on the "repressor region" of the *p27Kip1* promoter in αT3-1 cells (Fig. 5E) (see SOM) (*13*). Moreover, N-CoR, TBL1, HDAC3, and HDAC1 were also bound to the same region (Fig. 5E), which is consistent with the recruitment of at least two corepressor complexes through Dachs.

To test whether Six6 could directly regulate p27Kip1 expression in a biological context, we performed ChIP experiments using microdissected e13.5 wild-type retinas, showing that both Six6 and Dach2 were indeed recruited to the putative SE sites of the *p27Kip1* promoter (Fig. 5F), which is consistent with the correlation between *Six6* high expression and *p27Kip1* low expression at that developmental time (*13*). Despite the strong expression of *Sno* in the developing retina and its homology with Dach2, Sno was not present on the *p27Kip1* promoter (Fig. 5F), which is consistent with our finding of no detectable functional interactions between Six6 and Sno in transient transfection, two-hybrid, and microinjection studies (Fig. 4D) (*13*).

We therefore conclude that Six6/Dach complex binds directly to the *p27Kip1* promoter and represses its transcriptional activity in vivo, together with regulation of p19Ink4d and p57Kip2, to regulate proliferation. The Six6/CKI regulatory network likely serves as a molecular strategy for Six6-dependent regulation of the proper expansion of retinal and pituitary precursor cell populations. The strong coexpression of another highly related *Six* gene, *Six3*, during retinal development could partially compensate for the loss of Six6 (*5*). Six6/Dach repressive function in eye development is in contrast to the activation roles shown for Six1/Eya2 in muscle development (*11*), identifying a unique role of Six6 in terms of regulating downstream genes by interacting with specific partners. Together, these findings provide an organ-specific strategy for the expansion of precursor cell populations during development, a strategy that is likely used in other organ systems.

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Materials and Methods
Figs. S1 to S4

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Stochastic Gene Expression in a Single Cell

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Clonal populations of cells exhibit substantial phenotypic variation. Such heterogeneity can be essential for many biological processes and is conjectured to arise from stochasticity, or noise, in gene expression. We constructed strains of *Escherichia coli* that enable detection of noise and discrimination between the two mechanisms by which it is generated. Both stochasticity inherent in the biochemical process of gene expression (intrinsic noise) and fluctuations in other cellular components (extrinsic noise) contribute substantially to overall variation. Transcription rate, regulatory dynamics, and genetic factors control the amplitude of noise. These results establish a quantitative foundation for modeling noise in genetic networks and reveal how low intracellular copy numbers of molecules can fundamentally limit the precision of gene regulation.

Living cells possess very low copy numbers of many components, including DNA and important regulatory molecules (*1*). Thus, stochastic effects in gene expression may account for the large amounts of cell-cell variation observed in isogenic populations (*2, 3*). Such effects can play crucial roles in biological processes, such as development, by establishing initial asymmetries that, amplified by feedback mechanisms, determine cell fates (*4*). However, experimental evidence for stochasticity in gene expression has been circumstantial (*5, 6*). For any particular gene, it remains unknown whether cell-cell variation in the abundance of its product is set by noise in expression of the gene itself or by fluctuations in the amounts of other cellular components. The difficulty of experimentally distinguishing between these two possibilities has thus far precluded detection of intrinsic noise in living cells. The magnitude of the noise intrinsic to gene expression, and its relative importance compared with other sources of cell-cell variability, are fundamental characteristics of the cell that require measurement.

In general, the amount of protein produced by a particular gene varies from cell to cell. The noise (defined as the standard deviation divided by the mean) in this distribution is labeled η_{tot} and can be divided into two components. Because expression of each gene is controlled by the concentrations, states, and locations of molecules such as regulatory proteins and polymerases, fluctuations in the amount or activity of these molecules cause corresponding fluctuations in the output of the gene. Therefore, they represent sources of extrinsic noise (denoted η_{ext}) that are global to a single cell but vary from one cell to another. On the other hand, consider a population of cells identical not just genetically but also in the concentrations and states of their cellular components. Even in such a (hypothetical) population, the rate of expression of a particular gene would still vary from cell to cell because of the random microscopic events that govern which reactions occur and in what order. This inherent stochasticity, or intrinsic noise, denoted η_{int},

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Tissue-Specific Regulation of Retinal and Pituitary Precursor Cell Proliferation

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Mammalian organogenesis requires the expansion of pluripotent precursor cells before the subsequent determination of specific cell types, but the tissue-specific molecular mechanisms that regulate the initial expansion of primordial cells remain poorly defined. We have genetically established that Six6 homeodomain factor, acting as a strong tissue-specific repressor, regulates early progenitor cell proliferation during mammalian retinogenesis and pituitary development. Six6, in association with Dach corepressors, regulates proliferation by directly repressing cyclin-dependent kinase inhibitors, including the *p27Kip1* promoter. These data reveal a molecular mechanism by which a tissue-specific transcriptional repressor-corepressor complex can provide an organ-specific strategy for physiological expansion of precursor populations.

The mammalian retina has six different neuronal cell types that are generated in an evolutionarily conserved order from a population of retinal progenitor cells (RPC) (*1*). Studies in both *Drosophila* and mammalian systems have identified key nuclear factors that are required for formation and early determination of the eye, including *eyeless* (*ey*)/*Pax6* (*2*, *3*), *sine oculis* (*so*)/*Six* (*4*, *5*), *eye absent* (*eya*)/*Eya*, and *dachshund* (*dac*)/*Dach* (*6*–*8*). Genetic studies in *Drosophila* suggested the synergistic formation of a network with *so* as a DNA binding factor and *dac*/*eya* as transcription cofactors (*9*–*11*).

We investigated the role of mammalian *Six6*, an ortholog of *Drosophila optix*, which exhibits developmentally restricted expression in the eye, pituitary, and hypothalamus [see supporting online material (SOM) (fig. S1A)] (*12*). Detailed in situ hybridization analysis demonstrated that *Six6* expression is high in the optic vesicle at embryonic day 9.5 (e9.5), peaks in the retina around e13.5, and then progressively diminishes [see SOM (fig. S1A)]. At the early stage of Rathke's pouch, expression in the pituitary exhibits a dorsal-ventral gradient, with persistent expression in proliferating periluminal cells and reduced expression in differentiated cells [see SOM (fig. S1A)].

We took a genetic approach, using standard homologous recombination strategies in embryonic stem cells [see SOM (fig. S1, B and C)]. The expression pattern of the *lacZ* reporter, which was knocked into the *Six6* locus, recapitulated the endogenous *Six6* expression pattern, and Six6 protein absence was confirmed by Western blot (Fig. 1) (*13*). Although Six6 was not essential for survival, $Six6^{-/-}$ mice in a mixed genetic background exhibited a hypoplastic pituitary gland and variable degrees of retinal hypoplasia, often with absence of optic chiasm and optic nerve (Fig. 1) [see SOM (fig. S1D)], traits that resemble human defects associated with chromosomal deletions including the *SIX6* locus (*14*). In the 129sv (Stevens strain) background, the $Six6^{-/-}$ mice exhibited less severe but consistent defects in both the pituitary and retina.

Expression of early functionally important marker genes including *Pax6*, *Pax2*, *Six3*, *Vax1*, *Hes-1*, *Hesx1*, and *Rx* was unaltered (Fig. 2A) (*13*, *15*, *16*). Further analyses of cell-specific markers revealed the presence of all types, but at clearly decreased numbers in the more severely affected mice [see SOM (fig. S2)]. Similarly, immunostaining of pituitary hormones confirmed the presence of all six cell types (Fig. 2B) (*13*).

To investigate a potential cell proliferation defect in the $Six6^{-/-}$ mutants, the BrdU labeling index was measured 1.5 hours after peritoneal injection of pregnant females at e9.5, e10.5, e11.5, e13.5, e15.5, and e17.5; we found a reduction of labeling index in both retina and pituitary (Fig. 3A) (see SOM) (*13*). To further investigate the nature of this defect, the proliferation potential of RPCs was measured at different embryonic stages using mice with pure 129sv genetic background (see SOM). Such studies permit us to analyze changes in cell cycle number or length by evaluating cells exhibiting strong BrdU immunoreactivity, representing those that exit the cell cycle immediately, and cells exhibiting weak BrdU immunoreactivity, representing those that undergo one to three rounds of divisions before exiting the cell cycle after BrdU incorporation (Fig. 3B) [see SOM (fig. S3B)]. Consistent with the previous results, the majority of terminally mitotic RPCs labeled with BrdU at e11.5 were amacrine, cone, ganglion cells, and fewer horizontal cells (Fig. 3C) [see SOM (fig. S3B)] (*1*, *17*). A significant increase in the number of strongly labeled ganglion cells in the $Six6^{-/-}$ retinas was observed, indicating that RPCs destined to become ganglion cells prematurely exited the cell cycle. Consistent with this interpretation, we found dramatically lower numbers of weakly labeled BrdU-positive cells of all early-born cell types in the $Six6^{-/-}$ mutants (Fig. 3C). Further, analysis of the ganglion cell layer at postnatal day 35 (p35) revealed an ~20% decrease of total cell numbers in the $Six6^{-/-}$ mice, consistent with a decreased cell proliferation (Fig. 3D). Because analyses were performed at p35, allowing ample time for multiple rounds of cell division, the reduced numbers of weakly labeled cells in the mutant retina argue in favor of RPCs prematurely exiting the cell cycle. The difference was no longer detectable at later stages (Fig. 3E), consistent with the mild defect observed in 129sv mice. A terminal transferase–mediated dUTP-biotin nick end labeling (TUNEL) assay for e11.5 to e16.5 retinas and pituitary of mice with pure 129sv or mixed

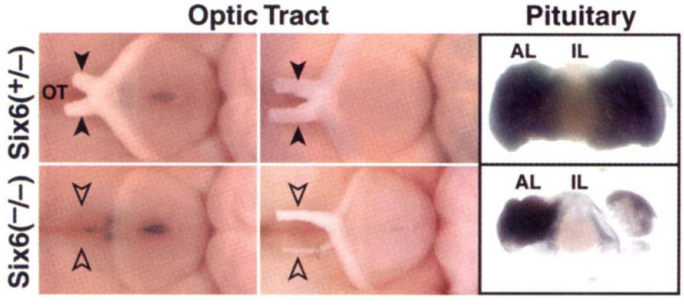

Fig. 1. Analyses of $Six6^{-/-}$ mutant phenotype optic nerve/optic tract (OT) and pituitary. The X-gal staining pattern (blue) shows normal expression in the hypothalamus (left panels). Solid arrowhead, normal size of OT; open arrowhead, reduction or absence of OT; AL, anterior lobe; IL, intermedia lobe.

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genetic backgrounds revealed no significant differences in apoptosis between *Six6*$^{+/-}$ and *Six6*$^{-/-}$ embryos (*13*) (see SOM). Taken together, all of our evidence suggests that Six6 regulates RPC proliferation potential during retinogenesis, consistent with *Xenopus xOptx2* function (*18*).

To study the transcriptional properties of Six6 and its potential coregulators that underline these effects on proliferation, we investigated its function in vitro and potential cofactors, such as Eya and Dach (*9*, *11*). Six6 fusion protein (Gal4DB/Six6) failed to activate an upstream activating sequence (UAS)–dependent reporter, even with cotransfection of *Eya* or *Dach* (Fig. 4A) (see SOM). However, it consistently repressed both 3xUAS/p36 and 3xUAS/tk reporters (Fig. 4, A and B). Deletional analyses proved that both the evolutionarily conserved Six domain and homeodomain (HD) were required for maximal repressive activity [see SOM (fig. S4A)].

To identify potential cofactors of Six6-mediated repression, we focused on Dach proteins, which exhibit structural and sequence similarity to corepressors Ski and Sno (*19*, *20*). Six6 strongly interacted with Dach1 (Fig. 4, B and C) (*13*), and Gal4DB/Dach1 fusion protein acted as a potent repressor in transient transfection (Fig. 4B). Further, Gst/Dach1 interacted directly with N-CoR (nuclear receptor corepressor) and histone deacetylase 3 (HDAC3) corepressors through its conserved NH_2-terminal domain and with Sin3A/B corepressor through its conserved COOH-terminal region (Fig. 4C) [see SOM (fig. S4B)], similarly to Ski/Sno (*19*). Mapping interaction domains revealed that Dach binds to the same region of N-CoR (–1469 to –1740) previously identified to associate with Su(H) and Ski [see SOM (fig. S4B)].

Whereas Six6 alone exerted weak repressive activity on the Six response elements (SE)–dependent reporter, comicroinjection of either Dach1 or Dach2 expression vectors strongly potentiated its repressive function (Fig. 4D) (see SOM) (*21*). We were unable to detect any synergistic interactions between Six6 and Sno (Fig. 4D). Furthermore, corepressor complexes capable of interacting with Dach were required, because microinjection of αHDAC1, αHDAC3, αSin3A/B, or αN-CoR immunoglobulin G (IgG) abolished the Six6/Dach-mediated repression (Fig. 4E). Together, these data suggest that Dach can function as a specific corepressor for Six6.

Because Six6 appears to function as a potent transcriptional repressor and *Six6*$^{-/-}$ mice exhibit hypocellularity consistent with early exit from the cell cycle of RPCs, we investigated the expression of genes inhibiting cell proliferation, such as the cyclin-dependent kinase inhibitors (CKIs). Systematic analyses of all CKI expression at e10.5, e13.5, and e15.5 demonstrated a consistent and significant two- to fourfold upregulation of *p27Kip1*, as well as *p19Ink4d* and *p57Kip2*, and their encoded proteins in *Six6*$^{-/-}$ retinas (Fig. 5, A and B) (see SOM) (*13*). We thus showed an upregulation of specific CKIs in the *Six6*$^{-/-}$ mutant mice, providing a direct mechanistic link to the hypoplastic retinal phenotype. Consistently, mutation of *p27Kip1* causes pituitary tumors and hyperplastic retina without affecting the balance of retinal cell types (*22*–*25*), the inverse of the *Six6*$^{-/-}$ phenotype, whereas overexpression of *p27Kip1* in retinal cells leads to premature cell cycle exit (*25*).

Previous in vitro studies indicated a potential "repressor region" between 0.9 and 2.2 kb in the *p27Kip1* promoter (*26*). Cotransfection of Six6/Dach into 293 cell line strongly repressed expression from 2.2-kb, but not 0.9-kb promoter (Fig. 5, C and D) (see SOM). In the pituitary cell line αT3-1, expressing

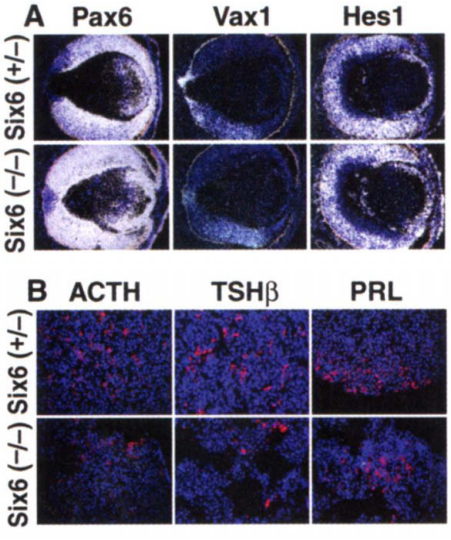

Fig. 2. Normal expression of early retinal–specific genes and pituitary hormones. (**A**) In situ hybridization of e13.5 retina and (**B**) immunohistochemistry of adult pituitary. ACTH, corticotropes; TSHβ, thyrotropes; PRL, lactotropes.

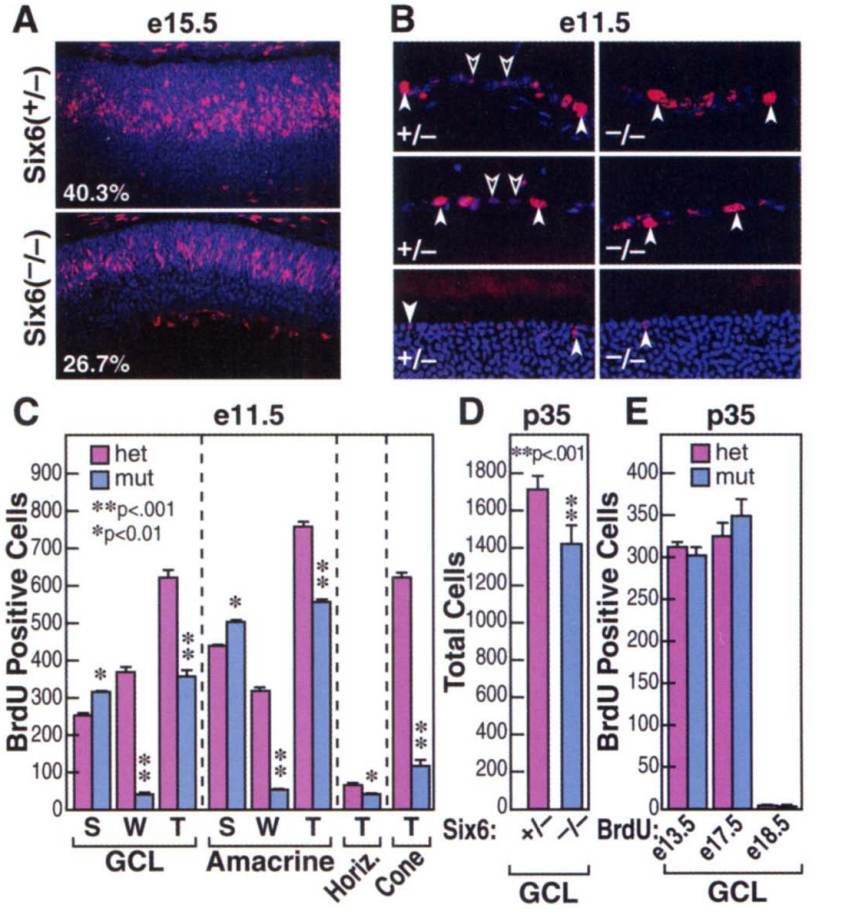

Fig. 3. Proliferation defect in the Six6 mutant retina. (**A**) BrdU labeling index after 1.5-hour injection at e15.5. (**B**) BrdU was injected at e11.5, and an analysis was performed on p35 retina. Top four panels, ganglion cell layer (GCL); bottom two panels, outer nuclear layer (ONL); solid arrowhead, strong (S)-labeled cells; open arrowhead, weak (W)-labeled cells. (**C**) Quantitative analyses of BrdU-positive ganglion at GCL, amacrine at inner nuclear layer (INL), horizontal at ONL, and cone photoreceptor at ONL. (**D**) The total number of cells from the GCL layer was counted from 5-week-old *Six6*$^{+/-}$ and *Six6*$^{-/-}$ littermates (n = 6). (**E**) Quantitative analyses of BrdU-positive ganglion cells at GCL at p35 when BrdU was injected at e13.5, e17.5, and e18.5. Results from >1000 cells were taken into account (mean ± SEM).

low levels of Dach2 and Six6, overexpression of Six6 alone is enough to repress the –2.2-kb *p27Kip1* promoter (*13*). Sequence analysis of *p27Kip1* promoter identified two conserved Six6 binding sites in this region (*26*), suggesting that *p27Kip1* might be a direct target for Six6. Indeed, chromatin immunoprecipitation experiments (ChIP) demonstrated that both Six6 and Dach2 were specifically bound

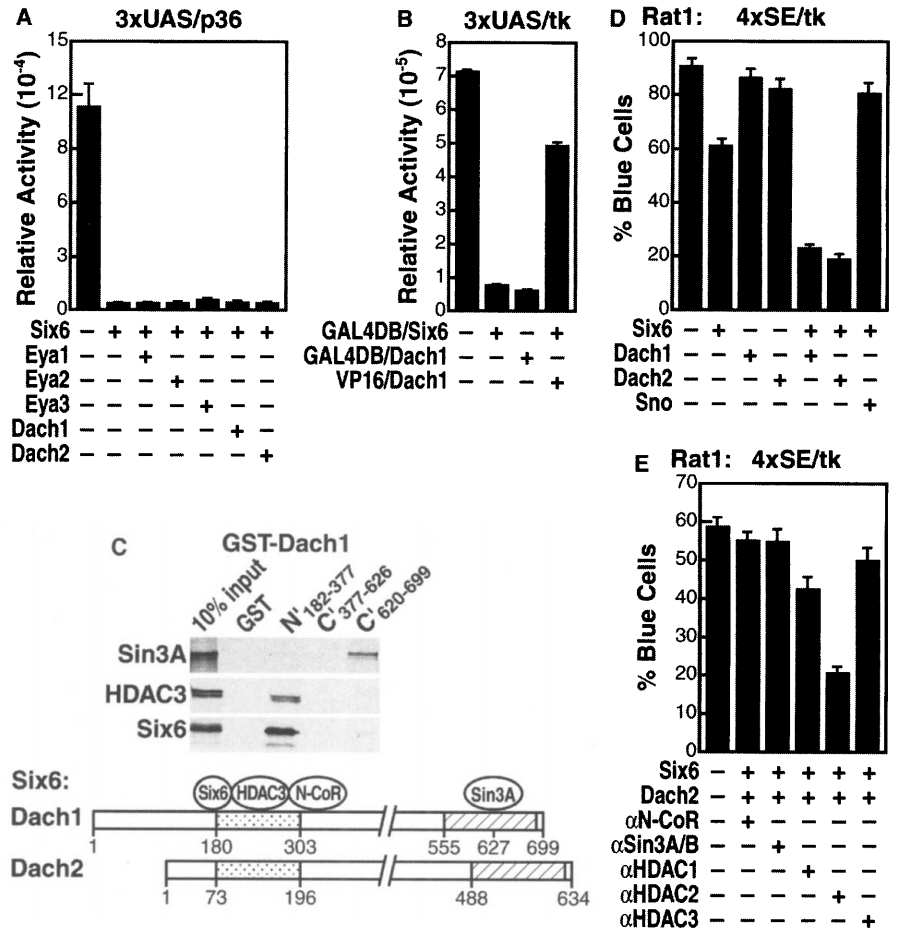

Fig. 4. Transcriptional properties of Six6 and Dach1/2. (**A**) Gal4DB/Six6 represses 3xUAS/p36 reporter, even with Eya or Dach in 293 cells. (**B**) Gal4DB/Six6 (compare first and second bar) and Gal4DB/Dach1 (compare third and first bar) repress 3xUAS/tk reporter. Six6 and Dach1 interaction in mammalian two-hybrid assay (compare fourth and first bar). (**C**) GST/Dach1 interacts with Six6 and HDAC3 through its conserved NH_2-terminus, with Sin3A/B through its conserved COOH-terminus. (**D**) Single-cell nuclear microinjection assay using Six binding element (4xSE/tk) reporter in Rat-1 cells. Six6 alone produces a mild repression, which is strongly potentiated by Dach1/2 but not by Sno. Dach1/2 alone has little or no effect. (**E**) Single-cell nuclear microinjection of specific IgGs against N-CoR, Sin3A/B, HDAC1, and HDAC3, but not HDAC2, reverses the repressive activity of Six6/Dach2. Results are the mean ± SEM; similar results were obtained in three independent experiments.

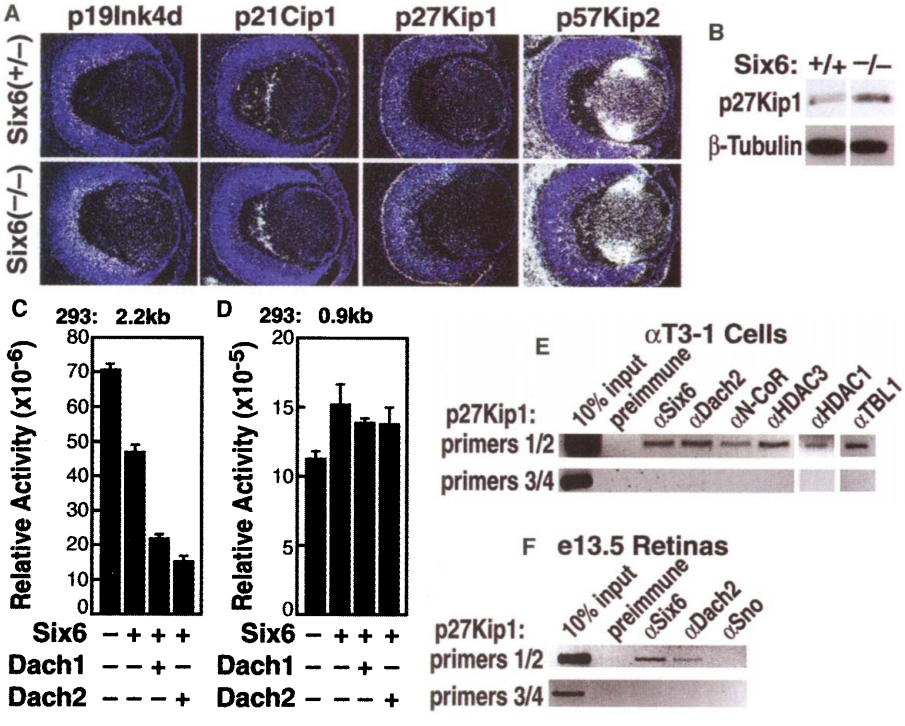

Fig. 5. Direct regulation of p27Kip1 by Six6. (**A**) In situ hybridization analyses of e13.5 retinas with specific CKI probes indicated upregulation of p19Ink4d and p27Kip1 in the Six6 mutant. (**B**) Increased p27Kip1 protein level in mutant e13.5 retina/lens was determined by Western blot analysis. β-tubulin was used as an internal control. (**C**) Six6 represses a 2.2-kb *p27Kip1* promoter in 293 cells, and repression is enhanced by coexpression of Dach1 or Dach2. (**D**) These factors exert no effect on the 0.9-kb p27Kip1 promoter. (**E**) ChIP assay of *p27Kip1* promoter on αT3-1 cells. (**F**) ChIP assay on e13.5 microdissected retinas from wild-type embryos, showing recruitment of Six6, Dach2, but not Sno, to the specific *p27Kip1* promoter region. Primer 1/2, "repressor region"; primer 3/4, coding region.