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A morphogenetic wave of *p27^{Kip1}* transcription directs cell cycle exit during organ of Corti development

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The molecular mechanisms coordinating cell cycle exit with cell differentiation and organogenesis are a crucial, yet poorly understood, aspect of normal development. The mammalian cyclin-dependent kinase inhibitor p27^{Kip1} is required for the correct timing of cell cycle exit in developing tissues, and thus plays a crucial role in this process. Although studies of p27^{Kip1} regulation have revealed important posttranscriptional mechanisms regulating p27^{Kip1} abundance, little is known about how developmental patterns of p27^{Kip1} expression, and thus cell cycle exit, are achieved. Here, we show that during inner ear development transcriptional regulation of *p27^{Kip1}* is the primary determinant of a wave of cell cycle exit that dictates the number of postmitotic progenitors destined to give rise to the hair cells and supporting cells of the organ of Corti. Interestingly, transcriptional induction from the *p27^{Kip1}* gene occurs normally in *p27^{Kip1}*-null mice, indicating that developmental regulation of *p27^{Kip1}* transcription is independent of the timing of cell cycle exit. In addition, cell-type-specific patterns of *p27^{Kip1}* transcriptional regulation are observed in the mature organ of Corti and retina, suggesting that this mechanism is important in differential regulation of the postmitotic state. This report establishes a link between the spatial and temporal pattern of *p27^{Kip1}* transcription and the control of cell number during sensory organ morphogenesis.

KEY WORDS: p27Kip1, Cell cycle, Development, Morphogenesis, Inner ear, Cochlea, Organ of Corti, Mouse

INTRODUCTION

Cell proliferation and differentiation are coordinated during the normal development of tissues and organs, although our knowledge of the molecular mechanisms underlying this coordination remains incomplete (Cremisi et al., 2003; Pagano and Jackson, 2004; Zhu and Skoultchi, 2001). During development, cell cycle exit is regulated by a family of cyclin-dependent kinases (CDKs), which, together with their positively acting co-factors, the cyclins, mediate the progression through the cell cycle (Sherr and Roberts, 1999). Regulation of the activity of the cyclin/CDK complexes occurs at several levels, including transcriptional and posttranscriptional regulation of cyclin genes, posttranslational modifications of the CDK molecule itself, and regulation by two families of CDK inhibitor proteins (CKIs): the Ink4 family, which includes p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c} and p19^{Ink4d}; and the Cip/Kip family which includes p21^{Cip1}, p27^{Kip1} and p57^{Kip2}. In particular, the developmental role of p27^{Kip1} has received some attention since it was shown that the loss of p27^{Kip1} leads to overall growth defects, multiorgan hyperplasia and other tissue-specific defects caused by a lack of timely cell cycle exit (Elledge et al., 1996; Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). Examples include developmental abnormalities in the inner ear (Chen and Segil, 1999; Lowenheim et al., 1999), retina (Dyer and Cepko, 2001a; Dyer and Cepko, 2001b; Levine et al., 2000; Nakayama et al., 1996), hematopoetic system (Ezoe et al., 2004), ovary (Tong et al., 1998) and nervous system (Casaccia-Bonnefil et al., 1999; Cunningham and Roussel, 2001; Doetsch et al., 2002; Durand et al., 1998; Zindy et al., 1999). The appearance of tissue-specific defects suggests that the timing of p27^{Kip1} expression during development is critical, not only for the generation of the correct relative numbers of constituent cell types in any given tissue, but also for the proper timing of cell cycle exit relative to other developmental events such as differentiation.

Development of the organ of Corti, the auditory sensory epithelium, provides an example of the need for the precise control of cell number during development, and the importance of p27^{Kip1} in coordinating cell cycle exit with differentiation and patterning. In *p27^{Kip1}*-null animals, the timing of cell cycle exit of auditory sensory progenitors is delayed, and supernumerary hair cells and supporting cells are produced (Chen and Segil, 1999; Lowenheim et al., 1999). Although hair cells and supporting cells in the *p27^{Kip1}*-null organ of Corti appear normal, and also appear to connect normally to neurons of the spiral ganglion, the animals are deaf, as assayed by a number of physiological parameters, including auditory brainstem responses and otoacoustic emissions (Chen and Segil, 1999; Lowenheim et al., 1999). While not ruling out the possibility that defects in other elements of the auditory system are responsible for the deafness of these mice, one possible explanation is that the delicate micromechanics of the organ of Corti (Nilsen and Russell, 2000) are disrupted by the presence of the supernumerary cells present in the mutant, and that the precise control of cell number by p27^{Kip1} during the formation of the organ of Corti is crucial to normal cochlear function.

Little is known about the mechanism by which the complex patterns of p27^{Kip1} abundance are regulated during embryonic development in vertebrates. Studies of p27^{Kip1} regulation have been conducted primarily in cell culture and have emphasized the importance of posttranscriptional (Agrawal et al., 1996; Hengst and Reed, 1996; Millard et al., 1997) and posttranslational (Muller et al., 1997; Nakayama et al., 2001; Pagano et al., 1995; Sheaff et al., 1997; Tam et al., 1997; Vlach et al., 1997) mechanisms for cell cycle

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regulation. Nonetheless, a growing number of reports, particularly in cancer cells, indicate a role for transcriptional regulation in response to specific physiological conditions and in a variety of isolated cells types (Servant et al., 2000; Chandramohan et al., 2004; Gizard et al., 2005; Inoue et al., 1999; Laub et al., 2005; Li et al., 2002; Medema et al., 2000; Murata et al., 2005; Stahl et al., 2002; Wang et al., 2005; Williamson et al., 2002). In addition, expression of the *Drosophila* *p27^{Kip1}* homolog Dacapo (DAP) (de Nooij et al., 1996; Lane et al., 1996) has been shown to be regulated transcriptionally by a complex array of tissue-specific and developmentally regulated enhancer/promoter elements (Liu et al., 2002; Meyer et al., 2002).

Thus, in spite of the emphasis on posttranscriptional mechanisms of p27^{Kip1} regulation during the cell cycle, the long-term transcriptional regulation of *p27^{Kip1}* during development may play a role in the tissue-specific pattern of cell cycle exit in mammals. Here, we report that a developmentally regulated wave of p27^{Kip1}-protein expression controls the spatial and temporal pattern of cell cycle exit in the embryonic cochlear duct. With the aid of a BAC transgenic reporter in which GFP is expressed under the control of the *p27^{Kip1}* locus, we show that this wave of p27^{Kip1} expression is regulated at the transcriptional level, and that this pattern of transcriptional regulation of *p27^{Kip1}* expression is independent of the cell cycle events in this tissue. In addition, tissue- and cell-type-specific patterns of p27^{Kip1} expression at later stages in the development of the inner ear and the retina suggest that the transcriptional control of p27^{Kip1} may be important for the ongoing control of the postmitotic state of a number of cell populations. Thus, the precise spatial and temporal control of *p27^{Kip1}* transcription is crucial for the normal development and homeostasis of the organ of Corti, and probably many other developing organ systems.

MATERIALS AND METHODS

Animals, cell birth dating and quantification of BrdU incorporation

Animal care was in accordance with institutional (AALARAC) guidelines and was approved by the Animal Care and Use Committee of the House Ear Institute. Animals for timed matings were put together in the evening and the next morning litters of those females that were discovered to have plugs were designated E0.5.

To analyze cell cycle events in the developing inner ear, timed pregnant female mice were injected with BrdU to label mitotic cells in the embryos. BrdU (5 mg/ml) was prepared in 7 mM NaOH buffer (pH 7.0) and injected intraperitoneally into pregnant mice at 50 μg/g of body weight. Two different injection regimens were used.

(1) To quantify cell cycle exit of hair cells, timed pregnant females (CD-1) (E12.5-E17.5) were injected three times (at 10.00, 12.00 and 14.00 h) on specific embryonic days and the embryos were allowed to survive until birth, at which time newborns were decapitated, and organ of Corti dissected, prepared as whole-mount surface preparations, and double-labeled with anti-BrdU and anti-Myosin VIIA to identify hair cells whose progenitors were still dividing at the time of BrdU injection. Double-labeled surface preparations were photographed along their entire length and computer reconstructed using Adobe PhotoShop. For quantification of BrdU$^+$ hair cells, the photographed organ of Corti was divided into seven segments of equal length, and the number of BrdU$^+$ hair cells, and the total number of Myosin VIIa$^+$ hair cells were counted for each segment. The percentage of BrdU-positive hair cells versus total hair cells was used to determine the percentage of hair cell progenitors still actively in the cell cycle at the time of BrdU injection (Fig. 2B).

(2) To analyze the ongoing pattern of cell division in embryos, timed pregnant females were injected once at 11:00 h and sacrificed two hours later (as in Fig. 2A,E,F and Fig. 4). Embryos were fixed by immersion in 4% buffered paraformaldehyde for 1-2 hours and the inner ears were then dissected either for whole mounts or for cryostat sectioning and immunohistochemistry as previously described (Chen and Segil, 1999).

Immunohistochemistry

Embryos or dissected bulla were submerged overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) at 4°C. Cryosections were prepared from whole embryos using standard procedures, as before (Chen and Segil, 1999). For whole-mount immunohistochemistry, cochleae were dissected in PBS, the lateral wall of the cochlea and tectorial membrane were first dissected free and the organ of Corti was then removed. For immunostaining, organ of Corti whole-mount preparations and cryosections were permeabilized in 0.2% Triton X-100/PBS for 20 minutes, blocked with 4% donkey serum/PBS for 30 minutes, then incubated with primary antibody (diluted in blocking solution) at 4°C overnight. Secondary antibodies conjugated with fluorescent dyes were used to visualize the labeled cells. The primary antibodies used were anti-BrdU antibody (Chemicon, mouse monoclonal, dilution 1:100), anti-p27^{Kip1} antibody (NeoMarkers, mouse monoclonal, dilution 1:100), anti-Myosin VIIa antibody (rabbit polyclonal, courtesy of Christine Petit and Aziz El-Amraoui, Pasteur Institute, dilution 1:1000) and anti-Glutamine Synthetase (rabbit polyclonal, Transduction Laboratories, dilution 1:100). Secondary antibodies were either FITC- or Rhodamine-conjugated (Jackson ImmunoResearch, dilution 1:200). For BrdU staining, the tissues were pre-treated in 2 N HCl for 1 hour at 37°C, followed by neutralization with 0.1 M boric acid (pH 8.5) for 30 minutes at room temperature. For anti-p27^{Kip1} staining, antigen retrieval was accomplished by boiling in 10 mM citric acid buffer for 10 minutes before permeabilization (Chen and Segil, 2002). Because the sodium citrate treatment attenuates EGFP reporter signal significantly, micrographs of native EGFP were captured prior to treatment. Confocal imaging of whole-mount cochlea was carried out under the *z*-sectioning mode at 10-μm intervals followed by 3D projection using a Zeiss LSM410 microscope.

p27^{Kip1}/BAC transgenic mice

Bacterial Artificial Chromosome (BAC) #485G10 containing the *p27^{Kip1}* locus was identified by screening a mouse BAC library (High density CITB Mouse BAC Colony DNA membranes, Catalog number 96050; Research Genetics, AL). Nucleotide (nt) numbering is based on NCBI contig number NT_039359. BAC end sequencing indicated that clone #485G10 contained a 129-kb insert from nt 4627867 to nt 4756781. This p27^{Kip1} locus is flanked by ~51 kb 5′ and ~78 kb 3′ relative to the start site of *p27^{Kip1}* transcription (Coleman et al., 2001).

Bacterial homologous recombination was used to modify the BAC (Yang et al., 1997; Yang et al., 1999). An IRES/EGFP reporter cassette (Yang et al., 1999) was targeted to the 3′non-coding region of the *p27^{Kip1}* gene using two homology arms of ~500 bp in length. The left homology arm corresponds to nt 4680198-4680727, 5′-GCAATTAATTAAACCCCCTATCCGACTG-CTTGCCCTG-3′ and 5′GCAAGGATCCGTCTGGCGTCGAAGGCC-GGGCTTC-3′; and the right homology arm corresponds to nt 4680728-4681233, 5′-GCAATTAATTAAACCCCCTATCCGACTGCTTGCCCTG-3′ and 5′-GCAATCTAGAGGCAGCTCTATTCACACTCCTAATC-3′. The first exon of the *p27^{Kip1}* gene was replaced in a second round of bacterial homologous recombination inserting the bacterial *neo* gene between nt 4679590 and 4680024. The *neo* gene coding sequence was not placed in frame to the translation start site of p27^{Kip1} gene. Modifications were subsequently verified by the Southern blot and sequence analysis (data not shown). BAC DNA was prepared as described (Yang et al., 1999), and transgenic mice were established using standard techniques by the Transgenic Mouse Core Facility (University of Southern California, Los Angeles, CA). The transgenic founders were generated in the genetic background B6D2F1 and mated to CD-1 mice to generate mice used for this study. Genotyping was performed using polymerase chain reaction (PCR) with PCR primer pairs: 5′-CGAAGGCTACGTCCAGGCGCGCA-CCAT-3′ and 5′-GCACGGGGCCGTCGCCGATGGGGGTGTTCTGC-3′, producing a 314 bp EGFP band; and 5′-ATGATTGAACAAGCTGGATT-3′ and 5′-TCAGAAGAACTCGTCAAGAAGGCG-3′, producing a 794 bp *neo* band.

RESULTS

The mouse inner ear develops from the otic placode between embryonic day 8.0 (E8) and E8.5 (Kelley and Bianchi, 2001; Kiernan et al., 2002). Starting on E9 the placodal epithelium

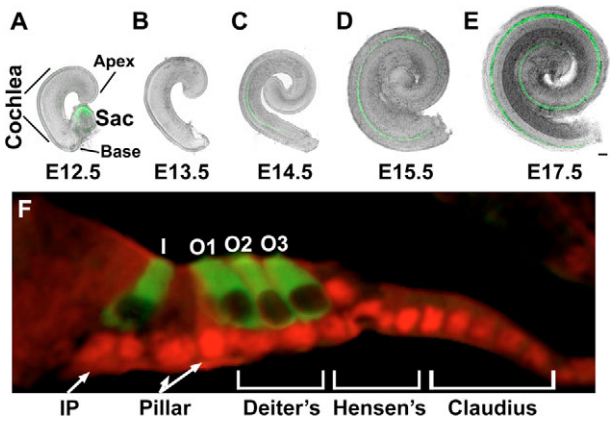

Fig. 1. Development and cellular anatomy of the mouse organ of Corti. (**A-E**) Cochlear epithelia from E12.5-E17.5 from mice carrying a transgenic Math1-GFP reporter (Chen et al., 2002; Lumpkin et al., 2003). Epithelia were dissected and photographed under brightfield conditions and fluorescence images of Math1/GFP expression (green) from the same preparations were superimposed to illustrate the basal to apical pattern of hair cell differentiation that initiates in the mid-basal regions of the cochlea at E14.5 (Chen et al., 2002) and spreads apically until ~E17.5 when the wave of differentiation is complete. Note that at E12.5 and E13.5 Math1/GFP expression is limited to the saccule where Math1-dependent hair cell differentiation begins around E10.5 (Bermingham et al., 1999). (**F**) Cross-section through a P1 organ of Corti illustrating the position of hair cells (green, stained with antibody to Myosin VIIa) and supporting cells (red, stained with antibody to $p27^{Kip1}$). The different varieties of hair cells and supporting cells are labeled: I, inner hair cell; O1-O3, outer hair cell-row 1-3; IP, inner phalangeal.

begins to invaginate to form the otic pit and by E9.5 an otic vesicle has formed. Starting around E10.5, the ventral portion of the otocyst begins to elongate and by E11.5 this elongating portion forms a rudimentary cochlear duct. In Fig. 1, a collection of dissected embryonic cochlear epithelial whole mounts illustrates the growth of the cochlear duct between E12.5 and E17.5. Coiling of the cochlear duct begins around E12, and the duct continues to grow and coil until it has reached a full 1.5 turns by around E18. Cellular differentiation within the cochlear duct requires the upregulation of the atonal homolog Atoh1 (Math1) (Bermingham et al., 1999). The induction of Math1 in hair cells occurs between E13.5 and E14.5 in the midbasal region of the cochlear duct (Chen et al., 2002; Lanford et al., 2000), which is the site where morphological evidence of hair cell differentiation first appears (Lim and Anniko, 1985; Sher, 1971). The wave of hair cell differentiation that ensues is illustrated in the cochlear preparations shown in Fig. 1, which are taken from a transgenic mouse in which a GFP reporter is expressed under the control of the Math1 enhancer, as previously described (Chen et al., 2002; Lumpkin et al., 2003). Prior to E14.5, the Math1 transgene is not visible in the cochlea (Fig. 1A,B), but is expressed in the vestibular system (part of the sacculus is included in the dissection in Fig. 1A), where hair cell differentiation begins several days earlier (Lim and Anniko, 1985; Sher, 1971). Subsequent to E14.5, Math1 expression is upregulated in a wave of differentiation that requires several days to spread from its origin in the cochlear base at E14.5 (Fig. 1C) to the apex of the cochlea on ~E17.5 (Fig. 1E). Math1 expression in differentiating hair cells slightly precedes the expression of other specific markers of more mature hair cells, including Myosin VIIa (Chen and Segil, 1999). The normal arrangement of one row of inner hair cells and three rows of outer hair cells in the differentiated organ of Corti is illustrated in a cross-section through the organ of Corti at P1, stained with the hair cell marker Myosin VIIa (Hasson et al., 1997; Sahly et al., 1997) (Fig. 1F). Each hair cell is separated by different supporting cells, which are stained with an antibody to $p27^{Kip1}$, whose expression is downregulated in hair cells during differentiation, but persists at high levels in differentiated supporting cells (Chen and Segil, 1999; Lowenheim et al., 1999) (Fig. 1F).

The onset of $p27^{Kip1}$ protein expression occurs in an apical to basal wave that precedes the wave of cell cycle exit

Previous studies have shown that by E14.5, the time when hair cell and supporting cell differentiation is beginning in the base of the cochlear duct, the cells that will form the entire organ of Corti have largely exited the cell cycle (Ruben, 1967). These cells form a zone of non-proliferating cells (ZNPC) whose borders represent the borders of the nascent organ of Corti (Chen et al., 2002; Chen and Segil, 1999). Based on analysis of [^{3}H]-thymidine labeling, Ruben (Ruben, 1967) suggested that cell cycle exit occurred in an apical to basal pattern starting around E12.5 and ending around E14.5. BrdU injections at E12.5, E13.5 and E14.5 confirm that, starting at E12.5, cells in the apical region fail to incorporate BrdU, and at E13.5 and E14.5 the region of BrdU-negative staining increases in area, which is indicative of a wave of cell cycle exit that is responsible for establishing the ZNPC (Fig. 2A). This wave of cell cycle exit was quantified in a second experiment in which BrdU was injected at E12.5, E13.5 and E14.5 and the embryos were allowed to survive until birth. At that time, whole mounts were stained with antibody to both Myosin VIIa and BrdU. Double-labeled hair cells ($BrdU^+$/Myosin $VIIa^+$) are indicative of hair cells that were still in the cell cycle at the time of BrdU injection. The percentage of double-labeled cells was compared with the total number of hair cells (Myosin $VIIa^+$) along the length of the cochlea (Fig. 2B). The progression of cell cycle exit is apparent from the rapid decrease in double-labeled cells that occurs from cochlear apex to base between E12.5 and E14.5.

To compare the wave of cell cycle exit with $p27^{Kip1}$ expression, whole-mount cochleae were dissected and stained with antibody to $p27^{Kip1}$. A steep wave of $p27^{Kip1}$ expression between E12.5 and E14.5 (Fig. 2C) parallels or slightly precedes the wave of cell cycle exit (compare Fig. 2A with 2C). A correlation between the wave of cell cycle exit and the expression of $p27^{Kip1}$ can also be seen in a tissue section double labeled for BrdU incorporation and $p27^{Kip1}$ expression, which is taken from an E14.5 embryo whose mother was injected with BrdU 2 hours earlier (Fig. 2E,F). The section was taken so that apical, middle and basal turns of the cochlear duct are simultaneously visible (the angle of cross-section is shown in Fig. 2D). The ZNPC is present in both the apical and middle turns of the cochlea and correlates with the presence of $p27^{Kip1}$ staining (compare brackets Apex with Middle, Fig. 2E with 2F). By contrast, BrdU-stained cells are present in the region at the base of the cochlea, where the ZNPC has yet to form at this time on E14.5, and this correlates with the absence of $p27^{Kip1}$ staining (compare Fig. 2E with 2F, Base).

Thus, formation of the postmitotic, prosensory domain of the cochlear duct is largely complete shortly after E14.5, the time of onset of hair cell differentiation. This means that unlike most other regions of the nervous system, where ongoing cell cycle exit is temporally correlated with cell differentiation, in the developing organ of Corti, a pool of postmitotic progenitors is established in toto

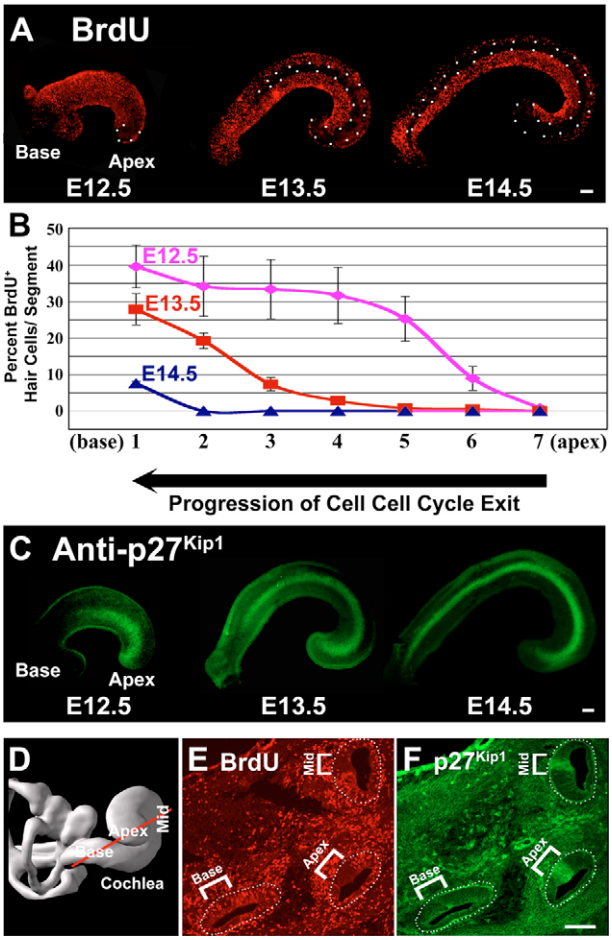

Fig. 2. A wave of cell cycle exit and p27^{Kip1} expression define the prosensory domain within the embryonic cochlea. (**A**) Cochlear whole mounts from mice labeled with BrdU at the time of sacrifice (E12.5, E13.5 and 14.5). BrdU was injected on indicated days 2 hours prior to euthanasia, and whole mounts were fixed and stained with antibody to BrdU. The base and apex of the cochlea are indicated. Dotted outlines indicate the region of the cochlear duct that is devoid of BrdU-labeled cells, indicating that cells in this region have stopped dividing prior to the time of the injection. (**B**) Quantification of hair cell cycle exit on E12.5, E13.5 and E14.5. Timed pregnant female mice were injected with BrdU at the indicated times (E12.5, E13.5, E14.5) and allowed to survive until E18.5, at which time hair cell differentiation along the entire length of the organ of Corti is complete. Each surface preparation is stained for BrdU, to indicate those cells in S phase of the cell cycle at the time of injection, and with antibody to Myosin VIIa, a marker for differentiated hair cells. The E18.5 preparation was divided into seven segments of equal length and myosin VIIa$^+$ hair cells were counted from each segment and compared with double-labeled Myosin VIIa$^+$, BrdU$^+$ hair cells in the same region. The percentage of BrdU$^+$ hair cells per segment is shown (error bars indicate s.d.). (**C**) Whole-mount preparations of embryonic cochlea at E12.5, E13.5 and E14.5 stained with anti-p27^{Kip1}. Antibody staining appears as a gradient with the highest intensity in the apical third of the nascent organ of Corti at E12.5. The gradient increases in length with the growing cochlear duct and the gradient of expression appears in approximately two-thirds of the length of the cochlea at E13.5. By E14.5 the p27^{Kip1} gradient of expression has nearly reached the base of the cochlea, coincident with the termination of cell division within the presumptive organ of Corti at this time. (**D**) Reconstruction of cochlear and vestibular components of the inner ear at E14.5 depicting the level of cross-section shown in E,F. (**E**,**F**) Cross-section through an E13.5-E14.5 cochlea stained for BrdU (E) and p27^{Kip1} (F). BrdU was injected three times at 2-hour intervals into a timed pregnant female mouse starting 6 hours prior to removing and fixing the embryos. The section was double-labeled to reveal both BrdU incorporation and p27^{Kip1} expression. The absence of BrdU staining in the apical and middle turns indicates the presence of a zone of non-proliferating cells in the apical and middle turns (E, apex and mid bracket). BrdU-labeled cells are seen in the basal turn, indicating that the ZNPC has not yet formed in this region. p27^{Kip1} staining is present in the apical and middle turns, but absent from the base (F), coincident with the apical to basal progression of cell cycle exit as revealed by the BrdU labeling (E). Scale bar: 100 μm.

prior to the onset of terminal differentiation. Indeed, the pool of sensory progenitors in the apical regions of the cochlear duct is maintained in an undifferentiated, but postmitotic state for 4-5 days before differentiation occurs.

The wave of p27^{Kip1} expression is regulated at the transcriptional level

In order to assay the mechanism of p27^{Kip1} regulation in the developing organ of Corti, we constructed a reporter using a Bacterial Artificial Chromosome containing 129 kb of genomic DNA surrounding the mouse *p27^{Kip1}* locus (Fig. 3A). An IRES-GFP cassette was inserted into the 3′ non-coding region of the p27^{Kip1} gene in the BAC to monitor gene transcription, and the coding region of *p27^{Kip1}* was deleted to avoid artifacts of overexpression (see Materials and methods). Two independent lines of transgenic mice were analyzed and the two lines expressed the transgene in the same temporal and spatial pattern; data from only one of the lines (*p27^{Kip1}*/GFP) is presented here.

Analysis of GFP expression showed that a gradient of *p27^{Kip1}*/GFP transcription (Fig. 3B) coincides with the temporal and spatial pattern of p27^{Kip1} protein expression (Fig. 3C). At E12.5, expression of p27^{Kip1} protein is detected in a small region in the apex of the organ of Corti and this is coincident with the expression of the GFP transgene. This is also the site where cell cycle exit is first observed (Fig. 2A,B). On E13.5, p27^{Kip1} protein expression has spread in a stripe through the apical third of the cochlear duct, paralleled by the expression of GFP (Fig. 3B,C). At this time quantitative differences in p27^{Kip1} levels are visible along the length of the cochlear duct at both the transcription (GFP) and protein (antibody) level, suggesting that a graded increase in the accumulation of *p27^{Kip1}* mRNA is driving a similar increase in protein levels. Because p27^{Kip1} acts quantitatively to bring about cell cycle exit, the leading edge of p27Kip1 expression is likely to slightly precede the leading edge of the wave of cell cycle exit. At E14.5, p27^{Kip1} protein and GFP transgene expression within the stripe of cells that will become the organ of Corti encompasses greater than three-quarters of the length of the cochlear duct (Fig. 3B,C). Alternate sections through the middle region of the cochlear duct reveal the spatial overlap of p27^{Kip1} protein expression (Fig. 3D) and that of the GFP transgene (Fig. 3E). Thus, the developmental regulation of p27^{Kip1} transcription correlates with both the increase in p27^{Kip1} protein levels, and the wave of cell cycle exit, suggesting that transcriptional control of p27^{Kip1} expression is responsible for limiting the number of postmitotic progenitor cells that will go on to form the mature organ of Corti.

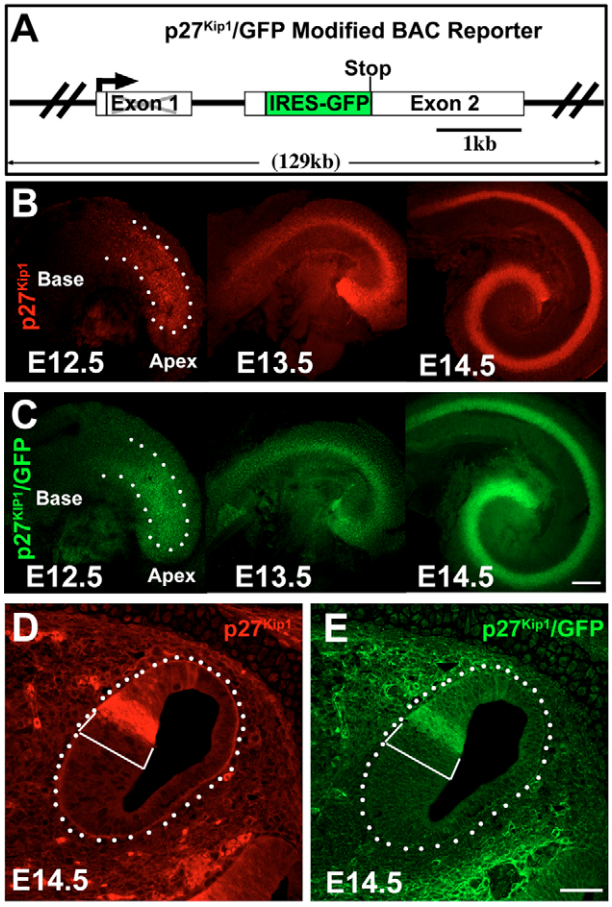

Fig. 3. The apical to basal wave of p27Kip1 expression is regulated at the transcriptional level. (**A**) Schematic of the modified p27Kip1/GFP BAC reporter showing the position of the IRES-GFP relative to exon 1 and exon 2 of the p27Kip1 mouse gene. BAC ends are located 51 kb 5′ and 78 kb 3′ to the start site of p27Kip1 transcription. The first exon of the p27Kip1 gene was removed to avoid overexpression of p27Kip1. See Materials and methods for further details. (**B**,**C**) Whole-mount preparations of the developing cochlear duct from E12.5 to E14.5 were photographed to reveal the endogenous p27Kip1/GFP transgene (C, green), and then labeled with antibody to p27Kip1 (B) to demonstrate the correspondence between the pattern of gene and protein expression at each age examined. In E12.5, apex and base are marked and dotted lines indicate the region of initiation of p27Kip1 and GFP expression. (**D**,**E**) Cross-section through the approximate middle cochlea stained to reveal anti-p27Kip1 (red) and expressing native GFP reporter (green) as above, to show the coincidence of p27Kip1-GFP transgene expression with p27Kip1 protein. Scale bar: 100 μm.

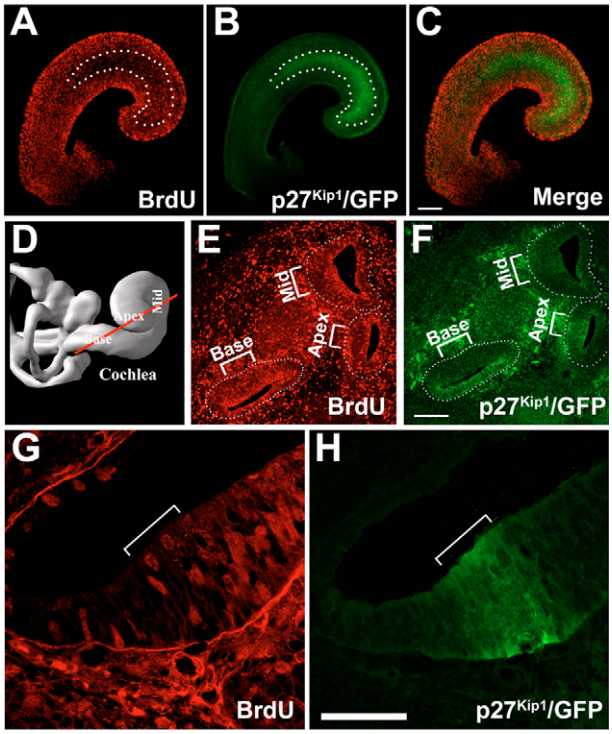

Fig. 4. p27Kip1 transcriptional regulation in p27Kip1-null mice is independent of cell cycle exit. (**A-C**) Whole-mount preparation of E13.5 cochlear epithelium from a p27Kip1-null embryo labeled in utero with BrdU. Timed pregnant female received three injections of BrdU every 2 hours, prior to sacrifice 6 hours after the first injection. BrdU staining (A, red) shows labeled cells throughout the epithelium, including in apical and middle regions, which are normally devoid of cycling cells in wild-type cochlea at this time (see Fig. 2). The GFP transgene (B, green) is expressed in an apical to basal gradient the extent of which matches that seen in wild-type embryos at this time (see Fig. 2). Merged image shows double-labeled cells (C, yellow) belonging to abnormally proliferating cells that are within the prosensory domain marked by p27Kip1/GFP transgene expression. (**D-F**) Surface reconstruction and cross-section through a p27Kip1-null E13.5 organ of Corti. (D) Surface reconstruction showing the approximate plane of section (red line) used in E and F. (E) Cross-section shows three turns of the E13.5 cochlea (apex, middle and base) stained for BrdU incorporation. (F) The same section as in E showing native p27Kip1/GFP reporter. Brackets indicate the site of presumptive organ of Corti formation. Note that BrdU+ cells (E, red) are present at all levels (apex, middle and base) in the p27Kip1-null embryo. GFP (F) can be seen strongly in the apical turn (bracket, apex) and weakly in the middle turn (bracket, mid) of the cochlear duct, but not in the basal turn (base), indicative of the gradient of transgene expression that occurs in spite of the failure of cell cycle exit due to the mutation of p27Kip1. (**G**,**H**) Cross-section through the middle turn of an E14.5 cochlear duct from a *p27Kip1*-null mouse, showing the presence of BrdU-labeled cells within the presumptive region of organ of Corti formation (bracket, G). (H) The same section as in G showing the overlapping pattern of p27Kip1/GFP transgene expression (bracket). Scale bar: 100 μm.

Transcriptional control of p27Kip1 is independent of cell cycle exit

Theoretically, p27Kip1 transcriptional induction could be dependent on cell autonomous developmental signals regulating its abundance, or, alternatively, on non-cell autonomous feedback mechanisms that react to the onset of cell cycle exit stimulated by alternate mechanisms. To test whether developmental regulation of *p27Kip1* expression is independent of cell cycle exit, we studied the expression of the *p27Kip1*/GFP transgene in the cochlear prosensory domain of *p27Kip1*-null mice, where cell cycle exit is delayed and the prosensory progenitors of the organ of Corti continue to divide (Chen and Segil, 1999) (Fig. 4). Pregnant *p27Kip1*/GFP reporter mice carrying p27Kip1-null embryos were injected with BrdU at E13.5 (Fig. 4A-F) or 14.5 (Fig. 4G,H), and two hours later p27Kip1-null embryos were dissected and chochleae were stained for BrdU incorporation indicative of continued presence in the cell cycle. BrdU labeling was present throughout the length of the E13.5 cochlear duct (Fig. 4A, BrdU), in contrast to the situation previously

described in wild-type embryos (Fig. 2A,B). Nonetheless, the *p27^{Kip1}*/GFP reporter is expressed in a pattern identical to wild-type cochleae (compare Fig. 4B with Fig. 3C) at E13.5, and overlaps with the region of BrdU incorporation (Fig. 4C, merge), indicating that transcription of the *p27^{Kip1}*/GFP transgene is regulated normally, even in the absence of p27Kip1 protein and in the presence of abnormal cell proliferation. Normal expression of the p27^{Kip1} transgene is also observed overlapping with BrdU-labeled cells in cross-sections through the developing E13.5 cochlea of p27^{Kip1}-null mice (Fig. 4D-F), as well as in cross-sections from *p27^{Kip1}*-null mice injected with BrdU on E14.5 (Fig. 4G,H). These results indicate that the apical to basal wave of *p27^{Kip1}* transcription is regulated cell autonomously and independent of the cell cycle state.

Cell-type specific regulation of p27^{Kip1} transcription in postnatal organ of Corti and retina

Mature *p27^{Kip1}*-null mice display cell-type-specific patterns of abnormal cell division (Fero et al., 1998; Kiyokawa et al., 1996; Nakayama et al., 1996), suggesting that p27^{Kip1} is involved in the cell-type specific regulation of the postmitotic state. In the inner ear, p27^{Kip1} protein is rapidly downregulated during hair cell differentiation, while being maintained at high levels in supporting cells (Chen and Segil, 1999). Transcriptional control of *p27^{Kip1}* might extend to this and other examples of cell-type-specific expression, or alternatively, posttranscriptional mechanisms could be entirely responsible. To test whether transcriptional regulation plays a role in cell-type-specific expression in the differentiated organ of Corti, we studied expression of the *p27^{Kip1}*-GFP reporter at P1 (Fig. 5). GFP disappears from differentiating hair cells embryonically within 24 hours of the appearance of Math1 (data not shown), and then remains absent from hair cells postnatally (Fig. 5), indicating that transcriptional downregulation has occurred. By contrast, p27^{Kip1} protein continues to be expressed at a high level in P1 supporting cells, as is the *p27^{Kip1}*/GFP reporter (Fig. 5A-C). These results indicate that the transcriptional control of *p27^{Kip1}* remains in force beyond the embryonic period and may play a role in maintaining the homeostatic state of hair cells and supporting cells.

In order to extend these observations to another sensory organ, we have studied the pattern of expression of the *p27^{Kip1}*/GFP transgene in the postnatal mouse retina (Fig. 5D-G). During retinal development, a dynamic pattern of p27^{Kip1} expression is involved in regulating cell cycle exit in several differentiating cell types (Dyer and Cepko, 2000; Dyer and Cepko, 2001a; Levine et al., 2000). However, in the mature retina, p27^{Kip1} expression has been downregulated in all cell types except for Muller glia (Dyer and Cepko, 2000; Dyer and Cepko, 2001a; Levine et al., 2000). We observed that in P21 retinas, the *p27^{Kip1}*/GFP reporter (Fig. 5E) is expressed in Muller glia, which are marked by expression of glutamine synthetase (Fig. 5D), as previously described (Vardimon et al., 1991). This pattern of *p27^{Kip1}*/GFP reporter expression (Fig. 5G) parallels the pattern of p27^{Kip1} protein expression (Fig. 5F). These observations indicate the importance of transcriptional control of postnatal patterns of p27^{Kip1} expression, and the importance of cell-type-specific regulation of the postmitotic state.

DISCUSSION

We have investigated the means by which p27^{Kip1} achieves the timely and ordered cell cycle withdrawal of organ of Corti precursors, and we now show that regulated transcription of *p27^{Kip1}* is largely responsible for driving the precise pattern of cell cycle exit required for normal development. Transcription of *p27^{Kip1}* is shown to follow a temporal progression from apex to base within the cochlear duct that prefigures a wave of cell cycle exit and establishes the extent of the postmitotic prosensory domain of the cochlea. In *p27^{Kip1}*-null mice, cell cycle exit is delayed and the wave of cell cycle exit does not form, leading to patterning abnormalities during subsequent differentiation (Chen and Segil, 1999; Lowenheim et al., 1999). Using a *p27^{Kip1}*/GFP reporter transgene, we show that *p27^{Kip1}*

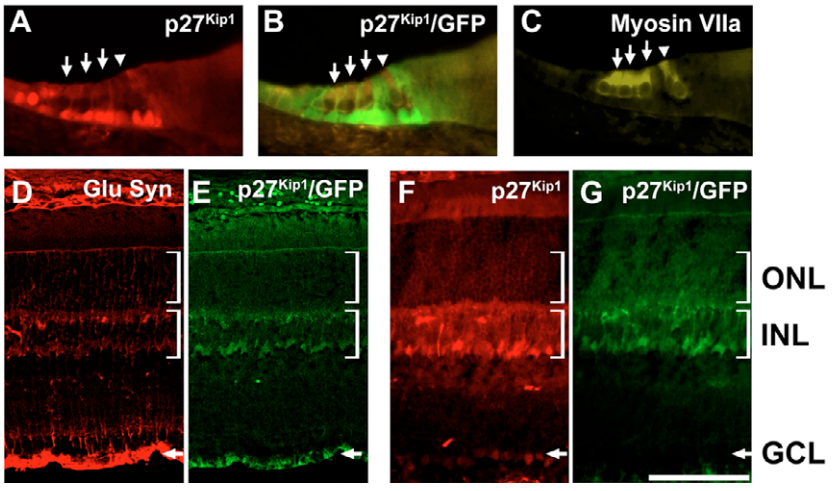

Fig. 5. Cell-type-specific transcriptional regulation of p27^{Kip1} in the postnatal organ of Corti and retina. (**A-C**) Sections through the postnatal day 1 (P1) organ of Corti triple-labeled with antibody to p27^{Kip1} (A), the native p27^{Kip1}/GFP transgene (B) and the hair cell marker Myosin VIIa (C). Note high levels of p27^{Kip1} expression in postnatal supporting cells and the lack of staining of differentiated hair cells, indicating that p27^{Kip1} is downregulated in hair cells at the transcriptional level. (**D-G**) Cross-sections through the P21 mouse retina. D and E are the same section double-labeled with antibody to glutamine synthetase, a marker of Muller glia in the retina (D), and the native p27^{Kip1}/GFP transgene (E). F and G are the same section double-labeled with antibody to p27^{Kip1} (F) and the native p27^{Kip1}/GFP transgene (G). Note the co-linearity of staining between glutamine synthetase, the p27Kip1 protein and p27^{Kip1}/GFP in the inner nuclear layer (INL), which is indicative of cell-type-specific transcriptional control in the postnatal retina. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar: 100 μm.

transcriptional regulation is unaffected in the $p27^{Kip1}$-null mouse, indicating that transcriptional timing of $p27^{Kip1}$ is controlled independently of cell cycle events. Transcriptional control of $p27^{Kip1}$ is also independent of differentiation within the organ of Corti, as the wave of cell cycle exit occurs prior to the onset of Math1-dependent differentiation (Bermingham et al., 1999; Chen et al., 2002). Finally, we show that the subsequent cell type-specific expression of $p27^{Kip1}$ in the mature organ of Corti and retina is also regulated at the transcriptional level, suggesting that differential regulation of $p27^{Kip1}$ transcription is important for maintaining cellular homeostasis in adult tissues (Fig. 5).

$p27^{Kip1}$ expression is responsible for enforcing the temporal separation between cell cycle exit and cell differentiation during cochlear development

During embryonic development, the processes that control cell division, and those that control cell differentiation are frequently difficult to separate experimentally because of tight temporal coordination between these events (see Cremisi et al., 2003; Fichelson et al., 2005; Zhu and Skoultchi, 2001). The temporal separation between cell cycle exit and cell fate decision during organ of Corti development (Chen et al., 2002) appears to be quite different from the normal pattern of development in other parts of the vertebrate nervous system, such as in the retina (Dyer and Cepko, 2001b; Levine et al., 2000) and the cortex (McConnell, 1990), where a kind of 'just-in-time' production of postmitotic cells is tightly linked to their differentiation. The reason for establishing a fixed number of postmitotic progenitors within the cochlear duct prior to the onset of differentiation is not clear; however, we hypothesize that it is related to the linear and iterative geometry of development within the confines of the elongating cochlear duct, wherein the same precise number and proportion of hair cells and supporting cells may be needed at each position along the cochlear length in order to support the final bio-mechanical function of the organ of Corti. By contrast, the retina develops circumferentially, and thus requires a continuously increasing number of progenitors as differentiation progresses from the center to the periphery (Dyer and Cepko, 2001b).

How is the temporal and spatial gradient of $p27^{Kip1}$ transcription achieved?

The molecular mechanisms governing $p27^{Kip1}$ gene transcription during development are unknown. We have used a 129-kb BAC containing the $p27^{Kip1}$ locus to study the pattern of transcriptional regulation responsible for controlling cell cycle exit in the developing organ of Corti. The $p27^{Kip1}$ regulatory regions included within this BAC are sufficient to recapitulate both the temporal and spatial pattern of $p27^{Kip1}$ expression within the cochlear duct (Fig. 3). Although several studies, involving a variety of cells grown in culture, have indicated that $p27^{Kip1}$ can be transcriptionally regulated in response to hormones, interleukins and other growth modulating conditions through response elements within sequences that lie 1-2 kilobases 5′ to the start site of $p27^{Kip1}$ transcription (Chen et al., 2005; Gizard et al., 2005; Stahl et al., 2002), preliminary results from our laboratory indicate that developmental regulation of $p27^{Kip1}$ expression is only partly recapitulated in transgenic reporter mice harboring a region of the $p27^{Kip1}$ gene including 9 kb of DNA 5′ to the start of transcription (data not shown). This indicates that the more complicated developmental control regions of the $p27^{Kip1}$ locus are likely to be complex and to span a considerable area around the $p27^{Kip1}$ gene itself. This is in keeping with the situation in *Drosophila*, where the $p27^{Kip1}$ homolog Dacapo has been shown to harbor a complex, tissue-specific and developmentally regulated array of enhancer elements (Liu et al., 2002; Meyer et al., 2002).

During the development of the cochlear duct, $p27^{Kip1}$ expression is confined to a narrow prosensory domain that represents the nascent organ of Corti (Chen et al., 2002; Chen and Segil, 1999). Although the mechanism by which the transcription of $p27^{Kip1}$ is confined to this region is unknown, one transcription factor that may prefigure the $p27^{Kip1}$ domain is Sox2. Sox2 is expressed broadly in the otocyst at E9.5 and comes to be expressed in the same domain as $p27^{Kip1}$ at E14.5 prior to the onset of hair cell differentiation (Kiernan et al., 2005b). Whether the narrowing of Sox2 expression to the $p27^{Kip1}$ domain precedes or follows the onset of $p27^{Kip1}$ expression is not known. In addition, although the exact role of Sox2 within the prosensory domain is unknown, its loss leads to a partial failure of $p27^{Kip1}$ expression and sensory development (Kiernan et al., 2005b).

The $p27^{Kip1}$ expression domain does not appear to encompass all regions of the cochlear duct that are competent to form hair cells and supporting cells. This is evident from Math1 transfection studies in which cells medial to the organ of Corti [the greater epithelial ridge (GER)] have been shown to be competent to differentiate as sensory cells (Izumikawa et al., 2005; Kawamoto et al., 2003; Shou et al., 2003; Woods et al., 2004; Zheng and Gao, 2000). Although the precise extent of this sensory-competent region is not known, it is possible that a 'competence' domain, including the region of the ZNPC and surrounding cells of the GER (and lesser epithelial ridge), is established prior to the establishment of the definitive prosensory domain that gives rise to the organ of Corti. Thus, the mechanisms responsible for $p27^{Kip1}$ expression may be part of a process that restricts a region of sensory competence to the exact number of cells needed to form the organ of Corti. Discovering the factors controlling $p27^{Kip1}$ transcription may lead us to the mechanisms by which this postmitotic prosensory domain is spatially defined.

Although we do not know which developmental pathways are responsible either for temporal or spatial aspects of this pattern, recent evidence suggests that Notch signaling may play a role in $p27^{Kip1}$ regulation at this time. The mutation of either of two Notch ligands, Jagged 1 or Jagged 2, or the Notch 1 receptor, leads to gene-specific patterning defects in the organ of Corti involving supernumerary hair cells, as well as apparent perturbations in $p27^{Kip1}$ expression (Kiernan et al., 2005a; Kiernan et al., 2006; Brooker et al., 2006), although $p27^{Kip1}$ expression is not completely eliminated. In addition, another recent report indicates that the Notch-responsive gene Hes1 is able to directly bind the $p27^{Kip1}$ promoter in HeLa cells (Murata et al., 2005). Hes1 does not appear to be expressed prior to or at the time of onset of $p27^{Kip1}$ expression in the cochlear duct (Zine et al., 2001) (A. Doetzlhofer and N.S., unpublished); however, the existence of a Hes-responsive binding site within the $p27^{Kip1}$-promoter suggests that different members of the Hes gene family may serve as a link between Notch signaling and $p27^{Kip1}$ transcription in the cochlea.

Our demonstration of the importance of transcriptional control of $p27^{Kip1}$ to the developmental timing of cell cycle exit does not preclude the possibility that posttranscriptional mechanisms governing the accumulation of $p27^{Kip1}$ protein are also partly responsible for the timing of these events. Indeed, our preliminary data indicates that loss of the F-box protein Skp2, which is involved in the posttranscriptional regulation of $p27^{Kip1}$, causes a subtle change in the timing of cell cycle exit in the organ of Corti (F.L. and N.S., unpublished), indicating that coordination between

transcriptional and posttranscriptional mechanisms regulating $p27^{Kip1}$-mediated cell cycle exit is likely to be crucial for normal development.

The importance of $p27^{Kip1}$ regulation to the maintenance of the postmitotic state of hair cells

Maintenance of cell-cell interactions (tissue architecture) is believed to be the reason that most cells of the nervous system are maintained in a lifelong postmitotic state. CKIs play a significant role in this process (Chen et al., 2003; Zindy et al., 1999). In CNS neurons, $p27^{Kip1}$ expression continues in the mature cortex, and along with the CKI $p19^{Ink4d}$, is part of the mechanism underlying the stability of the postmitotic state of neurons (Zindy et al., 1999). In mice wherein both *p19*^{Ink4d} and *p27*^{Kip1} have been mutated, the postmitotic state of mature neurons is compromised, and many cells re-enter the cell cycle and subsequently undergo apoptosis. However, if either $p27^{Kip1}$ or $p19^{Ink4d}$ is present, the postmitotic state of differentiated neurons is maintained. By contrast, *p19*^{Ink4d}-null mice suffer from a progressive hearing loss brought about by an inability of hair cells to maintain the postmitotic state (Chen et al., 2003). We have speculated that the developmentally controlled downregulation of $p27^{Kip1}$ in hair cells leads to a compromised ability to maintain the postmitotic state (Chen et al., 2003). Here, we show that the downregulation of $p27^{Kip1}$ in hair cells is controlled at the transcriptional level (Fig. 5). Similarly, $p27^{Kip1}$ levels are downregulated in all neuronal cell types in the mouse retina following differentiation (Levine et al., 2000), and we have observed that the *p27*^{Kip1}/GFP reporter is also downregulated in mature neurons of the retina (Fig. 5), indicating that this occurs at the transcriptional level as well. Interestingly, *p27*^{Kip1}/GFP is expressed exclusively in Muller glia of the mature retina (Dyer and Cepko, 2000; Levine et al., 2000), indicating that, as in the organ of Corti, cell-type specific expression of $p27^{Kip1}$ in the retina is regulated transcriptionally (Fig. 5). The reason that $p27^{Kip1}$ expression is maintained at different levels in different postmitotic cell types is not clear. Recent evidence that $p27^{Kip1}$ functions outside the context of the cell cycle to regulate the cytoskeleton (Besson et al., 2004) suggests that maintenance of the postmitotic state may not be the only reason for its differential expression in mature cell types.

Transcriptional regulation of *p27*^{Kip1} in supporting cells – relationship to regeneration

The differential expression of the *p27*^{Kip1}/GFP transgene in hair cells and supporting cells, as well as in the retina (Fig. 5), indicates that cell-type-specific expression of $p27^{Kip1}$ in the mature organ of Corti is maintained through transcriptional mechanisms. In non-mammalian vertebrates, the loss of hair cells leads to the proliferation of supporting cells and subsequent regeneration of functional hair cells (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). However, in mammals, the loss of cochlear hair cells does not lead to supporting cell proliferation, and hair cell regeneration does not occur (Chardin and Romand, 1995). The loss of hair cells is the major cause of deafness in the adult human population, making the failure of hair cell regeneration an important clinical problem. Although the generation of new hair cells in adult *p27*^{Kip1}-null mice is unclear, sub-populations of supporting cells are observed to divide spontaneously at postnatal times, indicating that supporting cells depend, at least partially, on $p27^{Kip1}$ for the maintenance of the postmitotic state, and that the persistence of high levels of $p27^{Kip1}$ in supporting cells may be one of the obstacles to regeneration (Chen and Segil, 1999; Lowenheim et al., 1999). Our observation that regulated transcription is at least partially responsible for the high level of $p27^{Kip1}$ expression in supporting cells provides a new set of potential targets for the transient manipulation of $p27^{Kip1}$ levels with the aim of stimulating the therapeutic proliferation of supporting cells.

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