INTRODUCTION

The cell cycle is a fundamental cellular process that governs the ability of cells to divide. Control of the cell cycle is crucial for the generation of tissues from dividing stem cells, such as the development of the nervous system. Exit from the cell cycle is associated with the control of differentiation because differentiated cells tend to be postmitotic. Re-entry of differentiated neurons into the cell cycle leads to apoptosis (Folch et al., 2012), although cells tend to be postmitotic. Re-entry of differentiated neurons is associated with the control of differentiation because differentiated cells have a lower differentiation potential of these cells. However, how polarization might be directly linked to the kinetics of the cell cycle is not understood. Here, we report that apicobasally polarized neuroepithelial cells in Xenopus laevis have a shorter cell cycle than nonpolar progenitors, consistent with mammalian systems. We show that the apically localized serine/threonine kinase aPKC directly phosphorylates an N-terminal site of the cell-cycle inhibitor p27Xic1 and reduces its ability to inhibit the cyclin-dependent kinase 2 (Cdk2), leading to shortening of G1 and S phases. Overexpression of activated aPKC blocks the neuronal differentiation-promoting activity of p27Xic1. These findings provide a direct mechanistic link between apicobasal polarity and the cell cycle, which may explain how proliferation is favored over differentiation in polarized neural stem cells.

SUMMARY

During the development of the nervous system, apicobasally polarized stem cells are characterized by a shorter cell cycle than nonpolar progenitors, leading to a lower differentiation potential of these cells. However, how polarization might be directly linked to the kinetics of the cell cycle is not understood. Here, we report that apicobasally polarized neuroepithelial cells in Xenopus laevis have a shorter cell cycle than nonpolar progenitors, consistent with mammalian systems. We show that the apically localized serine/threonine kinase aPKC directly phosphorylates an N-terminal site of the cell-cycle inhibitor p27Xic1 and reduces its ability to inhibit the cyclin-dependent kinase 2 (Cdk2), leading to shortening of G1 and S phases. Overexpression of activated aPKC blocks the neuronal differentiation-promoting activity of p27Xic1. These findings provide a direct mechanistic link between apicobasal polarity and the cell cycle, which may explain how proliferation is favored over differentiation in polarized neural stem cells.
Polarized and Nonpolar Neural Progenitors in *Xenopus Neuroectoderm Have Different Cell-Cycle Kinetics*

Dual-pulse S phase labeling (DPSL) analysis on outer layer apico-basally polarized progenitors and inner layer nonpolar neural progenitors (both Sox3+) showed that polarized progenitors have a significantly shorter cell cycle length and a shorter S phase length than nonpolar progenitors at open neural plate stage, NF13 (Tc, polarized versus nonpolar, mean ± SEM, 282 ± 14 min versus 411 ± 21 min, and Ts, polarized versus nonpolar, 40 ± 4 min versus 141 ± 14 min, Figure 1A). We also analyzed these progenitors for percentage of labeled mitoses (PLM) to estimate G2+1/2M phase length and compared them for their mitotic indices (Figure 1B). Putting the numbers together from these experiments (see Supplemental Experimental Procedures available online for details) showed that polarized progenitors have a shorter G1 phase (Tg1, 94 min versus 160 min for nonpolar cells) but a longer G2 phase than the nonpolar progenitors (Tg2, 132 min versus 89 min). The lengths of mitoses were marginally different between the two layers (Tm, 16 min versus 21 min) (Figure 1C). Thus, establishing the cell-cycle kinetic parameters for polarized and nonpolar progenitors showed that they differ significantly and that polarized progenitors cycle faster.

**aPKC Promotes Neural Progenitor Differentiation and Affects Cell Cycle Length**

Confirming our previous findings (Sabherwal et al., 2009), overexpression of constitutively active, membrane-targeted aPKC (aPKC-CAAX) suppressed neuronal differentiation, as judged by the reduction in the expression of a terminal differentiation marker N-tubulin. Conversely, nuclear dominant-negative aPKC (NLS-aPKC-Nt) promoted neuronal differentiation as N-tubulin was enhanced (Figure S1 available online). Extending these observations further, we found that on aPKC-CAAX overexpression, ElrC, a marker of committed neural progenitors (Carruthers et al., 2003), was also suppressed, whereas cells expressing the neural progenitor marker Sox3 showed expansion (Sox3+ area was increased on the injected side as shown by in situ hybridization) (Figure S1). This suggested that aPKC-CAAX suppressed neuronal differentiation by promoting neural progenitor expansion. In aPKC-CAAX-overexpressing embryos, the number of Sox3+ progenitors on the injected side was significantly higher (shown by immunostaining, Figure 2A) and cells on the injected side had significantly shorter Tc and Ts, than on the noninjected side, calculated by the DPSL technique. Control embryos overexpressing GFP-CAAX exhibited no such differences (Figure 2A).

To further see the effects of aPKC on different phases of the cell cycle, we treated and imaged HeLa Fucci cells (Saka-Sawano et al., 2008) for 48–60 hr in the presence of a myristoylated, cell-permeable inhibitor specific against aPKC (Sajan et al., 1999). Cells inhibited for aPKC showed significant lengthening of the total cell-cycle time (Tc, 25.32 ± 0.58 hr versus 19.38 ± 0.45 hr for controls). This was mainly attributed to the lengthening of the G1 phase (Tg1, 17.04 ± 0.47 hr versus 11.79 ± 0.51 hr for controls), with small effects observed in the early S phase (Tg2, 7.3 ± 0.78 hr versus 3.38 ± 0.40 hr for controls). M phase length (Tm) was not affected by the treatment. The effect on the length of S, G2, and M together (Tg2m) is significant (14.15 ± 0.67 hr versus 10.92 ± 0.43 hr for controls) indicated that the G2 phase was also largely unaffected by the inhibitor treatment (Figure 2B and Movies S1A and S1B).

Pseudosubstrate inhibitors against kinases have the highest specificity. The pseudosubstrate myristoylated inhibitor used here is highly specific and has no effect on classical and novel PKCs (Standaert et al., 1999). Nevertheless, these data were further substantiated by the experiment with a chemical inhibitor against aPKC, bisindolylmaleimide G66983. This inhibitor has been used previously to show that aPKC is involved in TE formation (Eckert et al., 2004) and can maintain ES cells in an undifferentiated state in the absence of LIF, through the inhibition of PKCζ (Dutta et al., 2011). G66983 showed similar effects on cell-cycle kinetics as shown by Myr inhibitor against aPKC (Figure 2B and Movies S1A and S1C).

The Fucci data along with DPSL data suggested that both gain and loss of aPKC signaling activity affects cell cycle length via G1 and S phases, with G2 and M phases remaining largely unaffected. These data suggested that the effects of aPKC on progenitors’ proliferation might be mediated via its effects on cell-cycle kinetics. To test this further, we investigated the interaction of aPKC with cell-cycle regulators.

**aPKC Directly Phosphorylates CIP/KIP Cell-Cycle Inhibitor p27Xic1 Both In Vitro and In Vivo**

G1 kinases (Cdk4/6) and G1/S kinase (Cdk2) are positively regulated by CyD and CyE/A, respectively, and negatively regulated by a CIP/KIP family cyclin-dependent kinase inhibitor (Cdkl) p27Xic1 (Ohnuma and Harris, 2003). Because cyclins D, E, A, and p27Xic1 are regulated by posttranslational modifications, mainly phosphorylations (Alberts et al., 2002), we analyzed them for being phosphorylation targets of aPKC. In vitro kinase assays showed that only bacterially expressed p27Xic1 is a direct phosphorylation target of GST-aPKC (commercially supplied) (Figure 3A and data not shown). The specificity of this in vitro phosphorylation was confirmed by performing the kinase assay in the presence of a pseudosubstrate inhibitor specific...
Figure 1. Polarized and Nonpolar Neural Progenitors Have Significantly Different Cell-Cycle Kinetics Parameters

(A) Dual pulse S phase labeling (DPSL) technique applied on wild-type NF13 Xenopus embryos (a) and analyzed by sectioning (b) shows that outer polar progenitors have shorter cell cycle (TC) and S phase (TS) lengths than inner nonpolar progenitors (c, mean ± SEM).

(B) Similar embryos were processed for percentage of labeled mitoses (PLM, to estimate TG2+1/2M) (a–c). Representative sections are shown in (b), in which white arrowheads show labeled mitoses for nonpolar progenitors, while yellow arrowheads show labeled mitoses for polarized progenitors.

(D) Mitotic index (in percentage) of Neural (Sox3+) progenitors

| Source    | Polarized progenitors | Nonpolar progenitors |
|-----------|-----------------------|----------------------|
| DPSL      | 262                   | 411                  |
| DPSL      | 40                    | 141                  |
| PLM       | 16                    | 21                   |
| PLM+PLM   | 132                   | 89                   |
| PLM+PLM   | 94                    | 160                  |
against aPKC (Sajan et al., 1999). This reduced the kinase signal in a dose-dependent manner (Figure 3B). To see if aPKC can phosphorylate p27Xic1 in a complex embryonic milieu as well, we performed in vitro kinase assay using embryonic lysate, instead of kinase buffer, as the medium for interaction between exogenous GST-p27Xic1 (bacterially expressed) and His-aPKC (commercially supplied). The result demonstrated that phosphorylated GST-p27Xic1 significantly increases in the presence of aPKC, whereas it drops down to basal level in the presence of pseudosubstrate inhibitor against aPKC (Figure 3C).

To further substantiate these observations in vivo, we performed in vivo kinase assay measuring the incorporation of γ-P32-labeled orthophosphate by p27Xic1, overexpressed in HeLa cells along with aPKC-CAAX or in the presence of myristoylated pseudosubstrate aPKC inhibitor. The experiment showed that p27Xic1 incorporated more orthophosphate when expressed along with aPKC-CAAX; the incorporation was reduced when the same cells were cultured in the presence of the inhibitor (Figure 3D). p27Xic1 and aPKC-CAAX also showed direct physical interaction in coimmunoprecipitation (coIP) assays. First, Flag-p27Xic1 and HA-aPKC-CAAX constructs were co-overexpressed in HeLa cells and HA-aPKC-CAAX was detected after IP of Flag-p27Xic1 using anti-Flag beads (Figure 3E). More importantly, the endogenous p27Xic1 and aPKC from embryonic lysate also showed direct physical interaction in coIP experiments where either p27Xic1 or aPKC is immunoprecipitated with antibodies and aPKC or p27Xic1 is detected by western blot in the pull-down, respectively (Figures 3F and S2).

The effect of aPKC inhibitors on HeLa Fucci cell-cycle length (Figure 2B) suggested that human p27Kip1 might also be negatively regulated by aPKC and may be a phosphorylation target of aPKC. However, our in vitro kinase assay using immunoprecipitated p27Kip1 from HeLa cells showed that human p27Kip1 is not a direct phosphorylation target of aPKC (data not shown). This suggests that aPKC negatively regulates p27Kip1 in an indirect manner.

**Regulation of p27Xic1 Activity by aPKC Likely Takes Place in the Cell Nucleus**

To gain some insight into where the interaction of aPKC and p27Xic1 may be taking place, we performed immunostaining on neurula stage embryos overexpressing Flag-p27Xic1 and HA-aPKC-CAAX. These showed that p27Xic1 was largely in the nucleus of the cells, and a portion of aPKC-CAAX, which was mostly localized to the cell cortex, was also found in the nucleus (Figure S3). These findings are consistent with previous reports showing nuclear enrichment of p27Xic1 (Chuang and Yew, 2001; Chuang et al., 2005) and some nuclear localization of aPKC-CAAX (Sabherwal et al., 2009) and lend support to the idea that aPKC interacts with p27Xic1 in the nucleus. In our previous publication (Sabherwal et al., 2009), we showed that aPKC-CAAX is more active and nuclear than aPKC and hypothesized that after getting activated in the membrane, a small proportion of aPKC-CAAX is translocated to the nucleus. This suggests that although aPKC-CAAX is predominantly membrane localized and weakly nuclear, it is highly active and sufficient to influence nuclear events like interacting with p27Xic1 and modulating its activity.

**aPKC Phosphorylates p27Xic1 in Its Cdk-Interaction Domain, Resulting in Its Reduced Binding to Cdk5**

To identify domains of p27Xic1 phosphorylated by aPKC, we performed immunocomplex kinase assays on Flag-tagged deletion constructs of p27Xic1. As shown in Figure 4A, the N-terminal and middle parts of the protein showed positive kinase signal whereas the C-terminal fragment showed no sign of phosphorylation during in vitro kinase assays. Phosphosite identification using liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis on Flag-p27Xic1 phosphorylated by aPKC in vitro (i.e., in vitro kinase reaction on immunoprecipitated/IPed Flag-p27Xic1 from HeLa cells) identified multiple phosphorylation sites with significant Ascore values (≥13, Ascore is a measurement of the confidence of phosphorylation; Beausoleil et al., 2006) within the N-terminal, Cdk-binding domain (Figure 4A). To see if the sites identified correspond to the phosphosites in vivo, similar LC/MS/MS analysis was carried out on Flag-p27Xic1 IPed from HeLa cells co-overexpressing it with HA-aPKC-CAAX. This analysis identified a phosphosite (T68) with a significantly high Ascore (Ascore = 27; Figure 4A), located within the Cdk interaction domain of p27Xic1; another site (T99, located immediately after the Cdk interaction domain) was picked with a low Ascore value (Ascore = 13; Figure 4A). None of these sites was identified on Flag-p27Xic1 IPed from HeLa cells overexpressing it alone. Xenopus p27Xic1 is 44% identical to human p27Kip1 and 40% identical to human p21Cip1 in the conserved N terminus. It also possesses a PCNA-like binding site characteristic of p21Cip1 in the C terminus (Su et al., 1995). The aPKC phosphosite in p27Xic1 (T68A) is not conserved in mammalian p27Kip1 but is conserved in mammalian p21Cip1 (although it has not been identified as an aPKC phosphorysate by others) and p27Xic1 from zebrafish (Figure 4B), suggesting some degree of functional conservation linking aPKC activity and cell-cycle regulation; the other phosphosites identified (T99) shows no conservation with other members of CIP/KIP Cdks.

Identification of aPKC phosphorylation sites prompted us to check if these phosphorylation events are functionally important by generating phospho(S/T to A) or phosphomimetic (S/T to E) mutants. Initial protein abundance experiments showed that the level of Flag-p27Xic1 protein is decreased in embryos injected with HA-aPKC-CAAX and increased in embryos injected with the dominant-negative HA-aPKC-NT (Figure S4A). Protein stability experiments using cycloheximide showed that the half-life of overexpressed Flag-p27Xic1 was reduced when it was co-overexpressed in HeLa cells along with HA-aPKC-CAAX (Figure S4B). However, nonphosphorylatable mutants (like T68A) were also destabilized in comparison to the wild-type protein, whereas phosphomimetic mutants (like T68E) of p27Xic1 appeared more stable (see Figures S4C and S4D for the experiment, neural plate cells converge to the midline. Percentage of mitotic indices for polarized and nonpolar progenitors showed no differences (d, mean ± SEM).

Table summarizes different cell-cycle parameters for the two types of progenitors. See the Experimental Procedures and Supplemental Experimental Procedures for details about the techniques and calculations of kinetic parameters. All scale bars represent 100 μM.
examples), suggesting that the effect of aPKC phosphorylation on p27Xic1 stability is complex and cannot be reproduced by single amino acid changes.

Not only the stability of CdkIs, but also the inhibition of Cdk kinase activity by CdkIs has been shown to promote differentiation (Hasan et al., 2013). We tested whether the binding of p27Xic1 to Cdns is affected by aPKC phosphorylation, which could explain the shortening of G1 and S phases of cell cycle. p27Xic1 interacts with Cdk2 and Cdk4 and inhibits their kinase activities (Finkielstein et al., 2001). CoIP assays using HeLa cells

Figure 2. Overexpressing Activated aPKC Causes Progenitor Proliferation and Shortens the Cell Cycle by Shortening G1 and S Phases
(A) DPSL analysis (a) shows that embryos on the aPKC-CAAX injected side show higher number of Sox3+ progenitors (b and c) and have shorter cell cycle (Tc) and S phase (Ts) (c, mean ± SEM). Control embryos overexpressing GFP-CAAX showed no such differences. The scale bar in (Ab) represents 100 μM. See also Figure S1.

(B) Schematic of Fucci cell cycle biosensor, which can be visually used to measure the lengths of various phases of cell cycle, is shown (a). When live imaged in the presence of either a pseudosubstrate inhibitor (MyrPSI) or a chemical inhibitor (Go6983/GO) against aPKC, HeLa Fucci cells showed a significant increase in cell-cycle length (Tc) due to elongated G1 and early S phases (b, mean ± SEM). Time-lapse movies are shown in Movies S1A–S1C.
co-overexpressing Flag-p27Xic1 along with either HA-Cdk2 or HA-Cdk4 showed that p27Xic1 binds to Cdk2 much stronger than to Cdk4 (data not shown). Similar coIP assays showed that phosphomimetic mutant T68E showed almost 50% reduction in its binding to Cdk2 compared to the wild-type p27Xic1 (Figures 4C, 4D, and S5), whereas the phosphomutant T68A showed enhanced binding (increased by almost 140%) to Cdk2 (Figures 4D, 4E, and S5). Other mutants showed binding similar to the wild-type p27Xic1 (Figures 4C, 4D, and S5). These assays suggested that a single phosphorylation event at amino acid T68 of p27Xic1 by aPKC is enough to affect its interaction with Cdk2. Using Histone H1 as a substrate for active Cdk2/CyclinA, we found that in comparison to the wild-type p27Xic1, phosphomutant T68A abolished the kinase activity of Cdk2 almost completely, while the phosphomimetic mutant T68E had a negligible effect on Cdk2 activity in the same assay.

Figure 3. aPKC Directly Phosphorylates p27Xic1
(A) In vitro kinase assays showing that aPKC phosphorylates bacterially expressed GST-p27Xic1. Myelin basic protein (MBP) was used as a positive marker for aPKC phosphorylation.
(B) Specificity of the kinase reaction was confirmed by using a pseudosubstrate inhibitor of aPKC.
(C) Kinase assay performed in an embryonic environment (using embryo lysate instead of kinase buffer) also confirmed p27Xic1 being a direct phosphorylation target of aPKC.
(D) In vivo kinase assay shows that p27Xic1 incorporates higher amount of P32 when it is co-overexpressed along with aPKC-CAAX; the amount of incorporated P32 is lower when p27Xic1 is overexpressed in HeLa cells in the presence of pseudosubstrate inhibitor against aPKC.
(E) CoIP of HA-aPKC-CAAX with Flag-p27Xic1 in HeLa cells shows that aPKC-CAAX and p27Xic1 interact physically.
(F) Physical interaction between p27Xic1 and aPKC was confirmed for endogenous proteins from embryonic lysates by a coIP assay using antibodies against endogenous p27Xic1 and aPKC. See also Figures S2 and S3.
Figure 4. aPKC Phosphorylates p27Xic1 in Its Cdk-Interaction Domain, Leading to Its Weaker Binding and Inhibition of Cdk2

(A) The schematic shows p27Xic1 protein with its key interaction domains. Boxes in the middle show N-terminal, C-terminal and middle fragments that were used for immunocomplex kinase assays that indicated that aPKC phosphorylates p27Xic1 in its N-terminal half of the protein. The amino acid sequence underneath represents the Cdk-interaction domain of p27Xic1. MS analysis of Flag-p27Xic1 post-kinase assays identified multiple phosphorylation sites within its Cdk interaction domain with significant confidence scores (Ascores > 13) after aPKC phosphorylation of p27Xic1 in vitro (indicated by arrows), whereas phosphosite (T68) was picked with Ascore of 27 after in vivo kinase assay; another phosphosite (T99) was picked with Ascore of 13 (both indicated by asterisks).

(B) Sequence alignment of the N-terminal half of Xenopus p27Xic1 with other CIP/KIP CdkIs shows that aPKC phosphosite T68 (with arrow) in p27Xic1 is conserved in mammalian p21Cip1 and fish p27Xic1; site T99 (with arrowhead) shows no conservation. Asterisks indicate fully conserved residues, whereas colons and periods indicate groups with strongly and weakly similar properties, respectively.
Taken together, these results mean that phosphorylation of p27Xic1 by aPKC essentially inhibits the activity of p27Xic1 by reducing its binding to Cdk2, which in turn results in failure to negatively regulate the Cdk2 kinase activity.

**p27Xic1 Increases Neuronal Differentiation, and This Effect Is Rescued by aPKC**

p27Xic1 overexpression by injecting mRNA has been shown to promote neurogenesis (Vernon et al., 2003), while its morpholino-mediated knockdown blocked neurogenesis and promoted progenitor proliferation (Carruthers et al., 2003). Here, we have used DNA injections, because in *Xenopus* the G1 phase appears in post-mid-blastula transition cell cycles. We found that such p27Xic1 DNA injections promoted neurogenesis (Figure 5A) in a cell autonomous fashion (Figure S6) and suppressed neuronal progenitor proliferation, consistent with results obtained previously with RNA overexpression.

A subset of p27Xic1-overexpressing embryos was used for fluorescence-activated cell sorting (FACS) cell-cycle profiling on isolated nuclei. Experimental embryos showed significantly higher percentage of nuclei in G1 phase than the control embryos (35.4% versus 27.4%), with a concomitant decrease of nuclei in S phase (40.7% versus 49.7% for control) and negligible effect on the proportion of nuclei in G2 phase (23.9% versus 22.9% for control, Figure 5B), indicating that G1 phase elongates and proliferation decreases upon p27Xic1 overexpression.

Whereas 89% of p27Xic1-injected embryos showed enhanced staining for Elc, the percentage dropped to 33% with a milder phenotype when p27Xic1 was coinjected with aPKC-CAAX (Figure 5C). Thus, p27Xic1’s overexpression phenotype of promoting neuronal differentiation was rescued by unilateral injection of aPKC-CAAX. The corresponding numbers for N-tubulin staining were 88% and 20% after overexpression of p27Xic1 alone or with aPKC-CAAX (data not shown). This supported the idea that aPKC promotes progenitor proliferation, at the expense of neuronal differentiation upstream of p27Xic1, by inhibiting its activity. The idea was further supported by the observation that the phosphomutant of p27Xic1 (T68A) could promote neuronal differentiation similar to p27Xic1, but aPKC-CAAX could not effectively rescue this effect (Figure 5C).

**Lengthening the Cell Cycle via CDK Inhibition by Chemical Means Promotes Differentiation**

To understand if the effects of aPKC and p27Xic1 activities on neuronal differentiation were mediated via their effects on the cell-cycle length, we manipulated the cell cycle of embryos with a corresponding decrease in the proportion of nuclei in S phase, suggesting a decrease in proliferation and an elongation of growth phases (Figure 6A). Experimental embryos also showed a significantly higher number of MyT1-positive cells (a marker of differentiated neurons, 12.53 ± 2.44 per section) than the DMSO controls (8.75 ± 0.38) (Figures 6B and 6C), supporting the idea that elongating G1 phase promotes differentiation.

**DISCUSSION**

Cell polarization and cell division are two fundamental biological processes that have been independently linked to cellular differentiation. In this work, we showed that apicobasal polarity and cell-cycle control are directly linked in neural progenitor cells in a way that leads to distinct differentiation potential of polarized versus nonpolar progenitors. Our work has uncovered a remarkable similarity in the endogenous cell-cycle kinetics of apicobasally polarized and nonpolar progenitors between *Xenopus* neuroectoderm and the mouse embryonic cortex (Arai et al., 2011). In both cases, the total length of the cell cycle and the length of G1 in apicobasally polar progenitors (apical progenitors in the mouse, superficial progenitors in *Xenopus*) are shorter than those found in nonpolar ones (basal progenitors in the mouse, deep progenitors in *Xenopus*). In addition, in both cases, the basal nonpolar progenitors have a higher propensity to differentiate than the apicobasally polarized ones (Chalmers et al., 2002; Chenn and McConnell, 1995). Thus, although the *Xenopus* neuroectoderm shows a much simpler structure, some of the basic principles relating to the cell-cycle control during neurogenesis are highly conserved.

We have used this system to specifically address the role of the apicobasal polarity in controlling the cell cycle via the apically localized key kinase aPKC. aPKC is a ubiquitous kinase that gets activated in cell cortex in a PI-3,4,5-trisphosphate (PIP3)-dependent manner (reviewed by Hirai and Chida, 2003). Polarized cells are thought to contain a highly active pool of aPKC because it is recruited to the apical cortex and/or the junctional complexes (Joberty et al., 2000; Lin et al., 2000) where it should get activated. Therefore membrane targeting of aPKC by attaching it to a CAAX motif makes it constitutively active and mimics the effects of apicobasal polarization on aPKC activity (Lee et al., 2006; Sabherwal et al., 2009).

aPKC was the first molecule shown to promote the proliferation of polarized neuroblasts in *Drosophila* and neural progenitors in *Xenopus* both by gain- and loss-of-function experiments (Rolls et al., 2003; Chabu and Doe, 2008, 2009; Sabherwal et al., 2009). From a mechanistic point of view, the interaction of apicobasal polarity kinase aPKC with the cell-cycle machinery in polarized cells was thought to be indirect, via the Hippo pathway (reviewed in Genevet and Tapon, 2011). For example, aPKC phosphorylates and negatively regulates KIBRA, an upstream positive regulator of the Hippo pathway (Büther et al., 2004;
Yoshihama et al., 2011). Overexpression of aPKC mislocalizes apical Hippo to the cytoplasm with its negative regulator RASSF, leading to the dampening of the Hippo pathway and resulting in enhanced cell proliferation (Grzeschik et al., 2010).

Our current results establish a direct link between aPKC and the cell cycle in context of cell polarity. In this study, we show that aPKC-CAAX directly phosphorylates the nuclear cell cycle/cyclin-dependent kinase inhibitor p27Xic1. Phosphorylation...
primarily takes place in T68, located in the N-terminal domain, although we cannot exclude contribution from other sites. aPKC-CAAX overexpression leads to a faster cell cycle in the neuroectoderm with shorter G1 and S phases, decreased neuronal differentiation, and enhanced neural proliferation (this work and Sabherwal et al., 2009).

aPKC has also been shown to have a role in cell proliferation of nonpolarized cells. For example, aPKC has been shown to directly phosphorylate and degrade p21Cip1 in human colorectal (HCT116) cells (Scott et al., 2002), to upregulate CyD1 transcription, and to reduce p27Kip1's nuclear translocation in a Ras-dependent manner in MCF7 cells (Castoria et al., 2004). We assume that these nonpolar cells have a basal activity of aPKC, which is further enhanced by recruitment to the apical membrane in polarized cells.

How does phosphorylation of p27Xic1 by polarity kinase aPKC lead to a faster cell cycle with shorter G1 and S phases? p27xic1 physically interacts with Cdk5 and cyclins through its Cdk/Cy interaction domains and reduces the activities of G1 kinase (Cdk4/CyD), G1-S transition kinase (Cdk2/CyE) and S progression kinase (Cdk2/CyA) with different half-maximal inhibitory concentration values (Su et al., 1995; Finkielstein et al., 2001). Our data show that phosphomimetic p27Xic1 has a reduced ability to bind and inhibit Cdk2. Enhanced Cdk2 activity through CyE overexpression causes the cells to cycle faster through G1 and enter S phase prematurely (Ohtsubo and Roberts, 1993), whereas inhibition of CyE/Cdk2 complex delays entry into S phase (van den Heuvel and Harlow, 1993). Thus, inhibition of Cdk2 kinase activity by CdkIs, like p27Xic1, is key in inhibiting the cycling of progenitors and promoting their differentiation. We suggest that in polarized progenitors, unrestricted kinase activities of G1/S-specific Cdk/Cy complexes due to lower p27Xic1 activity would shorten their G1 phase and concomitantly promote S phase entry, thereby promoting their cycling and interfering with the ability to differentiate.

The prolongation of total cell-cycle length, and G1 in particular, as cells approach differentiation seems to be a widespread phenomenon (Takahashi et al., 1995; Coronado et al., 2013; Roccio et al., 2013). Mechanistically, it is thought that elongation of the G1 phase promotes neuronal differentiation by providing sufficient time for the accumulation and posttranscriptional modification of differentiation-promoting factors, such as Neurogenin and NeuroD (reviewed in Lange and Calegari, 2010; Hardwick and Philpott, 2014). In support of this idea, it was recently shown that the activity of Neurogenin is controlled by time-dependent, rheostat-like sequential (de)phosphorylation events in a cell-cycle-dependent manner (Ali et al., 2011).

In conclusion, our findings provide mechanistic evidence for a direct link between apicobasal polarity and the cell cycle through aPKC and p27Xic1, which is used during development to endow polarized neural progenitors with lower propensity for differentiation than their nonpolar counterparts. p27Xic1 has a complex mode of regulation, which includes phosphorylation, ubiquitination, and degradation in the nucleus, and changes in the activity

---

Figure 6. Elongating G1 Phase by Nongenetic Means Promotes Neuronal Differentiation

(A) Growing Xenopus embryos from NF10 to 20 in the presence of 120 μM olomoucine, an inhibitor of cyclin-dependent kinases and G1 progression, may elongate the G1 phase of the cell cycle, as indicated by the cell-cycle profiling of the nuclei isolated from NF20 embryos. Experimental embryos showed a higher number of nuclei in the G1 phase of the cell cycle than the control embryos.

(B) Experimental embryos, when stained with neuronal differentiation marker MyT1, showed a significantly higher number of differentiated neurons in comparison to control (mean ± SEM).

(C) Examples of sections from control and experimental embryos stained with MyT1 antibody (green). Nuclei are blue and scale bar represents 50 μM.
through the cell cycle (Chuang and Yew, 2001; Chuang et al., 2005; Lin et al., 2006). Regulation of aPKC is likely to be equally complex, including shuttling between the membrane and the nucleus (Sabherwal et al., 2009). One challenge for the future is to understand the dynamic interaction between aPKC and p27Xic1 during the cell cycle.

EXPERIMENTAL PROCEDURES

Cell-Cycle Length Analysis

Dual-Pulse S Phase Labeling

This method enables the estimation of the total cell-cycle length (Tc) and S phase length (Ts) by injecting two different thymidine analogs (Martynyoga et al., 2005). Embryos collected at open neural plate stage (NF13) were injected with ethynyl deoxyuridine (EdU, 2 x 10 nl, 10 mM in DMSO [Life Technologies]) in the neural plate midline at two different positions. After 2 hr incubation, bromodeoxyuridine (BrdU, 2 x 10 nl, 10 mM in DMSO [Roche]) was injected in the same place (Figure 1A). Embryos were fixed in MEMFA 30 min later and processed for EdU, BrdU, and Sox3 (as a marker of both polar and nonpolar neural progenitors) staining as described elsewhere (Auger et al., 2012).

Percentage of Labeled Mitoses Analysis

This method involves labeling the mitotic (phosphohistone H3/PH3 +) cells with a thymidine analog (EdU or BrdU) and gives an estimate of the lengths of total cell cycle, S and G2+1/M phases, depending on the duration of the experiment (Shackney and Ritch, 1987; Peco et al., 2012). Embryos at NF13 were injected with EdU as described above and collected every 30 min until 240 min as the last point of our experiment (Figure 1B). Embryos were fixed, sectioned, and stained for EdU, PH3, and Sox3 as described elsewhere (Auger et al., 2012).

Cell-Cycle Profiling using Fluorescence-Activated Cell Sorting Analysis on Propidium Iodide-Stained Nuclei

Nuclei from NF15 embryos (20 per set) for FACS analysis were prepared as described elsewhere (Frederick and Andrews, 1994), followed by cell-cycle profiling using a CyAn ADP flow cytometer from Beckman Coulter running Summit v4.3 software. The modfit analysis on the data was carried out using FlowJo software.

Live-Cell Imaging of HeLa Cells-Based Fucci Biosensor for Cell-Cycle Length Analysis

HeLa Fucci cells were maintained and propagated as described elsewhere (Sakaue-Sawano et al., 2008). Control and experimental cells with inhibitors from HeLa cells (overexpressing the protein of interest) and 2–5 μg of mouse anti-Flag antibody (M2 clone [Sigma]). The tube was rotated overnight at 4°C. Washed beads were incubated with the kinase reaction mix as described above. Gel was run, dried, and exposed as described above.

For Histone H1 in vitro kinase assay, 0.5 μg of Histone H1 (Sigma), 100 ng of active Cdk2/CyclinA protein (Millipore), and 1 μl of γ[32P]-radiolabelled ATP (0.37 MBq) were mixed with immunoprecipitated wild-type or mutant p27Xic1 in 1x kinase buffer and incubated at 30°C for 30 min. The reaction was stopped and autoradiography was performed as described above.

Cell Culture, Transfections, Communoprecipitation, Whole Embryo Lysates, and Western Blot Analysis

HeLa cells were maintained and transfected as described elsewhere (Sabherwal et al., 2009). CoIP assays from HeLa cells overexpressing proteins of interest were performed as described elsewhere (Roth et al., 2010). Proteins were immunoprecipitated using 5–10 μg of the following antibodies: mouse anti-Flag (M2 clone [Sigma]), mouse anti-HA (Sigma), and mouse anti-Myc (Santa Cruz Biotechnology). CoIP from embryos was performed using the Pierce Crosslink IP kit, following the manufacturer’s protocol using rabbit anti-aPKC (C20 clone, Santa Cruz Biotechnology) and rabbit anti-p27Xic1 antibody (custom made against the antigen as described by Shou and Dunphy, 1996).

For making embryo lysates, embryos were dissociated in lysis buffer (50 mM Tris pH 7.5 + 150 mM NaCl + 0.5% NP-40 + 5 mM EDTA + 5 mM EGTA). Cleared lysates were used for western blot analysis. The following antibodies were used for detection: rat HA-HRP (Roche), mouse Flag-HRP (Sigma), mouse Myc-HRP (Santa Cruz Biotechnology), rabbit aPKC (C20 clone, Santa Cruz Biotechnology), rabbit anti-p27Xic1, and mouse anti-α-tubulin (DM1A clone [Sigma]).

Chemical Inhibitors

Myristoylated pseudosubstrate inhibitor against aPKC (Invitrogen Life Technologies; Sajan et al., 1999) was used at a working concentration of 12.5 μM. Chemical inhibitor against aPKC (Calbiochem) Szasz et al., 2013 was used at a working concentration of 5 μM. Olomoucine (Calbiochem; Calegari and Huttner, 2003) was used at a working concentration of 120 μM.

Whole-Mount Antibody Staining, Cryosectioning, and Antibody Staining on Sections

Antibody staining on sections and whole mounts was performed as described elsewhere (Sabherwal et al., 2009). The following primary and secondary antibodies were used: rat anti-HA (Roche), rabbit anti-Flag (Sigma), mouse anti-BrdU (MoBu clone [Life Technologies]), mouse anti-phosphohistoneH3 (Abcam) and rabbit anti-Sox3 (custom made; Zhang et al., 2003), rabbit anti-ElrC (mentioned in the figure legends) were chosen from the software and applied to the data.

Overexpression in Whole Embryos, X-Gal Staining, and Whole Mount In Situ Hybridization Followed by Sectioning

Animal experiments were approved by the University’s Ethical Review Panel and were undertaken under UK Home Office project license PPL 70/6484. Culturing and overexpression in Xenopus laevis embryos were carried out as described elsewhere (Sabherwal et al., 2009). Staging was made according to the Nieuwkoop and Faber table of development (NF stages; Nieuwkoop, 1967). The following amounts of mRNA or DNA were injected: aPKC-CAAX 0.25–0.5 ng, NLS-aPKC-NT 0.25–0.5 ng, and p27Xic1 0.15 ng (0.075ng x 2); 0.5 ng GFP or β-galactosidase (lacZ) mRNA was co-injected as lineage tracer/control. Antisense probes N-tubulin, EviC, and Sox3 have been described (Chalmers et al., 2002; Carruthers et al., 2003). Fixation, X-gal staining, in situ hybridization, and sectioning were carried out as described elsewhere (Bourguignon et al., 1996).
anti-MyT1 (custom made; Sabelwral et al., 2009), Alexa488-coupled anti-mouse, and Alexa647-coupled anti-rabbit (Life Technologies).

**Mass Spectroscopic Analysis**

For phosphopeptide mapping, protein bands were Coomassie stained, excised and in situ digested with trypsin. LC/MS/MS analysis of phosphosites was performed at the Taplin Biological Mass Spectrometry Facility (Harvard Medical School) as described (Roig et al., 2005). Please refer to the Supplemental Experimental Procedures for information regarding sample preparation for MS analysis.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.10.023.

**ACKNOWLEDGMENTS**

We thank Atushi Miyawaki (Brain Science Institute, RIKEN, Saitama, Japan) for kindly gifting us the HeLa Fucci biosensor. This work was funded by a Wellcome Trust Senior Fellowship grant (WT090868 to N.P.). N.S. and R.T are Wellcome Trust research associates and R.L. and P.S. are research assistants with the N.P. lab.

Received: September 16, 2013
Revised: September 5, 2014
Accepted: October 29, 2014
Published: December 8, 2014

**REFERENCES**

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002). Molecular Biology of the Cell. (New York: Garland Science).

Ali, F., Hindley, C., McDowell, G., Deibler, R., Jones, A., Kirschner, M., Guillemot, F., and Pippolot, A. (2011). Cell cycle-regulated multi-site phosphorylation of Neurogenin 2 coordinates cell cycling with differentiation during neurogenesis. Development 138, 4267–4277.

Arai, Y., Pulvers, J.N., Haffner, C., Schilling, B., Nüsslein, I., Calegari, F., and Huttnner, W.B. (2011). Neural stem and progenitor cells shorten S-phase on commitment to neuron production. Nat. Commun. 2, 154.

Auger, H., Thuret, R., Yakoube, W.E., and Papiolpulon, N. (2012). A Bromodeoxyuridin (BrdU) based protocol for characterizing proliferating progenitors in Xenopus embryos. In Methods in Molecular Biology (Totowa: Humana Press), pp. 461–475.

Barilà, D., Mangano, R., Gonzoli, S., Kretzschmar, J., Moro, M., Bohmann, D., and Superti-Furga, G. (2000). A nuclear tyrosine phosphorylation circuit: c-Jun as an activator and substrate of c-Abl and JNK. EMBO J. 19, 273–281.

Beausoleil, S.A., Villen, J., Gerber, S.A., Rush, J., and Gygi, S.P. (2006). A probability-based approach for high-throughput protein phosphorylation analysis and site localization. Nat. Biotechnol. 24, 1285–1292.

Bourguignon, L, Li, J., and Papalopulu, N. (1998). XBF-1, a winged helix transcription factor with dual activity, has a role in positioning neurogenesis in the developing nervous system. Biochem. J. 327, 703–707.

Calegari, F., and Huttnner, W.B. (2003). An inhibition of cyclin-dependent kinases that lengthens, but does not arrest, neuroepithelial cell cycle induces premature neurogenesis. J. Cell Sci. 116, 4947–4955.

Carruthers, S., Mason, J., and Papalopulu, N. (2003). Depletion of the cell-cycle inhibitor p27(Xic1) impairs neuronal neuronal differentiation and increases the number of Erc(+)-progenitor cells in Xenopus tropicalis. Mech. Dev. 120, 607–616.

Castoria, G., Migliaccio, A., Di Domenico, M., Lombardi, M., de Falco, A., Varricchio, L., Bilancio, A., and Auricchio, F. (2004). Role of atypical protein kinase C in estradiol-triggered G1/S progression of MCF-7 cells. Mol. Cell. Biol. 24, 7643–7653.

Chabu, C., and Doe, C.Q. (2008). Dap160/intersectin binds and activates aPKC to regulate cell polarity and cell cycle progression. Development 135, 2739–2746.

Chabu, C., and Doe, C.Q. (2009). Twins/PP2A regulates aPKC to control neuronal cell polarity and self-renewal. Dev. Biol. 330, 399–405.

Chalmers, A.D., Welchman, D., and Papalopulu, N. (2002). Intrinsinc differences between the superficial and deep layers of the Xenopus ectoderm control primary neuronal differentiation. Dev. Cell 2, 171–182.

Chenn, A., and McConnell, S.K. (1995). Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis. Cell 82, 631–641.

Chuang, L.C., and Yew, P.R. (2001). Regulation of nuclear transport and degradation of the Xenopus cyclin-dependent kinase inhibitor, p27Xic1. J. Biol. Chem. 276, 1610–1617.

Chuang, L.-C., Zhu, X.-N., Herrera, C.R., Tseng, H.-M., Pfleger, C.M., Block, K., and Yew, P.R. (2005). The C-terminal domain of the Xenopus cyclin-dependent kinase inhibitor, p27Xic1, is both necessary and sufficient for phosphorylation-independent proteolysis. J. Biol. Chem. 280, 35290–35298.

Coronado, D., Godet, M., Bourillot, P.-Y., Taponnier, Y., Bernat, A., Petit, M., Afnanassief, M., Markossian, S., Malashicheva, A., Iacone, R., et al. (2013). A short G1 phase is an intrinsic determinant of naive embryonic stem cell pluripotency. Stem Cell Res. (Amst.) 10, 118–131.

Dutta, D., Ray, S., Home, P., Larson, M., Wolfe, M.W., and Paul, S. (2011). Self-renewal versus lineage commitment of embryonic stem cells: protein kinase C signaling shifts the balance. Stem Cells 29, 618–628.

Eckert, J.J., McCallum, A., Mears, A., Rumsby, M.G., Cameron, I.T., and Fleming, T.P. (2004). PKC signalling regulates tight junction membrane assembly in the pre-implantation mouse embryo. Reproduction 127, 653–667.

Finkielewicz, C.V., Lewellyn, A.L., and Maller, J.L. (2001). The midblastula transition in Xenopus embryos activates multiple pathways to prevent apoptosis in response to DNA damage. Proc. Natl. Acad. Sci. USA 98, 1006–1011.

Folch, J., Junyent, F., Verdaguer, E., Auladell, C., Pizarro, J.G., Beas-Zarate, C., Pallas, M., and Camins, A. (2012). Role of cell cycle re-entry in neurons: a common apoptotic mechanism of neuronal cell death. Neurotox. Res. 22, 195–207.

Frederick, D.L., and Andrews, M.T. (1994). Cell cycle remodeling requires cell-cell interactions in developing Xenopus embryos. J. Exp. Zool. 270, 410–416.

Genevert, A., and Tapon, N. (2011). The Hippo pathway and apico-basal cell polarity. Biochem. J. 436, 213–224.

Grzeschik, N.A., Parsons, L.M., Allott, M.L., Harvey, K.F., and Richardson, H.E. (2010). Lgl, aPKC, and Crumbs regulate the Salvador/Warts/Hippo pathway through two distinct mechanisms. Curr. Biol. 20, 573–581.

Hardwick, J.I.A., and Philpott, A. (2014). Nervous decision-making: to divide or differentiate. Trends Genet. 30, 254–261.

Hasan, S.M.M., Sheen, A.D., Power, A.M., Lanevein, L.M., Xiong, J., Furlong, M., Day, K., Schuurmans, C., Opferman, J.T., and Vanderluit, J.L. (2013). Mc11 regulates the terminal mitosis of neural precursor cells in the mammalian brain through p27Kip1. Development 140, 3118–3127.

Hindley, C., and Pippolot, A. (2012). Co-ordination of cell cycle and differentiation in the developing nervous system. Biochem. J. 444, 375–382.

Hirai, T., and Chida, K. (2003). Protein kinase Czeta (PKCzeta): activation mechanisms and cellular functions. J. Biochem. 133, 1–7.

Joberty, G., Petersen, C., Gao, L., and Macara, I.G. (2000). The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. Nat. Cell Biol. 2, 531–539.

Kraenmobil, G., Scharnhorst, V., and Zentema, A. (1995). Inhibition of cyclin-dependent kinase activity triggers neuronal differentiation of mouse neuroblastoma cells. J. Cell Biol. 131, 227–234.

Lange, C., and Calegari, F. (2010). Cdk5 and cyclins link G1 length and differentiation of embryonic, neural and hematopoietic stem cells. Cell Cycle 9, 1893–1900.
Developmental Cell
Polarity Controls Cell Cycle Directly

Lange, C., Hutten, W.B., and Calegari, F. (2009). Cdk4/cyclinD1 overexpression in neural stem cells shortens G1, delays neurogenesis, and promotes the generation and expansion of basal progenitors. Cell Stem Cell 5, 320–331.

Lee, C.Y., Robinson, K.J., and Doe, C.Q. (2008). Lgl, Pins and aPKC regulate neuroblast self-renewal versus differentiation. Nature 439, 594–598.

Lin, D., Edwards, A.S., Fawcett, J.P., Mbanalu, G., Scott, J.D., and Pawson, T. (2000). A mammalian PAR-3-PAR-6 complex implicated in Cdc42/Rac1 and aPKC signalling and cell polarity. Nat. Cell Biol. 2, 540–547.

Lin, H.R., Chuang, L.C., Boix-Perales, H., Philpott, A., and Yew, P.R. (2006). A mammalian PAR-3-PAR-6 complex implicated in Cdc42/Rac1 and aPKC signalling and cell polarity. Nat. Cell Biol. 2, 540–547.

Lobios, V., Bel-Vialar, S., Trousse, F., and Pituello, F. (2008). Forcing neural progenitor cells to cycle is insufficient to alter cell-fate decision and timing of neuronal differentiation in the spinal cord. Neural Dev. 3, 4.

Mairet-Coello, G., Tury, A., Van Buskirk, E., Robinson, K., Genestine, M., and DiCicco-Bloom, E. (2012). p57(KIP2) regulates radial glia and intermediate precursor cell cycle dynamics and lower layer neurogenesis in developing cerebral cortex. Development 139, 475–487.

Martynoga, B., Morrison, H., Dijkgraaf, M.G., and Mason, J.O. (2005). Foxg1 is required for specification of ventral telencephalon and region-specific regulation of dorsal telencephalic precursor proliferation and apoptosis. Dev. Biol. 283, 113–127.

Nieuwkoop, P.D. (1967). Normal Table of Xenopus Laevis (Daudin). (New York: Garland Pub).

Ohnuma, S., and Harris, W.A. (2003). Neurogenesis and the cell cycle. Neuron 40, 199–208.

Ohtsubo, M., and Roberts, J.M. (1993). Cyclin-dependent regulation of G1 in mammalian fibroblasts. Science 259, 1908–1912.

Peco, E., Escude, T., Agius, E., Sabado, V., Medevielle, F., Ducommun, B., and Pituello, F. (2012). The CDC25B phosphatase shortens the G2 phase of neural progenitors and promotes efficient neuron production. Development 139, 1095–1104.

Roccio, M., Schmitter, D., Knobloch, M., Okawa, Y., Sage, D., and Lutolf, M.P. (2013). Predicting stem cell fate changes by differential cell cycle progression patterns. Development 140, 459–470.

Roig, J., Groen, A., Caldwell, J., and Avruch, J. (2005). Active Nercc1 protein kinase concentrates at centrosomes early in mitosis and is necessary for proper spindle assembly. Mol. Biol. Cell 16, 4827–4840.

Rolls, M.M., Albertson, R., Shih, H.P., Lee, C.Y., and Doe, C.Q. (2003). Drosophila aPKC regulates cell polarity and cell proliferation in neuroblasts and epithelia. J. Cell Biol. 163, 1089–1098.

Roth, M., Bonev, B., Lindsay, J., Lea, R., Panagiotaki, N., Hourt, C., and Papalopulu, N. (2010). Foxg1 and TLE2 act cooperatively to regulate ventral telencephalon formation. Development 137, 1553–1562.

Sabherwal, N., and Papalopulu, N. (2012). Apicobasal polarity and cell proliferation during development. Essays Biochem. 53, 95–108.

Sabherwal, N., Tsutui, A., Hodge, S., Wei, J., Chalmers, A.D., and Papalopulu, N. (2009). The apicobasal polarity kinase aPKC functions as a nuclear determinant and regulates cell proliferation and fate during Xenopus primary neurogenesis. Development 136, 2767–2777.

Saiz, N., Grabarek, J.B., Sabherwal, N., Papalopulu, N., and Plusa, B. (2013). Atypical protein kinase C couples cell sorting with primitive endoderm matura-

Sajjan, M.P., Standaert, M.L., Bandyopadhyay, G., Quon, M.J., Burke, T.R., Jr., and Farese, R.V. (1999). Protein kinase C-zeta and phosphoinositide-dependent protein kinase-1 are required for insulin-induced activation of ERK in rat adipocytes. J. Biol. Chem. 274, 30495–30500.

Sakaue-Sawano, A., Kurokawa, H., Morimura, T., Hanyu, A., Hama, H., Osawa, H., Kashiwagi, S., Fukami, K., Miyata, T., Miyoshi, H., et al. (2008). Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. Cell 132, 487–498.

Scott, M.T., Ingram, A., and Ball, K.L. (2002). PDK1-dependent activation of atypical PKC leads to degradation of the p21 tumour modifier protein. EMBO J. 21, 6771–6780.

Shackney, S.E., and Ritch, P.S. (1987). Percent labeled mitosis curve analysis. In Techniques in Cell Cycle Analysis (New York: Springer), pp. 31–45.

Shou, W., and Dunphy, W.G. (1996). Cell cycle control by Xenopus p28Kix1, a developmentally regulated inhibitor of cyclin-dependent kinases. Mol. Biol. Cell 7, 457–469.

Standaert, M.L., Bandyopadhyay, G., Sajjan, M.P., Cong, L., Quon, M.J., and Fareese, R.V. (1999). Okadaic acid activates atypical protein kinase C (Cζ) in rat and 3T3-L1 adipocytes. An apparent requirement for activation of Glut4 translocation and glucose transport. J. Biol. Chem. 274, 14074–14078.

Su, J.Y., Rempel, R.E., Erikson, E., and Maller, J.L. (1996). Cloning and characterization of the Xenopus cyclin-dependent kinase inhibitor p27XIC1. Proc. Natl. Acad. Sci. USA 92, 10187–10191.

Takahashi, T., Nowakowski, R.S., and Caviness, V.S., Jr. (1995). The cell cycle of the pseudostratified ventricular epithelium of the embryonic murine cerebral wall. J. Neurosci. 15, 6046–6057.

van den Heuvel, S., and Harlow, E. (1993). Distinct roles for cyclin-dependent kinases in cell cycle control. Science 262, 2050–2054.

Vernon, A.E., Devine, C., and Philpott, A. (2003). The cdk inhibitor p27Xic1 is required for differentiation of primary neurones in Xenopus. Development 130, 85–92.

Wilcock, A.C., Swedlow, J.R., and Storey, K.G. (2007). Mitotic spindle orientation distinguishes stem cell and terminal modes of neuron production in the early spinal cord. Development 134, 1943–1954.

Yoshihama, Y., Sasaki, K., Horikoshi, Y., Suzuki, A., Ohtsuka, T., Hakuno, F., Takahashi, S., Ohno, S., and Chida, K. (2011). KIBRA suppresses apical exocytosis through inhibition of aPKC kinase activity in epithelial cells. Curr. Biol. 21, 705–711.

Zhang, C., Basta, T., Jensen, E.D., and Klumpp, M.W. (2003). The β-catenin/VegT-regulated early zygotic gene Xnr5 is a direct target of SOX3 regulation. Development 130, 5609–5624.