博士論文（要約）

Analysis of the function of *Fbxl11* in retinal development

（網膜発生における *Fbxl11* の機能解析）

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The vertebrate retina is composed of six neurons and one glial cell, and all of these cell types differentiate from common retinal progenitor cells (RPCs) during development by tightly controlled gene expression. Epigenetic regulation has been attracting attention as one of the mechanisms to control gene expression. Genome-wide analyses have shown that the epigenetic landscape changes dynamically in the developing retina. In particular, it has been demonstrated that the histone methylation status plays an essential role in retinal development, mainly through the loss of function of histone methyltransferases and demethylases. However, there have been no reports about the role of H3K36 methyltransferase or demethylase in retinal development. *Fbxl11* (*Kdm2a*) is an enzyme that catalyzes the demethylation of H3K36me1/2. Systemic *Fbxl11* knockout mice manifest reduced cell proliferation, increased apoptosis, neural tube defects and are embryonic lethal, while the function of *Fbxl11* in retinal development had not been documented.

Fbxl11 binds to unmethylated CpG islands. In the developing retina, the methylation pattern of CpG islands differs between photoreceptors and other cell types. DNA demethylation by TET enzymes plays an essential role in the development of the retina and eye in zebrafish and Xenopus. These previous studies indicate the importance of TET enzymes in retinal development and suggest that TET enzymes also have important function in mammalian retinal development. Furthermore, they raise the possibility that the DNA methylation pattern generated by TET regulates *Fbxl11* recruitment and H3K36 methylation thereby establishing the chromatin state necessary for normal retinal development.

This study aimed to elucidate the importance of H3K36 methylation status, especially *Fbxl11*, in retinal development. By knocking out *Fbxl11* in the retina, I investigated the function of *Fbxl11* in retinal development. I also examined the effect of the loss of function of TET, which is estimated to contribute to the generation of unmethylated CpG islands to which *Fbxl11* can bind, on retinal development.

1. Retina-specific *Fbxl11* knockout led to upregulated apoptosis, reduced RPC pool, and abnormal rod photoreceptor and bipolar cell development

First, I examined the expression of *Fbxl11* at the mRNA and protein levels in the developing retina by reanalyzing publicly available RNA-seq data and immunohistochemistry, respectively. *Fbxl11* mRNA was consistently expressed in the retina during development. On the other hand, FBXL11 was mainly expressed in the neuroblastic layer (NBL) at the protein level at E14 and postnatal day 1 (P1). In contrast, at P7 and P17, it was
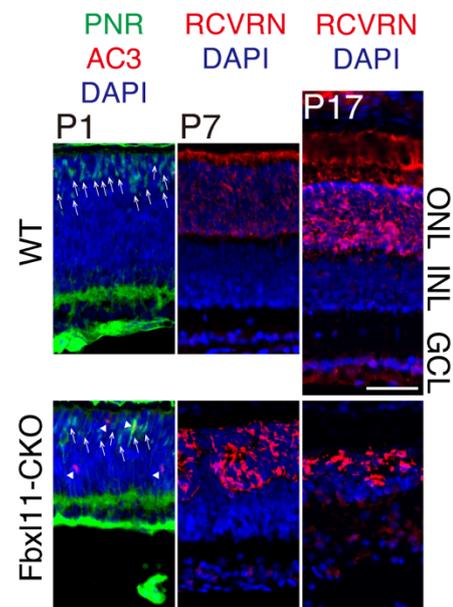

Figure 1. IHC of Fbxl11-CKO retinas at P1, P7, and P17. Arrows indicate PNR+ rod photoreceptorer cells. Arrowheads indicate apoptotic cells. Scale bar = 50 μm.

expressed primarily on the inner nuclear layer (INL) and outer nuclear layer (ONL), respectively. These results indicate that the spatial expression pattern of FBXL11 changes dynamically through retinal development. Next, I assessed the retinal phenotype of retina-specific *Fbxl11* knockout (Fbxl11-CKO) mice over time (Figure 1). At P1, Fbxl11-CKO retinas were thinner, with fewer PNR+ rod photoreceptors and more AC3+ apoptotic cells. At P7, rosette formation was observed in the ONL, and at P17, the ONL containing RCVRN+ rod photoreceptor cells had almost completely disappeared, and the INL was also thinner. At P1, KI67+ RPCs were reduced, and PH3+ mitotic cells were unchanged. The number of ganglion, amacrine, and horizontal cells, which mainly differentiate before birth, did not decrease. Bipolar cells, which, as do rod photoreceptors, differentiate mainly after birth, showed a decreasing trend at P17, but this was relatively mild compared to the decrease in rod photoreceptor cells. Evaluation of Fbxl11-CKO retinas at earlier embryonic stage showed increased apoptosis as early as E14. In addition, rod photoreceptor cells were reduced as early as E17, just after the beginning of rod photoreceptor differentiation. These results indicate that in the Fbxl11-CKO retina, apoptosis is enhanced from the embryonic stage, suppressing the survival of RPCs and resulting in abnormalities in the development of retinal cell types which differentiate mainly after birth, especially rod photoreceptor cells.

To evaluate the transcriptional effects of knocking out *Fbxl11*, I performed qPCR on the retinas at embryonic and early postnatal stages. At E14, there was no change in the expression of RPCs or rod photoreceptor genes, while at E17, photoreceptor marker genes *Otx2* and *Nr2e3* were decreased in Fbxl11-CKO, and the expression of more rod photoreceptor genes was decreased at P1. To comprehensively clarify the transcriptional changes, I performed transcriptome analysis by RNA-seq at P7. I identified 1259 downregulated genes and 1066 upregulated genes as differentially expressed in Fbxl11-CKO retinas. The expression of rod photoreceptor markers and bipolar cell markers was decreased in Fbxl11-CKO retinas. Gene ontology analysis revealed that the downregulated genes were enriched for terms such as vision and ion transport. On the other hand, terms such as cell cycle and alternative splicing were enriched for the upregulated genes. I hypothesized the relationship between the methylation status of H3K36 and alternative splicing, I found that intron retention was dramatically increased in Fbxl11-CKO retinas.

2. TET enzymes contribute to bipolar cell differentiation

Next, I analyzed the effect of TET loss of function on the development of the mouse retina. Among the *Tet* genes, *Tet3* has been reported to contribute to the maturation of mouse retina by altering gene expression through DNA methylation and the methylation state of H3K36. Therefore, I generated retina-specific *Tet3* knockout (Tet3-CKO) mice and evaluated their retinal development. At P29, when retinal maturation was completed, the number of CHX10+/ISL1- OFF- and PKCα+ rod bipolar cells was significantly reduced in Tet3-CKO mice (Figure 2A). The electroretinogram performed to evaluate the effect of *Tet3* knockout on

visual function showed that the a-wave, which reflects photoreceptor cell function, was unchanged. In contrast, the b-wave, which reflects bipolar cell function, showed a marginally significant attenuation in Tet3-CKO, especially under photopic conditions. These results indicate that *Tet3* contributes to visual function mainly through regulating the differentiation of OFF- and rod bipolar cells. Finally, to investigate the function of *Tet1* and *Tet2* in retinal development, I performed experiments using
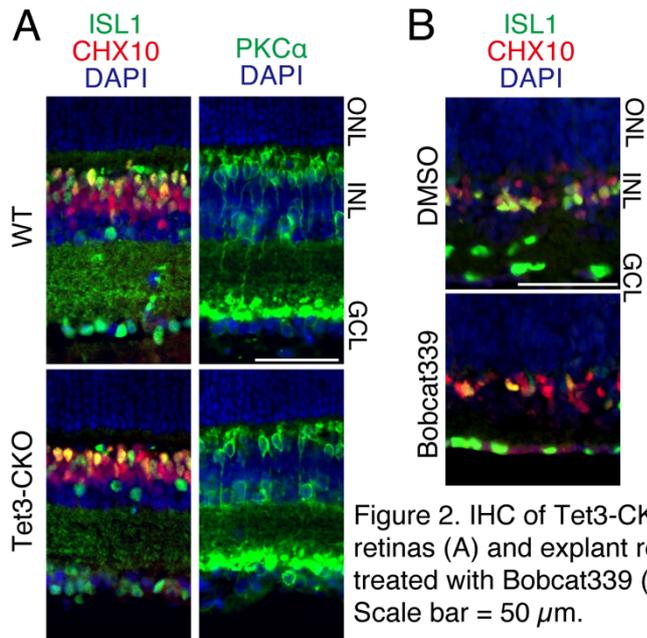

Figure 2. IHC of Tet3-CKO retinas (A) and explant retinas treated with Bobcat339 (B). Scale bar = 50 μm.

Bobcat339, a TET1/2 inhibitor. The amount of 5hmC was dramatically reduced rapidly when retinal explants were cultured with Bobcat339 in the medium, indicating that TET1 and TET2 are involved in the demethylation of 5mC during retinal development. When TET1 and TET2 were inhibited from E14 and cultured to the stage equivalent of P9, CHX10+/ISL+ double-positive ON-bipolar cells were significantly reduced (Figure 2B). On the other hand, there was no apparent effect on rod photoreceptor development, nor was there an increase in apoptosis.

In the present study, histological analysis suggest that *Fbxl11* is essential for cell proliferation and survival not only in embryogenesis but also in retinogenesis. The transcription of rod photoreceptor and bipolar cell genes is markedly reduced in Fbxl11-CKO retinas, and together with the results of morphological analysis, rod photoreceptor cells are particularly susceptible to loss of *Fbxl11*. Given that *Fbxl11* mRNA was expressed throughout embryonic retina, the cell type-specific transcriptional regulation by *Fbxl11* is not transcription-driven but is likely to be coordinated with other factors such as other histone modifications and transcriptional factors.

The transcriptome analysis revealed that *Fbxl11* affects the expression of various genes. H3K36me3 is an active histone mark that accumulates in the gene body region, and *Fbxl11* de-methylates H3K36me1/2 near unmethylated CpG islands. Considering the results of this study together with previous reports, it is still inconclusive how *Fbxl11* regulates the transcriptional state through histone modifications. To further elucidate how *Fbxl11* alters histone modifications and transcriptional state in the developing retina, it will be necessary to perform, for example, a multi-omics analysis of chromatin state and transcriptome for each cell type.

Both *Tet3* knockout and TET1/2 inhibition caused a decrease in bipolar cells. In contrast, the absence of developmental abnormalities in rod photoreceptor cells observed in

Fbxl11-CKO retinas indicates that TET enzymes and their DNA demethylation activity are not directly upstream of *Fbxl11* function. Another possible factor for the demethylation of CpG islands in rod photoreceptor cells is the passive dilution of 5mC by mitosis. DNMT1-mediated DNA methylation maintenance may be transiently depressed in a temporal or localized manner in rod photoreceptor cells. Moreover, there are differences in the subtypes of bipolar cells affected by *Tet3* knockout and TET1/2 inhibition. It remains to be clarified how each *Tet* gene regulates DNA methylation and gene expression.

In conclusion, I revealed that the H3K36me1/2 demethylase *Fbxl11* is essential for retinal progenitor cell survival and normal development of rod photoreceptors. This study also suggests that the function of *Fbxl11* in rod photoreceptor development occurs in an active DNA demethylation-independent manner, and that the active DNA demethylation may rather be required for bipolar cell differentiation and maturation.