Epigenetic regulation of retinal development and disease

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Abstract Epigenetic regulation, including DNA methylation, histone modifications, and chromosomal organization, is emerging as a new layer of transcriptional regulation in retinal development and maintenance. Guided by intrinsic transcription factors and extrinsic signaling molecules, epigenetic regulation can activate and/or repress the expression of specific sets of genes, therefore playing an important role in retinal cell fate specification and terminal differentiation during development as well as maintaining cell function and survival in adults. Here, we review the major findings that have linked these mechanisms to the development and maintenance of retinal structure and function, with a focus on ganglion cells and photoreceptors. The mechanisms of epigenetic regulation

are highly complex and vary among different cell types. Understanding the basic principles of these mechanisms and their regulatory pathways may provide new insight into the pathogenesis of retinal diseases associated with transcription dysregulation, and new therapeutic strategies for treatment.

Keywords Chromatin modifications · Cell type-specific transcription · Neuronal development and disease

Abbreviations

HKM	Histone lysine methylation
HKMTase	Histone methyltransferase
HKDMase	Histone lysine demethylase
DNMTase	DNA methyltransferase
HAT (KAT)	Histone lysine acetyltransferase
HDAC	Histone deacetylase
CT	Chromatin territory
TxF	Transcription factory

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Introduction

Appropriate regulation of gene expression is essential for retinal development and maintenance. Under- or over-expression of certain genes can lead to retinal developmental defects or degeneration. For example, loss of expression or over-expression of the *rhodopsin* gene in mouse retina leads to photoreceptor degeneration [1, 2]. Many retinal diseases, regardless of the primary insults, often show changes of gene expression in target cells, such as abnormal ganglion cell gene expression in glaucoma [3–5]. Over the years, researchers have identified many specific transcription factors expressed by different retinal cell types, which bind to their responsive DNA targets and regulate transcription [6–8]. However, recent studies have shown that

transcription regulation is not as simple as direct interactions between transcription factors and their responsive elements on naked DNA. Epigenetics, which refers to changes in gene expression that do not involve changes in DNA sequence, represents a new regulation of retinal development and maintenance.

Human genomic DNA is organized as the nucleoprotein polymer structure known as chromatin. The genetic information that is stored in the primary sequence of the DNAs generally remains stable. While individual retinal cells contain essentially identical complementary DNA sequences, their differences in gene expression regulation determine the diversity of cellular phenotypes and functions. As illustrated in Fig. 1, the primary mechanisms of epigenetic regulation are

controlled at three different levels: (1) DNA methylation; (2) histone modifications, and (3) chromosomal organization.

Level 1—DNA double helix

This includes methylation of cytosine bases at CpG sites of specific genomic regions during final cell division by DNA methyltransferases (DNMTases), which marks the genomic regions for transcription repression in a cell-type specific manner (e.g., genomic imprinting) [10]. This level also includes linear organization of gene clusters containing multiple functionally related genes that are co-regulated by a common enhancer, such as the locus control region *LCR* (e.g., the *red/green opsin* gene cluster [11–13]), or gene

Fig. 1 Genomic organization and epigenetic regulation in mammalian nucleus (adapted from Zhou et al. [9]). Different levels of chromatin organization are generally associated with inactive (off) or active (on) transcription. Level 1: Genomic DNA is methylated (Me) on cytosine bases in specific contexts, turning transcription off; Level 2: Genomic DNA is packaged into nucleosomes, which vary in histone composition and histone modifications [e.g., histone H3 lysine 9 trimethylation (H3K9me3); histone H3 lysine 9 acetylation (H3K9ac)]. Here, different histone modifications are indicated by colored dots and histone variants

such as H2A.Z are brown. DNA in chromatin may remain accessible to DNA-binding proteins such as transcription factors (TFs) and RNA polymerase II (RNAPII) or may be further compacted, which can be regulated by ATP-dependent nucleosome positioning in addition to histone modifications; Level 3: Chromatin can also organize into higher-order structures through intra- and inter-chromosomal interactions, such as nuclear lamina-associated domains and transcription factories shown here

clusters that are dynamically regulated during development (e.g., the β -globin locus)[14].

Level 2—nucleosomes

The core histones, H2A, H2B, H3, and H4, two of each, form octamers to package DNA double helix into structural units called nucleosomes. During cell division, differentiation, maintenance, and replacement, the N-terminal tails of core histone proteins are “marked” with any of several post-translational modifications, including methylation, acetylation, ubiquitination, and SUMOylation on the lysine residue, phosphorylation on serine and methylation on arginine (see Tables 1, 2, 3, 4, and 5). These modifications ensure that the multi-protein complexes that regulate transcription, replication and repair of DNA are directed to the correct region of the genome at the appropriate time. Histone marks are dynamically regulated by sets of enzymes that covalently couple or remove chemical groups on amino acid residues within the histone peptides. Some marks, particularly acetylation of lysine residues in histone H3 and H4 and methylation of lysine 4 (K4) in histone H3, are associated with transcriptionally active genes. Other marks, such as trimethylation of K9 and K27 of histone H3 and K20 of histone H4, are generally associated with transcriptional silencing. The enzymes responsible for these histone marks can be highly specific for a particular amino acid residue in one histone protein, and they are often components of a macromolecular complex that is part of the transcription or silencing “machinery” recruited to a particular gene locus by tissue-specific transcription factors. In general, histone marks are evidence of genetic regulatory mechanisms, although not necessarily the regulatory mediators themselves [47].

In addition to the highly conserved major histone species, some minor histone variants are also involved in transcription regulation in specific cell types, such as H2A.Z [48]. Nucleosomes do not evenly distribute to genomic sequences, and their locations are subject to repositioning by transcription regulators and ATP-dependent remodeling complexes, which affect the accessibility of a given genomic region to the transcription machinery [49]. For example, the transcription start site of an active gene can be nucleosome-free to allow the transcription machinery associated with RNA polymerases to access their sites for active transcription (Fig. 1).

Level 3—higher-order chromosomal organizations

Nucleosomes are further packaged into 30 nm chromosomal fibers. Various genomic regions in the fiber undergo intra- and inter-chromosomal contacts to form chromosomal territories (CTs) with distinct active (open) and repressed

(closed) domains. These domains are spatially organized within the nucleus (subnuclear compartments), including transcription factories (TxFs) formed by active CTs (for reviews, see [50, 51], and the section “Higher-order chromosomal organizations and transcription”).

All these three levels of genetic organization regulate transcription without changing the sequences of DNA, and thus fall into the category of epigenetic regulation. In addition to these three levels of epigenetic regulations, emerging evidence also indicates a specific group of micro-RNAs that can directly target effectors of the epigenetic machinery, such as DNA methyltransferases, histone deacetylases, and polycomb repressive complex genes. The interaction between non-coding RNA (including long and micro RNAs) and the epigenome represents a new layer of complexity in gene regulation [52–57]. To date, our knowledge of epigenetic regulation mostly comes from studies in non-visual systems. However, in recent years, significant progress has been made in understanding eye-specific epigenetic regulation, particularly in mammalian retinas. Here, we review this recent progress with an emphasis on chromatin modifications and the biological relevance of these epigenetic changes to normal function and disease of the mammalian retina. This is an exciting and rapidly evolving area of research, with much promise for potential clinical intervention for treating retinal diseases and reversing blindness.

Chromatin modifications in retinal ganglion cells and choroidal melanocytes

Adult retinal ganglion cells (RGCs) are postmitotic neurons located within the innermost nuclear layer of the mature retina. As the only output neurons of the retina, RGCs possess the unique ability to process and project information from the retina to the visual centers in the brain. Originating from multipotent, heterogeneous progenitor cells, RGCs are the first retinal neurons to be produced during vertebral retinogenesis. These neurons originate between embryonic day 13 (E13) and E19 in rats and E11 to postnatal day 0 (P0) in mice. Specification of cell types and functions ultimately rests on epigenetic regulations which make the transition of cell differentiation irreversible. These include DNA and histone modifications on the promoters of genes and chromatin remodeling. Epigenetic modification of RGCs particularly is of value in various ophthalmic conditions that include glaucoma, optic neuritis, ischemia, trauma, and neurological degenerative conditions in the CNS.

Histone methylation in retinal ganglion cells

Histone lysine methylation (HKM) marks show dynamic expression patterns throughout inner retinal and RGC

Table 1 Histone H3 methylation sites, and enzymes active in retina

Lysine (K) residue	Methyltransferase (HKMTase)	Effect	Demethylase (HKDMase)	Chemical HKMTase inhibitor	Example
K4	SET1/MLL1 [15]	Activation	LSD1 [16, 17]		Present in all layers in adult murine retina [16], associated with transcription of <i>ATH5</i> in newly born RGCs in chick retina [18].
K9	G9a [19], SUV39H [20], PRDM8 [21, 22]	Repression	LSD1	BIX-01294 [23] Chaetocin [24]	Present in newly born RGCs from E11-PO in murine retina [16]. Inhibition of G9a impairs RGC survival [16].
K27	EZH2 [25]	Repression		DZNep [25, 26]	Enriched in newly born RGCs, inner neuroblastic layer cells, and later, INL neurons [16]. Inhibition of EZH2 impairs RGC survival [16].

development. The histone lysine methyltransferases (HKMTases), via the SET domain, catalyze methylation of lysine residues present on amino-terminal tails of histones 3 and 4 (H3 and H4). Recent studies have implicated histone methylation as an important regulator of RGC development and survival.

In order to identify possible patterns of HKM during retinal development, a recent study used a variety of methylated lysine-specific antibodies to probe retinal sections during different periods of murine retinogenesis [16]. An antibody against tri-methylated K4 of H3 (H3K4me3), an HKM mark associated with active gene transcription and euchromatin, was detected throughout the inner retina and outer retina during the proliferative phases of retinogenesis. This mark was also observed in the more sharply demarcated nuclear layers (ganglion cell layer, inner nuclear layer

and outer nuclear layer) in the postnatal period and adulthood (P0 and 6 months of age), when most RGCs have exited the cell cycle. Two HKM marks associated with transcriptional repression, H3K27me3 and H3K9me2, demonstrated patterns more spatial-temporally regulated. H3K27me3, a repressive mark associated with X-chromosome inactivation [58], body patterning [59], stem cell pluripotency [60] was preferentially expressed in the inner retina and RGCs throughout E16 to 6 months of age. H3K9me2, a repressive euchromatic mark, was restricted largely to newly born RGCs present E16 and E18 mouse retina. Interestingly the loss of expression of the H3K9me2, after E18, parallels the loss of the ability of murine RGCs to extend axons [61].

Another report demonstrated the regulation of atonal homolog 5 (*Ath5*), a member of the basic helix–loop–helix

Table 2 DNA methyltransferases active in retina

DNA Methyltransferase	Function	Example
DNMT1	Maintenance of DNA methylation at CpG islands in mitotic and postmitotic cells; associated with transcriptional repression [27]	Localizes to postmitotic RGCs in developing retina [28]. Deletion results in retinal folds, pseudorosette formation and neuronal degeneration [29, 30].
DNMT3a/3b	De novo DNA methylation at CpG islands; associated with transcriptional repression [31]	Expressed in postnatal RGCs [28].

Table 3 Histone lysine acetyltransferases (KAT) active in retina

KAT designation [32]	Name	Specificity [33, 34]	Component of complex	Function
KAT3A	CREB binding protein (CBP)	H3K14, 18, 27, 56 H4K5, 12		Required for transcription activation by nuclear receptors [35]; DNA incorporation into chromatin after replication/repair [33]; overexpression stimulates axon regeneration [36]
KAT3B	p300	H3K14, 18, 27, 56 H4K5, 8		Required for transcription activation by nuclear receptors [35]; DNA incorporation into chromatin after replication/repair [33]; overexpression stimulates axon regeneration [36]
KAT2A	GCN5	H3K9, 14, 18, 23	STAGA/TFTC, ATAC	Associated with (but expendable in) transcription activation by nuclear receptors [35]
KAT2B	PCAF	H3K9,14	PCAF complex	Competes with viral E1A for CBP/p300 binding; associated with (but expendable in) transcription activation by nuclear receptors [35]

superfamily of transcription factors, and the beta-3 subunit of a neuronal nicotinic acetylcholine receptor ($\beta 3$ -*nAChR*) in RGCs by HKM marks. *Ath5* is expressed almost exclusively in RGC precursors and newly born RGCs; targeted disruption of the mouse gene results in retinas that lack RGCs and optic nerve [62]. *ATH5* regulates the expression of $\beta 3$ -*nAChR*, which is also specific to RGCs [63]. Both genes are temporally regulated during early retinogenesis. Di-methylated lysine 4 on histone H3 (H3K4me2), like H3K4me3, is associated with transcriptional activity. Chromatin immunoprecipitation (ChIP), using an antibody against H3K4me2, indeed captures the promoters of *Ath5* and $\beta 3$ -*nAChR* among RGCs in the developing chick retina. The ChIP signal of H3K4me2 and regulatory elements of these genes is proportional to their highly abundant expression during RGC genesis and specification (E9) and declines later (E12) in chick retinal development [18]. These data highlight the tight correlation between permissive HKM marks and expression of RGC-defining genes.

The intra-nuclear distribution of the H3K4me3 and H3K27me3 in adult RGCs, Müller glia, and other inner nuclear layer cells corresponds to euchromatic regions outside of highly compacted, heterochromatic regions (that stain intensely with DAPI). In contrast to RGCs, murine photoreceptors postnatally redistribute their euchromatin to the nuclear periphery (see below, “Chromatin territories and nuclear architecture of mammalian rod photoreceptors” section), and HKM marks are distributed accordingly [16, 64].

The HKMTases that catalyze the formation of the repressive H3K9me2 and H3K27me3 marks, G9a and EZH2,

appear to be developmentally regulated in RGCs. EZH2, the HKMTase that establishes H3K27me3, is a known epigenetic regulator of neural cell development [65, 66]. EZH2 was detected in newly differentiated and perinatal RGCs (E14-P0), but its expression rapidly extinguished at later points in development [16]. Similarly, G9a, the HKMTase that catalyzes dimethylation at H3K9 with known roles in neural development and neuronal plasticity [67, 68], was expressed in RGCs at early time points (E14-P0) but its expression was barely detectable by adulthood [16]. PRDM8, another HKMTase-like protein known to methylate H3K9 residues [21], contains the PR (PRDI-BF1 and RIZ homology) domain, which displays similarity to the SET domain [69], has also been reported to be expressed in postmitotic RGCs from E18.5 to P3 [22].

How do the HKMTases and histone methylation regulate RGC development and functions? To investigate the role of histone methylation at H3K27me3 and H3K9me2, specific chemical inhibitors against the EZH2 and G9a, 3-deazneplanocin (DZNep) and BIX-01294 (diazepin–quinazoline–amine derivative), were added to P0 RGC cultures. These inhibitors decreased H3K27me3 and H3K9me2 marks and induced RGC apoptosis [16]. Given the temporal expression pattern of G9a, H3K9me2, and EZH2, it is tempting to speculate that overexpression of these HKMTases might regulate the expression of genes responsible for RGC survival or axonal growth and regeneration. While overexpression of PRDM8 has been shown in vitro to methylate H3K9 and repress transcription of genes associated with steroidogenesis [21], a functional role for PRDM8

Table 4 Histone deacetylases (HDACs) and inhibitors [37, 38] [39]

HDAC	Other names	Class	Site	Inhibitors	Function
HDAC1	HD1	I	Nuclear	NaB, Val, TSA, SAHA	Interacts with RB protein; control of cell prolifer and differen
HDAC2		I	Nuclear	NaB, Val, TSA, SAHA	Transcription regulation; Important for chromatin compaction and maintenance of silencing; role in learning and memory
<i>HDAC3</i>		I	Nuclear	NaB, Val, TSA, SAHA	Transcription repression (NCOR component); up-regulated in RGC after nerve crush, responsible for H4 deacetylation [40]?
HDAC8		I	Nuclear	NaB, TSA, SAHA	Transcription co-repressor
HDAC11		I/IV		SAHA	Alternative splicing of IL-10; co-IPs with HDAC6
<i>HDAC4</i>	HDAC-A	IIa	Nuc and cyto	NaB, Val, TSA, Phenylbutyrate, SAHA	Interacts with 14-3-3 proteins; regulated via compartmentalization; the cytoplasmic form is implicated in rd1 photoreceptor survival [41]
HDAC5	HDA1	IIa	Nuc and cyto	NaB, Val, TSA, Phenylbutyrate, SAHA	Interacts with 14-3-3 proteins—regulated via compartmentalization
HDAC9	MITR	IIa	Nuc and cyto	NaB, Val, TSA, Phenylbutyrate, SAHA	Involved in homologous recombination; implicated in immune functions
HDAC7		IIa	Nuc and cyto	NaB, Val, TSA, Phenylbutyrate, SAHA	Involved in SMRT complex-mediated repression
HDAC6	HDA2	IIb	Cytoplasm	NaB, TSA, SAHA	Tubulin deacetylase; role in autophagy; primarily cytoplasmic
HDAC10		IIb		NaB, SAHA	Involved in homologous recombination
SIRT1		III	Nuclear	Sirtinol?	Acts on euchromatin - p53 is substrate; role in aging, neurodegeneration
SIRT2		III	Cytoplasm	Sirtinol	Cytoplasmic
SIRT3-5		III	Mitochondria		Mitochondrial; involved in regulating energy metabolism
SIRT6		III	Nuclear		Acts on heterochromatin-p53 is substrate; involved in genome stability, metabolic regulation, and inflammatory responses
SIRT7		III	Nuclear		Nucleolar

Entries presented in italics indicate HDACs that have been implicated in visual systems

NaB sodium butyrate, *SAHA* suberoylanilide hydroxamic acid (vorinostat), *TSA* trichostatin A, *Val* valproic acid

in RGCs has not yet been described. While not performed in this study [16], ChIP remains important in determining the mechanism by which HKMTase-mediated repression of gene expression stimulates RGC survival. Nevertheless, these data indicate that EZH2 and G9a support RGC viability and may represent novel epigenetic targets to regulate RGC survival in traumatic and neurodegenerative diseases such as glaucoma, and other causes of optic nerve atrophy.

DNA methylation in retinal ganglion cells

DNA methylation and histone methylation are intimately related. The methylation of DNA at CpG islands of regulatory elements is associated transcriptional repression [16, 70]. Recent evidence has indicated that DNA methylation is better correlated to histone methylation than genome sequence context [71]. Specifically, H3K9me and DNA methylation are directly related, while H3K4me and DNA methylation are inversely related [72]. The family of enzymes responsible for DNA methylation is known as the DNA methyltransferases (DNMTases) (Table 2) [73]. The interdependence of

DNA and histone methylation is illustrated by the fact that chemical inhibition of DNMTase by 5-azacytidine results in the loss of H3K9 methylation [74]. Conversely, deletion of the H3K9 HKMTases *G9a* and *Suv39h* results in DNA demethylation at specific loci [75, 76]. Furthermore, the H3K4/K9 demethylase (HKDMase) LSD1 is essential for maintaining proper global DNA methylation [77]. Finally, G9a and LSD1 can methylate and demethylate lysine residues within DNMT1 and DNMT3a, respectively, allowing direct regulation of the DNMTases by HKMTases and HKDMases [78, 79].

Similar to HKMTases, the expression of DNMTases appears to be temporally regulated throughout retinal development (Table 2). A recent study [28] described the spatio-temporal expression patterns of DNMT1, a DNMTase responsible for maintenance of DNA methylation in mitotic and postmitotic cells [27], as well as DNMT3a/3b, related DNMTases with de novo DNA methylation activity [31]. During E10.5–E11.5, when the retina is comprised of mitotic progenitors, DNMT1 is expressed ubiquitously. By E15.5, DNMT1 expression becomes restricted to the inner retina (presumptive RGCs and INBL), similar to H3K9me2

Table 5 Histone H2A deubiquitination

Protein	Enzyme	Substrate	Function	Examples
BAP1	Ubiquitin carboxy-terminal hydrolase (deubiquitinase) [42]	Monoubiquitinated H2A [43]	Tumor suppressor [42]; Deubiquitinase activity required for repression of Polycomb target genes [43]	Inactivating somatic mutations enriched in metastatic uveal melanoma samples [44]; germline mutations underlie multisystem cancer syndromes [45]
Ubiquitin-Specific Protease-22 (USP22)	Deubiquitinase [46]	Monoubiquitinated H2B>H2A [46]	Component of STAGA complex; require for full activation of STAGA-regulated genes [46]	Studied in cell lines [46]; not yet investigated in visual systems

and H3K27me3 [16, 28]. At P0.5, DNMT1 is confined to postmitotic Brn3a+ RGCs, similar to H3K27me3 [16, 28]. The expression of the de novo DNA methyltransferases, DNMT3a was expressed throughout the E11.5 retina, but was not detected at E17.5 and P0.5. At later time points, P6.5 and P10.5, DNMT3a/b becomes colocalized with DNMT1 in RGCs and INL neurons [28]. This postnatal expression pattern is similar to H3K27me3 [16]. A functional role for DNMT1 in retinal development has been recently reported following Cre-mediated excision of *Dnmt1* under control of *Rx* and *Six3* promoters. While all retinal cell types were produced, these conditional retinal knockouts showed disordered lamination, retinal folds and pseudorosettes, and central retinal degeneration, with abnormalities in present in the ganglion cell layer as well [29, 30].

How might DNA methylation regulate particular RGC genes? To date, only one study described DNA methylation changes—via bisulfite specific sequencing (a modified PCR method by which methylated CpG regions in primer-specific sequences can be amplified and identified)—in an RGC-expressed gene, *EphA5* [80]. *EphA5* is a member of the ephrin receptor family of tyrosine kinase receptors, which induce intracellular signaling cascades via binding of ephrin ligands. Through graded ephrin signaling, RGCs extend axonal processes to their retinotopic targets in the lateral geniculate nucleus and superior colliculus [81]. Increased methylation of the *EphA5* promoter in the nasal retina from E17 to P0 to 8 months (adult) resulted in a decrease of *EphA5* transcripts in the nasal retina. Unfortunately, these authors did not assay *EphA5* promoter methylation status in purified RGCs isolated at different ages. In a Müller cell line, the *EphA5* promoter is highly methylated; 5-azacytidine-induced demethylation allowed upregulation of *EphA5* mRNA. The authors propose that DNA demethylation may “unlock” the neurogenic potential of Müller cells—considered by some researchers to be multipotent progenitors—and

coax these cells to differentiate to RGC-like cells in injury or neurodegeneration [80].

Histone acetylation in retinal ganglion cells

Acetylation of lysine residues in amino-terminal histone tails of H3 and H4 is associated with transcriptional activation; conversely deacetylation of these residues corresponds to transcriptional repression [82]. Addition of three-carbon acetyl groups to core histone tails has been suggested to “open” chromatin by decreasing the nucleosome packing density and/or “loosening” histone–DNA interactions by neutralizing the positive charges of the unmodified lysine residues, allowing transcription machinery access to the DNA [35, 83, 84]. Indeed, lysine acetylation of histone tails at 9th and 14th positions (H3K9ac and H3K14ac) is associated with activating histone methyl marks (e.g. H3K4me) and hypomethylated CpG (DNA) islands [85, 86]. These histone marks also serve as docking sites for recognition domains on other proteins involved in transcription regulation and other nuclear activities [87]. Histone acetyltransferases (HATs or KATs) add the acetyl groups, and histone deacetylases (HDACs) remove them (see Tables 3 and 4). In vertebrates, many of these enzymes are members of gene families that show high degrees of homology and, in some cases, functional redundancy. This is true for the best-studied HATs in the retina, KAT3A/CBP and KAT3B/p300 [36, 88, 89], and KAT2A/GCN5 [90–92] and KAT2B/PCAF. CBP and p300 acetylate both histone H3 and histone H4 (Table 3). Jin et al. [35] reported that these enzymes and their products, H3K18ac and H3K27ac, are essential for initiating transcription activated by several nuclear receptors in response to hormone stimulation. GCN5 and PCAF, which were also recruited and shown to acetylate H3K9, were dispensable for transcription initiation in this system [35], but may play a role in maintaining expression [93]. In addition, GCN5 and PCAF are components of multiprotein

complexes with additional functions [46, 94], and so their presence may indicate recruitment of other regulatory activities.

Genetic and pharmacologic targeting of HATs and HDACs in RGCs remains an exciting area of therapeutic investigation. A recent study showed that H3K14ac mark increased from P7–21 but decreased in the adult; this pattern correlated with the expression of the HAT CREB-binding protein CBP/p300. In a model of adult optic nerve injury crush, H3K14ac remained at low adult levels while CBP/p300 decreased in response to injury. Overexpression of *CBP/p300* via adenoviral delivery to RGCs results in enhanced axonal regeneration, and ChIP revealed increased p300 occupancy at regulatory regions of *Sprr1a*, *GAP-43*, and *coronin1b*, indicative of active transcription of these pro-axonal and neurite outgrowth genes [36].

The inhibition of histone deacetylases via non-specific HDAC inhibitors has emerged as a therapeutic target to stimulate RGC survival. Indeed, a report has demonstrated increased expression of *HDAC2* and *HDAC3* and reduced H4 acetylation after optic nerve injury in dying RGCs. ChIP analyses using an H4Ac antibody show decreasing promoter acetylation of several RGC genes including *Thy1*, *Brn3b*, *Nrn1*, *Fem1c*, and the anti-apoptotic genes *BclX*, corresponding to decreased transcription. Pretreatment with trichostatin A of RGCs following optic nerve crush reversed H4 deacetylation, and preserved *Fem1c* transcription [40]. Using similar models, others have shown that treatment with HDAC inhibitors such as valproic acid (Val), sodium butyrate and trichostatin A can promote RGC survival, neuronal outgrowth, after mechanical (crush) and ischemia/reperfusion injury of the optic nerve and retina [95–98]. The ability to modulate histone acetylation through the use of a relatively well-tolerated, FDA-approved agent, such as Val—an agent which has shown to be safe and possibly efficacious in preserving visual field loss in patients with retinitis pigmentosa—raises the possibility that clinical trials for Val-mediated survival of RGCs in optic nerve injury, hereditary RGC degeneration, or glaucoma could be initiated in the near future [99].

Histone ubiquitination in choroidal melanocytes

Composed of blood vessel-forming elements and melanocytes, the choroid is a mixed tissue adjacent to the retinal pigment epithelium and neural retina. This highly vascular structure primarily serves a nutritive function to the outer one-third of the retina and to the retinal pigment epithelium. The epigenetics of choroidal melanocytes, the primary parenchymal cell type of the choroid, is poorly understood, but remains of great clinical importance. Transformation of choroidal melanocyte can result in the formation of the most common intraocular tumor in adults, uveal melanoma.

Metastasis of uveal melanoma to the liver is the most common cause of mortality, and metastatic uveal melanoma is one of the few eye disorders that can be lethal.

A series of recent papers have identified somatic mutations in the BReastCAncer1-associated protein (BAP1), a histone H2A deubiquitinase [43], in metastatic uveal melanoma, cutaneous melanoma, malignant pleural mesothelioma, and other cancers [44, 100, 101]. Additionally, inactivating germline mutations of BAP1 underlie a new family of hereditary cancer syndromes that predispose to uveal and cutaneous melanoma, lung adenocarcinoma, meningioma, and malignant mesothelioma [45, 101–103].

The ubiquitination of histone H2A is required for repression of specific sets of genes, known as the Polycomb target genes, which include the *Hox* genes [104]. Interestingly, EZH2 is an HKMTase required for repression of Polycomb genes. The deletion of the tumor suppressor BAP1 [42] allows for continued monoubiquitination of H2A and derepression of Polycomb genes [43]. The specific genes that are derepressed and causally linked to uveal melanoma metastasis due to BAP1 mutation are not yet known, but may include a subset of “class 2” genes, a constellation of genes whose elevated expression levels in malignant choroidal melanocytes is associated with uveal melanoma metastasis [44]. These data highlight the importance of histone ubiquitination as a target for pharmacologic or gene therapy intervention, such as the introduction of wild-type BAP1 into BAP1-deficient uveal melanoma, in order to prevent or delay metastasis.

Chromatin modifications in retinal photoreceptors

Photoreceptors constitute 70% of the cells in the mammalian retina. Precisely regulated gene expression is essential for their specialized function and survival. It has long been known that the *opsin* genes in photoreceptor precursors are not expressed until terminal differentiation, so that a lag period exists between the birth of cones/rods and their terminal differentiation. Do progressive/sequential changes in epigenetic status of the photoreceptor genes occur during this period? Using several photoreceptor genes, particularly the *opsin* genes, as models, it is known that photoreceptor gene transcription is regulated at each of the three genomic levels (see Table 6 for specific examples). It is important to point out that Level I regulation occurs during cell division, and therefore is more likely involved in gene regulation in retinal progenitor cells, while Levels 2 and 3 can occur in photoreceptor precursors after terminal cell division and therefore are more likely involved in photoreceptor fate specification and terminal differentiation. These multi-level regulations require the action of photoreceptor-specific

Table 6 Transcription regulation of photoreceptor genes at epigenetic levels

Genes	Regulation	Level	Function in transcription/defect-associated disease	References
R/G opsin	DNA methylation	I	X-inactivation/anomalous trichromacy	[105, 106]
Rbp3	DNA methylation	I	Silencing in non-photoreceptors/down-reg in cancer cells	[107]
Recoverin	DNA methylation	I	Silencing in non-photoreceptors/down-reg in cancer cells	[108]
Opsins	Histone acetylation	II	Activation in photoreceptors/RD*, SCA7	[91]
R/G opsin	LCR loops in R/G cones	III	Activation/blue cone monochromacy	[11, 109]
Rhodopsin	RER loops in rods	III	Activation/loss of expression; RD*	[11]
Rhodopsin	Euchromatin CTs	III	Activation/abnormal nuclear architecture -SCA7, RB	[92, 110]

RD retinal degeneration, SCA7 spinocerebellar ataxia type 7, RB retinoblastoma, CTs chromatin territories

transcription factors, including CRX, NRL, and NR2E3 [6, 7], and general chromatin modulators as described below. Mutations which disrupt mechanisms that function at any regulatory level can cause photoreceptor developmental defects or degeneration.

Histone modifications in photoreceptor gene expression

As photoreceptor precursors begin to differentiate, the cone-rod homeobox transcription factor CRX recruits histone acetyltransferases (HATs/KATs) to photoreceptor gene promoters [89]. CRX protein binds CBP and p300 directly [88, 89], and GCN5 indirectly via interaction with ataxin-7, a polyglutamine-containing component of the STAGA complex [90–92]. CRX recruitment of these HATs to photoreceptor gene promoters leads to increased H3 and H4 acetylation and transcription activation of CRX target genes. Using conditional knockouts driven by the *Rhodopsin* promoter, CBP and p300 were found to be redundant for maintaining photoreceptor phenotype. Loss of both greatly reduces histone acetylation on, and transcription of many CRX target genes [111], consistent with a role for CBP/p300 in maintaining transcription of these genes. Double conditional knockout retinas lose the characteristic densely packed chromatin in rod nuclei, the outer nuclear layer forms whorls and rosettes, and visual function is lost ([112]; Hennig, Peng, and Chen, unpublished data). This supports the idea that transcription regulation in photoreceptors requires a dynamic balance between histone acetylation (which activates transcription) and deacetylation (which represses it) [113], and that CBP/p300 plays a crucial role in this regulation.

The importance of GCN5 in photoreceptor gene transcription came to light in studies of a polyglutamine-expansion disease called spinocerebellar ataxia type 7 (SCA7), featuring progressive photoreceptor degeneration in addition to the brain defects. The disease protein ataxin-7 (ATXN7) is a component of a large chromatin remodeling complex that is highly conserved from yeast

(SAGA) to man (STAGA/TFTC). Among a number of general transcription factors/cofactors, the STAGA complex contains two key chromatin remodeling factors: GCN5 HAT for acetylation of histone H3 (Table 3); and USP22 (ubiquitin-specific protease 22) that removes ubiquitin from monoubiquitinated histone H2B and H2A (Table 5), required for full activation of STAGA-dependent inducible genes [46, 114, 115]. Using transgenic and knockin mouse models with retinal degeneration beginning at 4 weeks of age, it was found that assembly of the STAGA complex is impaired, reducing GCN5 activity on photoreceptor gene promoters, [91] and severely decreasing photoreceptor gene transcription [116]. These two contradictory reports nevertheless both show that changes of H3ac/H4ac levels on photoreceptor genes correlate with their transcriptional defects in *Sca7* mouse retina. *Sca7* mice carrying *Gcn5* alleles with decreased activity show accelerated photoreceptor degeneration and other symptoms [117], evidence of a genetic interaction between *Atxn7* and *Gcn5* that supports the role of GCN5-HAT in SCA7 pathogenesis.

A common feature of inherited retinal degeneration diseases is increased activity of all HDAC classes just prior to activation of the cell death machinery [118]. As a result, histones, especially H4, become hypoacetylated, and normally expressed genes are silenced [40, 119]. Subsequently, pro-apoptotic and stress-response genes show increased histone acetylation as they are activated. Interestingly, there is no increase in cell death in the *Cbp/p300* double conditional knockout mice ([112]; Hennig, Peng, and Chen, unpublished data), suggesting that CBP/p300 may be involved in up-regulating pro-apoptotic genes. Overexpression of HDAC4 in electroporated rd1 mouse retinas rescued rods from apoptosis; the protective form of HDAC4 was found to be retained in the cytoplasm where it stabilized hypoxia-inducible factor 1 α (HIF1 α) by deacetylating it [120]. Thus, acetylation/deacetylation regulatory mechanisms can have broader target ranges than just histone proteins.

Higher-order chromosomal organizations and transcription

General features

Spatial chromosomal organization is emerging as an important regulatory mechanism for gene expression. Chromosome folding within the nucleus has been known since the 1970s and 1980s [121–123]. Biochemical experiments revealed daisy-like chromosomal loops tethered at the base to the nuclear scaffold [124]. These loop attachment sites, called scaffold or matrix attachment regions (S/MARs, typically 200–1,000-bp-long and AT-rich), are found in both genic and intergenic regions of the genome. They correspond to boundaries between chromatin domains that contain active chromatin hubs and transcription factories (TxFs, see below). In the past 10 years, chromosome conformation capture (3C) [125] and 3C-related technologies [50, 126–131] have uncovered ultrastructural levels of genome organization. These assays take a snapshot of the 3D organization of chromatin architecture of chemically fixed cells/tissues. This 3D organization includes long-range physical contacts between DNA sequences located in the same (*cis*) or on different (*trans*) chromosomes (for reviews see [132, 133]). These techniques combined with three-dimensional multi-color DNA/RNA fluorescence in situ hybridization (FISH) [134, 135] and electron microscopic techniques allow visualization of intra- and inter-chromosome loops and chromatin territories within the individual nucleus (nuclear compartmentalization) [136]. Furthermore, genome-wide DNaseI hypersensitivity assays [137] for “open” or “closed” configuration (accessibility to regulatory proteins) of genomic regions, assessments of active and repressive histone marks [9, 138], and gene expression profiling studies have connected different phenomena together to allow functionally meaningful interpretations of epigenetic modulations and genomic organizations. The main features of higher-order genome organization as we currently understand it are summarized below:

1. Individual chromosomes in mammalian cells form different chromosome territories (CTs): i.e., the tendency of distant loci on the same chromosome to be near one another in space through intra-chromosomal interactions [130]. A classic example is the looping interactions between the immunoglobulin heavy-chain (*IgH*) gene clusters [130, 139]
2. These territories can be generally categorized as transcriptionally active (open, gene-rich regions) and inactive (closed, gene-poor regions) domains. Individual CTs (of the same category) on different chromosomes have a tendency for self-association with each other through inter-chromosomal interactions or other mechanisms. Multiple active CTs often assemble a transcription

factory at their peripheries. For example, many erythroid-specific genes on different chromosomes frequently colocalize with the globin locus *Hba* or *Hbb* in the erythroid nucleus where the transactivator KLF1/RNAPolymerase II-containing transcription factory mediates/coordinates their expression [135].

3. Active CT-associated transcription factories (TxFs) are generally located closer to the center of the nucleus than inactive CTs [50, 131]. At the ultra-structural level, active CTs and TxFs are often associated with electron-translucent euchromatin, while highly packed inactive CTs are associated with electron dense heterochromatin [140]. However, this rule is completely reversed in the rods of nocturnal mammals, where transcription factories are localized to the nuclear periphery and transcriptionally inactive CTs are in the center of nucleus (see below).
4. Higher-order genomic organizations are cell-type specific and dynamically regulated during development to coordinate spatial and temporal gene expression. For example, the *Hox* gene loci undergo a series of genomic organization changes during body axis formation in early mouse embryonic development [141], correlating with appropriate gene expression.
5. Both specific transcription factors and general epigenetic regulators acting at all three levels are involved in governing specific genomic organizations. The *opsin* chromosomal organization is a good example (see below).
6. Abnormal genomic organizations are reported to be associated with some human diseases, although it remains to be determined whether the observed changes are causal or consequential. The nuclear hallmarks used to recognize cancer cells in biopsies are one example [142].

Intra-chromosomal loops of opsin genes and subtype-specific transcription

In addition to gradual changes of histone acetylation levels on *opsin* gene promoters during photoreceptor differentiation [89], *opsin* genes also undergo looping organizations where the enhancer loops a distance of several kilobases to contact the promoter for transcription activation. Using transgenic mouse studies, specific interaction between *opsin* enhancer and promoter elements is known to be required for high-level expression of *rhodopsin* [143] and red/green cone *opsins* [13, 144]. However, biochemical evidence for physical interactions between *opsin* enhancer and promoter has been obtained only recently using the chromosomal conformation capture assay (3C) [11]. 3C captures in vivo interacting DNA fragments via formaldehyde crosslinking, restriction digestion and ligation. Quantitative PCR across

the ligation junction using primers selected for specific genomic fragments reveals their interaction frequency in a cell population (Fig. 2A) [125, 146]. To determine the cell-type specificity of whole retina 3C, Peng and colleagues used genetic tricks to enrich rod and cone cell populations in the mouse retina (Fig. 2A) [11]. The results of these 3C assays showed that the *opsin* enhancer forms looping contacts with its promoter (and coding regions) specifically in the photoreceptor subtype that actively expresses that *opsin*. For example, *rhodopsin* enhancer loops occur only in rods while cone *opsin* enhancer loops are only seen in cones (Fig. 2B). Thus, *opsin* intrachromosomal loops appear to represent an active genomic organization. This conclusion is

Fig. 2 Chromosomal conformation capture (3C) reveals *opsin* organization. **a** Diagram of steps involved in 3C assays using mouse retina as starting material. Photoreceptor subtype-specificity is achieved using different mouse lines to enrich rod or cone cell population (light blue box). **b** Model for differential chromosomal organization of *opsin* genes in rods vs. cones, and R/G *opsin* loci in human red vs. green cones, including the action of several transcription factors specifically expressed by rods (CRX, NRL, NR2E3) and/or cones (CRX, TRβ2, SALL3 [145])

supported by the observations that the *opsin* loops and transcription are correlated with the binding and regulatory function of three key photoreceptor transcription factors: CRX, NRL, and NR2E3. In *Crx* deficient mice, the pattern and frequency of *rhodopsin* intrachromosomal loops are altered, coinciding with significantly reduced *rhodopsin* transcription. Two rod-specific factors, NRL and NR2E3, are also required for regulating rod vs. cone *opsin* looping organizations. The authors further showed that *opsin* looping also occurs in rods and cones of human retinas. More interestingly, in retinas of two male donors, the red/green *opsin* enhancer LCR makes contacts with either *red* or *green* gene (Fig. 2B, bottom panel) in a 3:1 frequency that closely resembles the mean red/green cone ratio reported for normal males. In this case, the human red/green *opsin* loops correlate with both transcription activation and repression: when the LCR loops with the *green* promoter, it activates *green* gene expression but represses the looped out *red* gene (Fig. 2B, bottom panel). This study indicates that higher order genomic organization and cell-type specific transcription are connected in photoreceptors. However, this study also raised many unanswered questions: First, it is unclear if the looping organization leads to transcription or occurs as a consequence of transcription. This requires correlating looping with transcription during photoreceptor differentiation. Second, it remains to be determined why the loop conformation stays within the specific gene boundaries. For example, what prevents LCR-*red* gene looping from spreading to the *green* gene? Third, why do the enhancer loops spread beyond the promoter into the coding regions? Is this related to transcription elongation or pre-mRNA splicing? Fourth, what are the other transcription factors involved in the looping organization, such as cone-specific transcription factors in cones, or general transcription factors that mediate loop formation in different cell types? Fifth, what is the relationship between Level 2 and Level 3 regulation, e.g., are histone marks involved in the looping process? Lastly, what is the relationship between photoreceptor gene loops and the specific rod nuclear architecture (see below)?

Chromatin territories and nuclear architecture of mammalian rod photoreceptors

During the late stages of photoreceptor development, mice and other nocturnal mammals redistribute their rod photoreceptor chromatin to minimize light scatter [64]. Dense, gene-poor heterochromatin found towards the nuclear periphery in other cell types is repositioned at the center of mouse rod cell nuclei, surrounded by a peripheral ring of euchromatin containing gene-rich DNA (Fig. 3). Localization of transcription factors [92], activated RNA polymerase [147], splicing machinery and nascent rhodopsin transcripts [64] to the nuclear periphery indicates that this is the site of

Fig. 3 Chromatin organization in rods and ganglion cells. Schematic representation of the distribution of chromatin in mouse rod (*left*) and ganglion cell (*right*) nuclei. Constitutive heterochromatin containing late-replicating, gene-poor, major satellite repeat DNA from chromosomal regions near centromeres is found in chromocenters (*dark gray*) and localizes with “repressive” histone marks H3K9me3 and H4K20me3. Heterochromatin that is rich in long interspersed repeats (*medium gray*) surrounds these chromocenters in both types of nuclei, but in ganglion cells and most other cell types is also found at the nuclear periphery; H3K27me3 repressive marks are distributed in these areas. Euchromatin containing actively transcribed genes (*light gray*) and associated with “activation” histone marks such as H3K4me3 and acetylated histones H3 and H4 is found at the periphery of rod nuclei but in the internal regions of ganglion cell and most other nuclei (based on [64, 147, 148] and Hennig AK, unpublished observations)

active gene transcription in rods. Kizilyaprak and colleagues [147] have found that histone acetylation and methylation marks associated with gene activation and repression define concentric chromatin domains in wild-type mouse rod photoreceptor nuclei, with “repressive” marks (H3K27, H3K9, and H4K20 trimethylation) associated with the dense core and marks associated with active transcription (H3 and H4 acetylation and H3K4 methylation), localized to the nuclear periphery where transcription takes place.

The mechanisms responsible for establishing and maintaining this nuclear rearrangement are not known [64, 149, 150], but may involve the retinoblastoma (RB) family of pocket proteins, which are active in developing mouse rod photoreceptors at the time when nuclear compaction occurs. In the absence of RB, chromatin condensation fails to occur [151]. The RB protein directs pericentric and telomeric heterochromatin formation [148] as well as recruiting HDACs to active gene promoters to silence transcription (see review [152]). Longworth and Dyson proposed that RB functions as a “master regulator of chromatin structure” [153]. Since rod nuclear chromatin de-condenses in mice with defects in STAGA complex (containing GCN5) [92] or conditional knockout of CBP/p300 [111], transcription coactivators with HAT activity seem to be important for maintaining this chromatin architecture. Recent evidence that linker histones such as H1c might also play an important role in maintaining appropriate rod nuclear architecture

has come from *Sca7* mouse studies. Electron microscopy revealed expansion of euchromatin domain but reduced and disorganized heterochromatin territory [110]. DNA-FISH showed that rod-specific genes become mis-localized to heterochromatin regions within rod nuclei [92], indicating loss of specific gene compartmentalization. The more recent study, using immunogold labeling, stereology, and electron tomography demonstrated changes in chromatin organization in *Sca7* mice, including decondensation and fragmentation of rod heterochromatin and redistribution of heterochromatin towards the nuclear envelope, resulted in a twofold increase in nuclear volume. Unexpectedly, the amounts and distribution of acetylated histone H3/H4 in the nucleus are unchanged. However, a significant reduction of a major linker histone variant H1c in rods was observed. The authors therefore argue that altered histone acetylation is not the primary cause of chromatin decondensation in *Sca7* rods. However, it is unclear if the H1c change directly causes SCA7 retinal degeneration or is a consequence of the disease. Interestingly, conditional knockout of two other HATs, CBP/p300 also does not alter overall cellular levels of H3ac/H4ac, but does alter their levels on individual photoreceptor gene promoters tested by ChIP-PCR as well as photoreceptor nuclear chromatin organization [111]. These findings together illustrate the complexity of epigenetic regulation but point out that maintaining homeostasis is important for normal genomic organization, nuclear architecture, and transcriptional regulation. Teasing out primary and secondary players is a challenge for epigenetic regulation investigations, but studying the characteristic nuclear organization of mouse rod nuclei will provide insights into more general chromatin regulatory mechanisms.

Concluding remarks

Over the past decade, research into the regulation of transcriptional potential through epigenetic regulatory mechanisms or modification of DNA and chromatin structure has exploded. Here, we have summarized the major findings that have linked these mechanisms to the development and function of the mammalian retina.

The mechanisms of transcriptional and epigenetic regulation are themselves varied and highly complex, and future studies are needed to catalogue the vast number of regulatory events that occur as well as to understand the precise underlying mechanisms that are involved. Key questions include what controls the formation and maintenance of underlying epigenetic state of an individual gene; what are the intracellular signaling cascades that transduce the initial environmental cues at the receptor level to the cell nucleus to regulate the epigenetic state of specific subsets of genes; and how the dynamic regulation of the epigenetic mechanisms

contributes to cell fate specification and terminal differentiation. Genome-wide chromatin configuration and epigenetic studies with gene expression profiling in a cell type-specific manner, in combination with advanced bioinformatic analysis, are crucially needed in our further understanding of the epigenetic regulatory mechanisms. Development of the technology of high resolution live cell imaging, on the other hand, provides a powerful tool for visualizing 3D organization in real-time under the native conditions. As information on transcriptional and epigenetic mechanisms accumulates, it is essential to integrate it with equally important information regarding post-transcriptional (translational and post-translational) regulation to identify the initiators, effectors and targets. These studies will offer a complete picture of gene regulation and better understanding of how environmental signals change retinal development and function to cause diseases. The ultimate goal of these researches is to understand basic principles of retinal neuron development and behavior and, ultimately, to establish epigenetic “biomarkers” for human diseases and to identify new targets for the treatment of retinal degenerative disorders and new methods for their prevention.

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