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$p27^{Kip1}$ participates in the regulation of endoreplication in differentiating chick retinal ganglion cells

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Nuclear DNA duplication in the absence of cell division (i.e. endoreplication) leads to somatic polyploidy in eukaryotic cells. In contrast to some invertebrate neurons, whose nuclei may contain up to 200,000-fold the normal haploid DNA amount (C), polyploid neurons in higher vertebrates show only 4C DNA content. To explore the mechanism that prevents extra rounds of DNA synthesis in these latter cells we focused on the chick retina, where a population of tetraploid retinal ganglion cells (RGCs) has been described. We show that differentiating chick RGCs that express the neurotrophic receptors p75 and TrkB while lacking retinoblastoma protein, a feature of tetraploid RGCs, also express $p27^{Kip1}$. Two different short hairpin RNAs (shRNA) that significantly downregulate $p27^{Kip1}$ expression facilitated DNA synthesis and increased ploidy in isolated chick RGCs. Moreover, this forced DNA synthesis could not be prevented by Cdk4/6 inhibition, thus suggesting that it is triggered by a mechanism similar to endoreplication. In contrast, $p27^{Kip1}$ deficiency in mouse RGCs does not lead to increased ploidy despite previous observations have shown ectopic DNA synthesis in RGCs from $p27^{Kip1-/-}$ mice. This suggests that a differential mechanism is used for the regulation of neuronal endoreplication in mammalian versus avian RGCs.

Introduction

In eukaryotes, the cell cycle is regulated by Cyclins and their specific cyclin-dependent kinases (Cdks). The complex formed by Cyclin D and Cdk4/6 phosphorylates Retinoblastoma protein (Rb), thus leading to the release of the transcription factor E2F1,[1] which in turn induces the synthesis of the proteins necessary for DNA replication.[2] G1/S progression is then regulated by the activation of Cdk2 by Cyclin E, which phosphorylates additional residues of Rb,[3] thus facilitating cell cycle progression. DNA synthesis is driven by the association of Cdk2 with Cyclin A, and the Cdk1/Cyclin A complex regulate late G2 phase progression.[4-6] Finally, G2/M transition is regulated by the formation of the Cdk1/Cyclin B complex.[7] The transitions between the different cell cycle phases are further regulated by 2 families of Cdk inhibitors (CKI): the Ink and Cip/Kip protein families. The Ink family, composed by $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Ink4d}$, regulates the quiescent state by specifically binding to Cdk4/6 thus preventing its union with Cyclin D.[8] The members of the Cip/Kip family, constituted by $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, preferentially inactivate Cyclin/Cdk complexes but they can also modulate their activity.[3,9] Through all these mechanisms, cell cycle is strictly regulated to obtain 2 daughter cells with the same chromosomal number as that of the original cell.

Ploidy can be defined as the number of chromosome sets that are present in the cell nucleus and it is expressed as the N value (i.e., the set of chromosomes present in a gamete, or haploid cell). In contrast, DNA content (expressed as the C value) represents the amount of DNA present in a gamete or haploid cell. While the C value increases during S-phase, remaining duplicated during G2, the N value remains unaltered as the cell progresses through the cell cycle.[10-13] Polyploidy, defined as the presence in the cell nucleus of more than 2 sets of chromosomes, can affect all the cells of the organism. In this case, it is referred to as germinal polyploidy. A strategy to increase the body size, based on germinal polyploidy, has been widely used by plants,[14,15] and also by protozoa (Tetrahymena),[16] fish,[17] as well as by amphibians including *Salamandra salamandra* and *Xenopus laevis*.[18,19] Although germinal polyploidy cannot be observed in higher vertebrates because it compromises their viability, polyploidy affecting to specific cell types or tissues (i.e. somatic tetraploidy) can be widely observed in these organisms. In this case, the term polyploidy is used in a broad sense as a synonym of the

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C value, since the actual number of chromosomes in the affected cells remains usually unknown. Somatic polyploidy has several advantages for the affected cells including a better response to metabolic and/or genotoxic stress and a lower sensibility to apoptotic stimuli.[10,11,20,21] In mammals and higher vertebrates different cell types have been shown to be polyploid, including trophoblast giant cells,[10,11,,22] hepatocytes,[23,24] megakaryocytes,[25] keratinocytes,[26] and vascular smooth cells.[27] Endoreplication represents a major mechanism leading to developmental programmed somatic polyploidy.[10-12] This process consists of successive rounds of DNA synthesis in the absence of mitosis (endocycles), thus leading to cells with a DNA content higher than 2C. Although the mechanism of endoreplication is far from being completely understood, it seems to be dependent on the levels and activity of the Cdk/Cyclin complex after the completion of every S phase, the oscillations of Cyclin E and Geminin levels, and the localization of the minichromosome maintenance protein complexes.[10-12,28] Endoreplication can be mimicked by inhibition of Cdk1 activity,[11] and the subsequent prevention of the mitotic process. Moreover, cyclin E/Cdk2 is the G1 Cdk complex crucial for DNA synthesis initiation during the endoreplicative process.[29-32]

Somatic polyploid neurons generated by endoreplication are very common in the nervous system of lower vertebrates and invertebrates.[33,34] For instance, the nucleus of giant neurons from *Aplysia californica* has been shown to contain 200,000-fold the normal amount of haploid DNA (i.e., 200,000C).[33] These neurons have routinely been subjected to electrophysiological analyses,[35] proving that they are fully functional. In humans, around 10% of the cortical neurons show DNA contents higher than 2C, being tetraploid around 1% of these neurons.[36] Tetraploid neurons have also been found in the murine retina and cerebral cortex,[37,38] as well as in the retina, optic tectum, dorsal root ganglia, cerebellum, telencephalon and spinal cord of the chick.[37,38] In the chick retina, tetraploid ganglion cells are generated through cell cycle reactivation as they migrate to the ganglion cell layer, soon after their final mitosis[37] (see **Fig. 1**). Cell cycle reactivation in neurons fated to become tetraploid occurs in response to the interaction of nerve growth factor (NGF) with the neurotrophin receptor p75 ($p75^{NTR}$).[37-40] Tetraploid RGCs remain in a G2-like state in the presence of brain-derived neurotrophic factor (BDNF), which activates the TrkB receptor to decrease Cdk1 expression and activity in these neurons, thus blocking G2/M transition.[41] In contrast, in the absence of

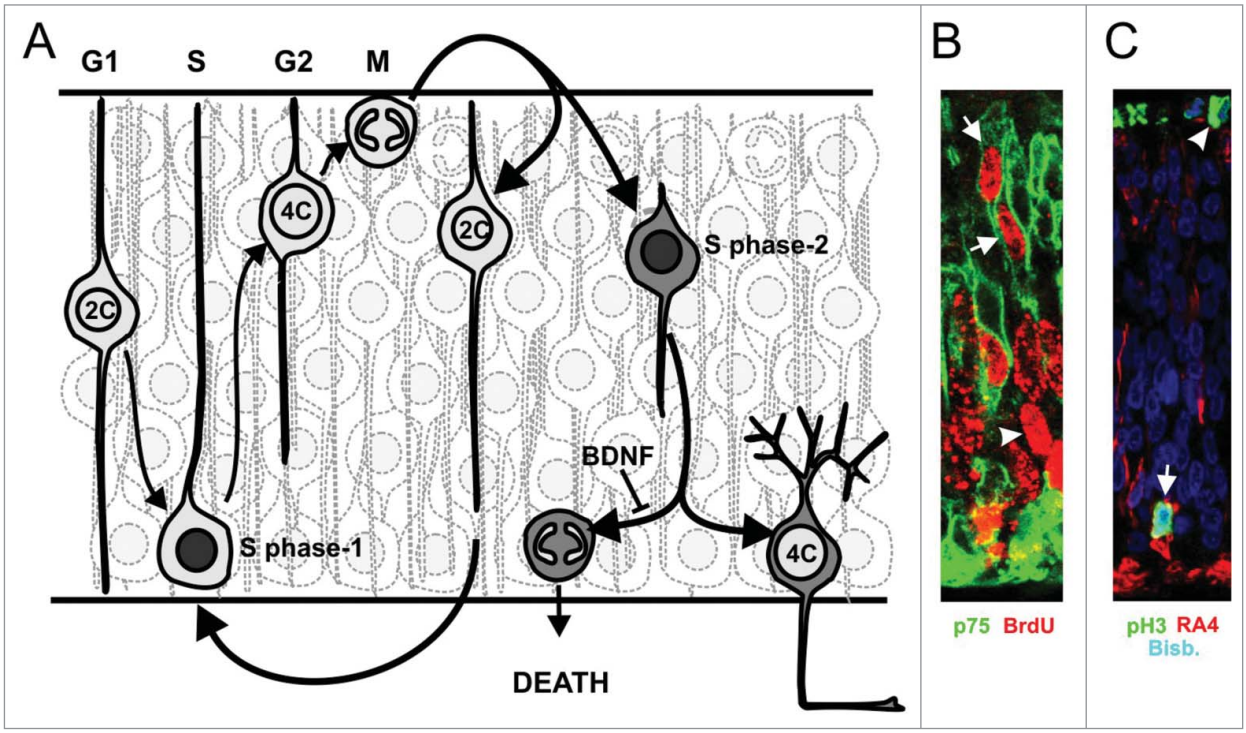

Figure 1. Scheme of the mechanism inducing tetraploid RGCs in the chick retina. (**A**) Retinal precursors undergo S-phase (dark gray nucleus) at the basal neuroepithelium (S-phase-1), and they displace their nuclei to the apical neuroepithelium during G2, showing 4C DNA content. Then, they undergo mitosis at the apical portion of the neuroepithelium. This division gives rise to precursors with 2C DNA content that undergo a new round of interkinetic nuclear movement (see ref.[78]). Alternatively, daughter cells may undergo neuronal differentiation (gray cytoplasm). Some of the differentiating RGCs can reactivate the cell cycle (S-phase-2) in response to NGF as they migrate to the basal neuroepithelium, where the GCL will be located. In the presence of BDNF, RGCs remain with 4C DNA content (i.e. tetraploid neurons), whereas in its absence they undergo ectopic mitosis at the basal neuroepithelium and die. (**B**) An illustrative image showing $p75^{NTR}$-positive differentiating RGCs undergoing S-phase-2 at the apical neuroepithelium (arrows). In contrast, precursors undergoing S-phase-1 (arrowhead) are located basally. (**C**) An illustrative image showing an RA4-positive differentiating RGC undergoing ectopic mitosis, revealed with phosphoHistone H3 immunolabeling (pH3), at the basal neuroepithelium (arrow). In contrast, precursors undergo mitosis at the apical neuroepithelium (arrowhead). Bisb.: bisbenzimide.

BDNF these neurons undergo mitosis followed by apoptosis[37] (**Fig. 1**).

So far, no polyploid neurons with DNA levels above 4C have been found in the normal brain of higher vertebrates.[37,42] Furthermore, Rb-deficient neurons have been shown to undergo cell cycle re-entry *in vivo*, remaining alive with a 4C DNA content but not higher.[43] Therefore, the question that arises is why tetraploid neurons cannot undergo successive rounds of endoreplication similar to those performed by giant neurons from lower vertebrates. As there should be a mechanism that prevents tetraploid neurons from undergoing successive rounds of endoreplication we have hypothesized $p27^{Kip1}$ as being a potential candidate for this function. In this study we provide evidence that this CKI is expressed by all RGCs in the developing chick retina, and that the downregulation of this CKI in cultured RGCs results in DNA synthesis and increased DNA content in these neurons. This mechanism seems to be specific for chick RGCs as the retina of $p27^{Kip1-/-}$ mice[44] lack RGCs with a DNA content higher than 4C.

Results

$p27^{Kip1}$ is expressed by layered RGCs in the developing chick neural retina, including those that become tetraploid

To determine whether $p27^{Kip1}$ may be involved in preventing successive rounds of S phase in tetraploid neurons, the expression pattern of $p27^{Kip1}$ was characterized in the E5 chick retina, a stage at which tetraploid RGCs are being generated in this tissue.[37] This analysis demonstrated that $p27^{Kip1}$ presents a conspicuous expression pattern. On the one hand, $p27^{Kip1}$-specific immunoreactivity can be clearly observed in the retinal pigmented epithelium (arrows in **Fig. 2**), as previously shown by ref.[45]. On the other hand, $p27^{Kip1}$ colocalizes with the RGC differentiating marker $p75^{NTR}$ in the basal region of the neural retina [99,4 ± 0,6% (n = 2) of the $p27^{Kip1}$-positive cells express $p75^{NTR}$] (**Fig. 2A**), thus suggesting that $p27^{Kip1}$ is expressed at the end of the migration of all differentiating RGCs. This assumption is consistent with the co-localization of $p27^{Kip1}$ with G4 (**Fig. 2B**), a marker that preferentially detects differentiating layered RGCs.[46] Therefore, the majority of the $p27^{Kip1}$-positive cells present in the retina at this stage are differentiating neurons localized in the ganglion cell layer (GCL), as previously described by ref.[47]. All cells that express TrkB receptor, known to prevent G2/M transition in tetraploid RGCs,[41] show $p27^{Kip1}$ immunoreactivity (**Fig. 2C**), suggesting that tetraploid RGCs also express $p27^{Kip1}$. In this regard, most laminated RGCs that express $p27^{Kip1}$ lack Rb [99,2 ± 0,4% (n = 3)] (**Fig. 2D**), a feature of RGCs that re-enter cell cycle and become tetraploid.[37] In sum, we conclude that $p27^{Kip1}$ is expressed by all layered RGCs, including those that become tetraploid.

$p27^{Kip1}$ expression was also analyzed by immunocytochemistry in E6 chick retina cultures maintained under neurogenic conditions, known to mimick RGC differentiation *in vivo*.[37,39,41,48-50] As expected from its expression pattern *in vivo*, $p27^{Kip1}$-specific immunoreactivity was detected only in the nucleus of G4-positive neurons (i.e. differentiating RGCs) (**Fig. 3**).

Knock-down of $p27^{Kip1}$ expression with specific interfering RNA constructs

To verify the participation of $p27^{Kip1}$ in preventing tetraploid neurons from undergoing successive rounds of endoreplication, specific shRNA vectors against $p27^{Kip1}$ were constructed following the procedure described by ref.[51]. Two independent vectors were designed, one targeting the 5′ region (1p27i) and another one targeting the 3′ region (2p27i) of the *Cdkn1b* mRNA. A shRNA vector known to interfere with *luciferase*[51] was used as a control (Luc-i). All these vectors co-express RFP. To evaluate the efficiency of the interfering RNAs, E6 chick retinal cells were electroporated with the shRNA vectors and then cultured under neurogenic conditions to give rise to differentiating chick retinal neurons (DCRNs).[48,49] After 20 h, these neurons were subjected to double immunostaining with G4- and $p27^{Kip1}$-specific antibodies. $p27^{Kip1}$ expression levels were quantified by image analysis in G4/RFP positive cells. Both interference vectors (1p27i and 2p27i) decreased the expression levels of $p27^{Kip1}$ by 50% and 67%, respectively, in comparison with the expression levels observed in RGCs transfected with the control vector (Luc-i) (**Fig. 4A**).

$p27^{Kip1}$ knock-down facilitates DNA synthesis and increased ploidy in differentiating RGCs

The interfering RNAs described above were used to test whether $p27^{Kip1}$ knock-down could induce BrdU incorporation in differentiated RGCs. To increase the proportion of these latter neurons in our cultures we employed a procedure previously described by ref.[52], based on the centrifugation of E7 chick retinal cells through a Percoll gradient. **Fig. 5** shows an example of a neurogenic culture enriched in RGCs obtained with this protocol and immunostained with βIII tubulin, a marker that is expressed at high levels by the RGCs.[53] After 20 h in culture, RGC-enriched cultures were lipofected with the 1p27i, the 2p27i, or the Luc-i vectors, and treated with BrdU during an additional 20 h period. BrdU incorporation was then quantified in lipofected cells (i.e., RFP-positive cells) expressing the neuronal marker NeuN. This analysis demonstrated a statistically significant increase of BrdU incorporation in neurons transfected with any of the $p27^{Kip1}$ shRNA vectors (**Fig. 4B**).

According to our hypothesis, the increase of DNA synthesis in RGCs triggered by $p27^{Kip1}$ knock-down should result in a concomitant increase of DNA content in these neurons through an endoreplicative mechanism. In accordance with this assumption, none of the neurons lipofected with any of the shRNA vectors (1p27i, n = 266; 2p27i, n = 188) as well as with the control vector (n = 188) did show mitotic figures at the end of the culture period, thus suggesting that the increase of DNA content in RGCs is not reduced as a result of cell division. To directly prove that the $p27^{Kip1}$ knock-down in RGCs actually results in an increase of the C value, DNA intensity level, evidenced by DAPI staining, was quantified by image analysis in the lipofected NeuN-positive neurons. In all these cultures 8.73 ± 1.12% (mean ± s.e.m.; n = 6) of the lipofected NeuN-positive cells were observed to be tetraploid, a proportion in the range to that previously observed in E6 chick retinal cells cultured under neurogenic conditions as well as in the posthatch chick retina in

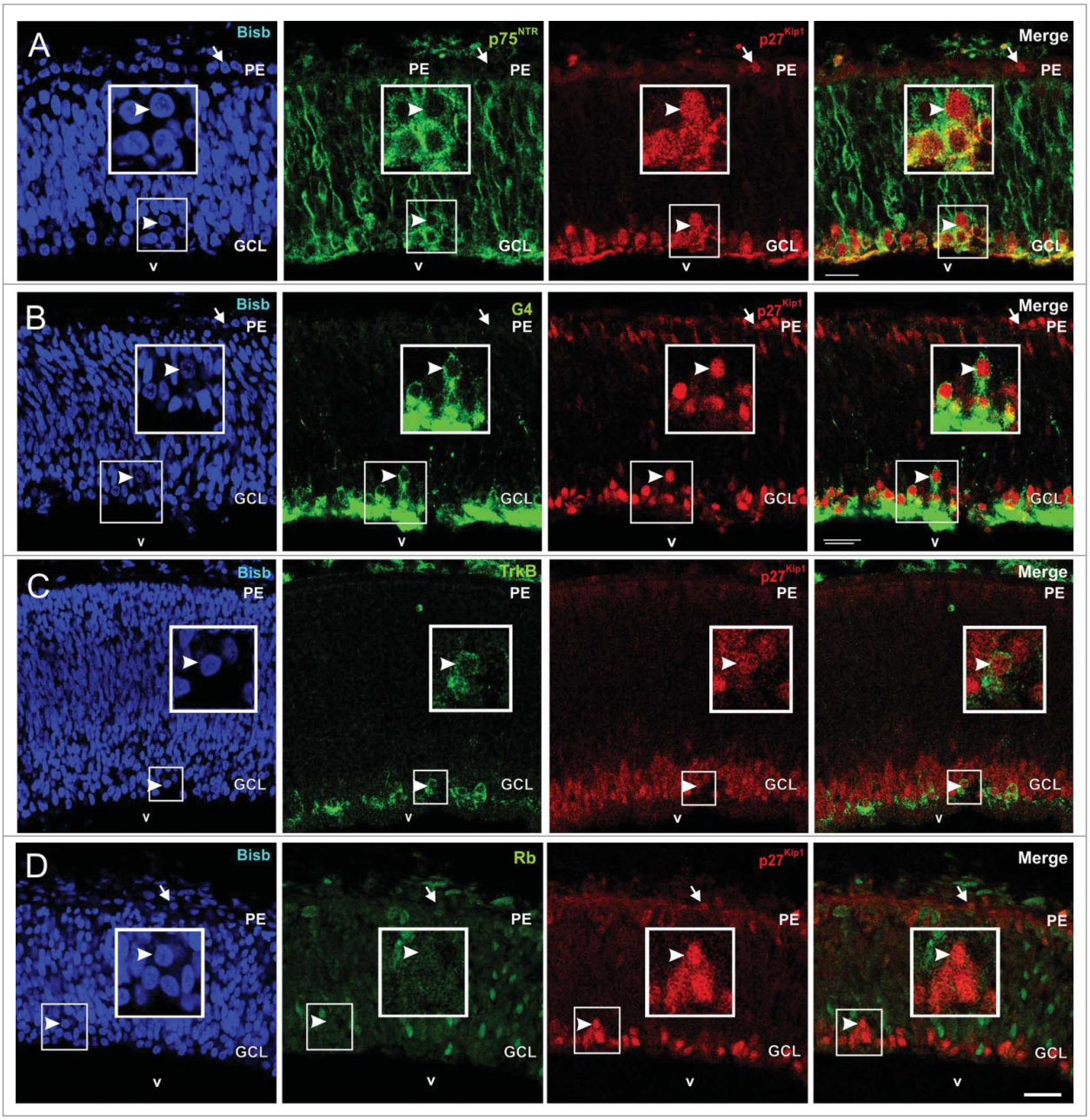

Figure 2. p27^{Kip1} expression in the E5 chick retina. Confocal sections from retinas of E5 chick embryos double labeled with an anti-p27^{Kip1} specific antibody (red) and antibodies (green) specific for p75^{NTR} (**A**), G4 (**B**), TrkB (**C**), or Rb (**D**) are shown. Nuclear staining with bisbenzimide in blue (Bisb.). p27^{Kip1} expression can be detected in the pigment epithelium (arrows) and layered differentiating RGCs in the GCL (arrowheads). (**A**) p75^{NTR}-positive cells co-localize with p27^{Kip1}. (**B**) p27^{Kip1} is expressed in differentiated CGRs expressing G4. (**C**) TrkB expressing cells also express p27kip1. (**D**) p27kip1 is expressed by RGCs lacking Rb. Boxes: high magnification of the framed areas. GCL: ganglion cell layer; PE: pigment epithelium; v: vitreous body. Bar: 20 μm.

vivo.[37] This analysis also indicated that around 10% of the more-than-diploid (>2C), p27^{Kip1}-knocked-down neurons showed DNA content higher than 4C and below 8C irrespective of the shRNA vector used. In contrast, >2C neurons transfected with the Luc-i control vector did not show DNA levels above 4C (**Fig. 4C**).

In the differentiating chick retina, tetraploid neurons that undergo mitosis finally die by apoptosis.[37,41] Therefore, the lack of mitotic figures in the neurons lipofected with the shRNA vectors is consistent with the absence of apoptosis in these cells. In this regard, none of the neurons transfected with either the 1p27i (n = 266) or the 2p27i (n = 188) vectors showed pyknotic

nuclei. A similar situation was observed in those neurons transfected with the Luc-i vector (n = 188).

DNA synthesis in RGCs expressing p27^{Kip1}-specific shRNAs is independent from Cdk4/6 activity

To test whether the increase of BrdU incorporation in neurons expressing p27^{Kip1}-specific shRNAs was due to endoreplication or, alternatively, to conventional cell cycle progression, a potent and specific inhibitor against Cdk4/6 (PD0332991) was used. As previously shown,[39] PD0332991 was able to prevent cell cycle progression in retinal precursor cells ($P < 0.05$) (**Fig. 6A**), thus indicating that Cdk4/6 is required for normal cell cycle progression in these proliferating cells. In contrast, PD0332991 was unable to inhibit DNA synthesis in cultured RGCs lipofected with the p27^{Kip1}-specific shRNA vectors and treated for 20 h with BrdU ($P > 0.05$, n = 3; ANOVA test). For this latter analysis, RGCs were identified by using 2 different criteria: expression of Brn3a (**Fig. 6B**), a known marker of a subpopulation of RGCs,[54] or just by morphology (monoaxonal cells with rounded cytoplasm were identified as RGCs) (**Fig. 6C**). In both cases, the increase of BrdU incorporation in response to the p27^{Kip1}-specific shRNA vectors was similar to that obtained in the experiments illustrated in **Fig. 4B**. Overall, these data indicate that the mechanism triggering DNA synthesis in p27^{Kip1}-knocked-down RGCs is different from the classical Cdk4/6-dependent mechanism leading to cell cycle progression in retinal precursor cells.

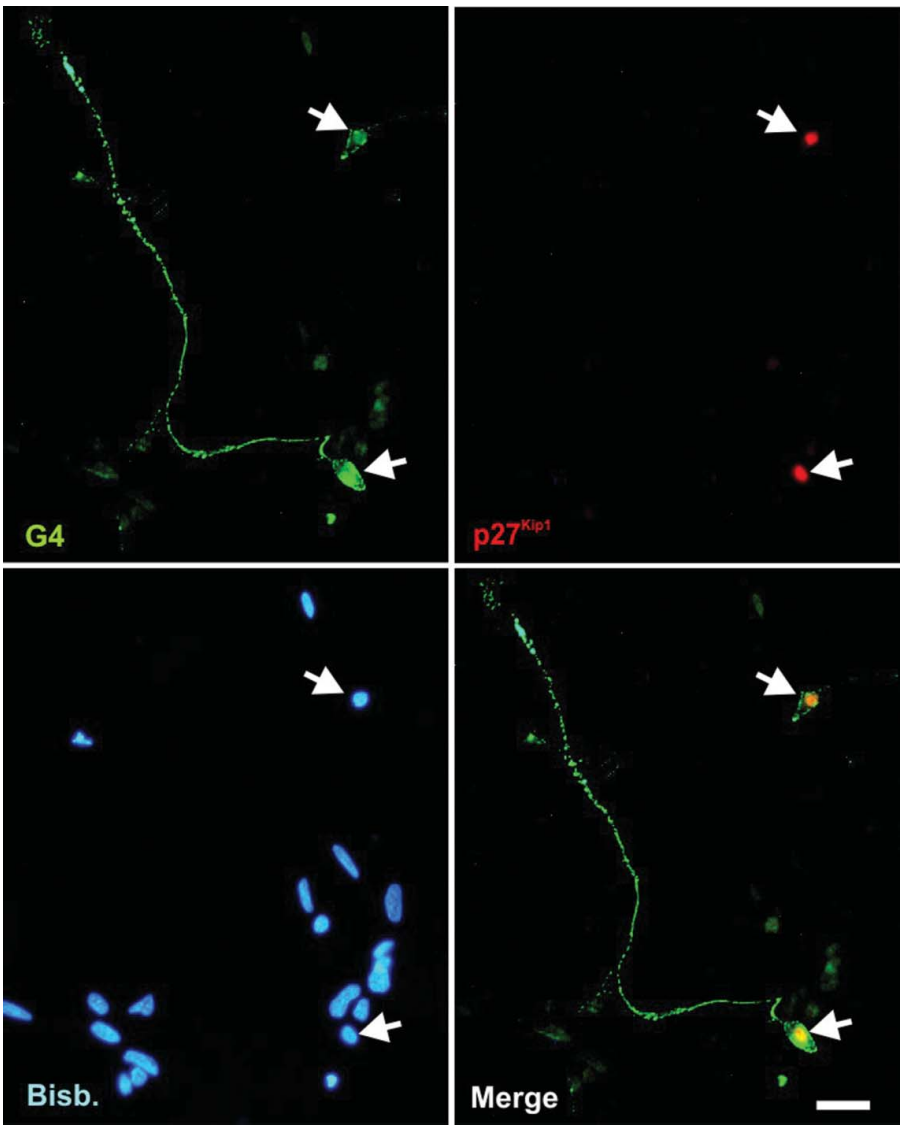

Figure 3. p27^{Kip1} is expressed by RGCs *in vitro*. E6 chick retinal cells were cultured under neurogenic conditions for 20 h, fixed and immunostained with antibodies against G4 (green) and p27^{Kip1} (red). Only differentiated RGCs neurons (G4+ cells) express p27^{Kip1}. Nuclear staining with bisbenzimide (Bisb.) is shown in blue. Bar: 20 μm.

No increase of ploidy in RGCs from p27^{Kip1} knock-out mice

A previous study has demonstrated that differentiated RGCs from p27^{Kip1} knockout mice are able to undergo DNA synthesis.[44] Moreover, p27^{Kip1} interacts with MCM7, a DNA replication licensing factor, to inhibit initiation of DNA replication.[55] These findings make p27^{Kip1} a candidate to block further rounds of endoreplication in mouse tetraploid RGCs. To directly test this hypothesis, the DNA content of Brn3a-positive cell nuclei isolated from the retina of wild-type and p27$^{Kip1-/-}$ mice at E20-P2 was analyzed by flow cytometry. At this developmental stage, RGCs have already been born,[56] and most of them express Brn3a.[57] This analysis confirmed previous results indicating that a proportion of mouse RGCs are tetraploid.[37] In contrast, no Brn3a-positive neurons were observed showing DNA contents higher than 4C in both wild-type and p27$^{Kip1-/-}$ retinas (**Fig. 7**). Similarly, the proportion of Brn3a-negative nuclei with a DNA content above 4C did not significantly differ in p27$^{Kip1-/-}$ vs. wild-type retinas [wild-type: 0.47 ± 0,12% (n = 3); p27$^{Kip1-/-}$: 0.23 ± 0.07% (n = 3)], thus indicating that the absence of p27^{Kip1} is not translated into hyperploidy in any retinal cell type.

Discussion

In this study we have demonstrated that p27^{Kip1} is expressed by layered, differentiating chick RGCs, including those previously shown to be tetraploid. In addition, we provide evidence

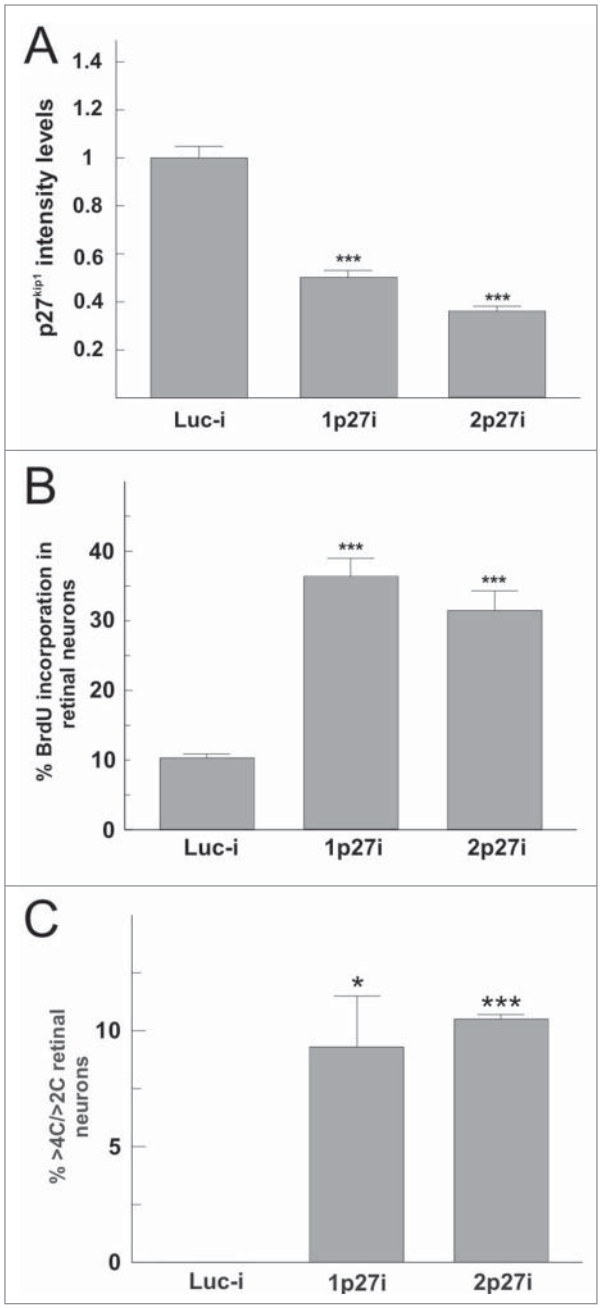

Figure 4. p27^{Kip1} downregulation increases BrdU incorporation and DNA amount in cultures enriched in RGCs. (**A**) E5 chick retinal cells were transfected with shRNA vectors designed against different regions of the *Cdkn1b* gene (1p27i and 2p27i) or a control shRNA vector against luciferase (Luc-i), and then cultured for 20 h under neurogenic conditions. Then, p27^{Kip1} expression levels were quantified by image analysis in differentiated chick retinal neurons transfected with the shRNA or control vectors. Both *Cdkn1b*-specific constructs were able to significantly decrease the expression levels of p27^{Kip1} as compared with the control construct. (**B**) RGC-enriched retinal cultures were obtained from E7 chick embryos as described by ref.[52]. These cells were then lipofected with Luc-i, 1p27i, or 2p27i vectors and then treated with 0.5 mg/ml BrdU for 20 h. The percentaje of neurons (NeuN+ cells) incorporating BrdU was significantly increased in the presence of p27^{Kip1}-specific shRNA compared to the control (RFPi). (**C**) The frequency of non-diploid neurons (>2C) with a DNA content higher than 4C (>4C) was significantly increased in the presence of p27^{Kip1}-specific shRNA compared to the control (RFPi). *$P < 0.01$; ***$P < 0.001$ (Student's *t* test, n = 3).

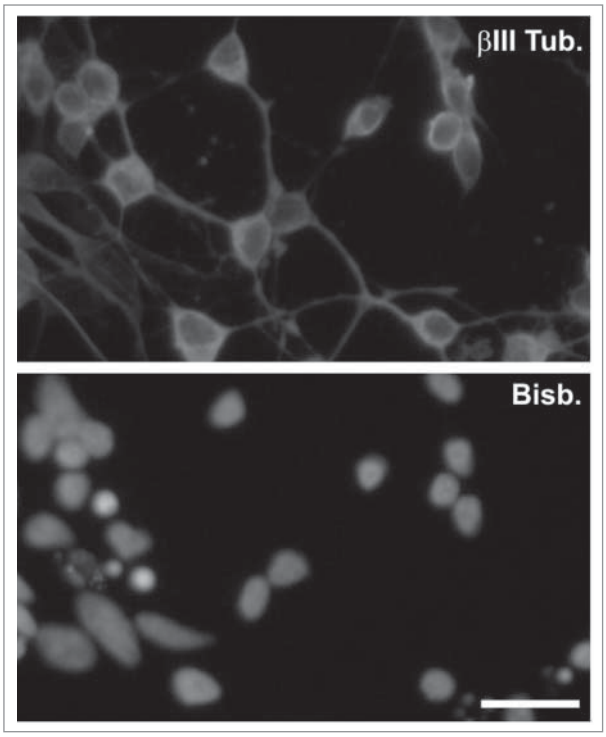

Figure 5. Enrichment of RGCs through a Percoll gradient. βIII tubulin staining (βIII Tub.) performed in a culture enriched in RGCs. Nuclei were stained with bisbenzimide (Bisb.). Bar: 20 μm.

that p27^{Kip1} downregulation induces DNA synthesis and hyperploidy in chick RGCs. We propose that p27^{Kip1} participates in the prevention of extra rounds of genome duplication in tetraploid avian RGCs. This mechanism may also keep diploid chick RGCs with normal DNA levels.

Some populations of vertebrate projection neurons replicate its DNA content and become tetraploid in the context of a normal developmental program.[37,38,42] This process results in neurons with increased size and extensive dendritic arbors.[37] In contrast, neurons with an elevated degree of ploidy have been observed both in invertebrates and in lower vertebrates.[33,34] Polyploidization in these latter neurons takes place through successive endoreplicative cycles that seem to be abolished in higher vertebrates, in which neurons are maintained with 4C DNA content. Our results suggest that p27^{Kip1}, a CKI known to prevent Cdk2 activity and block G1/S transition,[3,9,58] is likely involved in the mechanism that prevents tetraploid neurons to undergo successive rounds of endoreplication in the chick retina. This conclusion is consistent with a previous study showing that p27^{Kip1} can modulate endoreplication in *Solanum lycopersicum* L.[59]

We have demonstrated that p27^{Kip1} is expressed by differentiating chick RGCs in the E5 chick neural retina, an observation consistent with previous published work.[47] This conclusion is supported by the co-localization of p27^{Kip1} with both G4, a neuronal marker mostly expressed by layered, differentiating chick RGCs,[46] and p75^{NTR}, known to be an early marker of RGC differentiation in the chick retina.[37] In the neural chick retina, a subset of p27^{Kip1}-expressing neurons lack Rb, known to be

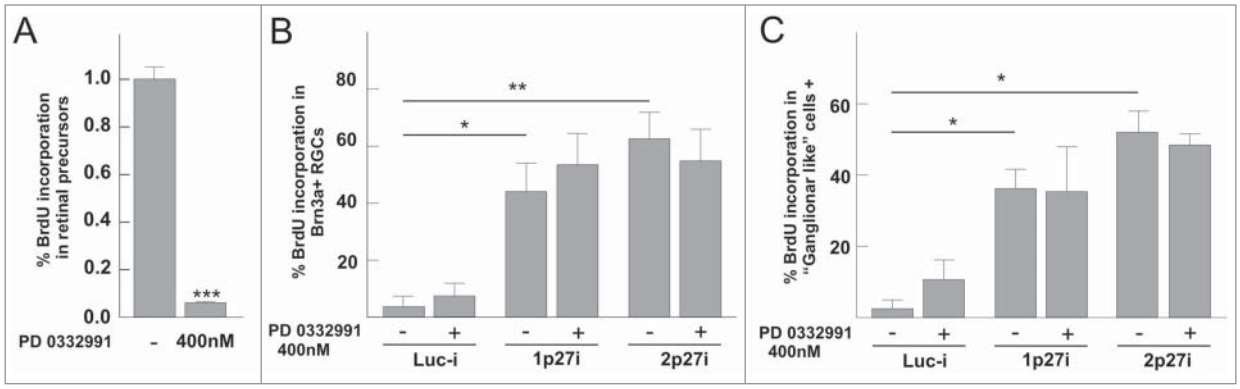

Figure 6. BrdU incorporation in RGCs, but not in retinal precursors, is independent of Cdk4/6 activity. (**A**) The selective Cdk4/6 inhibitor PD 0332991, used at 400 nM, prevents the capacity of E6 retinal precursor cells to incorporate BrdU after a short pulse (30 min). The analysis was performed after 20 h of inhibitor treatment. (**B**) RGC-enriched retinal cultures were lipofected with Luc-i, 1p27i, or 2p27i vectors and treated with 0.5 mg/ml BrdU for 20 h in the presence (+) or absence (−) of 400 nM PD 0332991. BrdU incorporation was evaluated in Brn3a-positive cells (a subpopulation of RGCs). (**C**) RGC-enriched retinal cultures treated as described above. BrdU incorporation was evaluated in cells that display RGC morphology (monoaxonic cells with round cytoplasm). $*P < 0.01$; $**P < 0.005$; $***P < 0.001$ (Student's *t* test, n = 3).

absent in tetraploid neurons,[37] and many of the $p27^{Kip1}$-positive neurons co-express TrkB, the BDNF receptor known to prevent ectopic mitosis in tetraploid chick RGCs.[41] Overall, these observations suggest that $p27^{Kip1}$ is expressed by all chick RGCs, including those that become tetraploid. Importantly, $p27^{Kip1}$ initiates its expression once that DNA duplication is already finished in migrating, tetraploid chick RGCs (see **Fig. 1**), as it would be expected for a blocker of extra rounds of endoreplication. In older stages of chick retinal development such as E12, $p27^{Kip1}$ is also expressed by horizontal and amacrine cells,[47] following a pattern coincident with the onset of differentiation of the different retinal neuronal types, from which RGCs are the first ones in coming out from the cell cycle.[60] This observation suggests that $p27^{Kip1}$ can maintain the tetraploid state of horizontal cells previously shown to remain with duplicated DNA levels in the chick retina.[61,62]

We have shown that $p27^{Kip1}$ downregulation causes an increase of BrdU incorporation in chick RGCs that does not lead to cell division, thus suggesting that $p27^{Kip1}$ prevents DNA synthesis and the subsequent increase of DNA amount in these neurons. This observation is consistent with the capacity of $p27^{Kip1}$ to inhibit a MCM7, necessary for the duplication license.[55] For this analysis, we used chick RGC-enriched cultures containing tetraploid neurons and maintained under conditions that mimic the *in vivo* situation.[37,39,41,48-50] Different criteria for neuron identification, including the expression of the neuronal marker NeuN in chick RGC-enriched cultures, the presence of the RGC marker Brn3a, or just by identifying RGCs in base to their morphology, yielded similar results, thus stressing the robustness of our analysis. Our results provide direct evidence that $p27^{Kip1}$ downregulation actually leads to an increase of DNA amount in at least 10% of the tetraploid chick RGCs. As this effect is not blocked by the Cdk4/6 selective inhibitor PD0332991, which prevents cell cycle progression in chick retinal precursors, we conclude that the canonic mechanism leading to S-phase in the chick retina is not involved in this process. This suggests that this new DNA synthesis upon $p27^{Kip1}$ downregulation could be due to a mechanism similar to endoreplication, where what is crucial is the activity of Cdk2.[63]

BrdU incorporation in chick RGCs triggered by $p27^{Kip1}$ downregulation is reminiscent of the phenotype observed in the retina of adult $p27^{Kip1}$ knock-out mice, where differentiated RGCs also undergo DNA synthesis.[44,64] Nevertheless, we could not find evidence for an increase of ploidy in mouse RGCs depleted of $p27^{Kip1}$. In mice, the effect of $p27^{Kip1}$ is synergic with that of $p19^{Ink4d}$ since the proportion of mouse RGCs that incorporate BrdU is enhanced in the retina of double $p27^{Kip1}$/$p19^{Ink4d}$ knockout mice.[44] In contrast, there is no evidence for

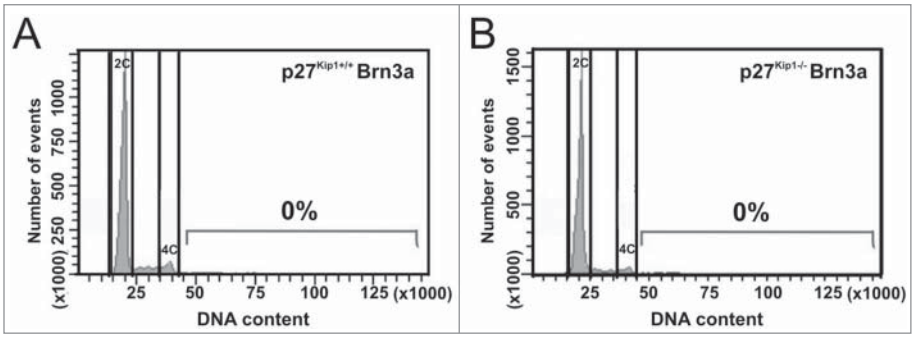

Figure 7. RGCs with a DNA content higher than 4C are not present in the $p27^{Kip1-/-}$ mouse retina. Cell nuclei isolated from the retina of either $p27^{Kip1+/+}$ (**A**) or $p27^{Kip1-/-}$ (**B**) P2 mice were immunostained for the RGC marker Brn3a, treated with PI (DNA content), and then analyzed by flow cytometry (n = 3 for each genotype). Representative DNA content histograms are shown for Brn3a+ nuclei. No net increase of RGCs with a DNA content above 4C can be seen in $p27^{Kip1-/-}$ retinas.

the existence of a $p19^{Ink4d}$ homolog in the chick genome, thus suggesting that this synergic mechanism does not take place in avian RGCs. Nevertheless, we cannot rule out that in the chick retina, a similar cooperative effect between $p27^{Kip1}$ and other members of the Ink family could actually take place. Indeed, homologs of mammalian $p15^{Ink4b}$, $p16^{Ink4a}$, and $p18^{Ink4c}$ have been identified in the chick (accession numbers: NP_989764, NP_989765, and XP_004936839, respectively). This may explain why only a relatively modest proportion of chick RGCs actually increases their DNA amount in response to $p27^{Kip1}$ downregulation. In contrast with the expression pattern of $p27^{Kip1}$ in the E5 chick retina, which is restricted to the differentiated RGCs (this study; ref.[47]), in the murine retina $p27^{Kip1}$ is also expressed by precursor cells during late G2/early G1.[65] The expression of $p27^{Kip1}$ in the mouse retinal precursors may explain both the increase of RGC axons in the optic nerve[66] and the retinal dysplasia[67-69] observed in $p27^{Kip1-/-}$ mice. Nevertheless, the increase of RGC number in the retina of $p27^{Kip1-/-}$ mice that would explain this phenotype is not dramatic,[66] and seems to be compensated by apoptosis.[44,65] In this context, interfering RNA against $p27^{Kip1}$ has been shown to induce cell death in cultured cortical neurons.[70] In contrast, no signs of apoptosis were evident in RGCs incorporating BrdU in our experiments, thus indicating that the mechanisms regulating endoreplication in the chick retina may differ from those in the mouse. This differential apoptotic response that allows the survival of avian RGCs with a DNA content above 4C may explain the presence of hyperploid RGCs in the chick but not in the mouse retina.

Our results suggest that $p27^{Kip1}$ is involved in blocking successive rounds of endoreplication in tetraploid chick neurons. Nevertheless, $p27^{Kip1}$ can also participate in the blockage of classical cell cycle progression[9] as well as neuronal differentiation and migration.[71] Therefore, $p27^{Kip1}$ is probably playing multiple roles in RGCs, including the blockade of tetraploid neurons to undergo extra rounds of endoreplication and the inhibition of cell cycle reactivation in diploid neurons that have not been programmed to become tetraploid.

Materials and Methods

Chick embryos

Fertilized eggs from White Leghorn hens (*Gallus gallus* L.) were obtained from a local supplier (Granja Santa Isabel, Spain) and incubated at 38.5°C in an atmosphere of 70% humidity. The embryos were staged as described previously.[72] All experiments were performed in accordance with the European Union and Spanish guidelines.

Mice

Mice of the $p27^{Kip1}$ mutant strain[67] were crossed and maintained at the University of Valencia animal core facility in accordance with Spanish regulations. Heterozygous mice were mated to produce all 3 genotypes. Embryo littermates were staged as described by ref.[73]. All experiments were performed in accordance with the European Union and Spanish guidelines.

Antibodies

The mouse monoclonal antibody (mAb) against neuron-specific βIII tubulin (clone 5G8; Millipore), which specifically recognizes RGCs from early stages of differentiation,[53] was diluted 1/2,000 for immunohistochemistry and 1/1,000 for immunocytochemistry. The rabbit polyclonal antibody (pAb) against G4, a chicken glycoprotein mostly expressed by differentiated RGCs,[46] was kindly provided by E.J. de la Rosa (Centro de Investigaciones Biológicas, CSIC). This antibody was used at 1/1,000 for immunohistochemistry and 1/500 for immunocytochemistry. The mouse mAb against Brn3a MAB1585 (Millipore), which specifically recognizes a subpopulation of RGCs in both chick[54] and mouse[57] retinas, was diluted 1/500 for immunohistochemistry and 1/300 for flow cytometry. The rabbit poylclonal antibody against NeuN ABN78 (Millipore) was used at 1/1,000 dilution for immunohistochemistry. The rabbit polyclonal antiserum [9650] against the extracellular domain of human $p75^{NTR}$ was a generous gift of Moses V. Chao (New York University), and it was diluted 1/1,000 for immunohistochemistry. The rabbit polyclonal antiserum against the extracellular domain of chick TrkB was a kind gift from Louis F. Reichardt (University of California, San Francisco), and it was used at 1/2,000 dilution for immunohistochemistry. The mouse monoclonal antiserum against BrdU (Developmental Studies Hybridoma Bank) was used at 1/4,000 dilution for immunohistochemistry. The rat monoclonal antibody against BrdU (AbD Serotec, MCA2060) was used at 1/200 dilution for immunocytochemistry. The anti-Rb pAb Ab39690 (Abcam) was used at 1/50 dilution for immunohistochemistry. The mouse monoclonal antibody against $p27^{Kip1}$ (BD Transduction Laboratories) was used at 1/500 for immunohistochemistry. The anti-phosphoHistone H3 rabbit polyclonal antiserum (Upstate Biotechnology) is a well characterized marker of mitosis and it was diluted 1/400 for immunohistochemistry. The mAb anti-RA4, a generous gift from Stephen C. McLoon (University of Minnesota), recognizes an epitope expressed by RGCs soon after their final mitosis,[74,75] and it was diluted 1/400 for immunohistochemistry. All secondary antibodies were purchased from Invitrogen. The Alexa Fluor 594 goat anti-rabbit antibody was used at a 1/1,000 dilution for immunohistochemistry. The Alexa Fluor 647 donkey anti-mouse and the Alexa Fluor 488 goat anti-rat antibodies were used at 1/1,000 for immunohistochemistry. The Alexa Fluor 488 goat anti-mouse antibody was used at a 1/1,000 dilution for immunocytochemistry and 1/500 for cytometric studies

Cell culture

Retinal cultures were performed under neurogenic conditions as described previously.[76] To this aim, neuronal precursors isolated from the E6 chick retina were cultured in the presence of laminin-1 and insulin to give rise to DCRNs.[48,49] Briefly, E6 retinas were dissected out of the pigmented epithelium and treated for 5 min at 37°C with 0.5 mg/ml trypsin prepared in PBS containing 3 mg/ml bovine serum albumin (BSA). The reaction was stopped with 1 mg/ml soybean trypsin inhibitor (Sigma-Aldrich) and cells were mechanically disaggregated by passing them 5 times through a Pasteur pipette whose tip has previously been

polished with a flame. Dissociated retinal cells were suspended in 50% Dulbecco's-modified Eagle's Medium (DMEM)/50% F12 (Sigma-Aldrich) plus N2 supplement (DMEM/F12/N2), and plated at a density of 10,000-200,000 cells/cm^2 on 10-mm round glass coverslips previously coated with 500 μg/ml poly (D-L) ornithine (PLO; Sigma-Aldrich) and 10 μg/ml laminin-1 (natural mouse laminin; Invitrogen). Cultures were maintained overnight (ON) in 4-well dishes (Greiner Bio-One, Germany). Cells were cultured in DMEM/F12/N2 for 20 h at 37°C in a water-saturated atmosphere containing 5% CO_2. BrdU (Roche) was used at 0.5 μg/ml. In some instances, cells were cultured for 20 h in the presence or absence of the Cdk4/6 inhibitor PD0339921 (Axon MedChem), used at 400 nM.

RGCs enrichment

RGCs enrichment was carried out following the protocol described by ref.[52]. For each experiment, 30 retinas from E7 chick embryos were dissected away from the pigment epithelium, and then treated for 5 min at 37°C with 0.5 mg/ml trypsin prepared in PBS containing 3 mg/ml BSA. The reaction was stopped with 1 mg/ml soybean trypsin inhibitor and then, cells were mechanically disaggregated as described above. Cell suspensions were diluted using DMEM/F12/N2 to reach 19.8 ml and distributed in 3 6.60 ml aliquots. Each aliquot was loaded as described below on a discontinuous Percoll gradient prepared in a 15 ml falcon tube. The bottom layer (d = 1.09) was obtained by mixing 1.12 ml DMEM/F12/N2 with 1.70 ml Percoll (Sigma-Aldrich); the second layer (d = 1.06) is formed by the mix of 1.80 ml of the cell suspension with 1.44 ml Percoll; the third layer (d = 1.04) was obtained by mixing 2.40 ml of the cell suspension with 0.52 ml Percoll; the forth layer (d = 1.02) was generated by mixing 2.40 ml of the cell suspension with 0.28 ml Percoll, and the top layer was composed by 2.70 ml DMEM/F12/N2. Then, discontinuous Percoll gradients were centrifuged at 351 × g for 30 min at 4°C. As a result of the centrifugation, 2 bands were generated. The top layer, enriched with RGCs, was isolated and washed with DMEM/F12/N2. After centrifuging the cells at 300 × g for 1 min, pellets were resuspended in DMEM/F12/N2 and plated on 10-mm round coverslips previously coated with PLO and laminin-1 (20,000–200,000 cells/cm^2). Cultures, performed in 4-well dishes, were maintained in DMEM/F12/N2 for 20–22 h at 37°C in a water-saturated atmosphere containing 5% CO_2.

shRNA

Two shRNA constructs targeting different regions of the gene *Cdkn1b* (accession number: NM_204256.2) were generated, based on the protocol described by ref.[51]. The 1p27i construct (1p27i) was generated by targeting the sequence found between 93 and 112 base pairs (bp) (GCCATGGAGGATTACACGAA). The 2p27i construct (2p27i) was generated by targeting the sequence found between 1043 and 1062 bp (GAGGAGGTTTCAGAAGACT). Once generated, these constructs were cloned into the pRFPRNAiC vector.[51] pRFPRNAi luciferase (Luc-i)[51] was used as negative control. All these plasmids co-express red fluorescent protein (RFP).

Lipofection

RGC-enriched cultures were lipofected with 3 μg of the desired plasmid using Lipofectamin 2000 (Invitrogen) following the manufacturer's instructions. Cultures were immediately treated with 0.5 μg/ml BrdU. In some cases, cultures were also treated with the Cdk4/6 inhibitor PD0339921, used at 400 nM, or vehicle. After 4–6 h, the culture medium was exchanged with fresh DMEM/F12/N2 containing 0.5 μg/ml BrdU and cells were cultured for 24 h at 37°C. 400 nM PD0339921 (or vehicle) was included in the replaced culture medium when necessary.

Explant electroporation

Electroporation of retinal explants was performed as described previously.[37,41] E6 chick retinas were dissected away from the pigment epithelium and fragmented into small pieces of around 10 mm^2. These retinal fragments were laid onto a glass coverslips and subsequently immersed in 4 μl PBS containing different plasmid combinations (0,75−1 μg/μl). Electroporation was performed with four 50 milliseconds pulses of 25–27 V, at a 200 milliseconds frequency. After electroporation, the explants were grown in suspension for 3–4 h in 50% DMEM/F12/N2. Explants were then collected, dissociated, and cultured as previously described.

In vivo BrdU treatment

E5-6 embryos were treated for 15 min with 40 μl of a solution containing 10 μg/μl BrdU, which was applied to the chorioallantoic membrane.

Immunocytochemistry

Immunocytochemistry was performed in cultures fixed with 4% paraformaldehyde for 15 min at room temperature (RT), and permeabilized for 30 min with phosphate buffered saline (PBS) containing 0.05% Triton X-100 (Sigma-Aldrich) (PBTx) and 10% Fetal Calf Serum (FCS; Invitrogen). Cultures were then incubated for 2 h (at RT) or ON (at 4°C) with PBTx containing 1% FCS and the appropriate primary antibodies. After five washes in PBTx, cultures were incubated for 1 h in PBTx containing 1% FCS and a 1/1,000 dilution of Alexa Fluor 488 goat anti-mouse antibody, Alexa Fluor 594 goat anti-mouse antibody, Alexa Fluor 647 donkey anti-mouse antibody, Alexa Fluor 647 goat anti-rabbit antibody, or Alexa Fluor 488 goat anti-rat antibody. All these secondary antibodies were purchased from Life Technologies.

For BrdU immunolabeling, cultures were previously subjected to DNA denaturation by incubation for 30 min at RT with 2N HCl/0.33× PBS, following by a neutralization step consisting of 3 15-min washes with 0.1 M sodium borate, pH 8.9, and 2 washes of 5 min each with PBS/0.05% Triton X-100. G4 immunostaining was performed in living cells as described previously.[50] To this aim, cultures were washed 4 times with Krebs-Ringer-Hepes buffer (KRH), and incubated for 20 min at RT with a 1/400 dilution of the anti-G4 antibody prepared in KRH. Cells were then washed 4 times with KRH and incubated for 20 min at RT with a 1/1,000 dilution of the Cy2-conjugated Affinipure Goat Anti-Rabbit IgG (H^+L) prepared in KRH. After 4 washes with KRH, cells were fixed with 4% PFA for 15 min. At this

stage, cells can be double immunolabeled using the standard protocol described above. Nuclear labeling was performed with PBS containing either 1 μg/ml bisbenzimide (Sigma-Aldrich) or 100 ng/mL DAPI (Sigma-Aldrich), and the preparations were then mounted in glycerol (Panreac)/PBS (1:1).

Immunohistochemistry

For immunohistochemistry, E5-6 chick embryos were fixed for 6–8 h at 4°C with 4% paraformaldehyde (Merck), cryopreserved ON at 4°C in PBS containing 30% sucrose (Merck), embedded in the OCT compound Tissue-Tek (Sakura), and cryosectioned (Cryostat 1950; Leica). Cryosections (12 μm) were permeabilized and blocked for 30 minutes at RT in 0.5% PBTx and 10% FCS, and they were then incubated ON at 4°C with the primary antibodies in PBTx plus 1% FCS. After 5 washes with PBTx, the sections were incubated for 1 h at RT with Alexa 594-coupled anti-mouse IgG (Invitrogen) and Cy2-conjugated Affinipure Goat Anti-Rabbit IgG (H^+L), each diluted 1/1,000. The sections were finally washed 5 times in PBTx, once in PBS, and they were then incubated with 1 μg/ml bisbenzimide (Sigma-Aldrich) in PBS before mounting in glycerol/PBS (1:1). Negative controls performed using only secondary antibodies resulted in lack of specific immunostaining (data not shown). For BrdU immunostaining, cryosections were incubated with the mouse anti-BrdU antibody as previously described.[38] Briefly, cryosections were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature, rinsed twice with PBS, and incubated for 1 h at 37 °C in 100 mM Tris-HCl buffer (pH 8.5) containing 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 10 μg/ml DNAse I (Roche). Then, cryosections were rinsed twice with PBS and subjected to immunohistochemistry as described above.

DNA quantification

DNA quantification was performed by measuring DAPI levels in lipofected cells, after being immunostained as described above. DAPI labeling was recorded with a Nikon E80i microscope equipped with a DXM 1200 digital camera (Nikon, Melville, NY), using a 40× magnification objective. The integral density of DAPI, obtained as arbitrary units, was quantified using the freely available ImageJ software (National Institute of Health, Bethesda, Maryland). Intensity values were normalized to control conditions using the mean value of the integral density of DAPI in the RNAi control. Cells with DNA amounts around the modal value were considered as diploid. The rest of the cells were classified as follows: cells with DNA amount above 1.5-fold the diploid level (>2C cells), cells showing 1.8-/2.4-fold the diploid level (4C cells), and cells containing more than 2.5-fold the diploid level (>4C cells). At least 88 cells were analyzed per treatment.

Flow cytometry

Flow cytometry of unfixed frozen tissue was performed as described previously.[42,77] E20-P2 mouse retinas, dissected out of the pigmented epithelium, were homogeneized with a Dounce tissue grinder in DNase-free PBS containing 0.1% Triton X-100 and 1× protease inhibitor cocktail (Roche) (2 retinas/600 μl, 3 strokes with the loose pestle and 3 strokes with the tight pestle). After addition of 5% FCS and 1.25 mg/ml BSA (final concentrations), nuclei were immunostained with anti-Brn3a (1/300) and Goat anti mouse alexa 488 secondary antibody (1/500), added both together. In the control sample (generated from small aliquots of each sample), the primary antibody was excluded. Finally, nuclei were incubated ON at 4°C in the dark, and then sieved through a 40 μm nylon filter to remove nuclear aggregates. Finally, propidium iodide (PI) (Sigma-Aldrich) and DNase-free RNase I (Sigma-Aldrich) were added at a final concentration of 25 μg/ml and 25 μg/ml, respectively, and incubated for 30 min at RT. In some instances, nuclei were sieved through a 100 μm nylon filter, with similar results. The quality of the nuclei and specificity of immunostaining signal was checked by fluorescence microscopy after labeling with 100 ng/ml DAPI. Flow cytometry was performed with a FACSAria cytometer (BD Biosciences) equipped with a double argon (488 nm) and helium-neon laser (633 nm). Data were collected by using a linear digital signal process. The emission filters used were BP 530/30 for Alexa 488 and BP 616/23 for PtdIns. Data were analyzed with FACSDiva (BD Biosciences) software.

Image analysis

$p27^{Kip1}$ levels were quantified using the equatorial confocal plane from cells transfected with either 1p27i, 2p27i, or Luc-i vectors. For each experimental point, $p27^{Kip1}$ levels were quantified in at least 200 electroporated cells.

Statistical analyses

Quantitative data are represented as the mean ± s.e.m. Statistical differences were analyzed using either Student's *t*-test or ANOVA.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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