cells grown for 48 h in SBI, SBI+OA, or serum-free medium (SFM) (differentiation control) inducing CAD cell neuronal differentiation (8, 59). Data are representative of those from three independent experiments. S, serum. B and C. Quantification of CAD cells immunostained with cyclin D (B) and cyclin A (C) in a time course after addition of the indicated inducers. Results are from three independent experiments, quantifying an average of 9,000 cells. Error bars indicate standard errors.

Increased p27^{Kip1} expression upon noradrenergic neuron differentiation. Since SBI treatment induces CAD cell neuronal differentiation by 48 h (Fig. 1C) and leads to their accumulation in the G₁/G₀ phase of the cell cycle (Fig. 2 and Table 1), we investigated whether these differentiation conditions induce expression of G₁-specific Cdk inhibitors. Since p27^{Kip1} is a key regulator of cell cycle exit for various neural progenitors, by employing real-time PCR we quantified p27^{Kip1} expression in RNA isolated over a time course following treatment of CAD cells with SBI with or without OA (Fig. 3A). Consistent with a role of Cdk inhibitors in CAD cell neuronal differentiation, expression of p27^{Kip1} is observed at 24 to 30 h after SBI addition. By contrast, with SBI+OA inhibiting differentiation, no difference is observed in p27^{Kip1} mRNA expression in comparison to the control (serum), with its expression further decreasing by 36 h (Fig. 3A). Since p27^{Kip1} expression precedes CAD cell differentiation, we propose that it is functionally linked to cell cycle exit of the neural progenitors.

The undifferentiated CAD cell line models neural progenitors. Accordingly, to directly demonstrate that p27^{Kip1} induction occurs in authentic noradrenergic neural progenitors, we employed primary cultures of NC cells. NC cultures treated

FIG. 3. p27^{Kip1} mRNA induction upon catecholaminergic neuron differentiation. A and B. Real-time PCR quantification of p27^{Kip1} mRNA from total RNA isolated from CAD cells (A) or NC cells (B) in a time course after addition of serum (S), SBI, or SBI+OA. C. Real-time PCR quantification of Phox2a mRNA using total RNA isolated from NC cells in a time course after addition of the indicated media. Results represent averages for three independent RNA preparations, with each PCR performed in identical triplicates. Data are normalized to 18S rRNA used as an internal control. Error bars indicate standard errors. AU, arbitrary units.

with SBI differentiate to the noradrenergic SA lineage, while SBI+OA treatment inhibits appearance of the neuronal phenotype (7, 12). It is important to note that direct comparison regarding the period of time required for differentiation in the CAD cell line versus the primary NC cultures cannot be made. For example, Phox2a expression occurs at day 2 of secondary NC culture, and noradrenergic markers appear at day 5 (7, 12). Employing real-time PCR, we demonstrated that in primary NC cells, SBI induces p27^{Kip1} mRNA expression at 72 h (day 3) of secondary culture (Fig. 3B). Importantly, induction of p27^{Kip1} mRNA at 72 h is preceded by Phox2a transcription at 48 h (day 2) of secondary NC culture (Fig. 3C), in agreement with our earlier observations (7, 12). This increase in p27^{Kip1} transcription is absent in NC cultures treated with SBI+OA (Fig. 3B and C), conditions shown to inhibit SA cell differentiation (12). Since SBI treatment of primary NC cells induces p27^{Kip1} transcription and differentiation to the noradrenergic SA lineage (7, 12), we conclude that p27^{Kip1} is involved in vivo during noradrenergic neural progenitor cell cycle exit and differentiation.

Cytoplasmic localization of p27^{Kip1} in differentiated noradrenergic neurons. Emerging evidence (14, 63, 66) supports the idea that the function of p27^{Kip1} depends on its subcellular localization. Nuclear localization of p27^{Kip1} is observed when it inhibits Cdk2 and Cdk1 (65). In addition, nuclear localization of p27^{Kip1} also occurs in proliferating cells, where p27^{Kip1} is in association with all cyclin/Cdk complexes (66), acting as an assembly or stability factor for cyclin D/Cdk4 and facilitating its nuclear import without inhibiting cyclin D/Cdk4 activity (13, 35). Moreover, during mitogenic stimulation, active cyclin E/Cdk2 in the nucleus associates with p27^{Kip1}, initiating the process of phosphorylation-dependent proteolysis of p27^{Kip1} (45).

By employing immunofluorescence microscopy, we monitored the subcellular localization of p27^{Kip1} in CAD cells at 24 h to 42 h following treatment with serum or with SBI with or without OA (Fig. 4A). With control treatment (serum), p27^{Kip1} is primarily in the nucleus during the 24- to 42-h interval (Fig. 4A) in association with either cyclin D or E (Fig. 4B), consistent with the role of p27^{Kip1} as a cyclin/Cdk assembly or stability factor (66). At 24 to 30 h after SBI addition, i.e., following the transcriptional induction of p27^{Kip1}, the localization of p27^{Kip1} is primarily nuclear (Fig. 4A). Interestingly, at 42 h after SBI addition, p27^{Kip1} is localized primarily in the cytoplasm of differentiated CAD cells. The nuclear localization of p27^{Kip1} following its transcriptional induction at 24 h of SBI treatment (Fig. 3A) is interpreted to mean that the increased p27^{Kip1} protein level results in inhibition of cyclinE/Cdk2 activity, thereby promoting G₁ arrest, cell cycle exit, and neuronal differentiation. By 42 h of SBI treatment, when neuronal differentiation has taken place (12), the cytoplasmic localization of p27^{Kip1} suggests that p27^{Kip1} is either on its way to degradation or has other functions (14, 63).

By contrast, with SBI+OA treatment, inducing neither neuronal differentiation nor increased p27^{Kip1} mRNA expression (Fig. 3A), p27^{Kip1} displays the same pattern as the control (serum), reinforcing the notion that the cytoplasmic localization of p27^{Kip1} observed with SBI treatment is linked to the differentiation process.

To confirm these observations, we examined p27^{Kip1} localization in NC cultures induced to differentiate to noradrenergic SA cells by SBI treatment (7). p27^{Kip1} localization is primarily nuclear in NC cells incubated for 24 to 72 h under control conditions (serum) or with SBI+OA, inhibiting SA cell development (12). NC cultures treated with SBI for 24 h display primarily nuclear p27^{Kip1} immunostaining, whereas by 72 h, p27^{Kip1} is primarily cytoplasmic (Fig. 4C). Since Phox2a is expressed in NC cells by 72 h but noradrenergic markers are detectable by day 5 of secondary NC culture, the cytoplasmic localization of p27^{Kip1} may turn out to be an early marker of neuronal differentiation. Thus, the same developmental mechanisms operate in the CNS- and NC-derived neuronal models, namely, cytoplasmic p27^{Kip1} localization concurrent with onset of neuronal differentiation.

Phox2a is required for p27^{Kip1} expression in CAD cells. To determine the causal link between activated Phox2a and p27^{Kip1} transcription, as well as the role of p27^{Kip1} in cell cycle exit required for neuronal CAD cell differentiation, we examined the effect of silencing Phox2a and p27^{Kip1} mRNAs. The specificity of the siRNAs for Phox2a and p27^{Kip1} was validated

FIG. 4. Cytoplasmic localization of p27^{Kip1} upon catecholaminergic neuron differentiation. A. p27^{Kip1} immunostaining of CAD cells in a time course after addition of serum (S), SBI, or SBI+OA. B. Confocal microscopy of CAD cells coimmunostained for p27^{Kip1} and cyclin D or cyclin E at 30 h of culture. C. p27^{Kip1} immunostaining of NC cells at day 1 and day 3 of secondary culture, grown with S, SBI, and SBI+OA. DAPI, 4',6'-diamidino-2-phenylindole.

by use of multiple controls. Briefly, nonspecific siRNAs displayed no effect on Phox2a and p27^{Kip1} mRNA levels as quantified by real-time PCR (Fig. 5A) or on CAD cell neuronal differentiation as monitored by peripherin immunostaining

(Fig. 5B). Importantly, the transfection efficiency of the introduced siRNAs was calculated to be nearly 90%, as assessed by transfecting the RISC-free-Glo siRNA detectable by fluorescence microscopy (Fig. 5B). In contrast to the case for the

FIG. 5. siRNA silencing of Phox2a or p27^{Kip1} in CAD cells. A. Real-time PCR quantification of Phox2a and p27^{Kip1} mRNAs with RNA isolated from CAD cells treated with SBI for 24 h after transfection of the indicated siRNAs. Results represent averages for three independent RNA

nonspecific siRNAs, transfection of siRNA for either Phox2a or p27^{Kip1} reduced the respective mRNAs by at least 50% (Fig. 5A). CAD cells transfected with siRNA for either Phox2a or p27^{Kip1} and induced to differentiate by SBI treatment (Fig. 5C) lack p27^{Kip1} immunostaining, in comparison to the untransfected control. Similarly, Phox2a siRNA transfection abrogates Phox2a immunostaining (Fig. 5C), demonstrating the effectiveness of the process. Increased concentration of transfected Phox2a or p27^{Kip1} siRNA suppresses appearance of the neuronal phenotype, as determined by peripherin (Fig. 5D) and TH (Fig. 5E) immunostaining as well as by quantification of TH mRNA by real-time PCR (Fig. 5F). These results demonstrate the regulatory importance of both Phox2a and p27^{Kip1} in neuronal differentiation.

To demonstrate the link between Phox2a and p27^{Kip1} expression, we quantified by real-time PCR the p27^{Kip1} mRNA levels following silencing of Phox2a mRNA. Knocking down Phox2a suppresses both p27^{Kip1} mRNA (Fig. 6A) and protein (Fig. 5C), thus demonstrating that p27^{Kip1} expression is dependent on Phox2a and suggesting that Phox2a regulates p27^{Kip1} transcription. Importantly, p27^{Kip1} silencing not only inhibits neuronal differentiation (Fig. 5D and E) but also results in increased proliferation of undifferentiated CAD cells (Fig. 6B). Furthermore, we examined whether p27^{Kip1} is sufficient to induce neuronal differentiation without Phox2a. Thus, we overexpressed p27^{Kip1} in the absence of SBI stimulation and looked for the appearance of neurites in transfected CAD cells. The results demonstrate that p27^{Kip1} on its own does not promote neuronal differentiation (Fig. 6C). Together, these observations (Fig. 5 and 6) support the idea that p27^{Kip1} regulates cell cycle exit of undifferentiated CAD cells (Table 1), a required step for neuronal differentiation.

Phox2a directly regulates p27^{Kip1} transcription. Since activated Phox2a is necessary for p27^{Kip1} mRNA expression (Fig. 3A) and Phox2a silencing suppresses p27^{Kip1} mRNA expression (Fig. 6), we investigated whether activated Phox2a directly regulates p27^{Kip1} transcription. CAD cells grown with SBI with or without OA were used in ChIP assays employing the Phox2a antibody (Fig. 7A and B).

Phox2a, a homeodomain transcription factor, interacts with the HD *cis*-acting element. Computer analyses identified five putative HD binding sites in the murine p27^{Kip1} promoter (Fig. 7A). Two HD elements located at the distal, 5' end of the promoter are 93 bp apart, while two HD elements proximal to TATAA box are 33 bp apart. The two distal and two proximal HD elements of the p27^{Kip1} promoter are each included in one PCR fragment for the ChIP assay analyses; these are referred to as site 1 and site 3, respectively (Fig. 7A).

In CAD cells grown in control conditions (serum) not inducing differentiation or in the melanoma B16 cell line, which does not express Phox2a, ChIP assays with the Phox2a anti-

body display only background Phox2a binding to sites 1 to 3 of the p27^{Kip1} promoter (Fig. 7B). Interestingly, with induction of CAD cell differentiation by SBI treatment, ChIP assays with the Phox2a antibody demonstrate that Phox2a binds preferentially to HD site 3, displaying background binding to sites 1 and 2 (Fig. 7B). Importantly, CAD cells treated with SBI in the presence of 1 nM OA display basal Phox2a binding to site 3 of the p27^{Kip1} promoter *in vivo* (Fig. 7B). OA inhibits a PP2A-like phosphatase required for activation of Phox2a DNA binding (1, 12).

To confirm these results, we cloned the mouse p27^{Kip1} promoter. Specifically, the region of the p27^{Kip1} promoter spanning nucleotides +50 to -1165, containing the proximal HD elements referred as site 3, was cloned upstream of the luciferase reporter. Transient transfections of p27^{Kip1}-luciferase reporter in CAD or NC cells treated with SBI with or without OA demonstrate that SBI treatment induces p27^{Kip1}-luciferase reporter expression, whereas treatment with OA inhibits this induction, in agreement with the results of the ChIP assays (Fig. 7C and D). Together, the results in Fig. 6 conclusively demonstrate that activated Phox2a directly regulates p27^{Kip1} transcription. We conclude that activated Phox2a induces noradrenergic neuron differentiation by inducing the transcription of the G₁-specific Cdk inhibitor p27^{Kip1}, which mediates cell cycle exit of neural progenitors, promoting differentiation.

Ectopic Phox2a expression induces p27^{Kip1} transcription and neuronal differentiation via a cAMP-dependent mechanism. The expression of p27^{Kip1} functioning as a Cdk inhibitor in diverse cell types is regulated by cell type-specific and ubiquitous transcription factors (9, 74). To further confirm that in noradrenergic precursor cells Phox2a mediates p27^{Kip1} expression in response to cAMP signaling, gain-of-function studies were performed, expressing Phox2a ectopically in CAD cells via the Tet-off expression system (26, 71).

Phox2a was cloned in the tetracycline-regulated expression vector (26) in frame with three copies of the FLAG epitope, enabling purification by immunoaffinity chromatography and immunodetection with the FLAG antibody. A time course of tetracycline-regulated Phox2a-FLAG expression is shown in Fig. S8E in the supplemental material, employing the tetracycline-regulated CAD-Phox2a-FLAG cell line. Ectopic expression of Phox2a-FLAG for 72 h by tetracycline removal is insufficient to induce neuronal differentiation of the CAD-Phox2a-FLAG cell line in the absence of cAMP stimulation, as assessed by peripherin immunofluorescence microscopy (Fig. 8A). Importantly, stimulation of cAMP signaling by forskolin (5 μ M) induced neuronal differentiation, demonstrating the requirement for activated Phox2a. Interestingly, activation by cAMP signaling of the ectopic and overexpressed Phox2a-FLAG accelerates neuronal differentiation, which occurs

preparations, with each PCR performed in identical triplicates. Data are normalized to 18S rRNA used as an internal control. B. Peripherin immunostaining of CAD cells at 48 h after transfection of the indicated control siRNAs, used at 100 nM. Transfection control siRNA is RISC-free-Glo, monitored by direct fluorescence microscopy. Following removal of transfection medium, CAD cells were incubated for 48 h in SBI. C. p27^{Kip1} or Phox2a immunostaining of CAD cells treated with SBI for 24 h after transfection of the indicated siRNAs. DAPI, 4',6'-diamidino-2-phenylindole. D and E. Peripherin and TH immunostaining, respectively, of CAD cells treated with SBI for 48 h after transfection of the indicated siRNAs. F. Real-time PCR quantification of TH mRNA, using total RNA isolated from CAD cells treated with SBI for 48 h after transfection of Phox2a or p27^{Kip1} siRNAs, as indicated. AU, arbitrary units. Error bars indicate standard errors.

FIG. 6. A. Phox2a silencing abrogates cAMP-mediated p27^{Kip1} transcription. Real-time PCR quantification of p27^{Kip1} mRNA, using total RNA isolated from CAD cells treated with SBI for 24 h after transfection of Phox2a siRNA, is shown. Results represent averages for three independent RNA preparations, with each PCR performed in identical triplicates. Data are normalized to 18S rRNA used as an internal control. Error bars indicate standard errors. AU, arbitrary units. B. Quantification of total cell number in CAD cell cultures transfected or not (-) with the indicated concentration of p27^{Kip1} siRNA and grown for 24 h with SBI. Control, untransfected CAD cells grown with serum (S). C. Immunofluorescence microscopy of p27^{Kip1}-transfected CAD cells grown in the presence of serum, using the indicated Myc or hemagglutinin (HA) antibodies. Fluorescent images are superimposed with corresponding phase-contrast images. Phase contrast of CAD cells grown in the presence of SBI is also shown. DAPI, 4',6'-diamidino-2-phenylindole.

within 12 h following cAMP stimulation (Fig. 8A). In control CAD-Phox2a-FLAG cells grown in the presence of tetracycline, i.e., not expressing Phox2a-FLAG, neuronal differentiation is not observed after 12 h of forskolin treatment (Fig. 8A). Moreover, in agreement with our earlier observations (Fig. 1) (12), cotreatment with forskolin and OA (1 nM) suppressed neuronal differentiation mediated by ectopic Phox2a-FLAG (Fig. 8A).

Employing real-time PCR, we monitored p27^{Kip1} expression, in RNA isolated in a time course, following forskolin stimulation with or without OA addition of CAD-Phox2a-FLAG cells grown without tetracycline for 72 h. Under these

conditions, p27^{Kip1} mRNA is detected at 4 h of cAMP stimulation, and this induction is inhibited by OA (Fig. 8B), in agreement with the results in Fig. 3. It is important to note that in CAD cells, endogenous Phox2a is not induced at 4 h of SBI treatment (data not shown).

To confirm that ectopic Phox2a-FLAG mediates the transcriptional induction of p27^{Kip1} transcription, ChIP assays monitoring the association of Phox2a-FLAG with the p27^{Kip1} promoter were performed (Fig. 8C). In the tetracycline-regulated CAD-Phox2a-FLAG cell line, Phox2a displays a cAMP-dependent and OA-sensitive association with the HD region of the endogenous p27^{Kip1} gene at 4 h following forskolin addition. These gain-of-function studies demonstrate that cAMP-activated Phox2a is necessary to induce p27^{Kip1} transcription.

Employing this tetracycline-regulated Phox2a-expressing CAD cell line, we have started to define the mechanism by which cAMP signaling activates Phox2a. Phox2a-FLAG, expressed from the tetracycline-regulated cell line grown without cAMP stimulation, was purified by FLAG immunoaffinity chromatography. According to our previous studies (12), Phox2a is constitutively phosphorylated and inactive in DNA binding to the HD site. Purified Phox2a-FLAG was dephosphorylated in vitro by treatment with λ phosphatase. Western blot analysis of the samples treated with or without λ phosphatase is shown in Fig. 8D (left panel). Phox2a-FLAG treated with or without λ phosphatase was used in EMSA with ³²P-radiolabeled HD probe (Fig. 8D, center panel). Dephosphorylated, λ phosphatase-treated Phox2a-FLAG exhibits enhanced HD binding in comparison to the untreated sample, in agreement with our earlier observations (12). Southwestern blot analyses employing the wild-type (wt) and mutant HD probes directly demonstrate that the λ phosphatase-dephosphorylated Phox2a-FLAG specifically binds to the wt HD DNA. Inactivation of λ phosphatase with EDTA abrogates wt HD binding to Phox2a-FLAG (Fig. 8D, right panel). These results are consistent with our earlier studies (12) and directly demonstrate the requirement for Phox2a dephosphorylation in binding to the HD site to mediate p27^{Kip1} transcription.

DISCUSSION

Here, we demonstrate that during noradrenergic neuron differentiation, activated Phox2a coordinates cell cycle exit and differentiation of neural progenitors by mediating transcription of the G₁-specific Cdk inhibitor p27^{Kip1}. Earlier, we have demonstrated that cAMP signaling in synergy with BMP2 induces noradrenergic neuron development by inducing Phox2a transcription (5, 7). cAMP signaling via PKA activation regulates a PP2A-like phosphatase required for Phox2a dephosphorylation (1, 12). This Phox2a dephosphorylation is necessary for Phox2a DNA binding to HD *cis*-acting elements and transcriptional activation (1, 12). Inhibition of the PP2A-sensitive step by 1 to 5 nM OA inhibits Phox2a DNA binding, Phox2a transactivation, and noradrenergic neuron differentiation (12).

Here, we demonstrate that cAMP signaling and BMP2 initially induce proliferation of undifferentiated CAD cells, followed by Phox2a binding to HD sites of the p27^{Kip1} promoter in vivo, p27^{Kip1} transcription, and neuronal differentiation. Importantly, OA inhibits the cAMP-mediated activation of Phox2a DNA binding (12) and all the Phox2a-dependent

FIG. 7. Activated Phox2a binds to the p27^{Kip1} promoter in vivo. A. Diagram illustrating the position of putative HD transcription factor binding sites, relative to the +1 start site, in the mouse p27^{Kip1} promoter. B. ChIP assays with Phox2a antibody and with Phox2a antibody in the melanoma B16 cell line are negative controls. Quantification of p27^{Kip1} DNA immunoprecipitated by Phox2a antibody or IgG was by real-time PCR employing mouse p27^{Kip1} gene-specific primers spanning HD site 1, site 2, and site 3 (see panel A). Data are expressed as fold change of p27^{Kip1} binding quantified relative to IgG and represent the averages from at least three independent experiments. Error bars indicate standard errors. C and D. Transient transfections of p27^{Kip1}-luciferase reporter in CAD and NC cell cultures, respectively, grown in S, SBI, and SBI+OA. Results represent averages from three independent transfections, each performed in identical triplicates.

downstream events, including p27^{Kip1} transcription, cell cycle exit of undifferentiated precursors, and differentiation. In vitro dephosphorylation of immunoaffinity-purified Phox2a-FLAG enhances DNA binding to the HD site, supporting the idea that OA inhibits p27^{Kip1} transcription by inhibiting the cAMP-mediated Phox2a dephosphorylation required for Phox2a DNA binding.

Figure 9 illustrates the mechanism by which the developmental signals of cAMP signaling and BMP2 induce transcription and activation of Phox2a. Activated Phox2a couples neural progenitor cell cycle exit and differentiation by inducing transcription of the Cdk inhibitor p27^{Kip1}. This is the first demonstration of a molecular mechanism by which specific developmental signals, i.e., those of cAMP signaling and BMP2, coordinate cell cycle exit and differentiation of neural progenitors, giving rise to noradrenergic neurons.

The CAD cell line is a model coupling cell cycle exit and neuronal differentiation. We have employed two neuronal noradrenergic models, the CNS-derived CAD cell line (68) and primary cultures of NC cells, both of which differentiate to noradrenergic cells (7, 8, 59). Despite the different developmental origins of the CNS-derived noradrenergic neurons and NC-derived SA cells, in vivo (50, 56, 67) and in vitro (5, 8, 12) studies support the idea that noradrenergic neurons differen-

tiate via similar mechanisms. Thus, comparative studies between the established CAD cell line and primary NC cells validate our observations, and importantly, the CAD cell line allows investigations not possible with the avian primary NC cell culture model.

Upon treatment with differentiation medium (SBI), CAD cells enter a proliferative phase within 24 h, independent of Phox2a activation, as demonstrated by the absence of an effect of OA. This proliferative phase displayed by the CAD cellular model is followed by the differentiation phase, which is completed within 48 h of SBI treatment. The differentiation phase requires activated Phox2a, as evidenced by the inhibition of differentiation by SBI+OA. Thus, the CAD cell model is ideal and amenable for study of the mechanism coupling cell cycle exit to neuronal differentiation. Flow cytometric analyses demonstrated that by 48 h of SBI treatment, the majority of CAD cells are in the G₁ phase and are differentiated neurons. By contrast, with SBI+OA treatment inhibiting neuronal differentiation, 40% of the cells are in G₁ and 40% are in the G₂/M phases of the cell cycle. The proliferative phase of neural progenitors, also modeled by the CAD cell line, is important for proper development of the nervous system, which is comprised primarily of postmitotic neurons. This principle is illustrated by mice null for the transcriptional repressor Hes1,

FIG. 8. Neuronal differentiation and p27^{Kip1} transcription by tetracycline-regulated expression of Phox2a-FLAG requires cAMP signaling. A. Immunofluorescence microscopy of Phox2a-FLAG and peripherin in CAD-Phox2a-FLAG cells grown with or without 5 μ g/ml tetracycline for 72 h and with or without 5 μ M forskolin and 1 nM OA for 12 h, as indicated. DAPI, 4',6'-diamidino-2-phenylindole. B. Real-time PCR quantification

which controls proliferation of neural progenitors during development. Hes1^{-/-} animals display severe developmental neuronal defects (31). Interestingly, the transcriptional repressor Hes1 binds to class C sites in the p27^{Kip1} promoter, repressing p27^{Kip1} expression (51).

cAMP and BMP2 promote noradrenergic neuron differentiation by inducing p27^{Kip1} transcription. In a number of neural differentiation models, cell cycle exit involves transcriptional induction of G₁-specific Cdk inhibitors, including p27^{Kip1}. In both CAD cells and primary NC cells, induction of differentiation by SBI induces p27^{Kip1} transcription. The subcellular localization of p27^{Kip1} changes during differentiation, as determined by the time course immunofluorescence analyses monitoring p27^{Kip1} localization. Following the p27^{Kip1} induction occurring 24 h after addition of SBI to CAD cells, p27^{Kip1} is in the nucleus; by 42 h of SBI treatment, p27^{Kip1} is localized in the cytoplasm in association with either cyclin D or E. Importantly, this nuclear-to-cytoplasmic movement of p27^{Kip1} is also observed in primary NC cells undergoing differentiation by SBI treatment to the SA lineage. Furthermore, in other neuronal differentiation models, p27^{Kip1} is also found in the cytoplasm upon differentiation (37). Cytoplasmic p27^{Kip1} has additional functions, including regulation of contact inhibition (58), migration (6, 47), and antiapoptotic effects (29, 54). The function of cytoplasmic p27^{Kip1} in differentiated (noradrenergic) neurons remains to be determined.

Activated Phox2a mediates p27^{Kip1} transcription during noradrenergic neuron differentiation. The causal link between Phox2a activation and p27^{Kip1} expression was demonstrated by both loss-of-function and gain-of-function approaches. Specifically, knockdown of Phox2a via siRNA transfection in CAD cells resulted in the absence of p27^{Kip1} mRNA induction, whereas overexpression of Phox2a accelerated p27^{Kip1} mRNA expression. Although other transcription factors acting via interaction with Sp1 induce p27^{Kip1} transcription in different model systems (9, 74), our results demonstrate that without Phox2a activated by cAMP signaling, these transcription factors, such as Sp1, are insufficient to promote p27^{Kip1} transcription in this noradrenergic cellular model. The link between p27^{Kip1} expression and neuronal differentiation by SBI treatment was demonstrated by knockdown of p27^{Kip1}; under these conditions, SBI treatment failed to mediate neuronal differentiation, while it resulted in an increase in the number of undifferentiated and proliferating cells. However, p27^{Kip1} overexpression is insufficient to mediate neuronal differentiation without Phox2a activation by cAMP signaling. We interpret these results to mean that the contribution of p27^{Kip1} in the

FIG. 9. Diagram illustrating how the developmental signals of cAMP signaling and BMP2 induce Phox2a transcription (5, 7). Phox2a is constitutively phosphorylated (1); cAMP-activated PKA via activation of a PP2A-like phosphatase, which is sensitive to inhibition by 1 nM OA, dephosphorylates Phox2a (12). The dephosphorylated Phox2a is transcriptionally active, coupling cell cycle exit and neuronal differentiation by inducing transcription of p27^{Kip1}.

process of neuronal differentiation is to enable neural progenitors to exit from the cell cycle.

ChIP assays employing the Phox2a antibody identified Phox2a bound to the proximal HD site 3 of the p27^{Kip1} promoter. Importantly, only with the SBI treatment, which is necessary for activation of Phox2a DNA binding, was Phox2a associated with HD site 3 (12). OA, which inhibits Phox2a DNA binding (1, 12), suppressed to nearly basal levels the *in vivo* Phox2a binding to HD site 3 of the p27^{Kip1} promoter. Similarly, in CAD and NC cells induced to differentiate, the p27^{Kip1}-luciferase reporter displayed enhanced expression, while OA addition inhibited this expression. These results are consistent with both the ChIP assays and our earlier observations of the OA effect on noradrenergic neuron differentiation (12). In support of these conclusions, we also directly demonstrate that dephosphorylation of Phox2a *in vitro* is necessary

of p27^{Kip1} mRNA from total RNA isolated from CAD-Phox2a-FLAG cells grown in the absence of tetracycline for 72 h, with or without 5 μ M forskolin and 1 nM OA for the indicated time course. Results represent averages from three independent RNA preparations, with each PCR performed in identical triplicates. Data are normalized to 18S rRNA used as an internal control. Error bars indicate standard errors. AU, arbitrary units. C. ChIP assays employing Phox2a antibody and CAD-Phox2a-FLAG cells grown without tetracycline for 72 h with or without forskolin and OA, as indicated, for 4 h. ChIP assays with IgG represent the negative control. Quantification of p27^{Kip1} DNA immunoprecipitated by Phox2a antibody or IgG was by real-time PCR employing mouse p27^{Kip1} gene-specific primers spanning HD site 3. Data are expressed as fold change of p27^{Kip1} binding quantified relative to IgG and represent the averages from at least three independent experiments. D. Left panel, Western blot (WB) analysis of Phox2a-FLAG purified by anti-FLAG M2 affinity gel (Sigma), treated with or without λ phosphatase (800 units) for 15 min at 30°C. Center panel, EMSA of Phox2a-FLAG treated with or without λ phosphatase, employing a ³²P-radiolabeled HD probe, as described previously (12). Right panel, Southwestern blot analyses of Phox2a-FLAG treated with or without λ phosphatase (800 units) for 2 h at 30°C, employing ³²P-radiolabeled wt and mutant (mt) HD probes. A Western blot of the Phox2a-FLAG samples run in parallel is also shown.

for enhanced Phox2a DNA binding to the HD site. Ongoing studies to determine the cAMP-regulated dephosphorylation(s) of Phox2a are in progress.

Our results, demonstrating the transcriptional induction of p27^{Kip1} in NC cell development to the SA lineage, agree with the expression pattern of p27^{Kip1} in the adrenal medulla (52). Mice lacking p27^{Kip1} exhibit marked hyperplasia of several organs and tissues which normally express p27^{Kip1}, including the adrenal medulla, retina, and pituitary (23, 34, 53). p27^{Kip1}^{-/-} mice also display a small incidence of adrenal medullary tumors, whereas animals that are double null for p18^{Ink4c} and p27^{Kip1} display a high incidence of adrenal medullary tumors (24). These results suggest that in addition to p27^{Kip1}, the Cdk inhibitor p18^{Ink4c} may also play a role in coupling cell cycle exit and NC cell differentiation to the SA lineage. In support of this hypothesis, it has been shown that maintenance of the postmitotic state in the postnatal mammalian brain is an active process, requiring both the Ink4 and Kip1 Cdk inhibitors (11, 16, 76). In the CAD cell neuronal differentiation model described here, coupling between proliferation and differentiation occurs within one cell cycle for 70% of the cells in culture. In the developing embryo, depending on the concentration of the extrinsic developmental signals, it is likely that there is a timed, hierarchical involvement of transcriptional induction of p27^{Kip1} and p18^{Ink4c}, ensuring precise regulation of progenitor proliferation versus differentiation (72).

In conclusion, this is the first report to identify a proneural transcription factor, Phox2a, induced and activated by the developmental signals of cAMP and BMP2, that mediates p27^{Kip1} transcription, thus coordinating cell cycle exit of noradrenergic progenitors and neuronal differentiation. Recent findings demonstrate that the tumor suppressor menin, via recruitment of the histone methyltransferase MLL, maintains *in vivo* expression of Cdk inhibitors, including p27^{Kip1} (32, 48). The involvement of menin and MLL in noradrenergic neural progenitor cell cycle exit and differentiation remains to be determined.

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