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Dnmt1-dependent DNA methylation is essential for photoreceptor terminal differentiation and retinal neuron survival

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Epigenetic regulation of the genome is critical for the emergence of diverse cell lineages during development. To understand the role of DNA methylation during retinal network formation, we generated a mouse retinal-specific *Dnmt1* deletion mutation from the onset of neurogenesis. In the hypomethylated *Dnmt1*-mutant retina, neural progenitor cells continue to proliferate, however, the cell cycle progression is altered, as revealed by an increased proportion of G1 phase cells. Despite production of all major retinal neuronal cell types in the *Dnmt1*-mutant retina, various postmitotic neurons show defective differentiation, including ectopic cell soma and aberrant dendritic morphologies. Specifically, the commitment of *Dmnt1*-deficient progenitors towards the photoreceptor fate is not affected by DNA hypomethylation, yet the initiation of photoreceptor differentiation is severely hindered, resulting in reduction and mislocalization of rhodopsin-expressing cells. In addition to compromised neuronal differentiation, *Dnmt1* deficiency also leads to rapid cell death of photoreceptors and other types of neurons in the postnatal retina. These results indicate that *Dnmt1*-dependent DNA methylation is critical for expansion of the retinal progenitor pool, as well as for maturation and survival of postmitotic neurons.

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Epigenetic regulation is essential for orchestrating tissue specific gene expression during mammalian development. Both histone modifications and DNA methylation contribute to the dynamic chromatin remodeling associated with distinct differentiation programs. Among the known DNA methyltransferases that catalyze the formation of 5-methylcytosine at the CpG dinucleotides, the activity of *Dnmt1* is required for maintaining DNA methylation patterns during DNA replication in newly synthesized daughter DNA strands,[1,2] whereas Dnmt3a and Dnmt3b are responsible for *de novo* DNA methylation.[3]

Establishing and maintaining DNA methylation patterns are important for proper development and normal function of the nervous system. In humans, defective DNA methylation is associated with a number of neurological disorders.[4] Mutations in *Dnmt1* have been linked to neurodegeneration in a form of hereditary sensory neuropathy and late-onset cerebellar ataxia, deafness, and narcolepsy.[5,6] In age-related macular degeneration, DNA hypermethylation has been associated with decreased expression of genes involved in reducing oxidative stress.[7] In the developing mouse brain, deletion of *Dnmt1* in progenitor cells impairs neuronal maturation and survival, and causes precocious astroglial differentiation.[8,9] Genome-wide analyses have revealed that Dnmt3a-dependent methylation in gene body facilitates transcription of neurogenic genes in adult neural stem cells by antagonizing Polycomb repression.[10] In postmitotic forebrain neurons, deletion of both *Dnmt1* and *Dnmt3a*, but neither one alone, leads to abnormal gene expression and synaptic plasticity,[11] suggesting that actively maintained DNA methylation is necessary for normal neural activities.

The vertebrate retina consists of six major types of neurons and the Muller glia derived from a common progenitor pool.[12] The mature retinal neurons are highly specialized cells. For example, photoreceptors display unique transcriptomes,[13–15] distinct cell morphologies, and nuclear structures.[16] Molecular analyses have shown that distinct mature retinal neurons have established cell-specific DNA methylation patterns correlating with neuronal type-specific gene expression.[17] In the developing zebrafish retina, morpholino knockdown studies have demonstrated that *Dnmt3* and *Dnmt1* have non-redundant functions and are both required for proper laminar layer formation and differentiation.[18,19] In addition, injury-induced retinal regeneration in zebrafish involves DNA demethylation in Muller glia and retinal ganglion cells (RGCs).[20] To determine the function of *Dnmt1* in mammalian retina development, we generated a retinal-specific *Dnmt1* gene deletion at the onset of mouse retinogenesis. Our analyses show that embryonic retinal development can still proceed after *Dnmt1* ablation, but maturation and

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Abbreviations: cKO, conditional knockout; *Dnmt1*, DNA methyltransferase 1; GFP, green fluorescent protein; GS, glutamine synthetase; IAP, intracisternal A-particle; ONL, outer nuclear layer; PCNA, proliferating cell nuclear antigen; PH3, phospho-histone 3; PKCα, Protein kinase C-α; RGC, retinal ganglion cell
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differentiation of postmitotic retinal neurons are severely disrupted in the absence of *Dnmt1* activity. Moreover, *Dnmt1* function is essential for retinal neuron survival, as *Dnmt1* conditional mutants show rapid postnatal retinal degeneration.

Results

Deletion of *Dnmt1* gene causes rapid degeneration of the postnatal retina. In the developing mouse retina, *Dnmt1* protein showed dynamic expression patterns as detected by immunolabeling. A higher level of *Dnmt1* was expressed in embryonic retinal progenitors and lens epithelial cells than in postmitotic retinal neurons (Supplementary Figures 1A–C). Furthermore, distinct nuclear distributions of *Dnmt1*, either diffused or as intensely aggregated puncta, were observed. As the retina approached maturity, *Dnmt1* was detected at high levels in retinal interneurons and RGCs, but nearly absent in the majority of photoreceptors (Supplementary Figures 1D–F). In adult retinas, a subset of photoreceptors, likely cone cells based on their location in the outer nuclear layer (ONL), retained a low yet detectable level of *Dnmt1* (Supplementary Figure 1).

To elucidate the function of *Dnmt1*, we generated a retinal-specific *Dnmt1* deletion using Cre-loxP recombination. Mice carrying a *Dnmt1* conditional allele with loxP sites flanking exons 4 and 5 were crossed with a Chx10-Cre driver strain expressing a Cre-green fluorescent protein (GFP) fusion protein in retinal progenitors at embryonic day 10.5,[21] to yield a mutant *Dnmt1* protein lacking the catalytic domain.[22] Immunocytochemistry showed that compared with the *Dnmt1* heterozygous mutant (*Dnmt1*$^{fl/+}$; Chx10-Cre) *Dnmt1* conditional knockout retinas (*Dnmt1*$^{fl/fl}$; Chx10-Cre, referred as cKO) contained drastically reduced *Dnmt1* at postnatal day 3 (P3) (Figure 1a). A few residual *Dnmt1*$^{+}$ cells were negative for GFP, due to the mosaic expression of Cre.[23] Furthermore, western blots showed more than a 90% reduction of *Dnmt1* protein in *Dnmt1* cKO mutants (Figure 1b). To assess the DNA methylation status, we performed immunolabeling for intracisternal A-particle (IAP), a retroviral protein expressed only under hypomethylation conditions.[8,22] In contrast to the complete absence of IAP expression in heterozygous controls, *Dnmt1* cKO mutants showed intense labeling of IAP (Figure 1c and Supplementary Figure 2).

Histological analyses showed that *Dnmt1* cKO retinas were reduced in size at P0 compared with controls (Supplementary

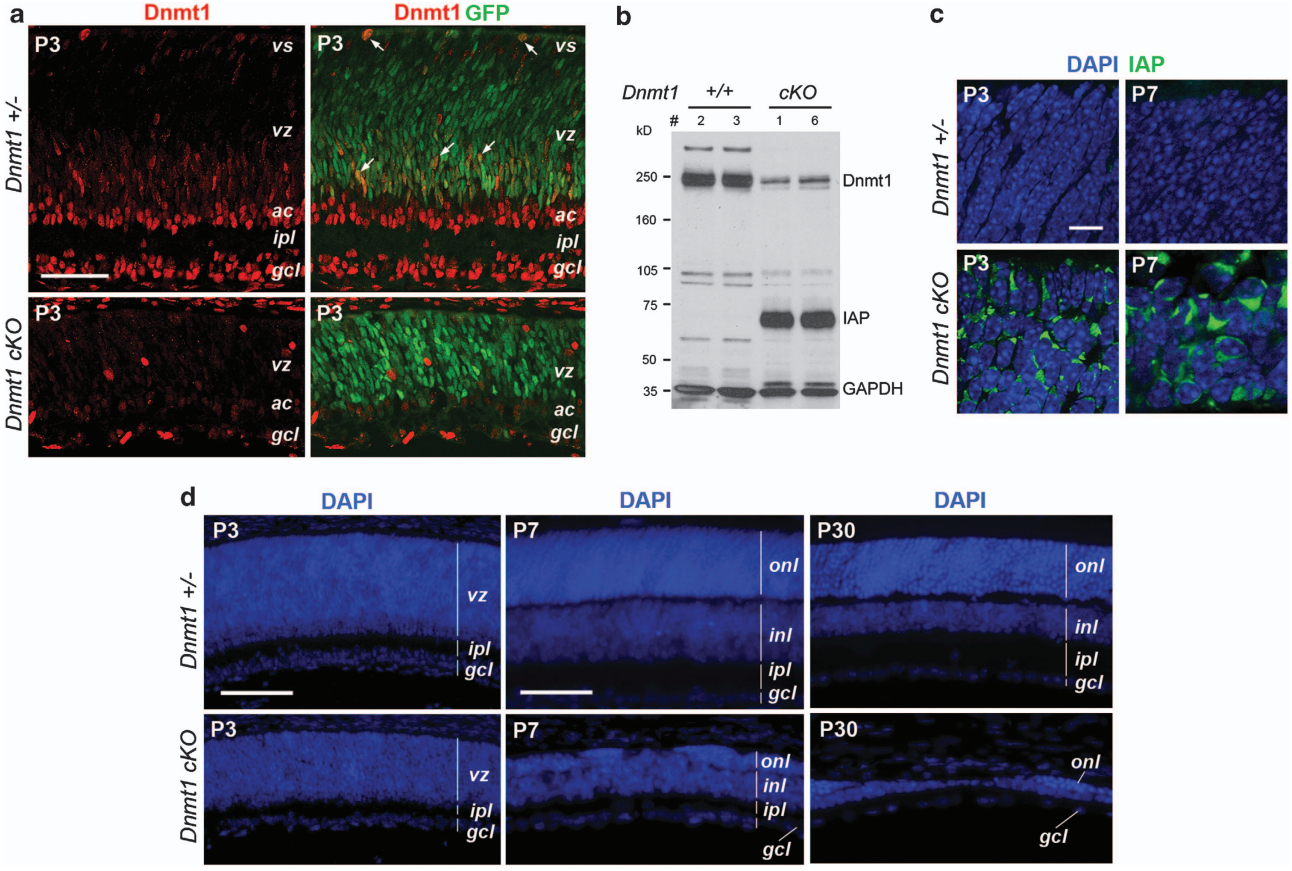

Figure 1 Effects of retinal-specific deletion of *Dnmt1* by Chx10-Cre. (**a**) Confocal micrographs of retinas co-labeled for *Dnmt1* (red) and GFP (green) at P3. The *Dnmt1* cKO retina shows reduced *Dnmt1* protein expression compared with the heterozygous control. White arrows point to cells co-expressing *Dnmt1* and Cre-GFP. (**b**) Western blots show reduction of *Dnmt1* protein in two individual P3 *Dnmt1* cKO mutant retinas that also express high levels of IAP. (**c**) Confocal images of the outer retinas co-labeled for DAPI and IAP at P3 and P7. *Dnmt1* cKO mutant retinas contain IAP^{+} cells with altered nuclear morphology. (**d**) DAPI labeling shows progressive thinning of *Dnmt1*-mutant retina at P3, P7 and P30. Scale bars, (**a**) 50 μm, (**c**) 10 μm, (**d**) P3, 100 μm; P7 and P30, 50 μm. *ac*, amacrine cells; *gcl*, ganglion cell layer; *inl*, inner nuclear layer; *ipl*, inner plexiform layer; *vs*, ventricular surface; *vz*, ventricular zone

Figure 3). Although *Dnmt1*-mutant retinas were only 20–30% thinner than heterozygous controls at P3, between P3 and P7, the mutant retina showed rapid reduction in thickness, especially in the ONL (Figure 1d). This trend continued till P30, resulting in the loss of most retinal cells (Figure 1d and Supplementary Figure 3).

***Dnmt1* gene deletion affects progenitor cell cycle progression and exit.** We next examined the effects of *Dnmt1* deletion on retinal progenitor. Immunolabeling for GFP$^+$ expressed by progenitors in Chx10-cre mice[23] and for the proliferating cell nuclear antigen showed abnormal distributions of progenitors in the ventricular zone in *Dnmt1* cKO mutants at P3 (Figures 2a–d). However, immunolabeling for the M-phase marker phospho-histone 3 (PH3) did not detect significant differences between the control and *Dnmt1* mutants, except for occasional ectopic PH3$^+$ cells (Figures 2e and f).

To assess progenitor behavior during the cell cycle, we performed fluorescence-activated cell sorting analyses of GFP$^+$ cells and the cyclin-dependent protein kinase inhibitor p27^{Kip1}, which is upregulated in cells poised to exit or already withdrawn from the cell cycle. The percentages of GFP$^+$ cells among total cells were not altered by the *Dnmt1* mutation at P2 (Figures 2g and h). Analyses of the cell cycle showed that in *Dnmt1* mutants, the distribution of GFP$^+$ progenitors were significantly increased in G1 phase from 57.6 ± 1.0 to 69.8 ± 2.75% and decreased in G2/M phase from 19.6 ± 0.38

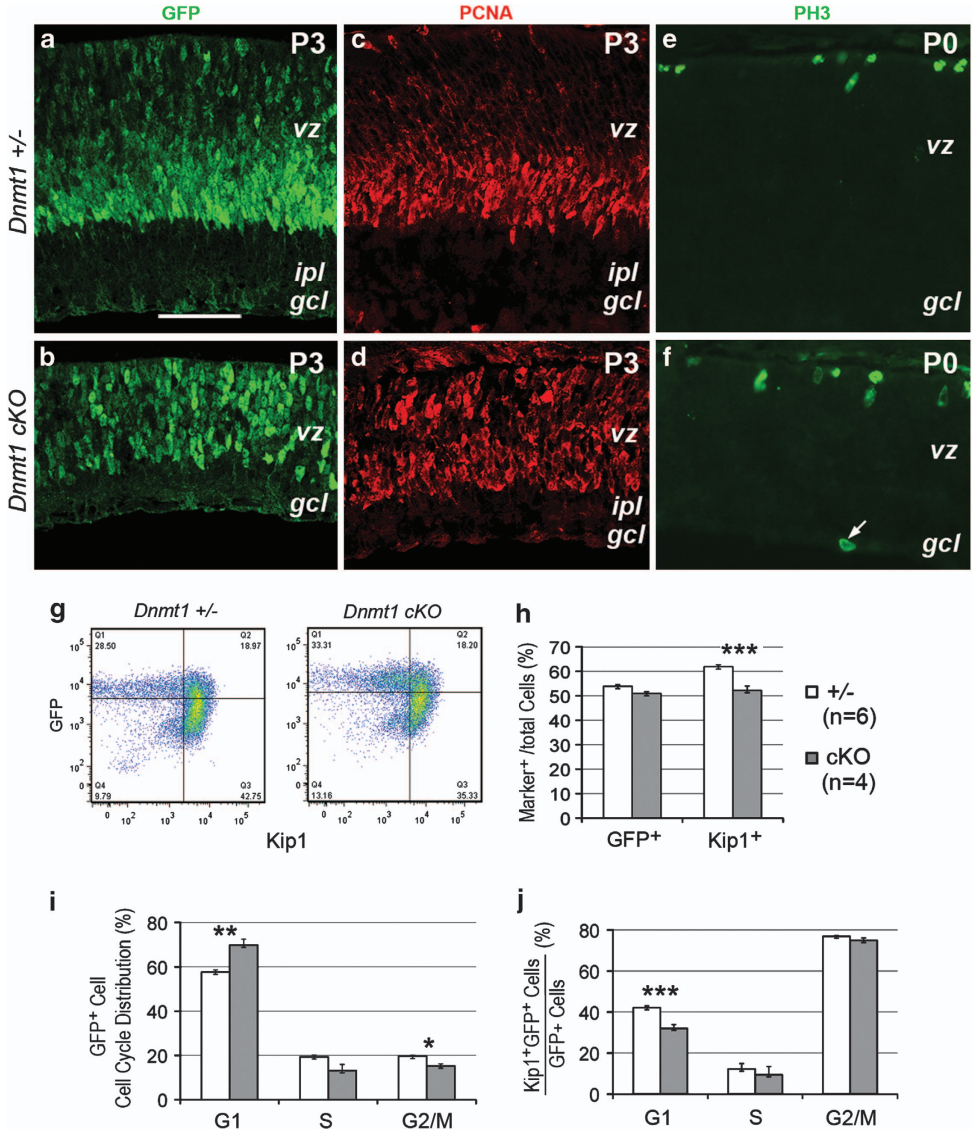

Figure 2 *Dnmt1* deletion alters progenitor distribution and cell cycle progression. (**a**–**f**) Immunolabeling of retinas show distribution of progenitor markers GFP (**a** and **b**) and proliferating cell nuclear antigen (PCNA, **c** and **d**) at P3, and M-phase marker PH3 (**e** and **f**) at P0. Arrow in **f** indicates an ectopic PH3$^+$ cell. Scale bar, (**a**) for (**a**–**f**) 50 μm. *gcl,* ganglion cell layer; *ipl,* inner plexiform layer; *vz*, ventricular zone. (**g**–**j**) Flow cytometric analyses of cell markers and cell cycle properties at P2. (**g**) Representative flow cytometry profiles for GFP and cyclin-dependent protein kinase inhibitor p27^{Kip1}. (**h**) Fluorescence-activated cell sorting quantification of GFP$^+$ cells and p27^{Kip1+} cells among total cells. (**i**) Cell cycle distributions of GFP$^+$ progenitors. (**j**) Quantification of p27^{Kip1+} GFP$^+$ cells among GFP$^+$ cells in different cell cycle phases. Numbers of individual retinas analyzed in (**h**–**j**) are indicated in **h**. *$P<0.05$, **$P<0.01$, ***$P<0.001$

to 15.2 ± 0.92% (Figure 2i), revealing defects in cell cycle progression and abnormal G1 accumulation.

In *Dnmt1* mutant retinas, the percentage of $p27^{Kip1+}$ cells among total cells was significantly reduced from 61.9 ± 0.72 to 52.2 ± 1.77% (Figure 2h), suggesting a reduction of either postmitotic neurons or progenitors exiting the cell cycle. Analyses of cell cycle distribution revealed that control and mutant retinas contained similar percentages (75–77%) of $p27^{Kip1+}$ cells among G2/M-phase GFP^+ progenitors at P2, indicating that mutant cells were able to upregulate $p27^{Kip1}$. However, *Dnmt1* mutant retinas showed a significant decrease of $p27^{Kip1+}$ GFP^+ cells from 42.1 ± 0.9% to 32.0 ± 1.85% among the 2n G0 cell population (Figure 2j), suggesting the defect occurred during or soon after the M phase to G0 phase transition. Together, these results demonstrated that the *Dnmt1* mutation affected progenitor cell cycle progression, and resulted in a defective cell cycle exit or faltered initial steps in becoming postmitotic neurons.

***Dnmt1* deficiency affects retinal neuronal production.** We next examined the effects of *Dnmt1* deletion on the production of embryonically born retinal neurons. Immunolabeling with the RGC marker Brn3a at P0 detected fewer RGCs, and many were mislocalized in the *Dnmt1* cKO mutant (Figures 3a and b). Interestingly, unlike the rest of the retina, less IAP was detected among RGCs (Figures 3i and j), indicating that either a small portion of RGCs were produced before *Dnmt1* deletion or this subset of RGCs had retained a higher level of DNA methylation. Another early born retinal neuronal type, the horizontal cells, exhibited abnormally exuberant dendritic arbors in the *Dnmt1* mutant as detected by neurofilament labeling (Figures 3e and f). Immunolabeling for the amacrine cell marker AP2α showed a reduced amacrine interneuron population due to *Dnmt1* deficiency at P3 (Figures 3c and d).

Among postnatally produced cell types, the bipolar cells were clearly present in *Dnmt1*-mutant retinas, as indicated by GFP signals driven by the Chx10 promoter and the rod bipolar

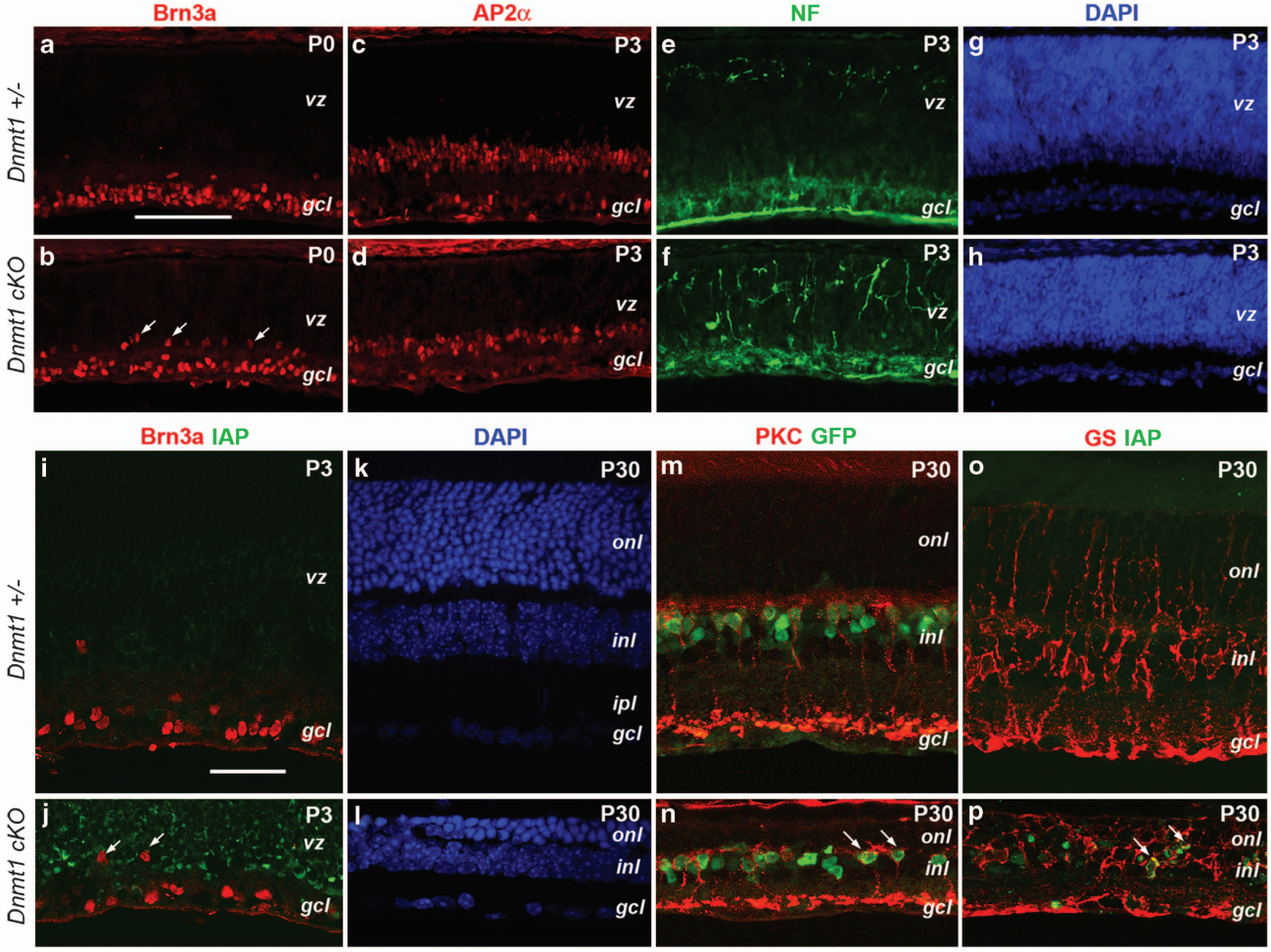

Figure 3 Effects of *Dnmt1* deletion on retinal cell type production. (**a–j**) Immunolabeling of early born cell types in the neonatal retina. Cell marker labeling for RGCs (**a** and **b**), amacrine cells (**c** and **d**), horizontal cells (**e** and **f**) are presented. **i** and **j** shows co-labeling of Brn3a and IAP. White arrows (**b** and **j**) point to mislocalized RGCs. DAPI staining (**g** and **h**) shows retinal laminar structures at P3. (**k–p**) Confocal images of P30 retinas for late-born retinal cell types. Cell marker labeling for bipolar cells (**m** and **n**) and Muller cells (**o** and **p**) are presented. Arrows in **n** point to rod bipolar cells that are double labeled for Cre-GFP and protein kinase C-α (PKCα), and **p** indicate mutant Muller cell double labeled for IAP and glutamine synthetase (GS). Scale bars, **a** for **a–h**, 100 μm; **i** for **i–p**, 50 μm. *gcl*, ganglion cell layer; *inl*, inner nuclear layer; *vz*, ventricular zone

cell marker protein kinase C-α (Figures 3m and n). We also detected the presence of cells co-labeled by the Müller glial marker glutamine synthetase and IAP, indicating that *Dnmt1*-deficient progenitors gave rise to Müller glia (Figures 3o and p). These results demonstrated that progenitors in *Dnmt1* cKO mutant retinas were able to generate most retinal cell types.

***Dnmt1* activity is essential for photoreceptor differentiation and survival.** The rapid demise of the ONL in *Dnmt1*-mutant retinas suggested that photoreceptor development was impaired. We first investigated the commitment of progenitors toward photoreceptor fates by detecting the homeobox protein Otx2, an essential positive regulator of photoreceptor production.[24] Similar expression patterns of Otx2 were detected among a subset of GFP$^+$ progenitors in *Dnmt1* heterozygous and cKO mutant retinas at P3 (Figures 4a–d). We also examined expression of Crx, a homeobox protein turned on in postmitotic photoreceptor precursors and required for both rod and cone photoreceptor differentiation.[25–27] Although Crx was detected in both control and *Dnmt1* cKO mutants, Crx$^+$ cells in the mutant retina were dispersed throughout the ventricular zone rather than localized near the future ONL (Figures 4e and f). Moreover, abnormal co-expression of Crx and the progenitor cell marker GFP was also observed in *Dnmt1* mutants (Figures 4f and f').

We next examined the onset of photoreceptor differentiation. At P3, *Dnmt1* mutant retinas showed a reduced number of cells expressing the early photoreceptor marker recoverin (Figures 4g and h). Furthermore, rod-specific rhodopsin

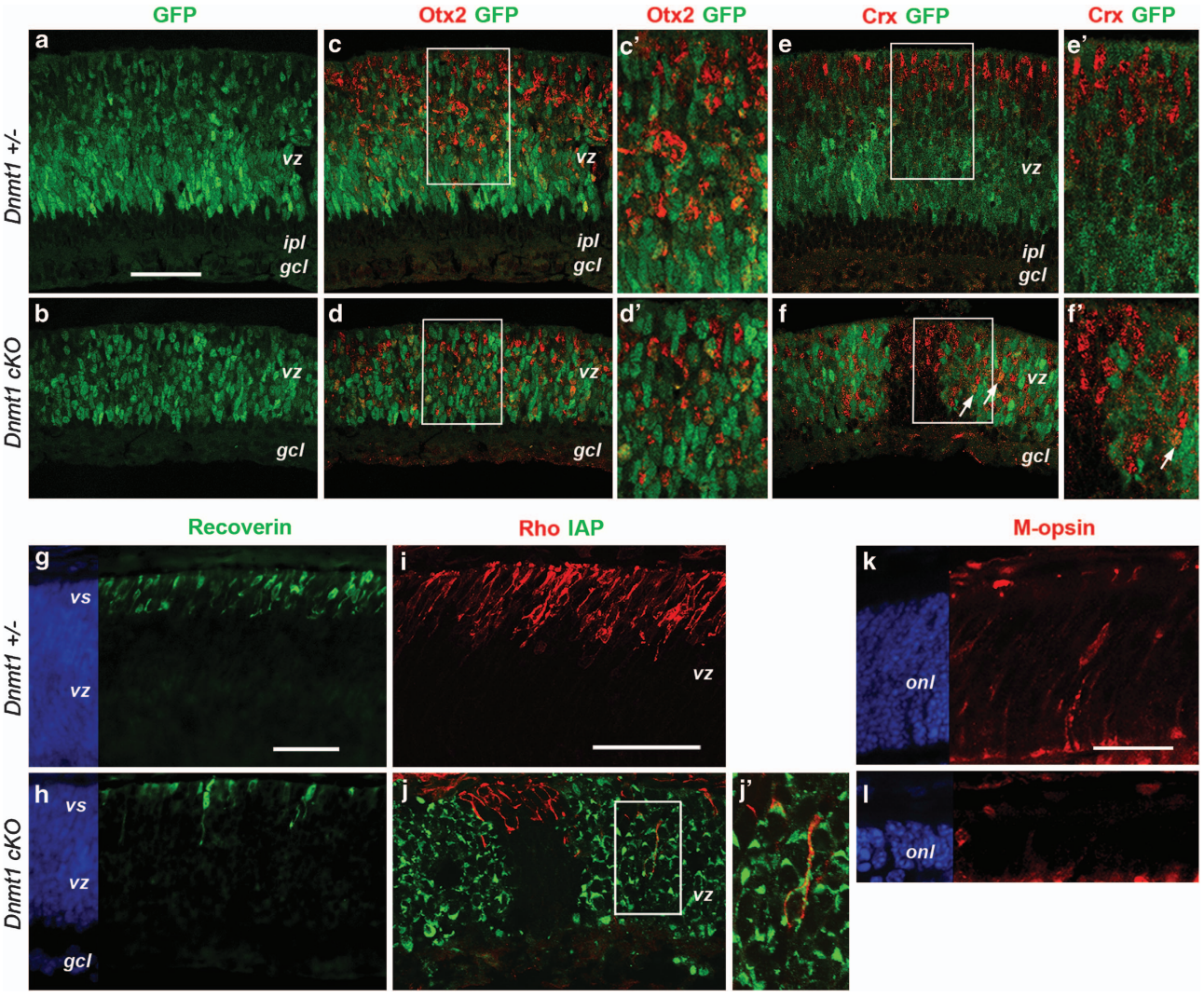

Figure 4 Effects of *Dnmt1* mutation on photoreceptor commitment and differentiation. (**a**–**f'**) Confocal images of co-immunolabeling at P3 for Otx2 and GFP (**a**–**d'**), or Crx and GFP (**e**–**f'**). **c'**, **d'** and **e'**, **f'** show twofold magnification of framed areas in **c**, **d** and **e**, **f**, respectively. The Crx$^+$ cells in control (**e** and **e'**) and in Cre$^-$ regions of the mutant (**f** and **f'**) are located near the ventricular surface; but *Dnmt1*-deficient Crx$^+$ cells are distributed throughout vz (**f** and **f'**). Arrows indicate co-labeled GFP$^+$ Crx$^+$ cells. (**g** and **h**) Immunolabeling for recoverin and DAPI at P3. (**i**, **j** and **j'**) Confocal images of co-labeling for Rho (red) and IAP (green) at P3. A lack of Rho$^+$ cells is seen in *Dnmt1*-deficient regions expressing IAP (**j**). A rare Rho and IAP co-labeled cell framed in **j** is shown in **j'** at twofold magnification. (**k** and **l**) Confocal images of immunolabeling for M-opsin at P3. Scale bars, **a** for **a**–**d**, **e** and **f**, **g** for **g** and **h**, **i** for **i** and **j**, 50 μm; **k** for **k** and **l**, 25 μm. *gcl,* ganglion cell layer; *ipl,* inner plexiform layer; ventricular surface; *vz,* ventricular zone

expression was significantly reduced in *Dnmt1*-deficient cells that were IAP$^+$ (Figures 4i and j). Confocal imaging detected very few rhodopsin and IAP double-positive cells (Figures 4j and j'). Similarly, M-opsin expression by cone photoreceptors was greatly diminished in *Dnmt1*-mutant retinas (Figures 4k and l). These data demonstrated that the commitment of progenitors toward a photoreceptor cell fate was not affected by DNA hypomethylation, but the onset and subsequent differentiation of photoreceptors were blocked.

Further evaluation of DNA methylation status of photoreceptor-specific genes using bisulfite sequencing showed a significant demethylation at CpG sites in both *Rhodopsin* and *M opsin* promoter regions in *Dnmt1* cKO mutants at P3 (Figure 5a). To determine whether the rapid reduction of retinal cell numbers was due to increased apoptosis, we labeled retinas for activated caspase 3. Compared with the heterozygous control retina, *Dnmt1* cKO mutants showed significantly increased caspase 3 signals throughout the retina (Figures 5b and c). Furthermore, increased numbers of pyknotic nuclei were observed among *Dnmt1*-deficient cells, which failed to express rhodopsin (Figures 5d-g), suggesting an increased apoptosis of postmitotic photoreceptors.

Discussion

We have addressed the role of *Dnmt1* during mammalian retinogenesis using retinal-specific gene ablation. The conditional *Dnmt1* mutant yields a truncated protein without most of the replication fork interaction and the entire methyltransferase catalytic domain.[22] Our results demonstrate that maintaining DNA methylation status by *Dnmt1* is critical for mammalian retinal development. Although progenitor proliferation persists in the embryonic retina, the proper differentiation of retinal neurons is severely impaired due to *Dnmt1* deficiency. Moreover, DNA hypomethylation leads to rapid retinal degeneration.

The high levels of *Dnmt1* protein expression in early retinal progenitors are consistent with its role in maintaining DNA methylation patterns as cells undergo rapid division. However, it is unexpected that mature retinal interneurons and projection neurons retain high levels of *Dnmt1* expression. Our

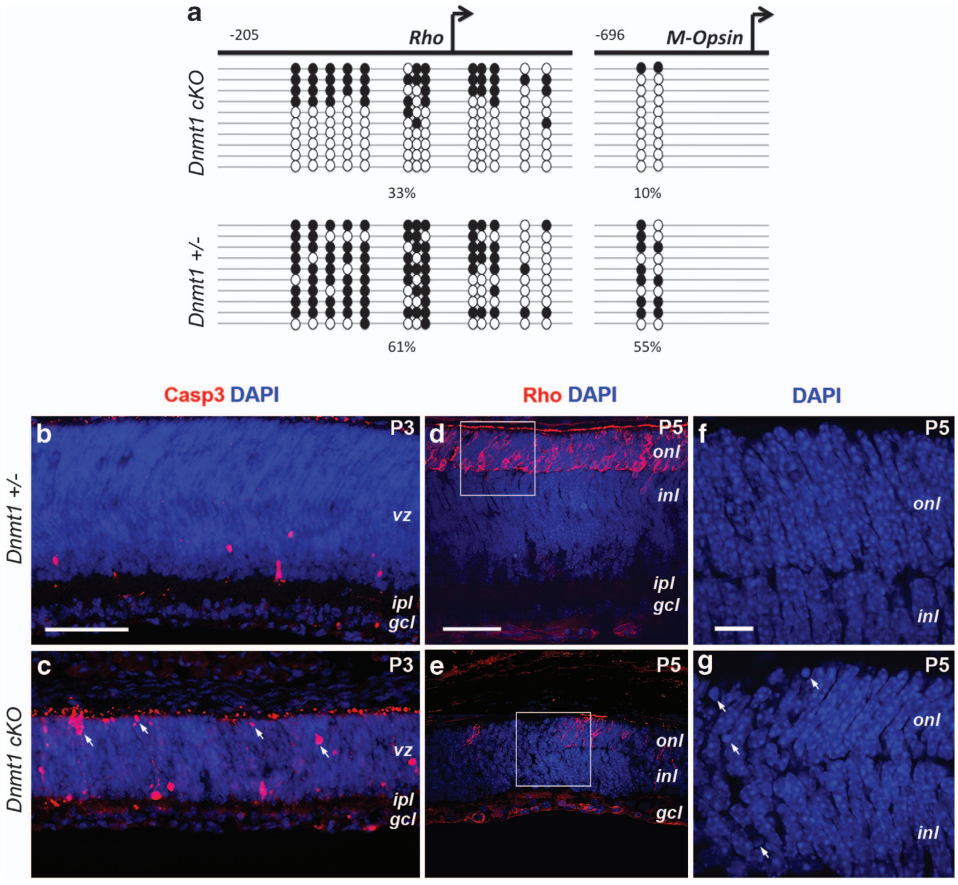

Figure 5 *Dnmt1* ablation causes DNA hypomethylation and cell death in the postnatal retina. (**a**) DNA methylation patterns at Rhodopsin and M-opsin promoters as determined by bisulfide sequencing. Significantly reduced CpG dinucleotide methylation is detected at P3 in the mutant retina. Columns correspond to CpG sites with the relative location from the transcription-starting sites (arrows), while rows correspond to sequenced clones. Solid circles indicate methylated CpG; open circles indicate unmethylated CpG. (**b** and **c**) Immunolabeling for activated caspase 3 shows increased apoptosis in *Dnmt1* cKO retinas at P3. Note that Casp3$^+$ cells are distributed throughout the mutant retina. (**d** and **e**) Co-labeling of DAPI and Rho at P5. **f** and **g** represent magnified regions framed in **d** and **e** to show condensed nuclei (arrows) present in mutant cells. Scale bars, **b** for **b** and **c**, **d** for **d** and **e**, 50 μm; **f** for **f** and **g**, 10 μm. *gcl,* ganglion cell layer; *inl,* inner nuclear layer; *ipl,* inner plexiform layer; *vz*, ventricular zone

results are consistent with a recent study,[28] which describes overlapping expression patterns of Dmnt3a and Dnmt3b with *Dnmt1* in RGCs, amacrine cells and cone photoreceptors. It is plausible that *Dnmt1* activity complements other DNA methyltransferases to maintain neuronal function. In zebrafish, both *Dnmt1* and Dnmt3 are required for retinal development, but engage distinct downstream chromatin modifiers.[18,19] Knocking down *Dnmt1* in zebrafish retina reduces differentiated photoreceptor marker, but without rapid ONL cell death. Accumulating evidence suggest that the activity of *Dnmt1* may not be limited to maintaining methylation patterns. For example, in the adult mouse brain, *Dnmt1* and *Dnmt3a* are both required for normal function and neural plasticity.[11] Furthermore, chromatin remodeling is clearly involved in brain and retinal stem cell potentiation.[10,20] Therefore, the potential function of *Dnmt1* in mature retinal neurons and during neural repair warrants further investigation.

Previous studies have shown that loss of *Dnmt1* activity results in progressive hypomethylation at CpG dinucleotides as progenitor cells undergo multiple rounds of cell divisions.[8,9] Although the onset of Chx10-Cre expression coincides with the initiation of retinal neurogenesis, effects of *Dnmt1* deletion may be cumulative. The low IAP expression in RGCs suggests that this early born cell type retains a certain level of DNA methylation. In contrast, another early born neuronal type, the horizontal cell, displays extensive IAP expression and dendritic abnormalities. Therefore, the lack of hypomethylation in RGCs may be due to compensation by other Dnmts expressed by RGCs.[28] Our results show that despite ablation of *Dnmt1* from the onset of retinogenesis, all retinal cell types are produced from *Dnmt1*-deficient progenitors. However, the impact of the mutation becomes increasingly severe, likely reflecting the increasing degree of DNA hypomethylation.

The effect of the *Dnmt1* deletion on embryonic retinal progenitor proliferation is relatively mild. However, in the postnatal stages, *Dnmt1* deficiency alters cell cycle progression resulting in an increased distribution of G1 phase progenitors. At P2, the majority of progenitors are typically ready to exit the cell cycle thus upregulating the $p27^{Kip1}$ as they enter the M phase. Our results show that *Dnmt1* mutant cells can upregulate $p27^{Kip1}$ during M, but then quickly suffer a significant loss as they transition into the G0 phase and become postmitotic. Therefore, the overall reduction of $p27^{Kip1+}$ cells in *Dnmt1*-mutant retinas is likely due to that many nascent neurons with hypomethylated genomes are unable to execute their specific differentiation programs and soon undergo apoptosis. This conclusion is consistent with the rapid cell loss in *Dnmt1* cKO retinas within the first postnatal week.

Photoreceptor cells are highly specialized sensory neurons with distinct transcriptomes. The nuclear structure of nascent rod photoreceptors undergoes extensive reorganization as they approach maturity,[16] and establish cell type-specific DNA methylation patterns.[17] Establishing a rod-specific transcription program is likely to involve coordination between DNA methylation and histone modifications under the direction of photoreceptor-specific transcription factors.[29] Commitment by proliferating progenitors towards a photoreceptor fate occurs in sequential steps[30] and is initiated by expression of Otx2, followed by upregulation of Crx as the committed cells withdraw from the cell cycle to become photoreceptor precursors.[24,27,31] Our results show that *Dnmt1* deficiency does not affect progenitors commitment to the photoreceptor fate, as Otx2 expression appears normal. Furthermore, we demonstrate that the onset of Crx expression is also not affected. However, the co-expression of Crx and progenitor markers and mislocalization of both Crx^+ and $rhodopsin^+$ cells indicate that DNA methylation defects interfere with further differentiation of photoreceptor precursors. Interestingly, bisulfide sequencing confirms that in *Dnmt1* cKO mutants, the CpG sites in both *Rhodopsin* and *M-opsin* promoters are hypomethylated at P3, and yet unable to sustain photoreceptor differentiation. This may reflect that hypomethylation alone is insufficient in sustaining and establishing photoreceptor-specific transcriptions. Moreover, failed neuronal differentiation results in rapid and massive cell death as previous observed in other neuronal cell types.[8]

Our study demonstrates the critical function of *Dnmt1* during retinal development, especially photoreceptor differentiation and survival. Recent evidence indicates that DNMT1 mutations can lead to progressive neurodegeneration and late-onset neuropathies in humans.[5,6] Furthermore, decline in DNA methylation has also been observed in age-related macular degeneration.[7] Future investigation on maintaining DNA methylation and epigenetic regulation in the mature retina may provide importance insights on disease mechanisms and treatment strategies.

Materials and Methods

Animals. $Dnmt1^{fl/fl}$ mice previously described[22] were crossed with heterozygous males carrying the $Dnmt1^{fl/+}$ allele and a Cre-GFP fusion transgene under the control of the *Chx10* locus.[21] The mouse lines were maintained in C57bl6 background. Genotypes were determined by PCR using primers for *Dnmt1* (forward: 5′-GGG CCA GTT GTG TGA CTT GG-3′, reverse: 5′-CTT GGG CCT GGA TCT TGG GGA TC-3′) and the *Cre* transgene (forward: 5′-GAC GAT GCA ACG AGT GAT GA-3′, reverse: 5′-AGC ATT GCT GTC ACT TGG TC-3′), respectively. All animal procedures followed NIH guidelines and were approved by the Animal Research Committee at University of California Los Angeles.

Immunocytochemistry. Cryosections fixed in 4% paraformaldehyde were processed for immunocytochemistry as described.[27] Antibodies used for immunolabeling are summarized in Supplementary Table 1. Immunofluorescent images were captured using Nikon E800 (Nikon, Tokyo, Japan) or Olympus FluoView 1000 confocal microscopes (Olympus, Tokyo, Japan). Samples from multiple animals (minimal, $n \geq 3$) of each genotype were analyzed.

Western blots. Cell lysates of P3 retinas were processed as described.[27] Signals of horseradish peroxidase conjugated to secondary antibodies were detected using ECL (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Multiple retinal extracts (minimal, $n \geq 3$) of different genotypes were analyzed.

Flow cytometry. Dissociated P2 retinal cells were immunolabeled and analyzed using an LSRII flow cytometer (BD Biosciences, San Jose, CA, USA) as described.[23] Individual samples from multiple heterozygous ($n = 6$) or cKO ($n = 4$) retinas were analyzed. Flow cytometry data were analyzed with FlowJo (Tree Star, Ashland, OR, USA). Quantification of markers is shown as mean ± S.E.M. after statistical analysis using Student's *t*-Test. P values < 0.05 were considered significant.

Methylation analysis. Genomic DNA isolated from P3 retinas were processed with bisulfite conversion using EZ DNA Methylation Kit (Zymo) by following the manufacture instructions. The Meth-primers are described previously.[17] Amplified PCR products were purified and cloned into the TOPO vector (Life Technologies, Grand Island, NY, USA). Ten individual control ($n = 10$)

and *Dnmt1*-mutant ($n = 10$) clones were sequenced to detect methylation patterns.

Conflict of Interest

The authors declare no conflict of interest.

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