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Methylation in cornea and corneal diseases: a systematic review

Yutong Xia[1,2], Kuangqi Chen[1,2], Qianjie Yang[1], Zhitong Chen[1], Le Jin[1], Liyue Zhang[1], Xin Yu[1], Liyin Wang[1], Chen Xie[1], Yuan Zhao[1], Ye Shen [1]✉ and Jianping Tong [1]✉

Corneal diseases are among the primary causes of blindness and vision loss worldwide. However, the pathogenesis of corneal diseases remains elusive, and diagnostic and therapeutic tools are limited. Thus, identifying new targets for the diagnosis and treatment of corneal diseases has gained great interest. Methylation, a type of epigenetic modification, modulates various cellular processes at both nucleic acid and protein levels. Growing evidence shows that methylation is a key regulator in the pathogenesis of corneal diseases, including inflammation, fibrosis, and neovascularization, making it an attractive potential therapeutic target. In this review, we discuss the major alterations of methylation and demethylation at the DNA, RNA, and protein levels in corneal diseases and how these dynamics contribute to the pathogenesis of corneal diseases. Also, we provide insights into identifying potential biomarkers of methylation that may improve the diagnosis and treatment of corneal diseases.

FACTS

- Epigenetic modifications have been increasingly linked to the pathogenesis of various ocular diseases, such as keratitis, glaucoma, age-related macular degeneration, and diabetic retinopathy, among others.
- Methylation is a key regulator in the pathogenesis of corneal diseases, including inflammation, fibrosis, and neovascularization, making it an attractive potential therapeutic target.

OPEN QUESTIONS

- What are the major alterations of methylation and demethylation at the DNA, RNA, and protein levels in corneal diseases and how these dynamics contribute to the pathogenesis of corneal diseases?
- Are there any potential biomarkers of methylation that can enhance the diagnosis and treatment of corneal diseases?

INTRODUCTION

As a phenomenon that is beyond genetics, epigenetic changes can dynamically manifest in response to developmental, environmental, and nutritional cues without altering the gene sequence [1, 2], they can influence the regulation of gene expression, phenotypes, and metabolic abnormalities [3]. Currently, three primary mechanisms are recognized to regulate gene expression: DNA methylation (and demethylation), histone modifications and non-coding RNA regulation [4]. With continuous advances in sequencing technology, epigenetics is now being used in various areas of research. Epigenetics offers a partial explanation for diseases [5] including cardiovascular [6], endocrine diseases [7], and autoimmune diseases [8] as well as many other complex pathophysiological processes such as inflammation [9], immunity [8] and neovascularization [10]. Epigenetic factors offer a partial explanation for diseases and may help to explain their onset and progression. Additionally, these factors support emerging epigenetic therapies for diseases [11, 12]. The past decade witnessed the increasing importance of epigenetics in eye development and ocular diseases [13–15]. Unlike inherited genetic modifications, which remain static, epigenetic changes are dynamic and can be influenced by environmental conditions, individual lifestyle, and diseases [16, 17]. Therefore, methylation modifications play a crucial role in the interaction between external factors and the genome.

Methylation is a crucial aspect of epigenetic modifications, whereby a methyl group is transferred from reactive compounds like S-adenosylmethionine (SAM) to other molecules by the action of methyltransferases. Specifically, these enzymes can modify diverse substrates such as DNA [18], RNA [19] and proteins [20]. And these biomolecules can undergo chemical modification through methylation processes, forming methylation products that impact protein functions and regulate gene expression and shutdown [21, 22]. Different forms of methylation can uniquely regulate epigenetic phenomena and play critical roles in cellular behaviors [21]. Clinically, epigenetic modifications have been increasingly linked to the pathogenesis of various ocular diseases, such as keratitis, glaucoma, age-related macular degeneration, and diabetic retinopathy, among others [12, 23, 24]. Furthermore, roles of methylation modifications in ocular physiopathology have also been pointed out [25].

The eye is a vital visual organ that comprises several essential structures, including the cornea, lens, vitreous, retina, optic nerve, and others (Fig. 1). The cornea is a transparent tissue located at

[1]Department of Ophthalmology, The First Affiliated Hospital of Zhejiang University, Hangzhou 310003, China. [2]These authors contributed equally: Yutong Xia, Kuangqi Chen. ✉email: idrshen@zju.edu.cn; idrtong@zju.edu.cn
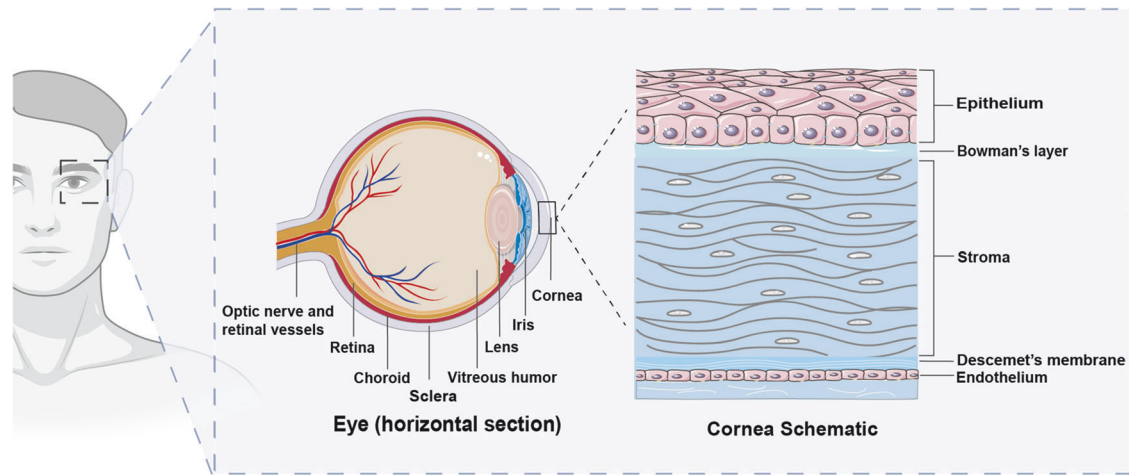
Fig. 1 The anatomy of the eye and cornea. The eye is an important visual organ that contains the cornea, lens, vitreous, retina, optic nerve, and other significant structures. The cornea is a transparent tissue at the front of the eye, which is composed of five distinct layers from anterior to posterior: CE, Bowman's layer, corneal stroma, Descemet's membrane, and the endothelium. Each layer of the cornea is crucial to its function since it not only acts as the first mechanical and immune barrier to the eye but also transmits and converges external light to the retina to produce vision. (Figure was created with BioRender.com).

the front of the eye and consists of five distinct layers, arranged from anterior to posterior: the corneal epithelium (CE), Bowman's layer, corneal stroma, Descemet's membrane, and the endothelium (Fig. 1). Functionally, the cornea serves as the ocular initial mechanical and immune barrier, transmitting external light to the retina to generate vision. Moreover, it not only transmits essential light for vision but also refracts light, providing focus to images [26]. Therefore, any damage to or illness of the cornea can result in severe vision loss or blindness [27]. Currently, although methylation has been extensively studied in posterior segment diseases, its potential role in anterior segment diseases has not been much emphasized. Moreover, recent researches have highlighted the impact of DNA methylation, N6-methyladenosine (m⁶A), and other methylation mechanisms on corneal functions, including cell migration, as well as corneal-related diseases, such as keratitis [28, 29], corneal would [14, 30, 31]. Despite the relative wealth of information on the transcriptional regulation of corneal cells and differentiation, reviews describing the potential of methylation in corneal diseases (CDs) are scarce and it is unclear how methylation modifications exactly affect corneal gene expression and CDs.

Our review aims to summarize the role of methylation in CDs, emphasizing the importance of DNA, RNA, and histone methylation in the cornea. Additionally, we provide insight into the pathogenesis of methylation-related CDs to offer new perspectives for their treatment.

METHYLATION MECHANISM AND FUNCTION
Classifications of methylation
The methylation pattern is determined by a dynamic balance of methylation and demethylation [32, 33], regulated by specific enzymes: "writers" or methyltransferases, "erasers" or demethylases, and recognized by "readers" or methylation-dependent binding proteins [21]. Various forms of methylation modifications, along with related modification factors, can play critical roles in corneal functions and health (Table 1).

DNA methylation. Actually, DNA methylation has been the most extensively studied epigenetic phenomenon to date and has been significantly translated into clinical applications for early diagnosis and therapy [21, 34]. Mechanistically, as a modification that does not alter the DNA sequence, DNA methylation plays a critical role in tissue-specific gene expression, genomic imprinting,

chromosome stability, and more [35]. DNA methyltransferases (DNMTs) are responsible for catalyzing DNA methylation, and act as the transfer of methyl groups from SAM to the fifth carbon of DNA cytosine residues [36]. This results in a chemical modification where methyl groups are covalently bonded to the cytosine residues [37]. Different types of DNA methylation include 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC), N6-methyladenine and others [38, 39], with 5mC being the most common type, and its DNA methylation status is influenced by specific regulatory molecules.

The "writer" proteins are key players in DNA methylation and include DNMT1, DNMT3A, DNMT3B and DNMT3L (Fig. 2). DNMT1 is critical for maintaining normal levels of DNA methylation. It targets double-strands DNA molecules that have only one methylated strand, playing a role in methylating the newly synthetic strand during semiconservative DNA replication [33]. DNMT3A and DNMT3B are two de novo methyltransferases that are capable of catalyzing the initial methylation of CpG sites [40]. The DNMT3L enzyme promotes DNMT3A/B but is itself catalytically inactive [35, 37, 41]. Previously, DNA demethylation was considered a passive process. However, the discovery of the Ten-eleven translocation dioxygenases (TETs) protein family reveals an active process that plays an important regulatory role (Fig. 2) [42, 43]. Regarding "reader" proteins, the three primary types of DNA methylation-binding proteins are members of the Methyl-CpG-Binding Domain (MBD), Kaiso, and Set and Ring Finger-associated (SRA) families [44–48] (Fig. 2).

Functionally, DNA methylation is crucial for preserving the stability of eukaryotic genomes and controlling vital physiological processes, such as the cell cycle and cellular development [33, 49, 50]. Numerous factors regulate gene expression, and the role of methylation in gene expression has been disputed. DNA methylation can regulate gene expression in many ways such as altering DNA conformation, enhancing DNA stability, and modifying chromatin structure. Specifically, promoter methylation can alter gene expression, leading to various pathogenic processes that ultimately result in numerous eye diseases, including CDs [31, 51].

DNA hypomethylation and hypermethylation. DNA hypomethylation is a significant DNA methylation state, alongside hypermethylation. It generally describes a relative situation where there is a decrease from the "normal" level of methylation [52]. Loss of methylation leads to euchromatin formation associated with highly transcriptionally active regions of DNA, increasing the risk

Table 1. Overview of various methylation studies in corneal diseases.

| Core event	Methylation type	Tissue type	Conclusion/Significance	Contributors
KTCN	DNA methylation	Human	It revealed unique DNA methylation patterns in human KTCN corneas.	Kabza [120]
Corneal wound	DNA methylation	Mice hCECs	1. DNMT1 and DNMT3B expression was significantly upregulated during CEWH. 2. miR-200a and CDKN2B were identified as molecular targets of DNA methylation and as having a causal connection with DNMT1.	Luo [62]
		Mice	The involvement of DNMT3B-madiated DNA methylation and PI3K/AKT/mTOR signaling modulation in Alkali burn.	Li [31]
	RNA methylation	Mice HUVEC	FTO regulates ocular angiogenesis in an m^6A-YTHDF2-dependent manner.	Shan [30]
		Mice HUVEC	METTL3 enhances the translation of specific target genes (Irp6, disheveled 1) which is mediated by YTHDF1.	Yao [134]
		Mice hCECs	NSUN2-mediated 5-Methylcytidine modification of UHRF1 mRNA modulates CEWH	Luo [176]
	Histone methylation	Human	Maspin synthesis is downregulated in corneal stromal cells by methylation of maspin promoters and histones.	Horswill [106]
		Mice HUVEC	Inhibition of EZH2 alleviated corneal angiogenesis by inhibiting FoxO3a/PI3K/AKT/mTOR signaling pathway.	Wan [182]
		Mice hCECs	Knockdown of SUV39H1 regulated the p27 expression level and reduced H3K9me3 marks at p27 promoter.	Yang [175]
		Mice CFs	EZH2 is a new target to inhibit corneal scarring by activating anti-fibrosis genes.	Liao [110]
Keratitis	RNA methylation	Mice	It represents the pioneering exploration of m^6A modification profiles in experimental fungal keratitis.	Hu [28]
	Histone methylation	Mice hCECs	Inhibition of Dot1L prevented corneal oxidative stress and inflammation through the p38 MAPK pathway in HSK.	Wan [107]
Corneal dystrophy	DNA methylation	Human	1. DNA methylation patterns play role in loss of corneal transparency. 2. Impaired fluid transport, cellular homeostasis, and cytoskeletal organization associated with gene methylation levels.	Khuc [186]
		Human	Snail and ZEB1 are upregulated by miR-199B hypermethylation	Pan [187]
	Histone methylation	Human	Knockdown of both MLL1 and SET7/9 significantly blocked the TGFβ1-induced gene expression and inhibited TGFβ1-induced changes in promoter H3K4me1/3 levels.	Maeng [185]
Brittle cornea syndrome	Histone methylation	Human	H3K9me2 at these PRDM5-target genes in fibroblasts and demonstrate that the BCS2 mutation p.Arg83Cys diminishes interaction of PRDM5 with repressive complexes.	Poter [205]
Diabetic keratopathy	DNA methylation	Human	1. Significant differential DNA methylation between diabetic and non-diabetic limbal epithelial cells. 2. Wnt-5a promoter was hypermethylated in diabetic limbal epithelial cells accompanied with markedly decreased Wnt-5a protein.	Shah [14]
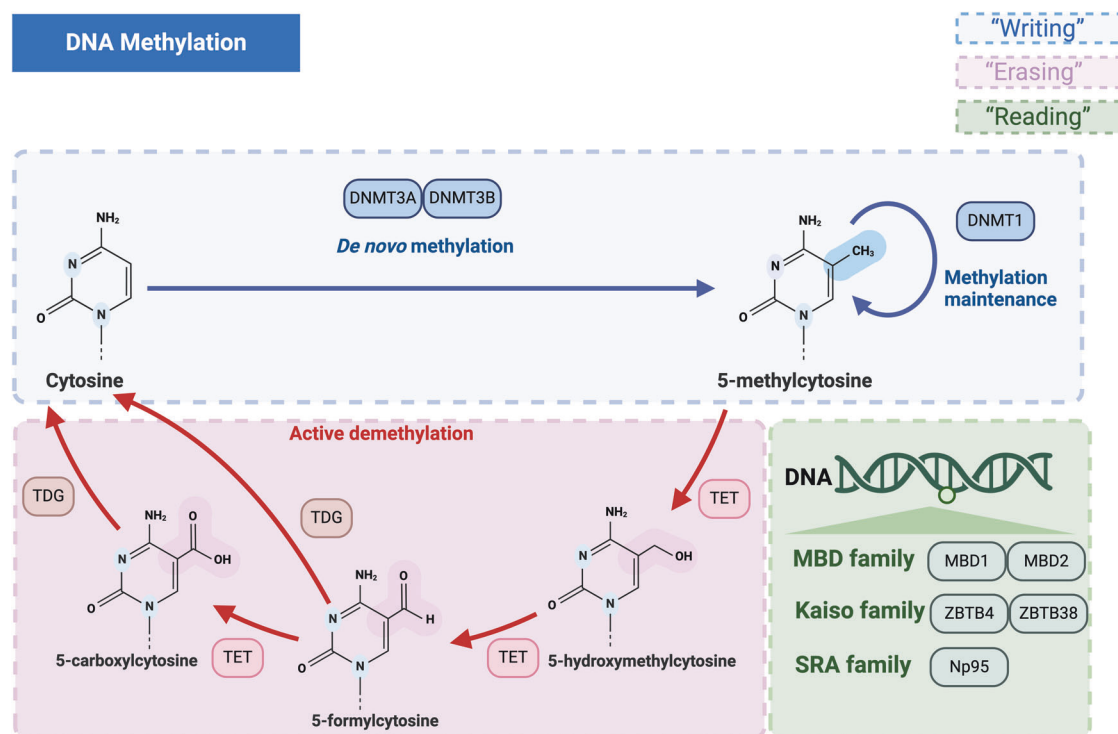
Fig. 2 The primary type and mechanism of DNA methylation. DNA methylation is catalyzed by DNMTs, facilitating the transfer of methyl groups from SAM to the fifth carbon of DNA cytosine residues. This results in the chemical modification of methyl groups through covalent bonding. The role of methylation modifiers such as "writers" (DNMT1, DNMT3A, DNMT3B), "erasers" (TETs) and "readers" (MBD1-2), have been widely reported, with some of them also participating in CDs. (Figure was created with BioRender.com).

of genomic instability [53]. In contrast to DNA hypomethylation, the addition of methyl groups to an excessive number of cytosine bases at gene promoters leads to repressed transcriptional activity [54]. Hypomethylation and hypermethylation play crucial roles in regulating gene expression, maintaining genome stability, and controlling important biological processes [55–57].

The role of CpG islands (CpGi) in DNA methylation. Methylated cytosines are non-randomly distributed in the genome. In mammals, DNA methylation primarily occurs at the cytosine 5' of the CpG dinucleotide [58]. CpG dinucleotides have been demonstrated to be scattered throughout the DNA of mammals or in clusters known as CpGi [59]. Over 70% of genes, including housekeeping genes, have their promoters located within CpGi [60, 61]. Methylation reduces the expression of downstream target genes [62], but has the opposite effect in specific cases [63]. Methylation levels at CpG sites can vary under different conditions. The density of CpG dinucleotides, the nature of the target gene, its location and degree of methylation all affect the outcome of methylation [64–66]. Methylation of CpGi in or around gene promoters is an essential pathway to gene silencing and imprinting [67–69]. In general, gene promoters of active genes have demethylated CpG regions, whereas silenced or low-expressed genes have hypermethylated regions. By specifically methylating certain CpG sites, certain circumstances may cause some genes to be transcriptionally suppressed [31]. DNA methylation was once thought to indicate transcription repression, and specific transcription factors (TF) are needed for gene transcription in eukaryotes. Methylation of CpGi can prevent TF binding and silence genes [40]. Conversely, TF binding can also prevent DNA methylation [70]. There is ongoing debate about how epigenetic modifications are inherited in mammals. A recent study revealed that methylation of CpGi can be passed down across generations by changing DNA methylation in mice. This discovery leads to new research on the role of methylation [71].

RNA methylation

As a crucial regulator of transcriptional expression, RNA methylation occurs in many types of RNAs, including messenger RNA (mRNA) (Fig. 3A) and transfer RNA. Functionally, RNA methylation and its associated signaling pathways are involved in numerous biological activities, such as cell differentiation, the stress response [72]. RNA methylation occurs in at least 150 forms across diverse RNA molecules [73]. Among them, m^6A and C5-methlcytidine are the most widely studied. m^6A refers to the methylation modification of the nitrogen atom (N) at position 6 of adenine (Fig. 3B), and it represents the most prevalent mRNA modification in humans and other mammals [74] (Fig. 3A), accounting for up to 50% of RNA methylation [75]. There is specific research on m^6A in the eye, making it a popular area of research in biology [76].

"Writer" proteins influence reversible RNA methylation similarly to how they regulate DNA methylation. Methyltransferase-like 3 (METTL3), Methyltransferase-like 14 (METTL14), and Wilms tumor 1 associated protein (WTAP) [77] (Fig. 3C) make up the majority of the m^6A methyltransferase complex. Their primary function is to catalyze the m^6A modification of adenosine on mRNA [78]. METTL3, as the pioneer RNA methyltransferase discovered, plays essential catalytic roles in m^6A methylation, and METTL14 may augment METTL3's catalytic activity [79].

The discovery of Fat mass and obesity-associated protein (FTO) as a m^6A demethylase sheds light on the fact that m^6A can be dynamically regulated, suggesting its importance in normal development and the pathogenesis of diseases [80]. Since then, FTO and AlkB homolog 5 (ALKBH5), both belonging to the AlkB family of Fe(II)/a-ketoglutarate-dependent dioxygenases, have been recognized as "eraser" proteins that remove m^6A RNA modifications [81, 82] (Fig. 3C). Demethylases remove methylation from m^6A-modified bases, regulating intracellular homeostasis and cellular damage repair [21, 81]. This highlights the dynamic and reversible nature of m^6A modification. Specific biological functions can be carried out through this process, m^6A-modified mRNAs necessitate particular
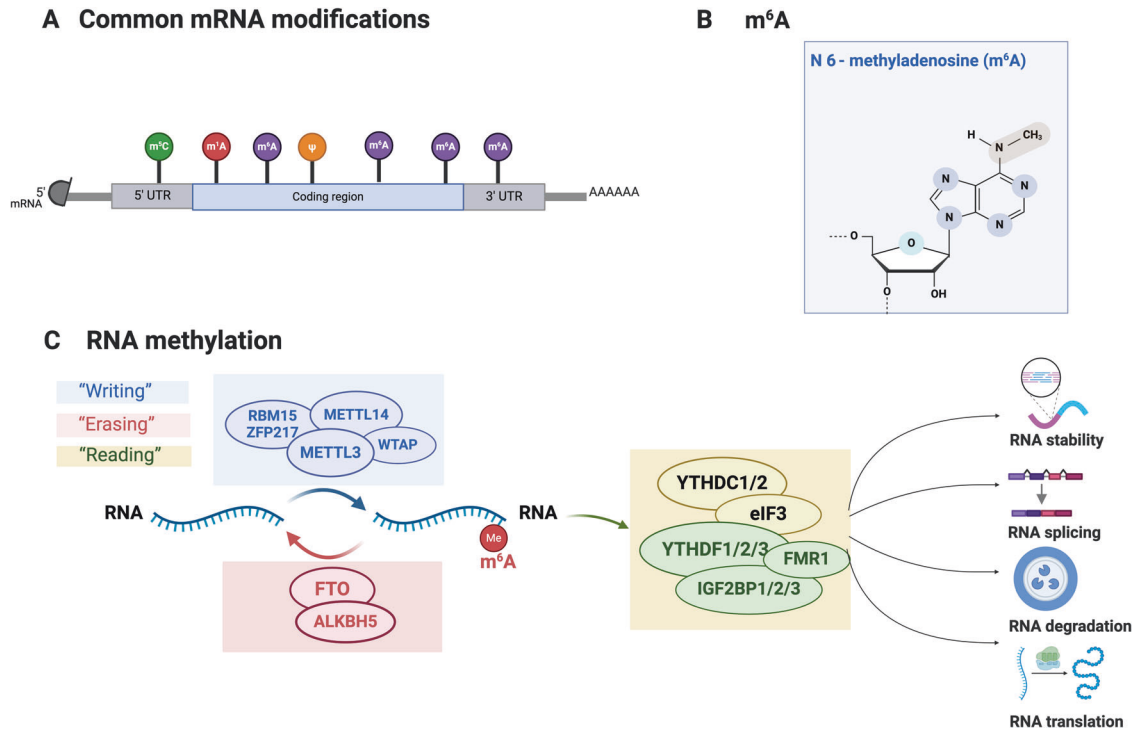
Fig. 3 The primary types and mechanisms of RNA methylation. A The methylation modifications occurring at different sites of mRNA reflect the diversity of RNA methylation. **B** The most representative RNA methylations are m⁶A. **C** m⁶A is catalyzed by the "writers" (METTL3/METTL14/WTAP) and removed by FTO and ALKBH5 "erasers". In addition, YTHDC1, YTHDC2 and YTHDF1/2/3 serve as m⁶A methylation-dependent binding proteins, acting as "reader" to regulate RNA stability, splicing, degradation, and translation functionalities. (Figure was created with BioRender.com).

RNA-binding proteins referred to "reader" proteins which include IGF2 mRNA binding proteins (IGF2BP1/2/3), the YTH domain protein family (YTHDC1/2, YTHDF1/2/3), eukaryotic initiation factor 3 (eIF3) and others [83] (Fig. 3C). The YTH domain, discovered within over 200 proteins, including YTHDC1, YTHDC2, YTHDF1, YTHDF2, and YTHDF3 [84], binds proteins and recognizes m⁶A-modified bases, initiating pathways for RNA degradation and miRNA processing. This is a common type of RNA base modification that mainly regulates RNA stability, splicing, degradation, translation, and other processes [85, 86] (Fig. 3C).

Histones methylation
Post-translational modifications (PTMs) are a frequent means of modifying proteins and changing their functions. Methylation, as a type of protein PTMs, significantly impacts cellular physiology and pathogenesis [87]. It affects the structure and activity of the modified protein as well as the interaction with other proteins [88], thereby regulating the translation, localization and signal transduction of the protein.

Indeed, methylation can occur on both histones and non-histones [87, 89]. Histones bind with DNA to create nucleosomes, which consist of 147 base pairs of DNA coiled around the histone core particles. Sequences of nucleosomes make up chromatin in eukaryotic cells. The histone core particle is composed of two molecules each of the histones H2A, H2B, H3, and H4. Histones are proteins that are highly conserved, having flexible N- and C-terminal domains along with a conserved globular domain (Fig. 4A). Most histone cores are globular, with less rigid N-terminus tails that can be modified by various types of modifications such as acetylation, methylation, and phosphorylation [90–93] (Fig. 4B).

The histone modifications are found in various residues of histone H3 and histone H4 [87]. It is regulated by histone methyltransferases (HMTs) [94] and histone demethylases (HDMs) (Fig. 4C). To be more specific, lysine methyltransferases (KMTs) are responsible for histone methylation, which trigger monomethylation, dimethylation, and trimethylation [95]. The majority KMTs contain the SET domain, which forms the methylation complex with the help of certain structural subunits and sustains the activity of KMTs [96]. HDMs can be roughly divided into two groups: Lysine-specific demethylase (LSD) and JmjC domain-containing family (JMJD) (Fig. 4C). Specifically, LSD1 can remove the mono-dimethylation modification of histones H3K4 and H3K9, while JmiC can remove the trimethylation modification of lysine [97, 98]. The discovery of the first histone demethylation modifying enzyme, LSD1, occurred in 2004 [99]. Henceforth, the dynamic regulation of histone methylation via histone methylases and demethylases was brought into the limelight. Methylated histones are recognized by proteins with methyl-binding domains [21, 100]. Functionally, as the most common protein modification, it can affect cell fate in ways other than just at the transcriptome or protein level [101]. Histone methylation can potentially repress or even activate transcription, depending on lysine that gets methylated. To regulate gene transcription, methylation of histones H3 and H4 occurs at distinct sites and varying degrees. The scientific community has widely accepted that the activation of genes is attributed to trimethylation or dimethylation of H3K4, H3K36, and H3K79, while methylation of histone H3K9 is known for its association with gene repression [102]. Additionally, protein methylation modifies arginine and lysine residues in non-histone proteins to regulate cellular signal transduction via MAPK, WNT, BMP, JAK-STAT, p53 and NFkB pathways [87, 103, 104]. These discoveries have furthered our understanding of epigenetic regulation. However, it is poorly understood how HMTs and HDMs act in the mammalian eye, multiple studies have investigated the connection between transcription and expression of specific genes during CDs, along with corresponding HMTs and HDMs involved [105–107] (Fig. 4C). For instance, the transcriptional activation of Enhancer of Zeste Homolog 2 (EZH2), which is
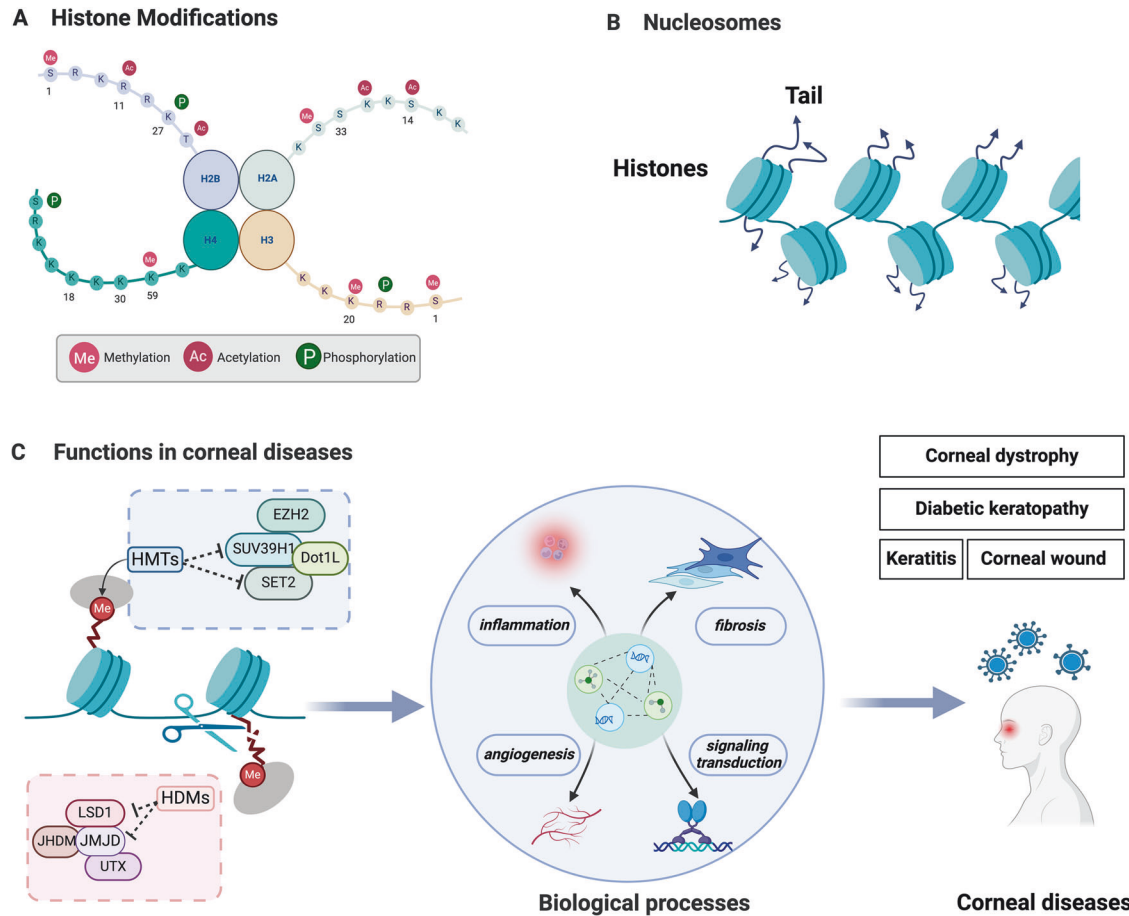
Fig. 4 The mechanisms of histone methylation. A Each of the histones H2A, H2B, H3, and H4 has two molecules in the histone core particle. Histones are highly conserved proteins with flexible N- and C-terminal domains as well as a conserved globular domain. **B** Histones combine with DNA to form nucleosomes, which then assemble into chromatin in eukaryotic cells. Most histones have globular cores, with flexible "tails" extending from their N-termini. **C** Histone methylation is regulated by HMTs such as SUV39H1, DOT1L, EZH2 and HDMs such as LSD1 and JMJD. Several studies have explored the association between the biological processes during CDs and corresponding HMTs and HDMs. (Figure was created with BioRender.com).

a fundamental element of HMTs, triggers histone H3 lysine 27 trimethylation [108, 109] which is recognized to have a significant impact on corneal scarring [110]. Similarly, altered expression levels of Disruptor of telomeric silencing-1 like (Dot1L) which is correlated with H3K79 that results in keratitis [107]. We strongly believe that further investigation into protein methylation modification should be undertaken in CDs.

Different methylation levels correlate with disease severity, and they can act as vital epigenomic markers for the development of effective diagnostic, prognostic and predictive biomarkers for diseases such as cancers [111], neurological diseases [112]. Recent studies have focused more on the effects of methylation modifications in ocular diseases [113]. Our review offers an in-depth exploration of the topic by covering a broad range of processes, including CE repair, ocular fibrosis, and the oxidative stress and inflammatory response caused by different methylation levels. we specifically highlight the significance of methylation modification in relation to the cornea.

METHYLATION IN CORNEAL PHYSIOLOGY AND PATHOLOGY
Modification factors vary in cornea
The role of methylation modifiers such as writers (DNMT1, DNMT3A, METTL1, METTL3), erasers (TET1, FTO and ALKBH5) and readers (Methylated CpG binding protein 2, YTHDF1-3, YTHDC1-2) in ocular tissues have been widely reported [81, 114–117]. The

expression of these methylation modifiers, namely DNMT1, DNMT2, DNMT3A, DNMT3L, and FTO, can vary during both normal corneal physiological activities and pathological processes [28, 30, 118] (Tab.1). Specifically, DNMTs catalyze DNA methylation modifications in the eye and are involved in various tissues of the preocular segment, including the human cornea, conjunctiva, lens anterior capsule, trabeculae, and related cells. In particular, DNMT1, 2, 3A, and 3L proteins are expressed in the human cornea, with DNMT2 preferentially present in corneal endothelial orientation [118]. Therefore, studying the function of DNMTs in the cornea is crucial. Moreover, different methylation regulators may also be expressed differently in different CDs [119]. Thus, it appears that factors that modify methylation can impact corneal function under both healthy and pathological circumstances. Additionally, these factors can also regulate corneal cell function by activating and expressing specific genes associated with methylation modifications (Fig. 5).

Regulation of corneal physiology and pathogenic processes
It is crucial to understand how methylation modification affects ocular cellular physiology, and pathology. The majority of ocular diseases in humans have also been linked to methylation dysfunction [120] and CDs continue to be a major cause of vision loss worldwide. The cornea, a transparent tissue with refractive capacity, has to maintain the homeostasis of five layers in order to function normally [121]. These layers are the epithelium,
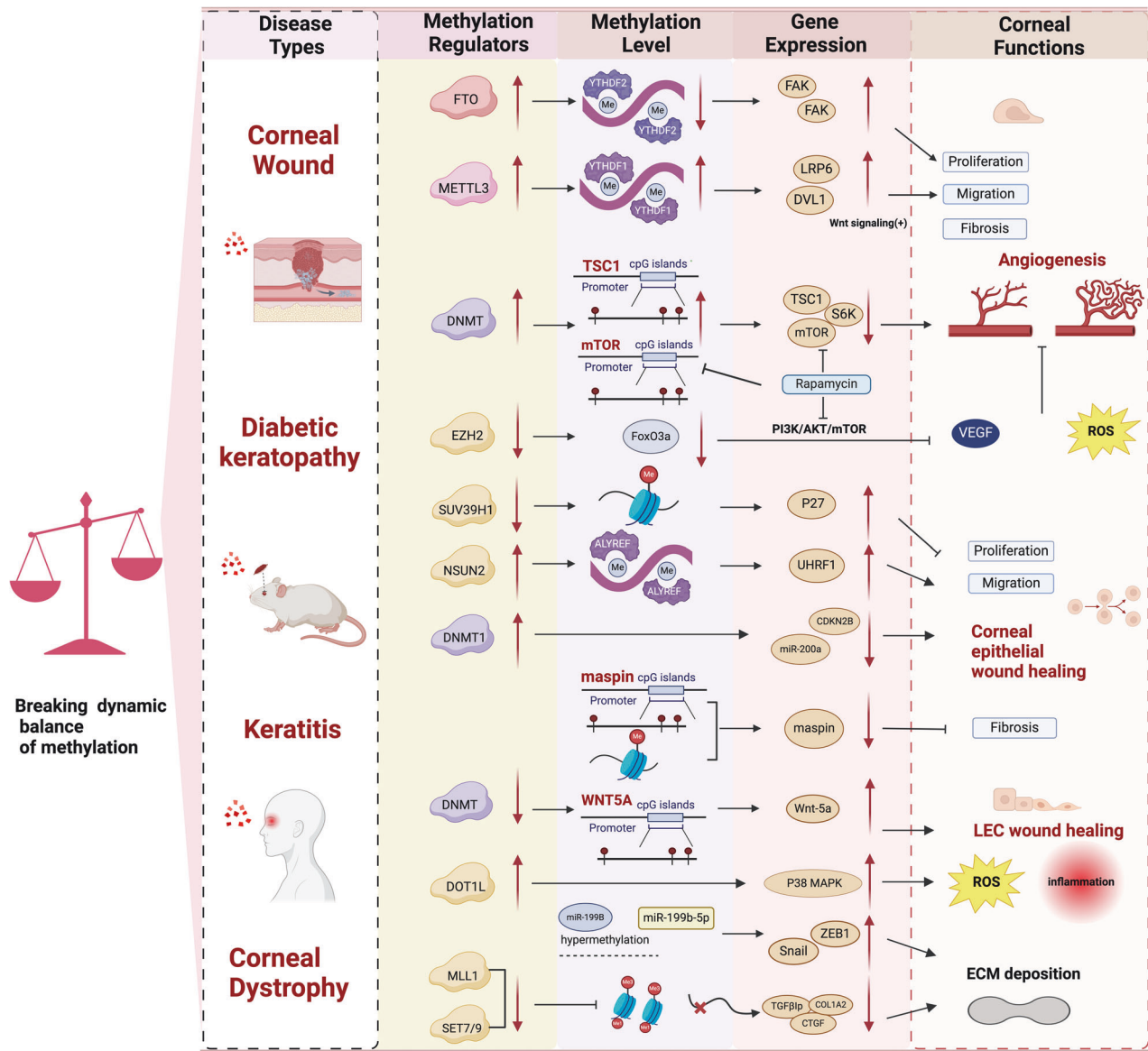
Fig. 5 The methylation balance is disrupted in corneal diseases. Maintaining the equilibrium between methylation and demethylation is therefore necessary for sustaining proper cornea function. In corneal diseases, methylation modifications can trigger changes in corneal function and related pathological processes. Regulatory factors for methylation modification can cause changes in methylation levels which, in turn, can affect gene expression. Ultimately, these changes can lead to neovascularization, activation of ROS, and increased expression of inflammatory-related factors. (Figure was created with BioRender.com).

Descemet's membrane, corneal stroma, Bowman's layer, and a monolayer of metabolically active but mitotically inactive endothelial cells [27, 122]. Every layer of cells is vital for the proper functioning of the cornea, the epithelium serves as a protective barrier and helps to maintain a smooth surface and Bowman's layer provides additional support to the stroma below it. The corneal stroma is the thickest layer and is responsible for the corneal strength and transparency. Descemet's membrane acts as a basement membrane and provides support for the endothelial cells, which regulate fluid balance in the cornea [27, 123, 124]. Without any one of these layers, the corneal normal functioning would be compromised.

Methylation regulators may affect the mechanisms of corneal physiology, including corneal differentiation, pathogenic processes, and homeostasis. According to Sasamoto et al., knockdown of *TET2* in CECs led to a considerable reduction in 5hmC peak distribution, and effected proteins involved in corneal differentiation, including KRT78, MYEOV and MAL [125]. Furthermore, recent research has shown that methylation-induced genetic differential status is linked to the differentiation of induced pluripotent stem cells into CECs [126]. Induced pluripotent stem cells produced from corneal limbal epithelium cells are more likely to differentiate into limbal-like stem cells than those derived from fibroblasts, possibly due to epigenetic methylation changes in genes related to limbal epithelium cells (such as *NTRK1*, which codes for TrkA) [127]. Furthermore, it appears that DNA methylation regulatory factors are closely related to corneal functions such as enhancing the expression of tight junction-related genes like claudin 6 and claudin 9 [128].

Methylation modifications play crucial roles in the pathophysiological processes of several complex CDs (Table 1 and Fig. 5), including inflammation [129], immunity [130], neovascularization [30] and stromal remodeling [131] (Fig. 5). For instance, Luo et al. discovered that upregulation of DNMT1 and DNMT3B during

corneal epithelial wound healing (CEWH) affected corneal cell proliferation and migration [62]. Regarding interferon's function in innate immunity, the cornea is not an exception [130]. In addition, excessive METTL3 promotes m^{6}A methylation to decrease interferon synthesis, implying that methylation negatively modulates interferon response [132].

Abnormal angiogenesis is a prominent feature of many CDs, including corneal dysgenesis. The central corneas are normally avascular, but they become vascularized when they are exposed to inflammation, infection, or hypoxia. Under certain pathological conditions, the level of FTO through m^{6}A mRNA demethylation is increased in neovascularized corneas, specifically, silencing *FTO* increases m^{6}A methylation levels in proangiogenic genes, such as *FAK*. This results in reduced RNA stability and faster RNA degradation via YTHDF2, which attenuates suture-induced neovascularization [30, 133]. In another study, the knockout of *METTL3* inhibited corneal neovascularization in vivo, *METTL3* enhances the translation of specific target genes, including *lrp6* and disheveled 1. This enhancement is mediated by YTHDF1. Moreover, the involvement of these genes suggests that they may play a role in regulating WNT signaling [134]. Additionally, knocking out *METTL3* in corneal limbal stem cells promotes the proliferation and migration of in vivo cells, resulting in fast repair of corneal injury [135, 136]. During corneal injury, CECs also migrate more rapidly. Corneal limbal stem cells possess the ability to regularly renew and differentiate, and they migrate to the central cornea to replace damaged or dead CECs [137]. This research highlight the crucial role of m^{6}A in regulating corneal injury repair and offers new insights for the treatment of CDs [136].

Furthermore, infection, trauma, chemical or surgical damage to the cornea can cause fibrosis in the cornea, leading to impaired vision. Some studies discovered that corneal fibrosis may be related to methylation levels [138, 139]. Promoter and histone methylation could regulate the differentiation of keratinocytes into wound-healing fibroblasts. Maspin enzyme, which is largely epithelial in origin but is also present in corneal stromal keratinocytes, is hypothesized to be downregulated during the transformation of keratocytes to fibroblasts. This involved promoter methylation on a CpGi and histone methylation of the *maspin* gene [140, 141]. The scarring of the cornea is caused by fibrosis of the tissue at the end of the process of corneal injury, which may result in vision loss [121]. However, the mechanism behind corneal scarring is still not well understood, and there is no specific treatment to alleviate or cure corneal scarring. Current treatment modalities are mainly corneal transplantation [142]. Recent efforts have focused on understanding the role of histone methylation in corneal scar formation. EZH2 has been shown to be upregulated in certain fibrotic diseases tissues [143]. A study conducted by Liao et al. revealed that the expression of EZH2 was upregulated in vitro (cellular models of corneal myofibroblasts), high-throughput transcriptome sequencing revealed that blocking EZH2 may inhibit corneal fibroblasts (CFs) activation by inducing the expression of antifibrotic genes [110]. This process suggests that effects of gene promoter and histone methylation may be associated with corneal fibrosis.

Methylation and demethylation in dynamic equilibrium
Methylation and demethylation processes are in a state of dynamic equilibrium. The methylation process can be influenced by various factors, including genetics, individual characteristics, and environmental influences such as aflatoxin B1 and air pollution. While genetic factors are known to affect susceptibility, non-genetic risk factors like DNA methylation modifications, histone modifications, and inflammatory risk factors are also gaining attention in this regard [144–147]. Methylation levels in tissues are variable and dynamic [148, 149], with a balance between establishing and eliminating methylation [33], which is mediated by methyltransferases and demethylases [150] (Fig. 5).

DNA methylation and demethylation. During DNA demethylation processes, the commonly occurring demethylase TET protein tightly regulates DNA methylation modifications by promoting active DNA demethylation and dynamically regulating the levels of 5mC and 5hmC [151], thereby regulating the activation of specific gene expression [152]. DNA methylation can turn off gene expression, and demethylation can turn on gene expression [153]. Indeed, specific TF binding sites are present after some promoter demethylation. These sites bind to non-coding DNA sequences surrounding or covering the promoter region, affecting RNA polymerase function, and blocking gene activation. In the case of mTOR gene promoter methylation caused by alkali burns (Fig. 5), rapamycin may erase or diminish the methylation so that certain TF binding sites become accessible to the transcription factor [31].

RNA/Histone methylation and demethylation. The dynamic character of methylation is implied by the dynamic changes in methylation regulatory factors such UHRF1 and TET3 [66, 154]. Regulatory molecules that maintain the dynamic balance of methylation (such as METTL3/FTO) are expressed in different tissues of the eye as well as in cornea [155]. Maintaining the equilibrium between methylation and demethylation is critical for proper cornea function. For instance, the methyltransferase METTL3 plays a biological role in maintaining homeostasis in mouse T cells and in differentiated T cell-mediated pathogenesis [156]. Disrupting this balance may contribute to the development of corneal immune diseases. The different sites and patterns of histone methylation can evolve many methylation modification patterns in cornea, which increase the complexity and diversity of gene expression (Fig. 5). It is the duty of HMTs and HDMs to maintain the level of histone methylation.

Furthermore, methylation is dynamic in different stages of the host. For instance, post-translational methylation modifications in aging ocular tissue are dysregulated. Several methylation metabolites can accumulate in aged corneas, and other ocular tissue [157]. The increased accumulation of methylated metabolites can potentially impact methylome metabolism.

Overall, understanding the dynamic nature of methylation and demethylation processes along with their regulation by factors like genetics, environment, and enzymatic activities is crucial for comprehending the complex mechanisms involved in gene expression and tissue function.

METHYLATION IN CDS
Keratitis
Current studies on methylation in keratitis focus mainly on Herpes simplex keratitis (HSK), which is caused by the highly prevalent Herpes simplex virus (HSV) [158]. Ocular disease brought on by HSV-1 infection is typically manifested as HSK [159]. In developed countries, HSV infection is a leading cause of corneal blindness [160]. Various pathological processes, such as inflammation, oxidative stress, neovascularization, and endothelial damage, can result in corneal damage, clouding, vision loss, and even blindness [159, 161]. Unfortunately, there are currently limited treatment options available. Recent studies have demonstrated that methylation-related mechanisms tightly regulate the establishment of latency and reactivation of HSV-1. Due to the continuous expression of latency-associated transcripts and simultaneous transcriptional suppression of lytic genes, histone methylation modifications are involved in the differentiation of active and inactive genomic regions. This suggests the significance of histone methylation in HSV-1 [105, 162]. As more is understood about the role that HKM plays in herpes virus, it could become an increasingly important epigenetic target for treating ocular HSV infections.

When the host is infected with the virus, the innate and adaptive immunity systems of the host inhibit HSV-1 replication

and act as anti-infective role. However, the virus also damages the host's immune system, leading to herpes-related immune inflammatory response in the corneal stroma that is mainly regulated by the pro-inflammatory CD4 Th1 and Th17 cells [163, 164]. A crucial balance exists between inflammatory T cells and regulatory T cells (Tregs), such as Foxp3$^+$ CD4 T cells between other pro-inflammatory CD4 T cell subsets [163, 165, 166]. Studies have demonstrated that the specific demethylation region in the highly conserved intron 2 of Tregs is vital for the transcription of the *Foxp3* gene. When this region is demethylated, transcription factors Ets-1 and CREB can bind to each other and act as enhancers of sequential transcription of the *Foxp3* gene [167, 168] and *Foxp3* gene expression is controlled by CREB/ATF sequence-specific binding and CpGi DNA methylation, etc. [168]. Moreover, 5-Azacytidine (5-Aza) covalently binds to cysteine residues at DNMT catalytic sites, ensuring the normal function of Tregs to reduce corneal inflammation. An interesting finding of this research was the 5-Aza therapy enhanced the function and suppressive activity of Tregs, reducing lesions and effectively controlling virus-induced inflammation [29]. Furthermore, oxidative stress is considered as one of the critical factors in the pathogenesis of CDs [169], including keratitis. In the progression of HSK, the activity of superoxide dismutase continues to decrease, while the levels of malondialdehyde and hydrogen peroxide continue to increase [170]. Several investigations have indicated that Dot1L may regulate oxidative stress, because Dot1L plays a specific role in catalyzing the methylation of H3K79 in the targeted genes [171, 172]. Moreover, Dot1L can be used to regulate the expression of pro-inflammatory factors that involved in the p38-MAPK pathway such as IL-1β, matrix metalloproteinases (MMP)-1, MMP-2, IL-6 and MMP-9 in HSK [107]. Therefore, reducing the formation of reactive oxygen species (ROS) decreased corneal oxidative stress and inflammatory response, suggesting that Dot1L could be a potential target for therapeutic interventions to treat HSK.

Furthermore, in a study of global m^{6}A levels, Hu et al. discovered that the expression of METTL3 was considerably higher in fungal-infected corneas than in normal corneas. This suggests that m^{6}A methylation may be implicated in the transduction pathways of fungal keratitis by regulating several critical signals, including the PI3K-Akt signaling pathway [28]. Above all, inhibiting methylation modification may limit pro-inflammatory and pro-oxidative stress in numerous ways, thereby preventing the onset of keratitis.

Corneal injury
The CE, which is the outermost layer of the cornea, can regenerate and is especially sensitive to physical, chemical, and pathological stimuli [173, 174]. CEWH is crucial for repairing the damage caused to the cornea and restoring its integrity and transparency.

Recent studies have demonstrated that DNA methylation, RNA methylation and histone methylation modifications can regulate CEWH [62, 175]. Luo et al. found that corneal epithelial injury led to higher levels of DNMT1 and DNMT3B expression, as well as significant overall DNA hypermethylation modifications. Upregulation of DNMT1 significantly increased the rate at which CE injuries healed, as well as the ability of hCECs to proliferate and migrate. The proposed mechanism is that DNA hypermethylation decreases miR-200a and Cyclin-dependent kinase inhibitor 2B (CDKN2B) expressions [62]. Furthermore, modifying mRNA with 5-Methylcytidine via *NSUN-2* also affects CEWH. Knocking down *NSUN-2* delayed CEWH and inhibited hCECs proliferation and migration in vitro [176].

DNA and RNA methylation, along with the upregulation of histone methylation transferase suppressor of variegation 3–9 homolog 1 (SUV39H1), are involved in CEWH following corneal injury. Specifically, SUV39H1 is crucial for controlling CECs proliferation by suppressing p27 through H3K9me3-mediated mechanisms during CEWH. Epigenetic modifications like SUV39H1 hold promise as potential therapeutic interventions to speed up the process of corneal repair [175].

Corneal chemical injury is a common ophthalmic emergency, and alkalis can penetrate ocular structures with high capacity, resulting in burns that have a significant impact on vision. Clinically, corneal alkali burns (CAB) can lead to a range of complications such as delayed epithelial healing, conjunctival scar formation, dry eye disease, vascularization and corneal clouding [177]. The major pathological features following CAB are corneal vascularization, inflammation, and fibrosis [178, 179]. Several studies have shown that rapamycin, a mTOR receptor inhibitor, can reduce corneal turbidity and neovascularization in CAB by various signaling pathways like TGF-1/ERK [180, 181]. An interesting study by Li et al. discovered that DNMT3B was primarily responsible for methylation of the *mTOR* gene promoter after CAB. This resulted in the activation of the PI3K/AKT/mTOR signaling pathway and overexpression of HIF-1α resulted in high Vascular endothelial growth factor (VEGF) expression [31]. Further research is needed to determine if *mTOR* can function as a practical therapeutic target to mitigate neovascularization during CAB therapy via the blockade of downstream pathways. Moreover, a recent study demonstrates that the histone methylation also takes part in CAB. Through lowering Forkhead-box protein O3a (FoxO3a) mediated oxidative stress, inhibition of EZH2 prevents corneal neovascularization [182]. In detail, EZH2, a core component of HMTs, is responsible for histone H3 lysine 27 trimethylation [108]. More evidence points to the possibility that methylation-inhibiting enzymes could slow the progression of several diseases.

Corneal dystrophies
Corneal dystrophies are rare genetic disorders that impact both eyes. They occur due to the accumulation of specific substances produced in various layers of the cornea. There are different classifications of corneal dystrophies depending on anatomical structure, clinical manifestations, and inherence patterns [183, 184]. Currently, methylation studies on corneal dystrophies are mainly conducted in Fuchs endothelial cell dystrophy (FECD), with a few in Granular corneal dystrophy type 2 (GCD2) [185–187].

The most prevalent corneal endothelial dystrophy, FECD, is a significant indicator and the leading cause of corneal transplant surgeries among patients worldwide [188, 189]. FECD is a highly prevalent, progressively bilateral disease [190]. Generally, the gradual and persistent loss and dysfunction of endothelial cells in both structures and function eventually result in corneal edema [142, 191]. The corneal endothelial cells (CEnCs) are derived from the neural crest and are in a specialized extracellular mesenchyme. Many diseases that impact CEnCs can compromise corneal function and visual acuity. Therefore, it is crucial to maintain a specific physiological range of stromal hydration for clear vision [192]. FECD is linked to several spontaneous and inherited mutations, characterized by abnormal accumulation of extracellular mesenchyme, but the underlying molecular pathogenesis of it is unknown [187, 193]. DNA methylation has recently been suggested to affect corneal endothelial metabolism, cytoskeletal structure, and ion transport [186]. Some investigators have speculated that DNA methylation patterns may contribute to corneal edema and the resulting loss of corneal transparency in FECD [12]. Besides, miRNA gene promoters are often affected by abnormal DNA methylation in FECD. miRNAs, which are tiny non-coding RNAs that have undergone extensive evolutionary conservation, regulate not only fundamental biological processes including development, stress, and metabolism but also the entire course of disease development [194, 195]. The extracellular matrix (ECM) inducible genes snail and *ZEB1* are highly expressed in FECD [119]. Therefore, a study confirmed that aberrant methylation of miRNA promoters also contributes to FECD. *MiR-199B* hypermethylation completely silences the maturation transcript miR-

199b-5p, increasing the expression of snai1 and ZEB1, as well as activating the transforming growth factor β (TGFβ) signaling pathway [119, 187]. These effects lead to increased ECM deposition in FECD, suggesting a methylation-regulated mechanism for ECM protein production and secretion by CEnCs.

Other CDs
In addition to the aforementioned CDs, methylation modification has also been explored in a few other corneal conditions such as keratoconus (KTCN), brittle cornea syndrome type 2 (BCS2) and diabetic keratopathy.

KTCN is identified by progressive corneal dilation and thinning, which causes vision impairment and significantly lowers patient's quality of life. The disease is influenced by a variety of factors, including complex gene, individual differences, and environmental effects such as ultraviolet radiation [196–198]. Nonetheless, the underlying cause or pathogenesis of KTCN remains unclear [199]. Additionally, there is growing interest in non-genetic factors, such as epigenetic factors like DNA methylation modifications, are gaining attention for their potential involvement in symptom development. To investigate the epigenetic role of KTCN, Kabza et al. performed DNA methylation sequencing and analysis of corneas affected by KTCN. The study confirmed 112 differentially methylated regions in the DNA, many of which overlapped with the sensitive sites of KTCN. Furthermore, 12 genes including *WNT5A*, *IQGAP2*, *PARVB*, *WNT3*, and *RB1* were downregulated [120, 200]. Intriguingly, a study showed that some of these genes had been downregulated in the corneas from patients with KTCN compared with non-KTCN [201]. Taken together, DNA methylation may be a possible explanation causing KTCN. However, the researchers discovered dysregulation of related genes, such as *TGFβ1*, *P4HB*, and *BCL2*, in patients with KTCN when they examined the mitochondrial DNA (mtDNA) methylation and sequencing of those genes. No differences were found in the mtDNA methylation sequencing, suggesting that the role of mtDNA methylation modifications in KTCN was not responsible for expression differences. The deeper mechanisms are worth exploring [202].

Furthermore, BCS2 is an inherited connective tissue disorder with *PRDM5* being one of the most often mutated genes [203, 204]. A study has identified H3K9me2 on the *PRDM5* target gene in CFs from BCS2 patients, suggesting that the mutation may be responsible for the activation of CFs through the histoplasmosis [205]. It appears that this mutation may exert epigenetic effects via histone methylation, resulting in irreversible ocular damage.

In addition, diabetic keratopathy is characterized by impaired CEWH, compromised barrier function, and diminished tear secretion, among other features [206]. Recent research comparing the DNA methylation patterns of limbal epithelial cells in primary cultures from diabetic and non-diabetic individuals reveals epigenetic changes in the diabetic cornea. These changes include dual inhibition of *WNT5A* through DNA methylation and miRNA activity suggesting that *WNT5A* serves as a novel stimulator for CEWH and could be a potential target for improving wound healing and stem cells in diabetic corneas [14]. Simultaneously, this provides new evidence for the significant role of methylation in CDs.

CONCLUSION AND PROSPECTS
Targeting risk factor genes
The identification of aberrantly methylated CpG loci and the characterization of their distribution patterns, which are complementary diagnostic methods widely used in oncological and immunological diseases [207, 208]. Similarly, the promoters of various methylation-related key genes are closely associated with CDs, such as *mTOR* gene in CAB and miRNA gene promoters in FECD, which may be target for detection [31, 187]. This means that

identifying the differentially methylated loci for some genetic CDs seems particularly crucial. Additionally, the methylation levels of specific genes in peripheral blood could serve as biomarkers for early disease diagnosis or for predicting drug efficacy. For example, this could involve conducting screenings to detect lung cancer at an early stage or evaluating the efficacy of VEGF-targeted drugs on cancer cells [209, 210]. We must acknowledge that more basic and clinical research are still needed for the application of epigenetic modifications as non-invasive biomarkers in ophthalmology diagnosis.

Targeting metabolic synthesis
Metabolic substances such as SAM, folic acid [211] and vitamin B12 [212] are necessary for DNA and chromatin alterations, serving as methyl donors for DNA and histone methylation. According to a study conducted by Lan et al., folic acid supplementation significantly reduced the toxic effects of drugs on the cornea [213], including in a clinical trial [214]. Moreover, the methylation level is closely connected to changes in folate intake [215]. Succinate and ferredoxin can also affect DNA and histone demethylation enzymes. Specifically, high levels of succinate can inhibit DNMT activity, leading to decreased DNA methylation levels in cells [216]. Furthermore, succinate is significant in signaling pathways related to inflammation, hypoxia, and metabolism [217] that may take part in CDs. Therefore, metabolic changes may result in global changes in the methylation-associated genome. The corresponding changes in triggered metabolites may imply potential therapeutic targets, and metabolic changes can modulate specific loci and induce genes, leading to persistent epigenetic modifications that can be inherited between generations.

Targeting enzymes
Methylation-related regulators or inhibitors can influence the expression levels of upstream and downstream factors by affecting methylation balance in their promoters. Currently, the treatment modalities for prevalent corneal pathologies primarily strive to manage inflammation, prevent infection, and improve visual acuity to the greatest extent feasible. Methylation regulatory agents have the plausible ability to supplement the extant therapies. Some researchers have hypothesized that combining anti-VEGF with DNMT inhibitors may improve the treatment of neovascularization-related ocular diseases [218, 219]. Clinically, epigenetic therapies are more targeted and cause fewer side effects than regular drugs. Some of these treatments have shown promise in treating systemic diseases by inhibiting DNMTs and may be a potential treatment for CDs. The DNMT inhibitors 5-Aza (azacytidine; Vidaza) and 5-Aza-20-deoxycytidine (decitabine; Dacron) have received FDA approval for the treatment of cutaneous T-cell lymphoma and bone marrow cancer, respectively [2, 220]. However, the use of epigenetic modalities to treat CDs is still in clinical trials. For instance, EZH2 inhibitors (EPZ-6438) are expected to become drugs for corneal scarring [110]. Future epigenetic advancements may lead to a deeper understanding of the pathophysiology of CDs. Since DNMT is associated with diseases, it is possible that DNMT modulators will help us treat CDs more effectively than current methods allow.

Perspectives
New technological advancements have renewed interest in using methylation modifications to treat ocular diseases. Methylation, a critical biochemical process, plays a crucial role in DNA and histone modifications, corneal gene regulation and cell fate. It also affects pathways associated with CDs. Some progress has been made in studying methylation modifications in the eye, particularly in the retina. There is a growing interest among researchers in PTMs, including methylation and ubiquitination. Therefore, it is necessary to examine more closely the specific role that

methylation modifications play in the mechanisms of CDs. Studies of methylation modifications on the cornea currently concentrate on corneal cell differentiation, changes in gene expression and its methylation sites in CDs, as well as changes in related regulatory factors. As a result, disease genes are enriched for important pathways and predictions. Lack of animal models for DNA methylation editing, which targets de novo DNA methylation editing by linking the action of CpGi, is the fundamental obstacle to DNA methylation investigations. To further advance our understanding of methylation modifications, in-depth studies can be conducted using single cell sequencing and triple sequencing techniques. On the one hand, it helps understand the importance of methylation modifications for eye development, which can be analyzed by methylation regulatory factors such as transferases and demethylases, as previous studies on zebrafish eye development clearly demonstrated the importance of DNMTs and dynamic expression patterns [221, 222]. On the other hand, it will enable better prognostic analyses, early screening, diagnosis, and targeted treatment of CDs. More importantly, it will provide novel treatment strategies for CDs, particularly those that are unresponsive to conventional therapies. Despite the progress made, challenges and questions remain. For example, what are the profiles of methylation regulatory factors in different types of corneal cells? How does each type of molecule change in corneal and related ocular diseases? What are the main contributing elements to complex CDs? Additionally, what roles do non-CpG methylation, m^6A-related RNA methylation, and histone methylation play in corneal development and diseases? Is there a close correlation between the effects of methylation modifications? Finally, are there any side effects associated with the concomitant use of related inhibitors for CDs treatment? These considerations should be considered in future studies. Furthermore, it is crucial to conduct more extensive research to identify target molecules and biomarkers, elucidate the pathogenesis of epigenetically linked diseases and evaluate the safety and effectiveness of new epigenetic therapies for treating CDs.

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The authors declare no competing interests.

ADDITIONAL INFORMATION

Correspondence and requests for materials should be addressed to Ye Shen or Jianping Tong.

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