博士論文

The role of H3K27 demethylation in retinal development

(網膜発生における H3K27 脱メチル化の役割)

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Abstract

The H3K27me3 repressive mark is written by the polycomb repressive complex 2 (PRC2) and erased by the Jumonji C (JmjC) domain-containing proteins Utx and Jmjd3 as such they antagonize the various functional roles of PRC2 including in cellular differentiation. There are many new insights into the roles of Utx and Jmd3 in development; in the retina, Jmjd3 was found to regulate the differentiation of rod bipolar cells through erasing H3K27me3 from the Bhlhb4 locus, a critical gene. Utx, also a major demethylase of H3K27me3 has some differing effector mechanisms to Jmjd3 in cellular differentiation, however, it's role in the development of the retina has yet to be studied.

In this study, by Utx knockdown in vitro and conditional knockout in vivo followed by immunohistochemical analyses, I demonstrated that Utx is expressed in retinal progenitor cells in development and in cells of the inner nuclear and ganglion cell layers in the mature mouse retina.

Also, I discovered that Utx may regulate the differentiation of Protein kinase C alpha (PKCα)-expressing rod bipolar cells found in the inner nuclear layer as its loss led to a reduction in their number. Utx loss did not affect other bipolar cell subtypes and did not affect retinal progenitor cell proliferation and apoptosis.

Further analyses using Utx/Jmjd3 conditional double knockout mice (DKO) to understanding the interrelationships of Utx and Jmjd3 in retinal development are underway. So far, a research collaborator found that DKO led to greater loss of rod bipolar cells compared to Utx or Jmjd3 single deletion, however, the PKCα loss was incomplete implying possibility of partial redundancy and involvement of a third regulator. In addition, further study of the molecular mechanisms by which Utx regulates rod bipolar cell differentiation is planned. Hopefully this knowledge can in future amount to

understanding the possible role of Utx in human retinal diseases such as congenital stationary night blindness caused by lack of rod bipolar cells.

Introduction

The vertebrate retina lines the posterior inner part of the eye. It is made of six major cell types; one glia type called Müller-glia that provides metabolic and homeostatic support and five neuron types namely: photoreceptor cells, bipolar cells, amacrine cells, horizontal cells and ganglion cells. The two types of Photoreceptors: rods and cones form synapses in the outer plexiform layer with bipolar and horizontal cells (1); bipolar, amacrine and ganglion cells synapse in the inner plexiform layer (IPL) (2). The photoreceptor cell bodies form the outer nuclear layer (ONL) while horizontal cells, amacrine cells and Müller glia cell bodies form the inner nuclear layer (INL) and ganglion cell bodies, the ganglion cell layer (GCL) (Fig. 1).

Photoreceptors when stimulated by light undergo changes in membrane potential which leads to glutamate release and transfer of signals to glutamatergic bipolar cells under the modulation of horizontal cells. Bipolar cells then relay this signal to ganglion cells via amacrine cells. Ganglion cells are the final output neurons of the retina that relay the processed information to higher centres of the brain for interpretation (2) (3).

Mouse retina development begins at around embryonic day 10. Ganglion cells are first to be formed prenatally followed by cone photoreceptors, horizontal cells and most amacrine cells. Development proceeds until eye opening which takes place at postnatal day 14, with bipolar cells; the rest of the amacrine cells and Müller glia being formed postnatally before this stage (4) (5). Although they may start to differentiate at different time points, there is an overlap in the development timeline of the cells of the retina (6) (Fig. 2).

All retinal cell types arise from a homogenous group of proliferating progenitor cells which progressively gain heterogeneity as they start and continue to differentiate until

they form distinct cell types. The timing of onset of differentiation and cell fate to which the progenitors differentiate are controlled by intrinsic cues from within the progenitor cells. These are proteins transcription factors which bind to specific DNA sequences in order to regulate the differentiation process (6).

Several discoveries have been made describing these transcription factors and more are still coming to light but, there is a group of core transcription factors of the basic helix-loop-helix (bHLH) such as Atoh7,Ptf1a, and, homeodomain-containing families such as Vsx proteins, which are known to regulate the production of retinal cells. bHLH proteins have two distinct domains; the basic one that binds to a consensus DNA sequence known as E box and the HLH domain which allows homo or heterodimer formation with other bHLH factors forming complexes that can regulate different developmental processes (7). Homeobox-containing proteins as their name suggests, have a homeobox domain that allows sequence-specific DNA binding to regulate gene expression and many aspects of development including cell lineage specification (8) (9).

In retinal development initially Vsx2 is expressed in all retina progenitor cells with the exception of a small population that differentiates later into a subset of BCs and into MG. Down regulation of Vsx2 allows for expression of Atoh7, Ptf1a and Vsx1 (Fig. 3) (10). Atoh7 specifies for GCs while preventing PRs generation. Ptf1a which is downstream of FoxN4 and a repressor of Atoh7; is needed for the formation of HCs and ACs at the expense of GCs, PRs and BCs. Finally, Vsx1 expression leads to generation of a subset of BCs that is different from the one specified by Vsx2 and MG (10).

Epigenetics is a phenomenon in which factors external to the genetic code that is DNA, can influence how this genetic code is read without altering its sequence and do so in a

mitotically or meiotically heritable way (11). This provides a link of how environmental influences like tobacco smoke, air pollutants and others can affect epigenetic factors and influence gene expression changes in pathophysiological processes especially when the exposures happen in developmental stages (12). An area of much research interest presently is how epigenetics play a role in disease states such as cancer. A well-known mechanism in cancer epigenetics is activation of oncogenes by hypomethylation of their DNA and or repression of tumour suppressor genes by hypermethylation of their DNA (13) (14).

Some of the known epigenetic regulators are DNA methylation, Noncoding RNAs(ncRNAs) and histone modification. DNA methylation occurs through addition of a methyl group (CH3) to a cytosine nucleotide and it normally occurs at cytosines followed by guanine i.e. at CpG dinucleotides. The methylation state is mitotically heritable in that after DNA replication DNA methyltransferase I recognizes hemi methylated DNA (methylated on the parent strand) and lays down a methyl group on the daughter strand (15)(16).

DNA methylation is associated with gene silencing. This can be either through recruiting protein complexes like histone modifiers to lay down silencing histone marks including histone deacetylases and histone methyl transferases (17) or by blocking transcription factor binding to the methylated sequence.

ncRNAs such as RNA interference and Xist also, have important roles in epigenetic control through participating in heterochromatin formation. Examples of this are genomic imprinting (parent of origin mono-allelic silencing) whereby long ncRNAs act in cis to bring about silencing of flanking genes by binding to histone methyltransferases such as G9a and polycomb repressive complex 2 (PRC2) which then silence the flanking genes.

Also, in X chromosome inactivation, inactive X-specific transcript (Xist) and X (inactive)-specific transcript, antisense (Tsix) are required to silence one of the X chromosomes in female mammals (18).

Histone modification is perhaps the most studied mechanism of epigenetic regulation. It is known as post-translational modification (PTM) of amino acid residues in the N-terminal tails of histones which are the proteins on which DNA is wrapped around in a nucleosome. The addition of PTMs to histone tails affects how histone proteins interact with DNA by strengthening or weakening the hydrostatic forces that bind histones to DNA. The presence of PTMs also can lead to recruitment of other proteins and complexes with enzymatic activities that alter chromatin orientation and thus affect different functions such as transcription of genes; DNA replication, repair and recombination (19).

There are many known ways histones can be modified such as acetylation, phosphorylation and methylation among others. PTMs interact with each other in order to fine-tune their overall effect on chromatin and hence on cellular processes (Fig. 4). This “crosstalk” happens in different ways including but not limited to; one type of modification depending on another for deposition for example methylation of H3K4 and of H3K79 are dependent upon the ubiquitylation of H2BK123; competitive inhibition can occur when more than one type of modification pathway happens on the same site exemplified by lysine residues which can be methylated, acetylated or ubiquitylated all of which may have opposing effects (19).

Histone methylation is the addition of a methyl(CH3) group to amino acid residues of histone tails. In relation to the other modifications it is the smallest in size and does not modify the charge of the histone proteins therefore, it does not mechanically shift the chromatin structure nor affect it through altering its hydrostatic interactions. The known

methylated amino acids are arginine (R) and lysine (K). Arginine residues can be mono(me1) or di (me2) methylated on their guanidinyl groups whereas lysines can have one (me1),two (me2) or three (me3) methyl moieties on their amine groups.

Lysine methylation involves moving a methyl group from S-adenosylmethionine to the Ɛ-amino group of lysine and is catalysed by histone methyl transferase enzymes (HMTs) (19)(20). HMTs are specific as to which lysine residue they target and how much they methylate the residue for example Neurospora crassa DIM5 specifically methylates lysine 9 on histone 3 (H3K9) whereas SET7/9 targets lysine 4 on histone 3 (H3K4), also DIM5 tri-methylates H3K9 but SET7/9 can only mono-methylate H3K4 (21).

Major histone methylation marks on histone 3 (H3) and histone 4 (H4) are found at H3K4, H3K9, H3K27, H3K36, H4K20 and H3K79. Gene expression studies have aided in revealing the correlation of some of these lysine methylation states with gene expression but only few of these consistently occupy repressed or active genes: Trimethyl-lysine 4 on histone 3 (H3K4me3) is universally found at the promoters of active genes while trimethyl-lysine 36 on histone 3 (H3K36me3) occupies their gene bodies; Trimethyl-lysine 27 on histone 3 (H3K27me3) was consistently associated with repressed gene bodies and their flanking regions (22) (fig.5).

The histone modification-H3K27me3 is only found in multicellular organisms and has been mainly linked to silencing of individual genes as opposed to repetitive genes which require H3K9me3 and H4K20me3 in addition to other silencing mechanisms (22). H3K27me3 is mediated by enhancer of zeste homolog 1 and enhancer of zeste homolog 2 (Ezh1 and Ezh2, respectively) of PRC2, a member of the polycomb group proteins (PcG). The other protein components of this complex are Eed, Suz12 and RbAp46/48 proteins. Ezh1 was found to have less methyltransferase activity as its knockdown did not

result in global reduction in H3K27me3 as was the case of Ezh2. However, Ezh1 has transcriptional repression activity which is known to be through chromatin compaction as is the case of PRC1 another PcG member and L3MBT-L1. It has also been found that Ezh1 is a stronger repressor in vitro whereas Ezh2 is more potent in vivo where it requires another factor PHF1 to stimulate its H3K27me3 activity, allowing the recruitment of PRC1 which needs binding to H3K27me3 to compact chromatin (23). Important to note is the difference in expression profile of these two enzymes; Ezh1 being found in adult tissues while Ezh2 was only found in proliferating cells (23) (24).

It has been established that the H3K27me3 repressive histone mark is important in X inactivation, a dosage compensation phenomenon in which the paternal or maternal X chromosomes is silenced in female mammalian cells; in some instances it is also involved in silencing imprinted genes but perhaps its most studied role is that of regulating the expression of developmental genes: inactivation of the primary H3K27 methyltransferase- Ezh2 in the hematopoietic lineage blocked formation of Pre-B from pro-B cells (25). Also, in skin development, deletion of Ezh2 led to the dramatic loss of H3K27me3 and accelerated epidermal differentiation. Gene expression analysis of the Ezh2-deleted epidermal precursor versus wild type cells revealed that 90 genes that are normally involved in the final maturation stages of the epidermis were upregulated on Ezh2 deletion (24). This points to the role of H3K27me3 in regulating the timing of differentiation by blocking transcriptional activators from accessing the relevant genes.

H3K27me3 is additionally known to play a role in establishing a so-called ‘bivalent’ state of chromatin. Bivalent chromatin occurs when genes carry both the active state modifcation-H3K4me3 and the repressed state modification-H3K27me3(26). This mostly occurs in embryonic stem cells where genes that regulate development exist in a

poised state with stalled RNA polymerase II. The active mark- H3K4me3 is found at the CpG islands (promoters) of these genes but due to the absence of activating or developmental cues, the KDM5 family of H3K4me3 demethylases displace this mark from the CpG rich areas. This allows the PRC2 complex to access the promoter areas and deposit H3K27me3 (27). Deposition of H3K27me3, has been suggested to recruit the PRC1 complex which, as previously mentioned, compacts chromatin and can further activate PRC2 in a feed forward mechanism. When the differentiation decisions are made, transcriptional factors and coactivators compete with PRC2 for binding at CpG rich areas of lineage-specific genes which depletes H3K27me3 and reverses chromatin compaction. This together with the presence of H3K4me3, reinforces the active state of the genes that are required to differentiate the specific cell type (28). In this context, H3K27me3 has been described as a regulator of a developmental transition where by committed progenitor cells carry the bivalent chromatin state but on receiving differentiation signals, the lineage-appropriate genes loose the repressive H3K27me3 modification as described above probably via the action of H3K27me3 demethylases (29). On the other hand, lineage-inappropriate genes are stably repressed by H3K27me3 in additional to other silencing mechanisms such as DNA methylation and other repressive histone marks.

Histone methylation was initially thought to be a static modification until the discovery of Histone demethylases. The first discovered demethylase was LSD1A (Lysine-specific demethylase 1A) that demethylates H3K4 and H3K9 by an oxidative reaction using flavin as a cofactor (30). Since then, more classes of demethylases have been discovered of the JmjC (Jumonji C) domain- containing proteins including the UTX/UTY group of proteins consisting of Ubiquitously transcribed tetratricopeptide repeat, X chromosome; its Y

chromosome homolog and Jumonji domain- containing 3 (UTX, UTY and JMJD3 respectively).

The UTX gene that is found in humans and mice, escapes X-inactivation (31) in the female mammalian genome and is located at position 11.2 of the short arm of chromosome 11. Its protein has six tetratricopeptide repeats (TPR) at the N-terminal which are thought to mediate protein-protein interaction with other proteins, and a JmjC domain on the C-terminal ; UTY is found on the Y chromosome and has 88% homology with UTX as it has the same TPR and JmjC domains. JMJD3 also found in mice and humans has high homology with UTX and UTY both inside and outside of the JmjC domain but lacks the TPR domains.

Folded into eight β-sheets, the JmjC domain is the catalytically active site found in the three proteins. Three residues within the domain bind an Iron Fe(II) cofactor and two bind an α-Ketoglutarate cofactor, allowing the domain to form a highly reactive oxoferryl species that hydroxylates the methylated histone and disposes of the methyl group as formaldehyde (32)(33).

In additional to high residue specificity, histone demethylases are also methylation status- specific. In one study using calf histones, UTX and JMJD3 were able to demethylate H3K27me1/me2/me3 and H3K27me2/me3 respectively without having any effect on methylated H3K4,H3K9 or H3K36.These results were also confirmed on a monkey fibroblast-like cell line (COS-7 cells) (34). In comparison, UTY was found to have lower catalytic activity compared to its UTX and JMJD3 counterparts due to point mutations that alter its substrate binding; instead it has many important non-catalytic functional roles through its intact tetratricopeptide repeat region (35) for example, UTY

is responsible for the association between loss of the Y chromosome and poor prognosis in a range of cancers (36).

UTX and JMJD3 counteract the actions of PRC2 in maintaining embryonic stem cell identity, establishing bivalent domains and in development, among others. In human embryonic kidney cells (HEK293 cells), UTX knockdown led to increased H3K27me3, PRC1 and PRC2 at transcription start sites of HOX genes (regulate body structure) (37) while, knockdown of the UTX zebrafish homolog zUTX1 led to posterior notochord truncation and abnormalities in posterior structures of the embryo (38). In addition, when lipopolysaccharide was added to macrophages, it induced an inflammatory response which led to accumulation of JMJD3 and in turn a change in expression of a subset of genes including Bmp-2: at the previously bivalent promoter of the Bmp-2 gene (a morphogen involved in bone development), JMJD3 accumulation led to depletion of H3K27me3 but no change in H3K4me3 marking the activation status of this gene (39).

Several studies have revealed the role of the H3K27me3 modification in different systems. Firstly, in late neocortical development PRC2 through the H3K27 methyltransferase activity of Ezh2 restricts the neurogenic potential of neural precursor cells allowing the transition from neuronal to astroglial fate. When Ezh2 was deleted in these neural precursor cells there was a prolonged neurogenic phase and a delayed astrogenic phase (40). Secondly in Xenopus retina, PRC2 mRNA was found to be expressed in retinal progenitor cells (RPCs) and down regulated in differentiated retinal cells while global H3K27me3 was upregulated upon differentiation. As the authors found no Ezh1 expression in xenopus retina, knockdown of Ezh2 in RPCs led to reduction in H3K27me3 in postmitotic cells. Also blocking of PRC2 reduced retina progenitor cells (RPCs) proliferation as well as expression of pro-neural genes. In keeping with this,

gliogenesis was favoured over neurogenesis with production of MG at the expense of GCs and BCs (41). Thirdly, deletion of Ezh2 and hence downregulation of H3K27me3 in mouse RPCs led to reduction in proliferation of these cells and upregulation of Cdkn2a(Arf/Ink4a) and Cdkn2b(Ink4b), which regulate cell cycle transition from the G1 to the S phase and apoptosis (42)(43). These genes are repressed by H3K27me3 in the new born mouse (44). There was also early differentiation of normally late born cells- rod PRs and MG in the early postnatal period with a later reduction in the rod PRs possibly due to low progenitor number caused by early cell cycle exit. The increase in number MG was thought to be due to a shift in favour of gliogenesis as seen in the Xenopus retina (43). Finally, a study using the mouse retina examined the role of H3K27me3 in the retina by blocking the demethylase action of Jmjd3 using shRNA targeting Jmjd3. Knocking down Jmjd3 in RPCs at E17 depleted PKC positive rod-ON BCs as well as recoverin positive cone-OFF bipolar cells. The loss of these retinal subtypes was accompanied by significant reduction of Bhlhb4 and Vsx1 transcripts that are known to mediate differentiation of rod-ON and cone-OFF BCs respectively. In addition, these gene loci were found to have low H3K27me3 in wild type Isl-1 positive ON BCs and when Bhlhb4 was over expressed together with shRNA targeting Jmjd3, it was able to rescue the PKC positive rod BC number to comparability with control. The authors were able demonstrated the important role of Jmjd3 in spatiotemporal regulation of retinal cell subtype-specific differentiation through its demethylation of H3K27me3 (45).

As previously mentioned, H3K27me3 demethylases behave differently in terms of their amino acid residue specificity and status of methylation. They could also behave differently based on the differences in their structures; despite the fact that Jmjd3 and Utx both have been found to have H3K27me3 demethylase-dependent and independent roles,

their demethylase-independent actions could be different because Jmjd3 does not contain the TPR domains contained in Utx.

In this study, I aim at eliciting the role of Utx in the development of the mouse retina which has not been studied before. I hypothesize that Utx and Jmjd3 may have similar target genes as has been found in some studies and thus Utx may also regulate the differentiation of bipolar cells. I also aim to uncover the possible functional interactions between Utx and Jmjd3 using the mouse retina as a model.

Materials and methods

Mice Strain:

Heterozygous Utx floxed male mice were kindly provided by Prof. Honda (Tokyo Women's Medical University) and were of C57BL/6J background. Homozygous Utx conditional knockout (CKO) mice were obtained by crossing heterozygous Dkk3-Cre;$Utx^{flox/Uty}$ male with homozygous $Utx^{fl/fl}$ female mice to give homozygous Utx CKO female mice. The Dkk3-Cre mouse line used was previously described (46). The presence of a vaginal plug was defined as E0.5 and the day of birth as P0. All animal experiments were approved by the Animal Care Committee of the institute of Medical Science, University of Tokyo and were conducted in accordance with the ARVO (Association of Research in Vision and Ophthalmology) statement for the use of animals in ophthalmic and vision research

Genotyping:

Genomic DNA was extracted from mice tails, PCR performed using KOD FX Neo reagents (Toyobo Co., Ltd., Osaka, Japan), then Agarose gel electrophoresis was done. Dkk3-Cre transgene PCR conditions: 98ºC, 2min; 98ºC, 10sec; 60ºC, 30sec all for 35 cycles then 68ºC, 1min. Primers used: Cre159 (5'-TCGATGCAACGAGTGATGAG-3'), Cre160(TTCGGCTATACGTAACAGGG-3'). Utx flox PCR conditions: 98ºC, 2min; 98ºC, 10sec; 68ºC, 1min for 35 cycles. Primers used: Kdm6a_loxP2-F(5'-GCCACATATTTCCCTAGAACACCCAGGCAG-3'), Kdm6a_GT_R(5'-AGCTAAGCTGCTTCCAGGGTTCTCATTAGC-3')

Total RNA extraction, reverse transcription and quantitative PCR (RT-qPCR):

The following reagents were used: Sepasol-RNA I super G for total RNA isolation (Nacalai tesque), ReverTra Ace with gDNA remover (TOYOBO), THUNDERBIRD florescent probe (TOYOBO). Total RNA extraction and reverse transcription were done according to the supplier's protocol. Conditions for the one-step qPCR programs: 1 Pre-incubation cycle - 95ºC for 60s ; 40 cycles of 3-step amplification- 95ºC for 10sec, 60ºC for 10sec, 72ºC for 30sec and 1 melting cycle- 95ºC for 10sec, 65ºC for 60sec, 97ºC for 1sec. Transcript levels of Bhlhb4, Tfap2a, Prkca and Vsx1 were normalized by Gapdh and Tbp, and then quantified using Fasmac primers as follows:

Gapdh_F: 5'-TGACCACAGTCCATGCCATC-3'

Gapdh_R: 5'-CATACCAGGAAATGAGCTTGAC-3'

Sdha_F: 5'-GTGTGAAGTAGGGCAGGTCC-3'

Sdha_R: 5'-ACAAGGCACTGGCTCGATAC-3'

mKdm6a_F: 5'-CTCTGGACTTGCAGCACGAA-3'

mKdm6a_R: 5'-ATTGGTAGGCTCCATGCCTC-3'

Bhlhb4-F: 5'-AAAATTACGCCGAGGTCCTT-3'

Bhlhb4-R: 5'-TCACCTCCTGGGATCTTCC-3'

mTfap2a_F: 5'-CGTTACCCTCCTCACGTCAC-3'

mTfap2a_R: 5'-TGGGATCGGAATGTTGTCGG-3'

Pkca-F: 5'-CAGGAAGGGGGTGCTTCT-3'

Pkca-R: 5'-GCGAGAGACTGAGAGACCAGA-3'

Vsx1-F: 5'-GGTTGAAATGGGTTCTTGCT-3'

Vsx1-R: 5'-TGAGGCAGGAGATCATGAAA-3'

Plasmids:

shRNA vectors for knocking down Utx and Jmjd3 were made as previously described (53). Target sequences were aagtcttgtgattggctaaat (shJmjd3 second), aagcaaatgttccagtgtata (shUtx first), aagtcagtttgtcttaatgcagc (shUtx second). Efficiency of shRNA for mouse Jmjd3 were checked using NIH3T3 cells (Iida et al 2014), and those of shRNA for mouse Utx were checked by immunostaining with Kdm6a antibody. Utx expression vector (pDONL-mUtx_FlagHis) was kindly gifted by Prof. Suzuki at Cancer Research Institute of Kanazawa University. The Utx-expressing component was digested using BamHI and NotI and then inserted into a CAG-KS vector. shUtx-resistant Utx was made by inverse PCR using the KOD-plus -Mutagenesis Kit (TOYOBO) according to the kit's instruction manual; the following primers were used: mUtx_Mut_F3 (with a point mutation)- ccctgtctacaggtttattcagcgacctgg and mUtx_Mut_R2- acatttgcttcatatagatcttc. PCR conditions used: preincubation at 94◦C for 2 min, 9 cycles of the 3 PCR steps at 98◦C for 10 sec, 50◦C for 30 sec and 68◦C for 9min then holding at 10◦C. PCR product was ligated and transformed in to E.coli, then the mutations were confirmed by sequencing.

Immunostaining:

Immunostaining was performed as previously described (54). Retinae were fixed with 4% PFA in PBS, washed three times in PBS and with 15% and 30% sucrose for cryoprotection prior to sectioning. Sections were incubated with primary antibodies over night at 4ºC then secondary antibodies for 1h at room temperature. Nuclei were counter stained with DAPI 1µg/µl and sections mounted in 50% glycerol/PBS. Primary antibodies were: PKC (1:200; Calbiochem), Isl1 (1:500; DSHB), Ki67 (1:200; BD Bioscience), GS (1:500; Abcam), Tfap2a (1:50; DSHB), Calbindin (1:500; Chemicon), Chx10 (Pan BP; 1:500; Exalpha), PNR (1:200; PPMX), GFP(1:2000; Abcam), Bhlhb5 (1:500; Santa Cruz

Biotechnology), Recoverin (1:1000,Chemicon), Kdm6a (Utx) (1:2000; GeneTex), ACTIVE caspase-3 (1:500; Promega), Kdm6b (Jmjd3) (1:2000; Abcam). Flourescent secondary antibodies conjugated to Alexa Flour 488 or Alexa Flour 594 (Molecular probes, Eugene, OR) were used.

Retinal Explants:

Retinal explants preparation and culture was done as previously described (54). Retinae with lenses extracted from ICR mice at P0, P1 or P2 were electroporated and retinae isolated as explants onto Millicell chamber filters (diameter- 30mm, pore size- 0.4µm) with the ganglion cell layer facing upwards. The retinal explants were cultured in six-well plates containing medium (1ml/retina) which constituted of : 50% MEM-HEPES (Gibco), 25% Hank's balanced salt solution (Gibco) and 25% heat-inactivated horse serum (JRH Biosciences) supplemented with 200 µM L-glutamine, and 5.75 mg/ml glucose, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco).

The medium was replaced on alternate days and explants were cultured for 3 days(P1) or 11 days (P2) after which they were harvested, cryo-sectioned and immuno stained with appropriate antibodies.

Results

Utx expression pattern in the mouse retina:

I examined the expression dynamics of Utx in the mouse retina. On immuno-histochemistry, Utx was expressed in retinal progenitor cells in the neuroblastic layer (NBL) co-staining with Ki67 and by cells in the ganglion cell layer (GCL) during early embryonic development (E14 to E18). As development progressed, Utx was more strongly expressed in the post mitotic cells of the inner NBL and the GCL, with weakening expression in the outer NBL (P1, P3). In mature retina (P14), Utx was expressed in the GCL and INL but not in the ONL. I also co-stained Utx with various INL and GCL cell type markers and found that Utx was expressed in all INL and GCL cells (Fig. 6 A & B). On the other hand, staining mature retina with Jmd3 marker showed almost no expression at this stage (Fig. 6 D). On RT-qPCR, Utx expression was stable from developmental to adult stages (Fig. 6 C) unlike the expressed of Jmjd3 that was found to be high in mid postnatal development and low before and after this stage (45). RNA sequencing data obtained in our lab showed that the expression levels of Utx and Jmjd3, are comparable in early embryonic development in whole retina. In post-natal development the expression level of Utx in photoreceptors (CD73 positive) and non-photoreceptor cells (CD73 negative) remains the same as the prenatal levels while that of Jmjd3 peaks in both cell types. In adult retina, Utx expression levels remains stable unlike Jmjd3 which decreases in adult compared to developmental retina (Fig. 6E). In addition, using in situ hybridization (ISH), Utx signals were detected in the INL at P8 and P12 (45).

ShRNA-mediated knockdown of Utx impaired the differentiation of rod bipolar cells:

In order to understand the role of Utx in the development of the retina, I performed loss of function experiments in retinal explants. I first checked the knock down efficiency of two different short hairpin RNA (shRNA) that target Utx by transfecting them or pU6 empty vector (control) in P1 retina along with an EGFP expressing plasmid and culturing for 3 days. I then examined the level of Utx expression by immuno-histochemistry of cryosections of the explants. At 3 days of culture, the number of EGFP and Utx double-positive cells was significantly decreased in first or second shRNA compared to that in the control (Fig. 7), indicating that both shRNA successfully knocked down Utx expression in transfected cells. To check the role of Utx knockdown in the retina, I transfected the two shRNA (shUtx first & second) into P1 retina and cultured them for 12 days. Immuno-staining with markers of various retina cell types revealed that of the EGFP expressing cells in the INL , the percentage of protein kinase C alpha (PKCα)-expressing rod BCs had decreased compared to that in the control retinae (Fig. 8). The percentage of Chx10 (pan bipolar cell marker) positive cells slightly decreased with knockdown of Utx using both shRNA (Fig. 9). The other bipolar cell subtypes- cone OFF and ON bipolar cells, were not significantly changed by knocking down Utx (Fig. 10). As shUtx first seemed to cause a stronger phenotype than the second, I used shUtx first in all subsequent experiments. Knock down of Utx did not affect the differentiation of other retinal cell types and the ONL thickness (reflecting photoreceptor cell number) (Fig. 11).

Utx overexpression rescues the reduction in rod bipolar cell number in shUtx-transfected retinae:

To confirm that the reduction of rod bipolar cells seen after knockdown of Utx was indeed due to down regulation of Utx, I checked if Utx overexpression could rescue the loss of bipolar cells after knockdown of Utx. First P0 explants were transfected with shUtx or control vector and an EGFP plasmid then cultured for 12 days and immune-stained as cryosections. At this stage also, PKCα positive cells were decreased by knockdown of Utx (Fig. 12 A & D). I then checked the effect of overexpressing Utx on its own. P2 retinae were transfected with the control vector or with Utx and cultured for 11 days as explants. After staining the explant sections with anti PKCα and EGFP markers, I did not find any difference in the structure of rod bipolar cells and there was no significant difference in their number between Utx-transfected and control explants. Thus, Utx on its own did not cause changes in rod bipolar cells (Fig. 12 B). To check if Utx overexpression could rescue rod bipolar cells after Utx knockdown; shUtx and EGFP or shUtx, Utx and EGFP plasmids (shUtx + EGFP or shUtx + Utx + EGFP respectively) were transfected into P0 retina which were then cultured as explants for 12 days. Immunohistochemistry with anti PKCα and EGFP revealed that Utx over expression rescued the number of PKC α-expressing cells, albeit not fully (Fig. 12 C & D).

Deletion of Utx in mouse retina also leads to a reduction in rod bipolar cell number:

To delete Utx, heterozygous Utx floxed male mice that express Dkk3 promoter regulated Cre recombinase (gifted by Professor Honda of the Tokyo Women's Medical University)

were crossed with heterozygous Utx floxed female mice to give homozygous Utx conditional knockout (Utx cKO) female mice. The deletion targeted exon 11 and 12 of Utx leading to a frame shift mutation and a premature stop codon that lead to truncation of the protein (Fig 13 A). I confirmed the deletion of Utx by genotyping using PCR for the presence of the cre recombinase gene and absence of both copies of Utx. Also, I confirmed the deletion of Utx by examining the mRNA expression level using RT-qPCR (Fig. 13 B). Further, I checked the protein level of H3K27me3 by immunostaining wild type (WT) and Utx cKO P14 retinae then quantified the H3K27me3 protein level using the AxioVision software: As expected the absence of Utx demethylase activity increased H3K27me3 levels in the Utx cKO compared to WT retinae mostly in the INL and GCL (Fig. 13 C). Next, I checked if deletion of Utx in the mouse retina in vivo would have the same effect on rod bipolar cells as knockdown of Utx in vitro. I carried out immunohistochemical analysis of WT versus Utx cKO retinae at P10, P14, P24 and P40. I found that the deletion of Utx in vivo at all these stages also causes a significant reduction in the number of PKCα positive rod bipolar cells in Utx cKO compared to WT retinae (Fig. 14 A & B). Overall bipolar cell number was also decreased to varying degrees at the different stages (Fig. 14 C & D). As was the case for Utx knockdown, when Utx was deleted, the number of other subtypes of bipolar cells were not significantly affected. P14 WT and Utx cKO retinae were stained with antibodies against: Isl1 which is a marker for ON bipolar cells (cone ON and rod ON), Pax6- pan amacrine cell marker, Recoverin and Bhlhb5- markers of con OFF bipolar subtype in the outer part of the INL. The average number of cone ON (weakly Isl1+Pax6-) and cone OFF (Recoverin+Bhlhb5+) bipolar cell subtypes was not significantly different between WT and Utx cKO retinae (Fig. 15).

Discussion

Utx may be important in maintenance of INL and GCL cells :

Utx is expressed retinal progenitor cells of the NBL in embryonic and early postnatal stages (E14 to P3) at both mRNA level and protein level. Surprisingly I did not find a difference in proliferation or apoptosis using Ki67 and active caspase 3 markers respectively, at P3 in Utx cKO versus WT retina (Fig 17). It is possible that proliferation and apoptosis may happen at earlier or later stages than examined which could be the cause of reduction in cell types such as rod bipolar cells. Further, Utx continued to be expressed stably and was maintained to adult stages on transcript level and, in the INL and GCL on protein level. It is possible that in addition to its role in differentiation, Utx may be involved in maturation and/or maintenance of INL and/or GCL cell types. On the other hand, the study on Jmjd3 previously mentioned, showed that Jmjd3 mRNA and protein expression peaked at mid postnatal stages and was low before and after that. I also found that Jmjd3 protein expression was very low in P14 explant retina (Fig 6D). This may mean that Jmjd3 is only required for differentiation of late born cell types such as rod bipolar cells.

Utx regulates retinal rod bipolar cell differentiation:

In vitro, knockdown of Utx decreased PKCα expressing rod bipolar cells at P12 when I analysed protein level by immunostaining. This knockdown phenotype of Utx was verified by the ability of Utx overexpression to rescue partially the number of rod bipolar cells; I observed that Utx over expression was very strong in the ONL but, only few

transfected cells in the outer part of the INL where rod bipolar cells are located, were able to express Utx (Fig. 12E control panels & Fig. 12F), therefore this could be the reason for the incomplete rescue of rod bipolar cell number when Utx was overexpressed in the shUtx transfected retinae. Deletion of Utx in vivo retinae also caused a similar phenotype to that of Utx knockdown in vitro with consistently significant decrease of PKC α expressing cells at P10, P14, P24 and P40 which confirms that Utx is important in the differentiation of rod bipolar cells. The overall bipolar cell number analysed using the pan bipolar cell marker- Chx10, was slightly decreased by knockdown of Utx and deletion of Utx also caused decrease of Chx10 to varying levels of significance at P10, P14, P24 and P40. This reduction represents the loss of rod bipolar cells which make up 38% of all bipolar cells (46) and may be the reason why the overall bipolar cell number did not decrease dramatically.

Utx is not required in the differentiation of other retinal cell types:

Utx did not seem to be required in the generation of other retinal cell types in vitro such as amacrine cells, horizontal cells, Müller glia and rod photoreceptors as its knockdown showed no changes in the protein expression level of Tfap2a, calbindin, GS and in the thickness of the outer nuclear layer at the P13 stage as shown in the above results.

In addition, Utx cKO retinae at an earlier stage (P3) did not show any defect in the differentiation of early born amacrine cells as Tfap2a protein level was unchanged (Fig 16); this together with the invitro results confirms that Utx is dispensable in the differentiation of early born retinal cell types. The role of Utx in differentiation of late born cells using Utx cKO retina will be examined in future.

Utx regulates differentiation in other systems:

The role of Utx in inducing the expression of lineage-specific genes and allowing cellular differentiation has been documented in several studies: Utx double knockout female mice exhibited severe cardiac defects and Utx knockout male mice lacked rhythmic contractions of cardiac cells due to failure to induce the appropriate cardiac specific genes (47); Utx is critical in the differentiation of red blood cells as Utx-deleted female mouse embryos at E10.5 were anaemic (48) and hematopoietic transcription factors were down regulated in the bone marrow of Utx KO mice (49). In neurogenesis, Utx was identified as a promotional transcription factor for Pten in cerebral cortical development through H3K27me3 demethylation at the Pten promoter (50) also Utx deficiency was found to cause defects in CNS neural morphology and hippocampal synaptic transmission (49).

Utx and Jmjd3 may be partially redundant and not the only regulators of rod bipolar cell differentiation:

I examined the interaction of Utx and Jmjd3 by many repeated rescue experiments of their knockdown phenotypes of reduced rod bipolar cell number but the experiments were unsuccessful because of the possibility that the CAG promoter could not efficiently express these genes in bipolar cells and/or at the mouse stage at which I transfected. A research collaborator therefore recently started to examine double conditional knock out mice (DKO) generated by crossing Dkk3-Cre; $Utx^{flox/Uty}$; $Jmjd3^{flox/flox}$ male with $Utx^{flox/flox}$; $Jmjd3^{flox/flox}$ female mice. Jmjd3 cKO like Utx cKO led to reduction but not complete loss of PKCα positive rod bipolar cells; DKO retinae seemed to cause more reduction of rod bipolar cells compared to single knockout of either gene but also did not lead to complete loss of PKCα positive cells (data not shown). This means that Utx and

Jmjd3 may be at least partially redundant in their role in the differentiation of PKCα expressing rod bipolar cells. In addition, the incomplete loss of PKCα expressing cells may be due to the presence of other mechanisms that additionally regulate rod bipolar cell development for example an as yet undiscovered demethylase gene or another transcription factor that may be indispensable for the differentiation of these cells.

Study limitations and future recommendations:

As mentioned above, Utx over expression using the CAG promoter was not successful in bipolar cells when transfection was done at the P0 (birth) stage. To confirm that Utx over expression can rescue rod bipolar cell number after shUtx; it is important in future to try a different promoter such as pHes5 which is also expressed in retinal progenitor cells and at a different stage if necessary. Also, to complete the confirmation that Utx did not affect other retinal cell types it is important to stain with more markers in Utx cKO and WT retinae.

To understand better the contribution of Utx and Jmjd3 to rod bipolar development and answer questions about redundancy or lack thereof, further analysis using conditional double knock out mice is important and ongoing.

It may be also interesting to check if Utx regulates rod bipolar differentiation in the same manner as Jmjd3 by demethylating H3K27me3 at the promoters of the appropriate genes This can be done by examining H3K27me3 levels at loci of candidate genes after knockdown of Utx also by comparing the rescue of PKCα positive cells using catalytically inactive Utx versus full length Utx proteins. In embryonic stem cells and in cardiac development Utx was found to have demethylase independent mechanisms of activating gene expression; the loss of Utx was found to disturb the interaction between

the Brg1-containing SWI/SNF chromatin remodelling complex and the core cardiac transcription factor- Tbx5, an effect that is thought to be independent of the demethylase activity of Utx (52).

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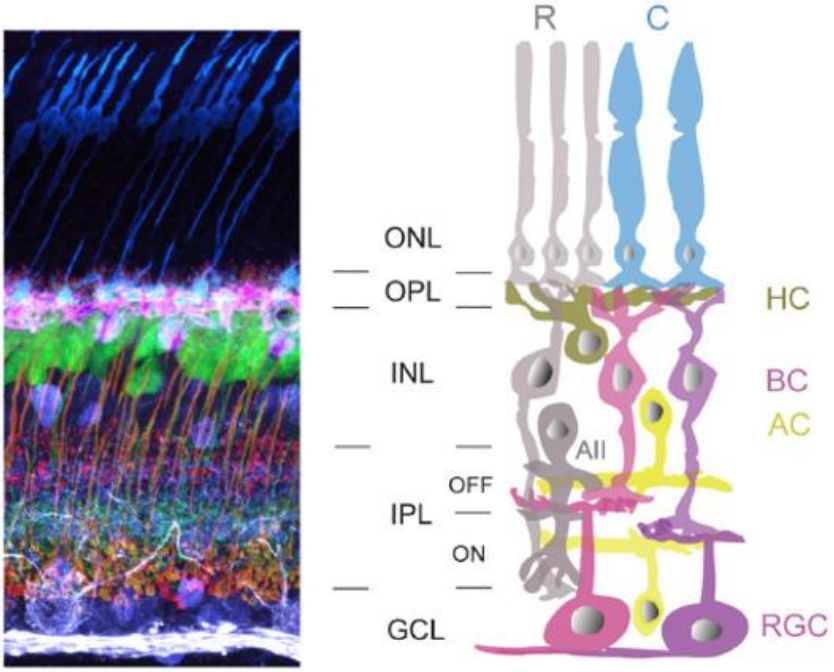


Figure 1. The basic structure of the mammalian retina:
Left: A vertical section of mouse retina showing labelling of the major neuronal cell types. Immunostaining for cone photoreceptors (anti-cone arrestin, blue), horizontal cells (anti-calbindin, pink), bipolar cell terminals (anti-synaptotagmin2 and anti-PKC, red), amacrine cells (anti-calretinin, purple), and ganglion cells (SMI-32, white).Right: A schematic showing the layers of the mouse retina corresponding to the left side image. PKC = Protein Kinase C, SMI-32 = anti hypo-phosphorylated neurofilament heavy chain, ONL = outer nuclear layer, INL = inner nuclear layer, GCL = ganglion cell layer, OPL = outer plexiform layer, IPL = inner plexiform layer, HC = horizontal cell, BC = bipolar cell, AC = amacrine cell, RGC = retinal ganglion cell. (adapted from reference 2)

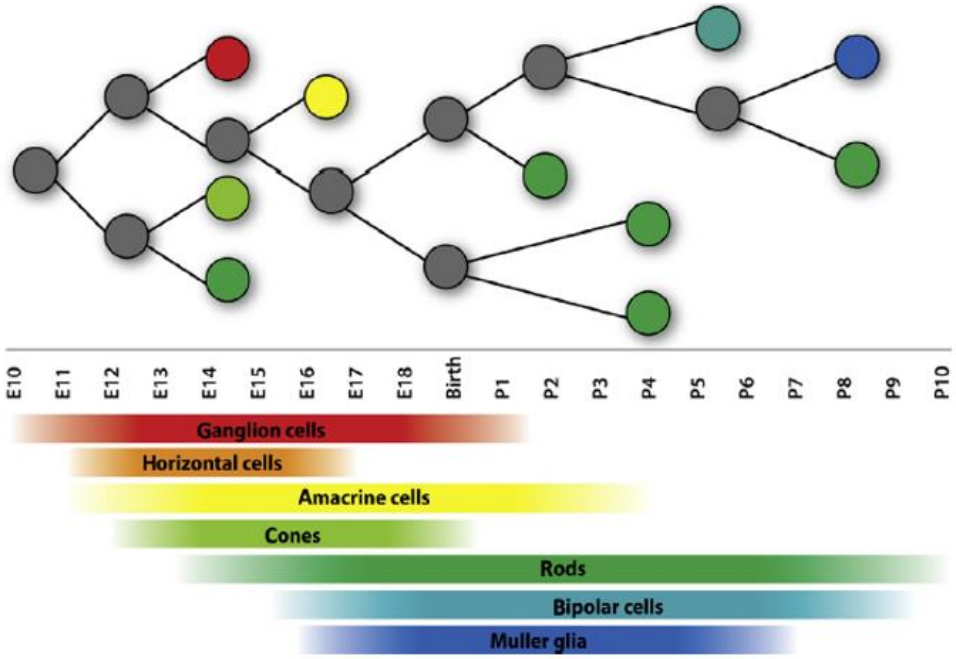


Figure 2. Development timeline of the retina:
Top: a depiction of the progressively changing competence of progenitor cells as they divide to form daughter progenitor cells, neurons or asymmetrically forming both progenitor and neuronal cells**.** Bottom: The overlapping time points at which different cell types of the retina are generated (adapted from reference 6).

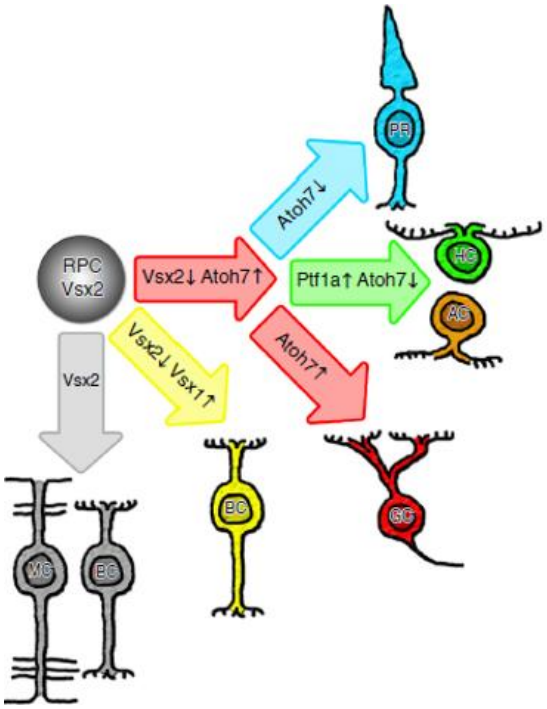


Figure 3. Basic helix-loop-helix and homeodomain core transcription factors regulate generation of retinal cell types:
Vsx2 is expressed in the common progenitor pool preventing differentiation by repressing Atoh7, Ptf1a and Vsx1. As development progresses Vsx2 expression in most RPCs ceases allowing formation of the different cell types. The remainder of the Vsx2-expressing RPCs generate a subset of BCs and MG . RPC=Retinal progenitor cell, BC = Bipolar cell, GC = Ganglion cell, PR = Photoreceptor cell, HC = Horizontal cell, AC = amacrine cell (adapted from reference 10)

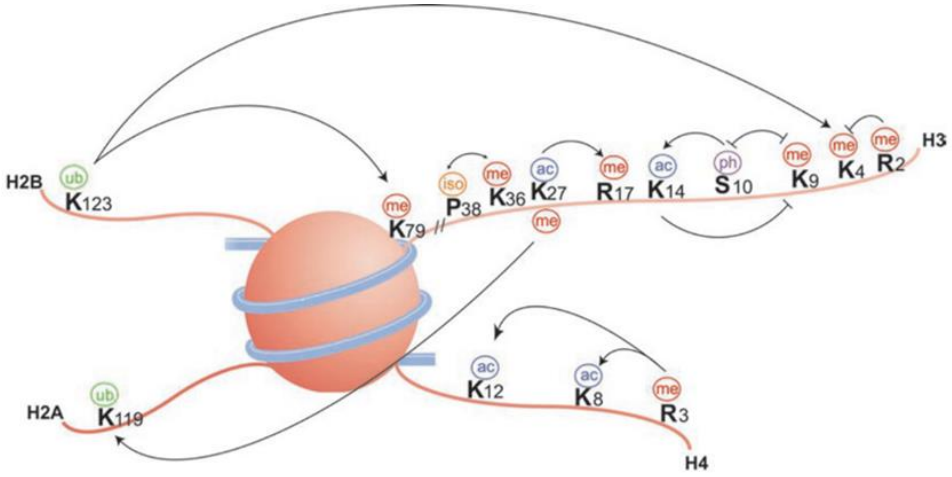


Figure 4. Histone modification crosstalk
Histone modifications on adjacent or distant sites interact in order to fine tune their effects on chromatin. Arrow ends indicate positive interactions while flat ends indicate negative interactions (adapted from reference 19).

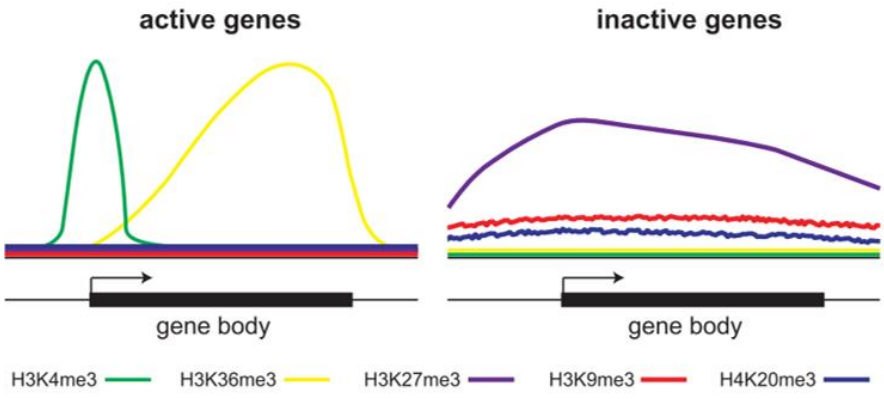
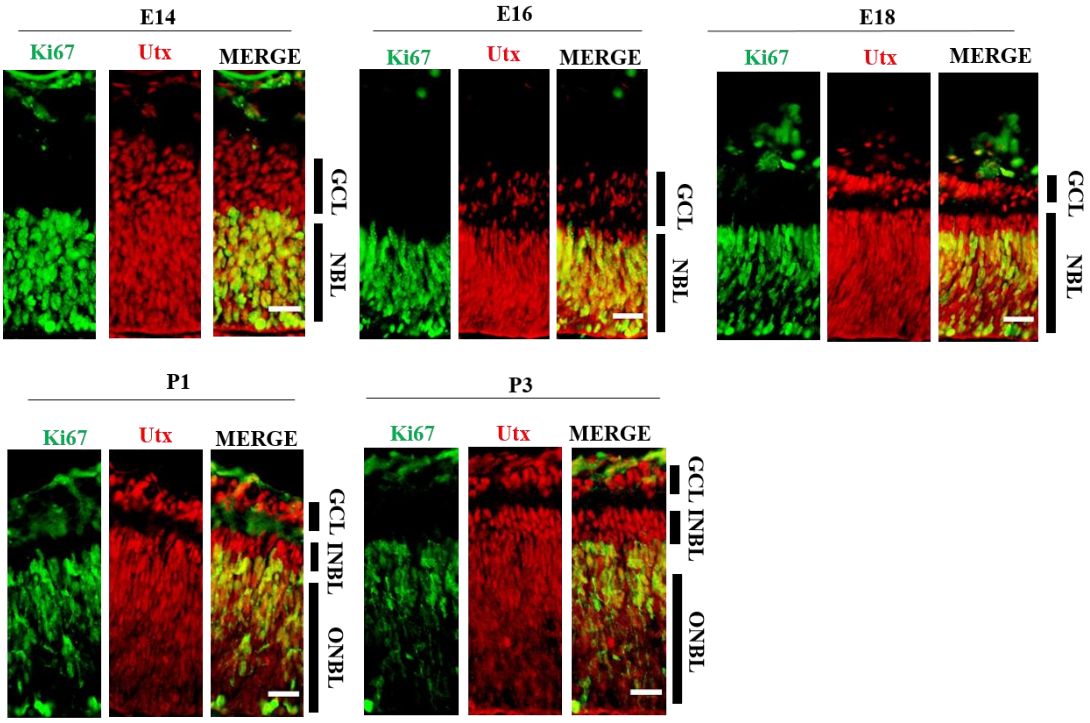
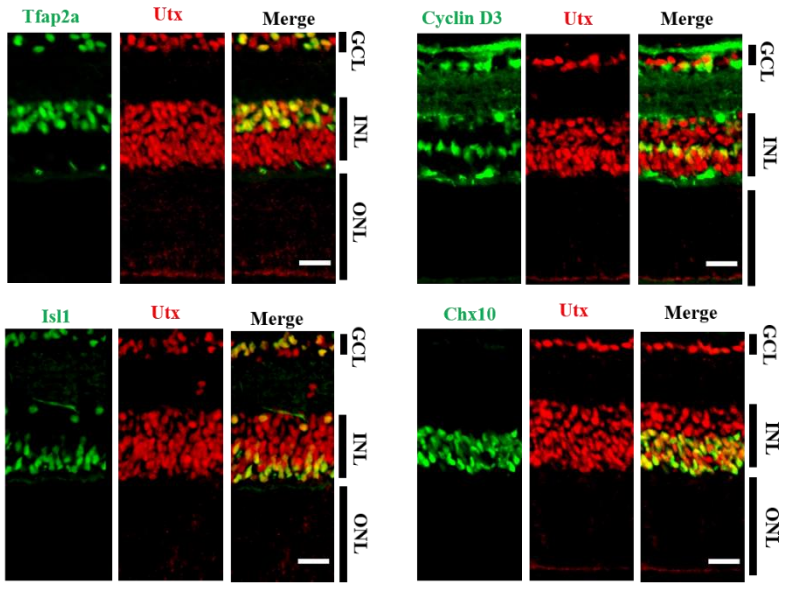


Figure 5. Histone methylation patterns at mammalian genes
H3K4me3 commonly occupies the promoters of active genes whilst H3K36me3 occupies their gene bodies. At inactive genes H3K27me3 is the major player occupying their gene bodies and flanking regions. H3K9me3 and H4K20me3 also occupy inactive genes but to a lesser extent (adapted from reference 22).

A


E14
Ki67
Utx
MERGE
GCL
NBL
E16
Ki67
Utx
MERGE
GCL
NBL
E18
Ki67
Utx
MERGE
GCL
NBL
P1
Ki67
Utx
MERGE
GCL
INBL
ONBL
P3
Ki67
Utx
MERGE
GCL
INBL
ONBL

B


Tfap2a
Utx
Merge
GCL
INL
ONL
Cyclin D3
Utx
Merge
GCL
INL
Isl1
Utx
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GCL
INL
ONL
Chx10
Utx
Merge
GCL
INL
ONL

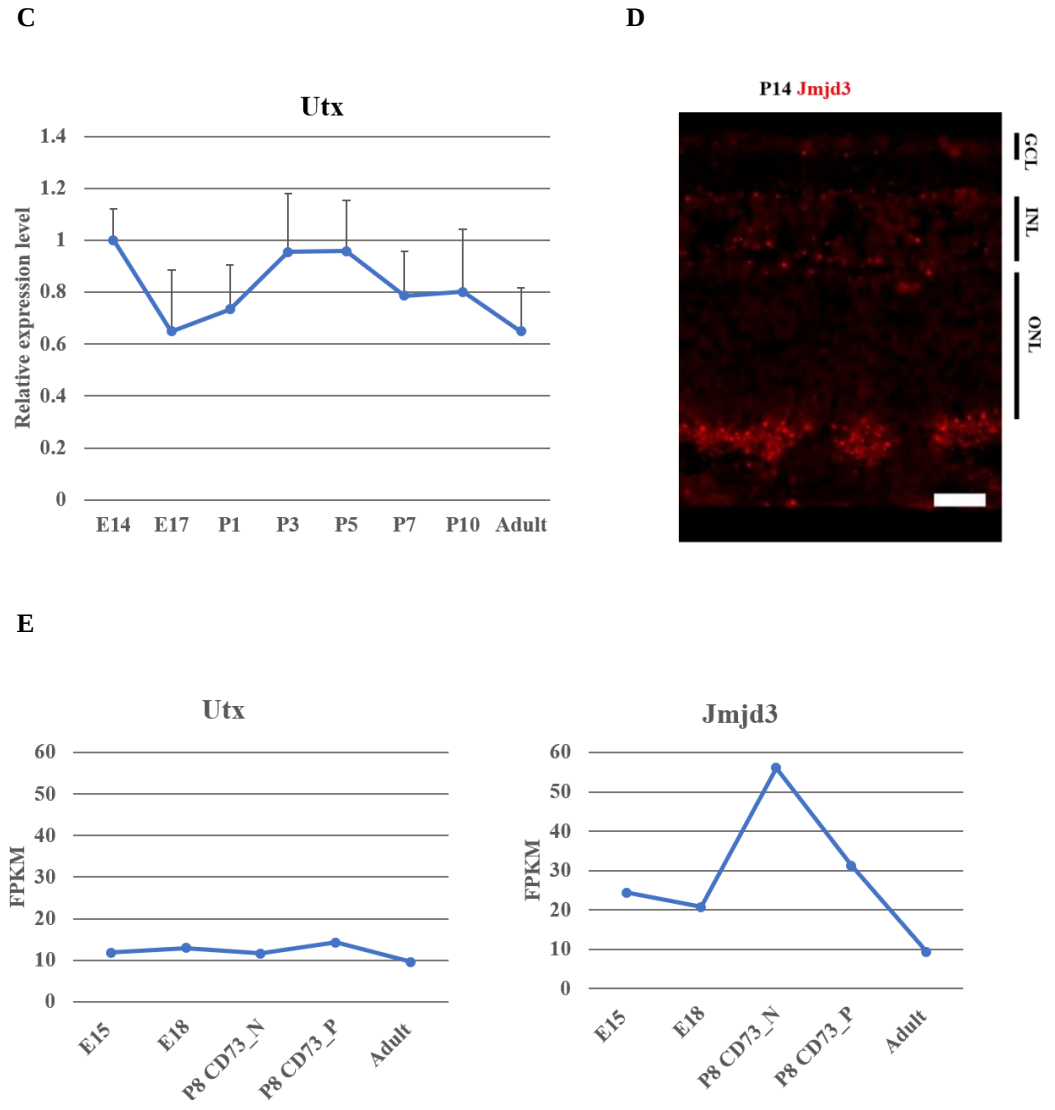


Figure 6. Utx expression pattern in the mouse retina

A: In prenatal stages of development (E14-E16) Utx is expressed in RPCs (Ki67 positive) of the NBL and in the GCL. Perinatally Utx is highly expressed in postmitotic cells in the INBL and GCL (E18-P3). B: In mature retina (P14) Utx is expressed in the INL and GCL and co-stains with markers of all cells in these layers. C: Utx mRNA expression in the retina is stable from developmental to mature stage on RT-qPCR. D: The expression of Jmjd3 in the mature retina (P14) is very low both in the INL and GCL; there is no expression in the ONL. E: RNA sequencing also showed that Utx is stably expressed while Jmjd3 expression peaks at mid postnatal stages and is low before and after that (accession number GSE71462). (NBL= neuroblastic layer, ONBL/INBL= outer/inner neuroblastic layer respectively, GCL= ganglion cell layer). FPKM = Fragments Per Kilobase of transcript per Million mapped reads. Scale bar =25 µm (A&B), 30 µm.

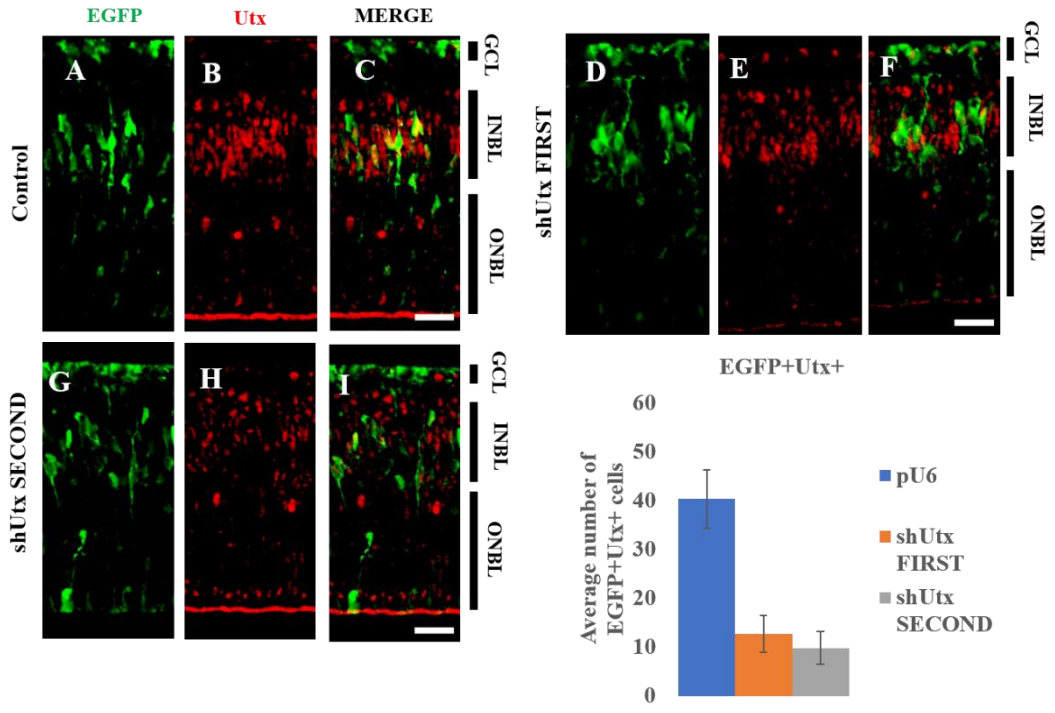


Figure 7. Utx expression was efficiently knocked down using two different shRNAs Utx expression decreased after knockdown with two different shRNA (D-I) in comparison to control (A-C). shRNAs or pU6 empty vector were transfected together with an EGFP-expressing plasmid in P1 retinae which were then cultured for 3days as explants. Utx expression was analysed by immunohistochemistry. EGFP and Utx double positive cells were counted over 440µm long sections. The average number of cells from three independent experiments is shown. ** p-value <0 .01, p-value > 0.05 = not significant (ns) by ANOVA followed by post-hoc Tukey's HSD test. Scale bar 25µm.

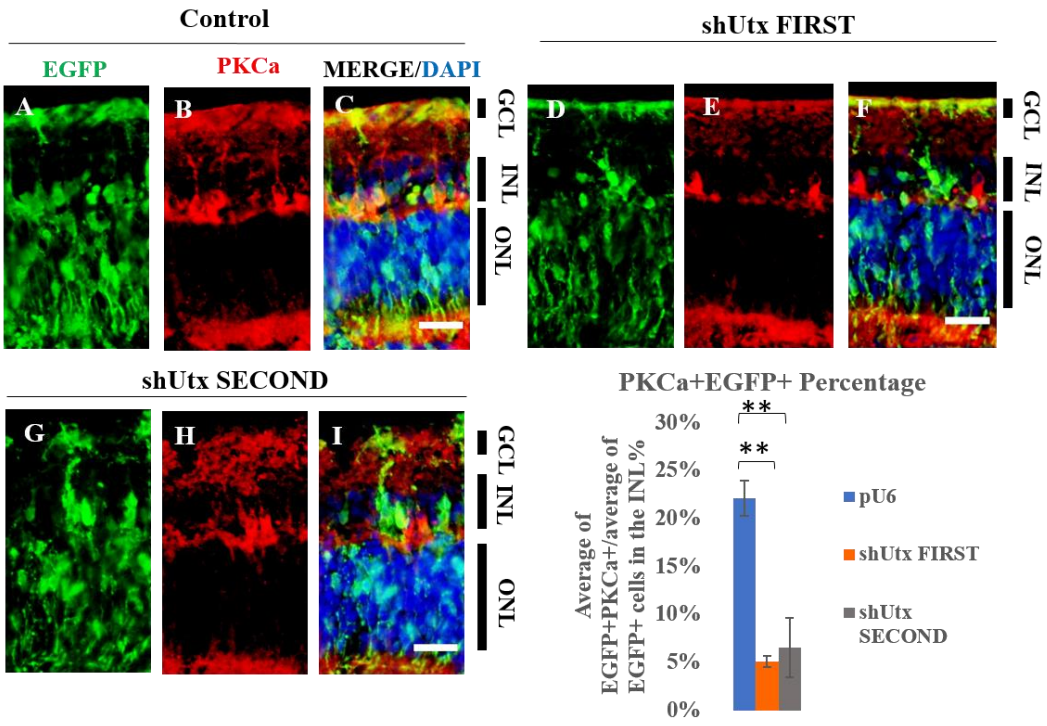


Figure 8. Rod bipolar cell number is reduced by knockdown of Utx.
Knockdown of Utx using the two shRNAs reduced PKC-expressing rod bipolar cells (PKC). A control vector (A-C) or shRNA(D-I) was transfected together with an EGFP-expressing plasmid at P1 then retinae were cultured for 12 days as explants. The percentage of EGFP+PKC+ cells in the INL was obtained from at least three independent experiments, ** p-value <0 .01 by ANOVA followed by post-hoc Tukey's HSD test. ONL = outer nuclear layer, INL= inner nuclear layer, GCL= ganglion cell layer, PKCα (Protein kinase C alpha). Scale bar = 25μm

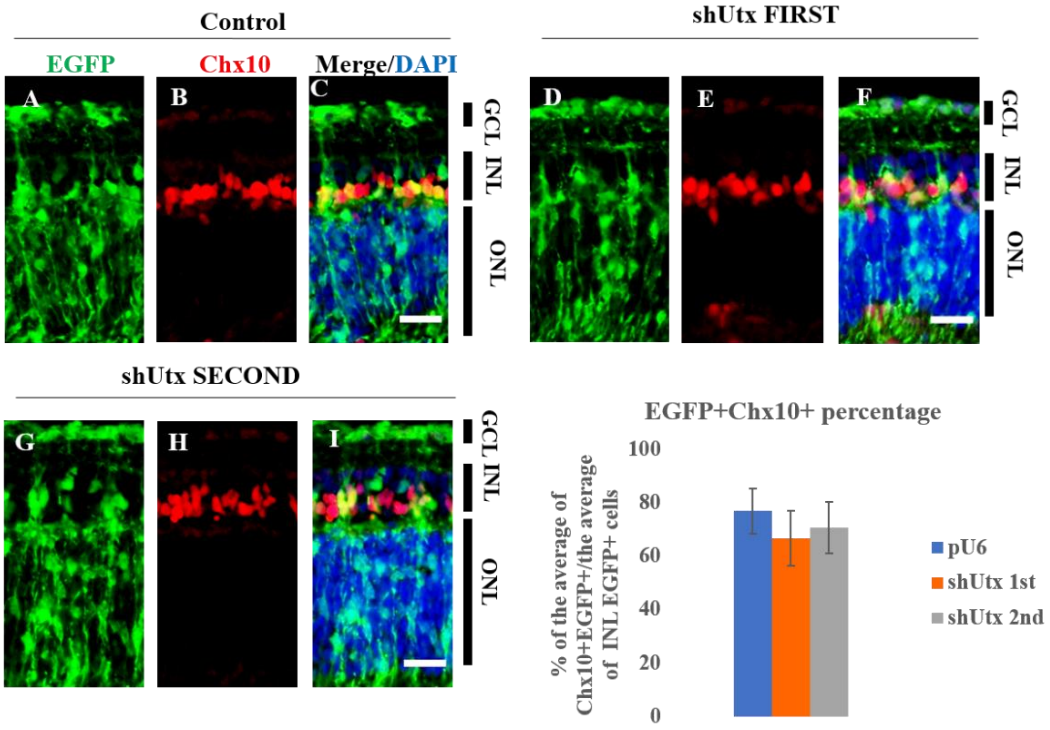
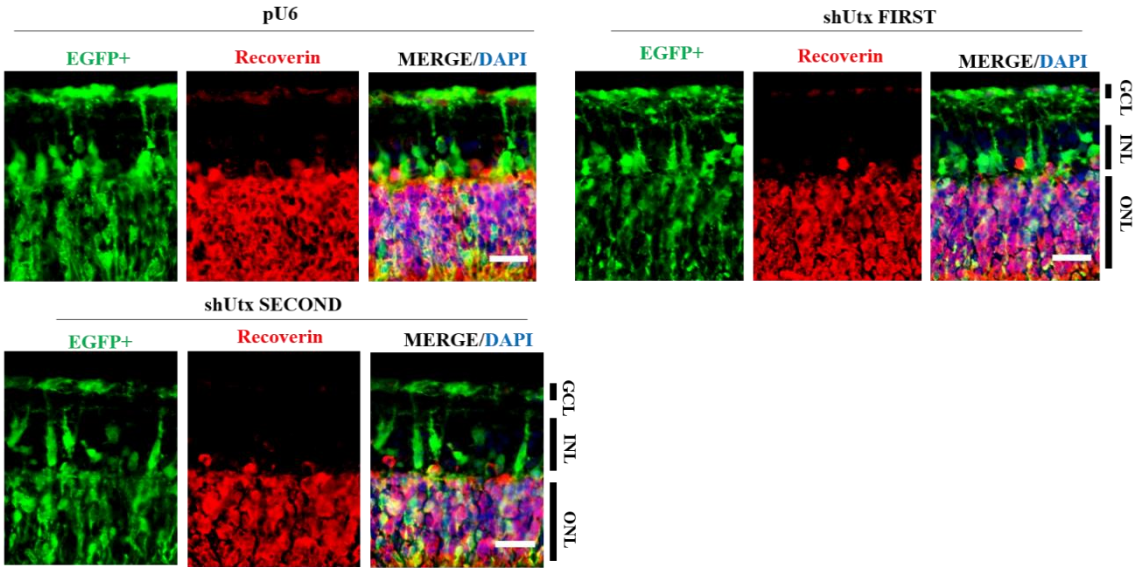
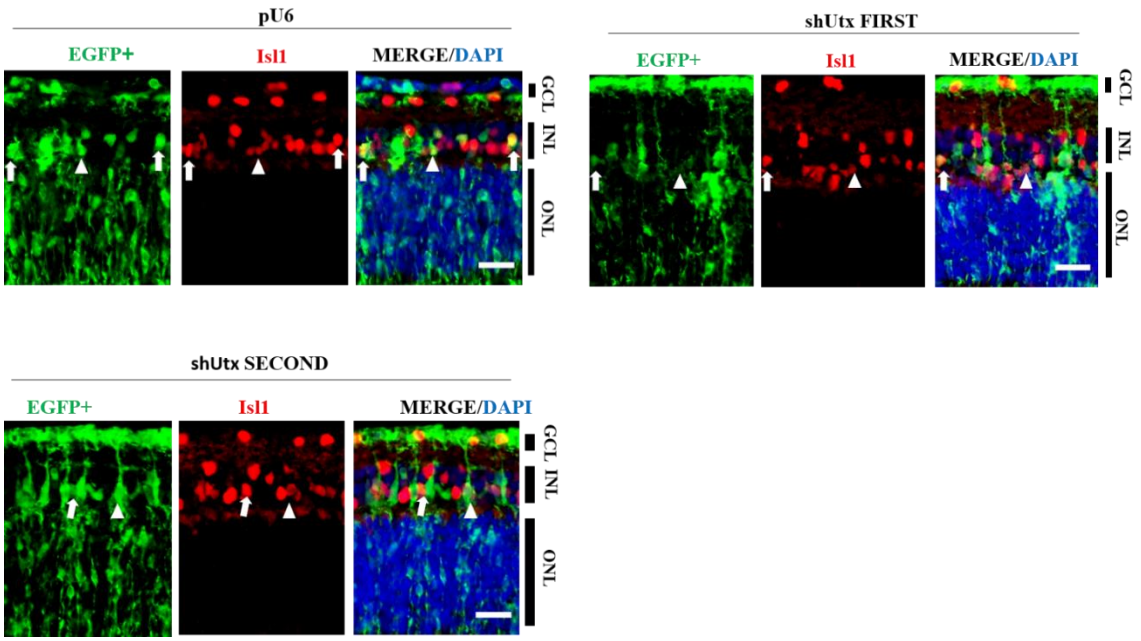


Figure 9. Overall bipolar cell number slightly decreased after knockdown of Utx
Overall bipolar cell number decreased slightly after transfection with shUtx . A control vector (A-C) or shRNA(D-I) was transfected together with an EGFP-expressing plasmid at P1 then, explants were cultured for 12 days. The percentage of EGFP+Chx10+ cells in the INL was obtained from at least three independent experiments. p-value > 0.05 by ANOVA followed by post-hoc Tukey's HSD test. ONL = outer nuclear layer, INL= inner nuclear layer, GCL= ganglion cell layer, Chx10 (pan bipolar cell marker). Scale bar = 25μm.

A


pU6
EGFP+
Recoverin
MERGE/DAPI
shUtx FIRST
EGFP+
Recoverin
MERGE/DAPI
GCL
INL
ONL
shUtx SECOND
EGFP+
Recoverin
MERGE/DAPI
GCL
INL
ONL

B


pU6
EGFP+
Isl1
MERGE/DAPI
shUtx FIRST
EGFP+
Isl1
MERGE/DAPI
GCL
INL
ONL
shUtx SECOND
EGFP+
Isl1
MERGE/DAPI
GCL
INL
ONL

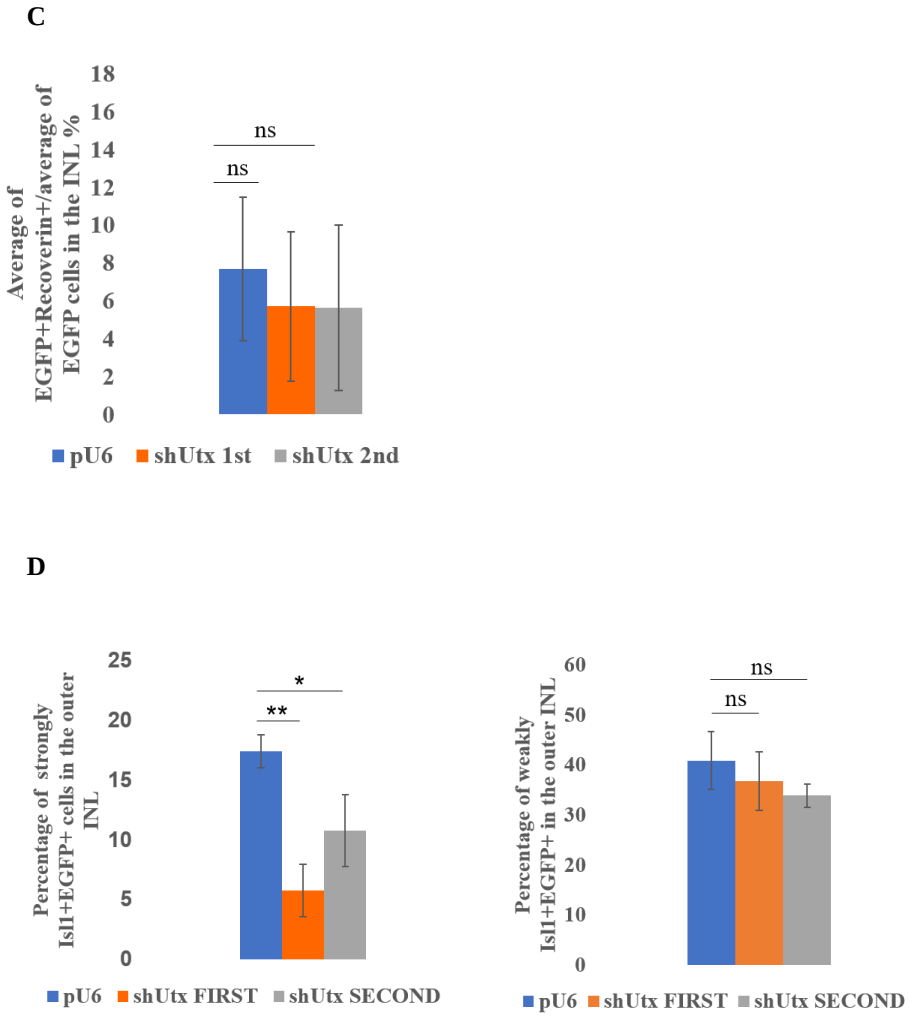
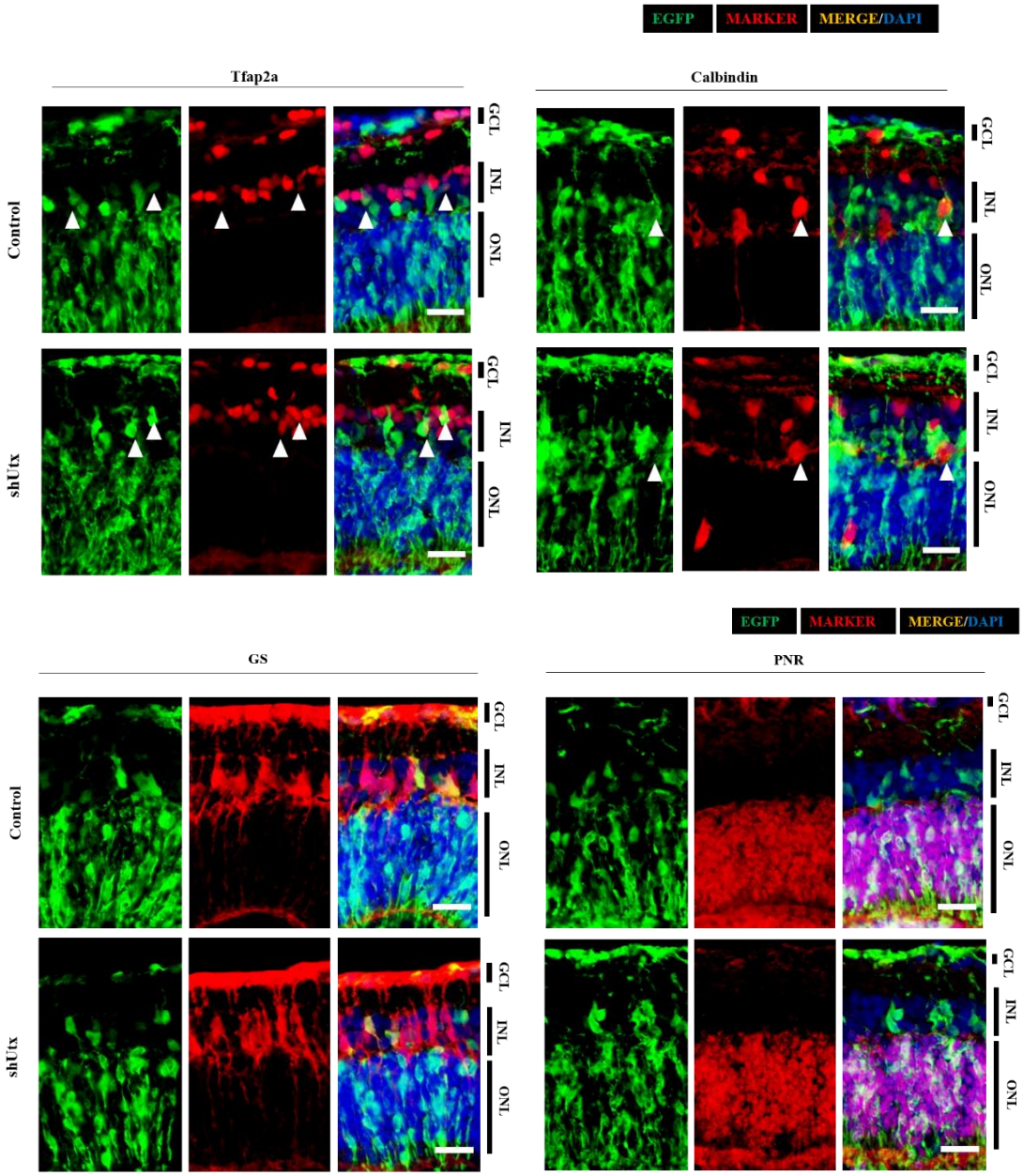


Figure 10. Other bipolar cell subtypes were not affected by knocking down Utx:

A: Cone OFF bipolar subtype number was not significantly affected by knock down of Utx . The U6 promoter (control) or shRNA targeting Utx was transfected together with an EGFP-expressing plasmid at P2 then, explants were cultured for 11 days. B: Cone ON bipolar cells represented by weak Isl1 signals (arrow heads) were not significantly changed by knockdown of Utx while rod ON bipolar cells represented by strong Isl1 signals (full arrows) were significantly reduced as expected. C & D : Counting results showed no significant difference in cone OFF bipolar and cone ON bipolar cell subtypes. Counting was done over 438µm long images; cells from two images were counted for each marker and the percentage of EGFP+marker+ cells in the INL was obtained from three independent experiments. *p-value < 0.05, **< 0.01 by ANOVA followed by post-hoc Tukey's HSD test. ns = not significant ONL = outer nuclear layer, INL= inner nuclear layer, GCL= ganglion cell layer, Recoverin = cone OFF bipolar subtype marker, Isl1 = cone ON and rod ON bipolar subtype marker. Scale bar = 25µm

A


EGFP
MARKER
MERGE/DAPI
Tfap2a
Calbindin
Control
shUtx
GCL
INL
ONL
GS
PNR

B

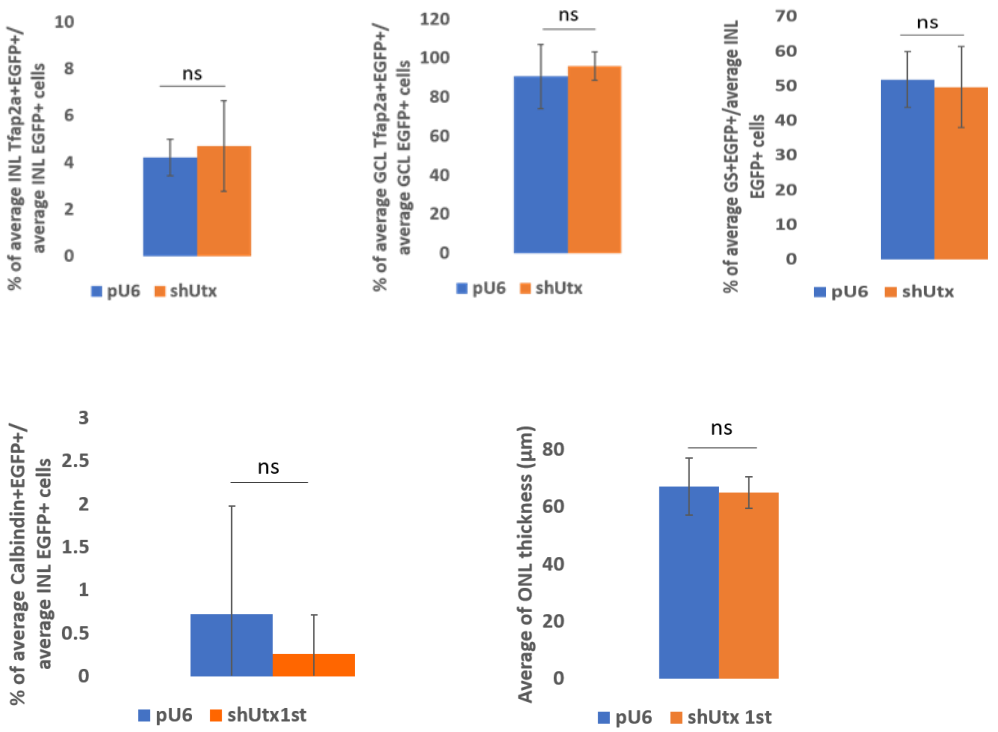
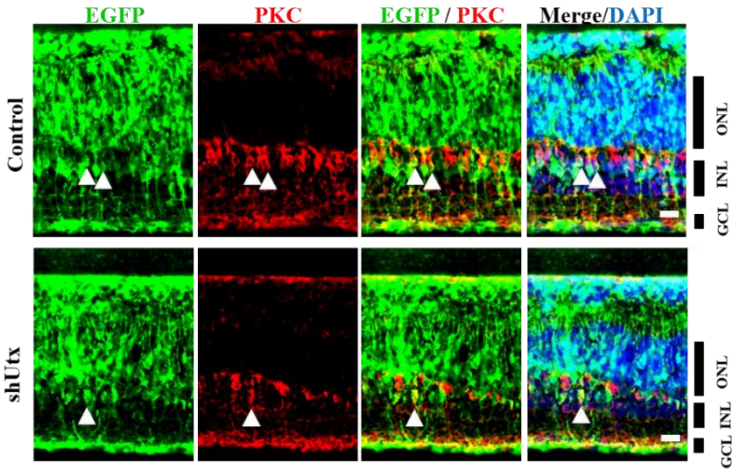


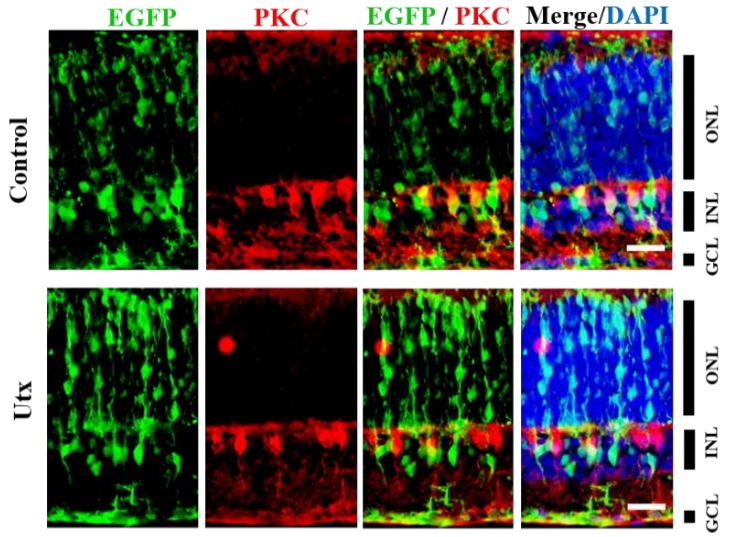
Figure 11. Knockdown of Utx does not affect the differentiation of other retinal cell types

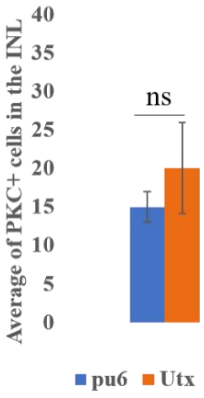
Immunohistochemical analysis of other cell types revealed no difference in cell number after knockdown of Utx. P1 retina were electroporated with shRNA targeting Utx along with an EGFP expressing plasmid then cultured for 12 days. A: amacrine cells stained by transcription factor AP-2; alpha (Tfap2a), horizontal cells stained by calbindin, Müller glia stained by glutamine synthetase (GS) and rod photoreceptors stained by PNR also known as nuclear receptor subfamily 2, group E, member 3 (Nr2e3). B: Counting results over 438µm long images; cells from two images were counted for each marker and the average number from three independent experiments, calculated. The thickness of the outer nuclear layer reflecting photoreceptor number was measured in the left, mid and right thirds of the outer nuclear layer and the lengths, averaged. Two images were measured in Utx knockdown and control retina. p-value> 0.05 = not significant(ns) by Student t test. Scale bar = 12µm

A

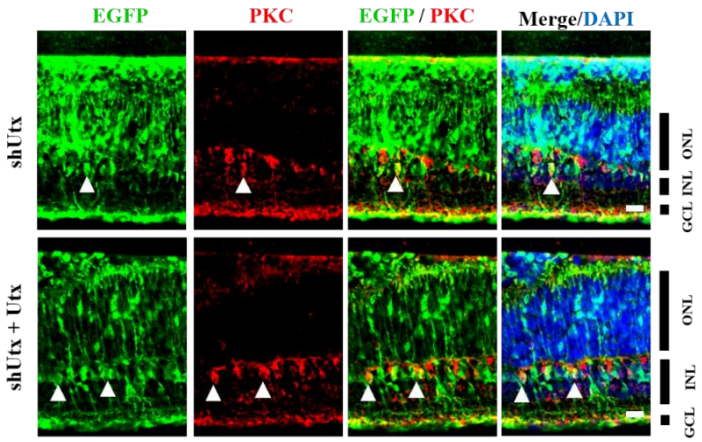

EGFP
PKC
EGFP / PKC
Merge/DAPI
Control
shUtx
ONL
INL
GCL

B

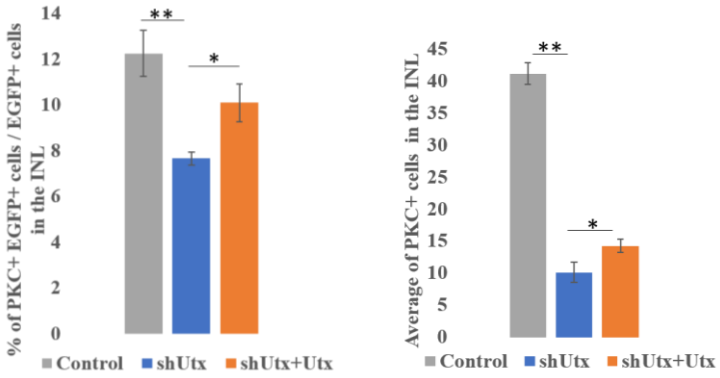

EGFP
PKC
EGFP / PKC
Merge/DAPI
Control
Utx
ONL
INL
GCL


Average of PKC+ cells in the INL
40
35
30
25
20
15
10
5
0
ns
pu6
Utx

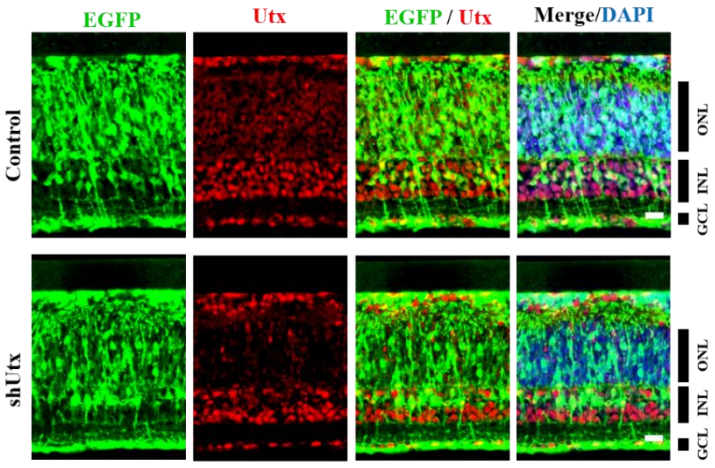
C


EGFP
PKC
EGFP / PKC
Merge/DAPI
shUtx
shUtx + Utx
ONL
INL
GCL

D


% of PKC+ EGFP+ cells / EGFP+ cells in the INL
Average of PKC+ cells in the INL
Control
shUtx
shUtx+Utx
**
*

E


EGFP
Utx
EGFP / Utx
Merge/DAPI
Control
shUtx
ONL
INL
GCL

F

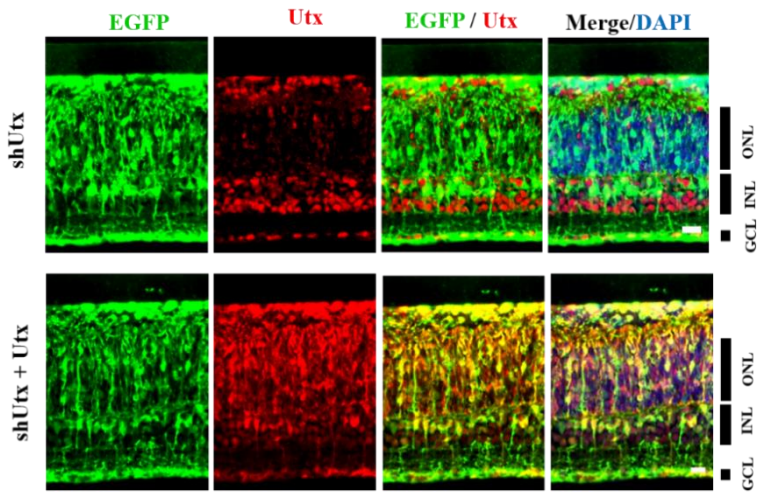
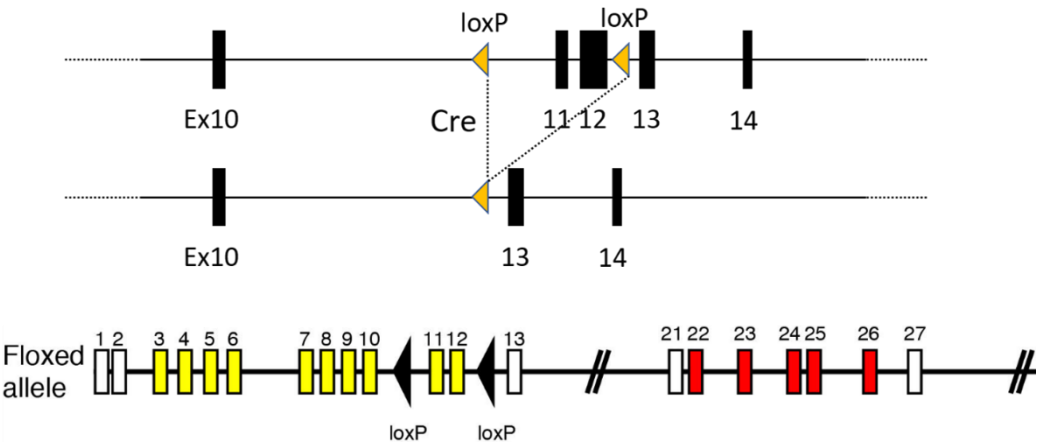
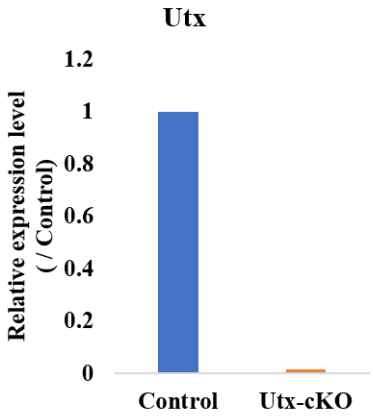


Figure 12. Utx overexpression rescues rod bipolar cells in shUtx transfected retina: A: Utx knockdown at P0 also leads to loss of PKC+ cells. B: Utx over expression on its own did not affect rod bipolar cell structure or significantly alter the PKC+ cell number. C: Overexpression of Utx after its knockdown rescued PKC+ cell number. D: The percentage of EGFP+PKC+ cells and the average number of PKC+ cells in the INL were obtained from three independent experiments. E: In the same explants, Utx expression in the INL was reduced by shUtx. F: Utx was successfully overexpressed in Utx-transfected retina with stronger signals in the ONL than in the INL; only some EGFP+ cells in the INL overexpressed Utx. P0 or P2 (Overexpression of Utx alone) retinae were transfected with shUtx or the U6 promoter as control(A), a Utx over expression vector or U6 promoter as control (B), shUtx or shUtx and Utx overexpression vector (C) along with the EGFP plasmid before they were cultured as explants for 12 days. Scale bar = 18µm (A,C,E,F), 25µm(B). **p< 0.01,*p< 0.05, ns= not significant. ONL = outer nuclear layer, INL= inner nuclear layer, GCL= ganglion cell layer, PKC α (Protein kinase C alpha).

A


loxP
loxP
Ex10
Cre
11 12
13
14
Ex10
13
14
Floxed allele
1 2
3 4 5 6
7 8 9 10
11 12
13
21 22
23
24 25
26
27
loxP
loxP

B


Utx
Relative expression level (/ Control)
1.2
1
0.8
0.6
0.4
0.2
0
Control
Utx-cKO

C

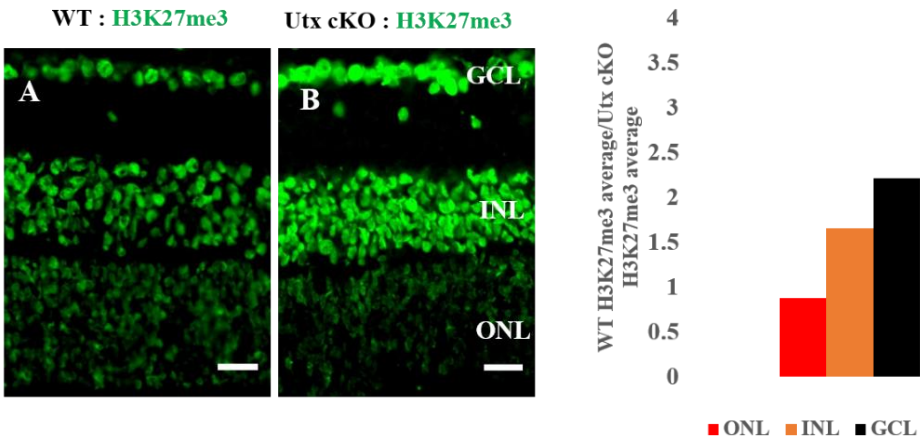
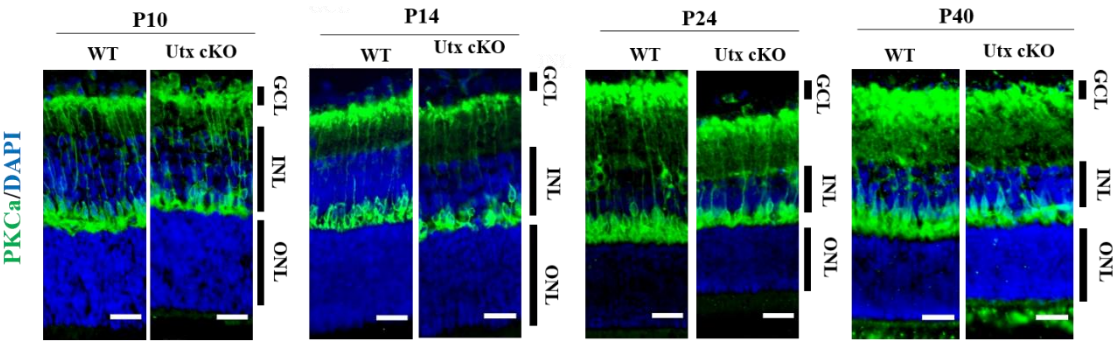


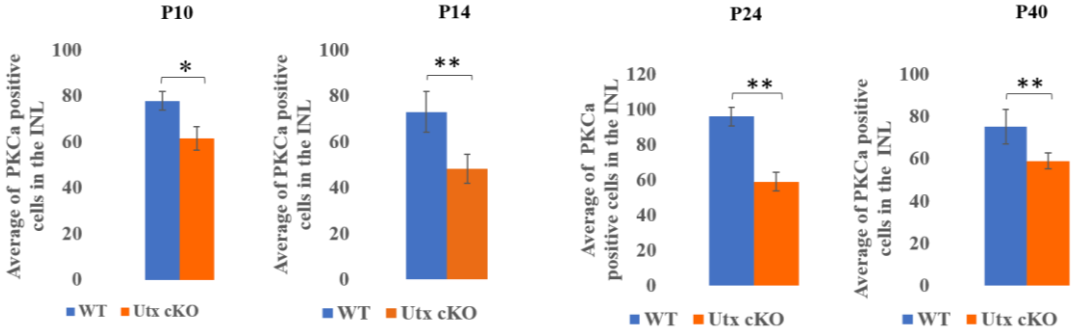
Figure 13. Generation of Utx conditional knockout mice

A: Top- deletion of exon 11&12 that encodes Utx including the region of the JmjC domain. Heterozygous Utx-floxed and Dkk3-cre expressing male mice (a gift from Prof. Honda) were crossed with homozygous Utx floxed female mice to give Utx conditional knockout (Utx cKO) female mice. The exons containing the TRP domain are shown in yellow, and the exons containing JmjC domain are shown in red. Bottom- schematic of the Utx (Kdm6a) gene showing the tetratricopeptide repeats (TPR) and the catalytic Jumonji C (JmjC) domain (adopted from reference 34) B: Utx transcript level was depleted in Utx cKO retinae. Total RNA extracted from cKO and WT retinae was quantified by RT-qPCR and cq values used to calculate relative expression levels. C: Left- immuno-staining for H3K27me3 showed increase of H3K27me3 in the INL and GCL after knocking out Utx (In-figure A & B). Right- H3K27me3 grey value was measured over 200µm (GCL), 100µm (INL), 50µm (ONL) of WT and cKO images. The ratio of the Utx cKO average grey to that of WT is shown. ONL = outer nuclear layer, INL= inner nuclear layer, GCL= ganglion cell layer, CKO = conditional knockout, WT = wild type. Scale bar = 25µm

A


P10
P14
P24
P40
WT
Utx cKO
PKCa/DAPI
GCL
INL
ONL

B


P10
P14
P24
P40
Average of PKCa positive cells in the INL
Average of PKCa positive cells in the INL
Average of PKCa positive cells in the INL
Average of PKCa positive cells in the INL
*
**
**
**
WT
Utx cKO

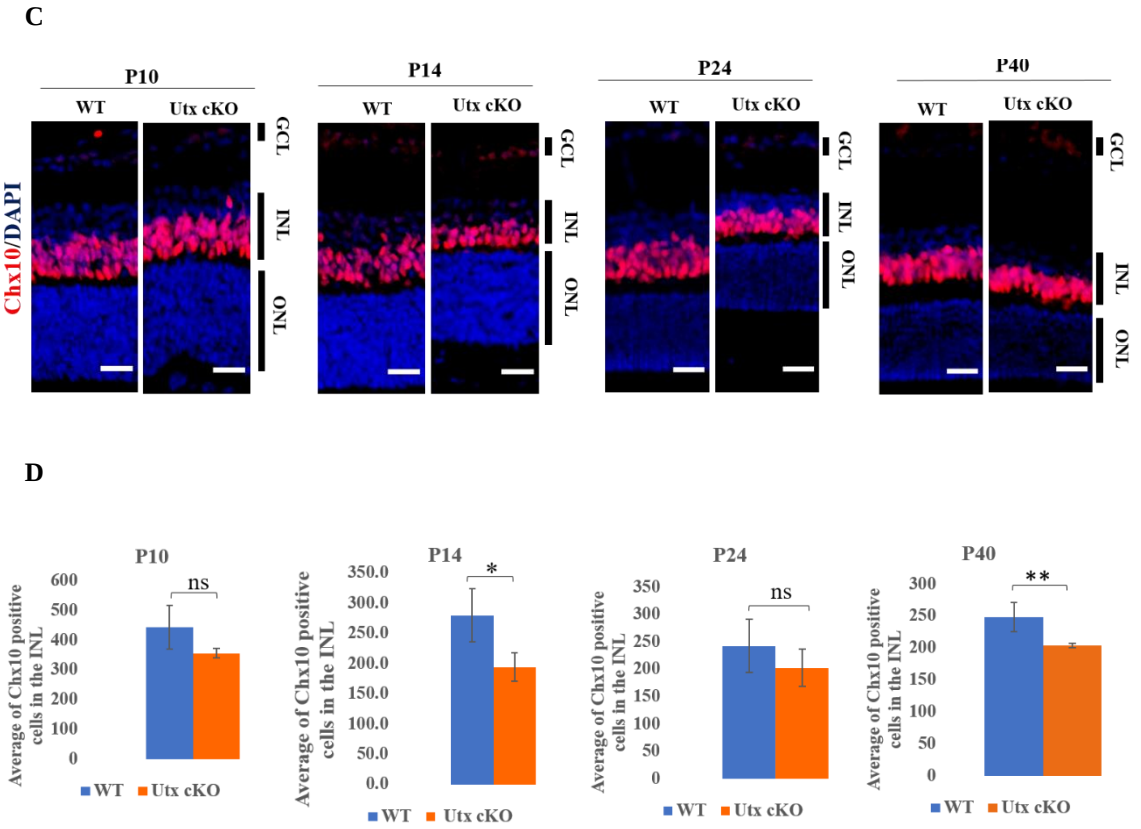
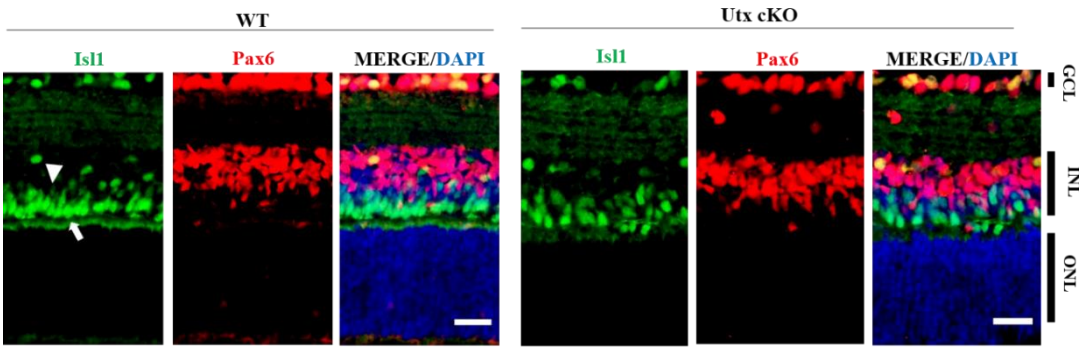


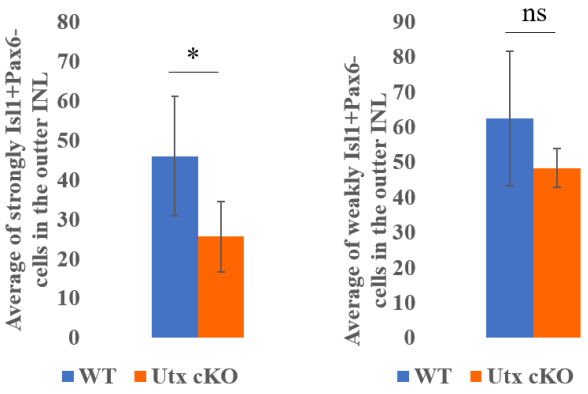
Figure 14. Rod bipolar cells decrease when Utx is knocked out

A: Rod bipolar cells were decreased by Utx cKO compared to WT in developing and adult retina at P10, P14, P24 and P40 stages. C: Overall bipolar cell number decreased with varying significance at different stages. B & D: Counting data corresponding to the images in A & B; PKCα was consistently decreased by Utx cKO while Chx10 expression also decreased to different extents. Two 438µm wide images were counted for both markers in WT and Utx cKO retinae. The average number of cells in three independent experiments is shown. *p<0.05, **p<0.01 by student t test, ns = not significant. ONL = outer nuclear layer, INL = inner nuclear layer, INL= inner nuclear layer, GCL= ganglion cell layer, CKO = conditional knockout, WT = wild type, PKC α = rod bipolar cell marker, Chx10 pan bipolar cell marker. Scale bar = 25µm

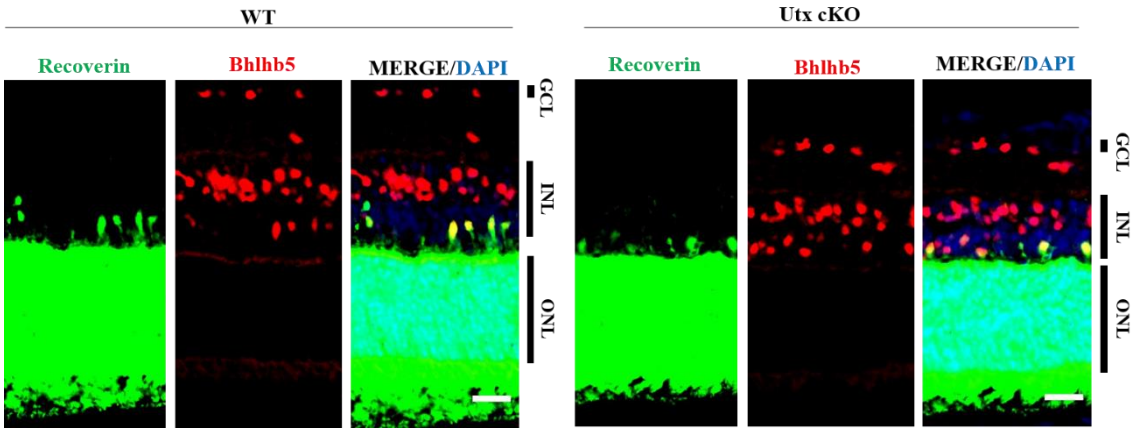
A


WT
Utx cKO
Isl1
Pax6
MERGE/DAPI
Isl1
Pax6
MERGE/DAPI
GCL
INL
ONL

B


Average of strongly Isl1+Pax6- cells in the outter INL
80
70
60
50
40
30
20
10
0
*
WT
Utx cKO
Average of weakly Isl1+Pax6- cells in the outter INL
90
80
70
60
50
40
30
20
10
0
ns
WT
Utx cKO

C


WT
Utx cKO
Recoverin
Bhlhb5
MERGE/DAPI
Recoverin
Bhlhb5
MERGE/DAPI
GCL
INL
ONL
GCL
INL
ONL

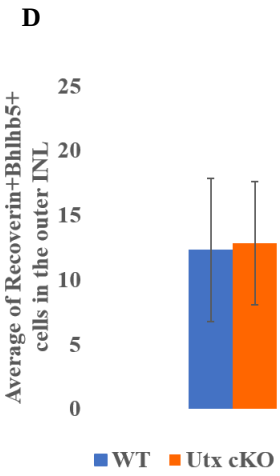


Figure 15. Other bipolar subtypes were not affected by loss of Utx:

A: Cone ON bipolar cell number was not significantly different between WT and Utx cKO at P14.The arrow head shows weakly Isl1+Pax6- cells which represent cone ON bipolar cells and their number was the same after Utx cKO, while the full arrow shows strongly Isl1+Pax6- cells in the outer most part of the inner nuclear layer that represent rod ON bipolar cells and were significantly decreased as expected. B: Graphical representation shows a significant decrease in rod ON bipolar cells (left side) and no significant change in cone ON bipolar cells after cKO of Utx. C: Cone OFF bipolar cell number was not affected by knockout of Utx. D: The average number of cone OFF bipolar cells shown in C was not different between WT and Utx cKO retinae. Counting was done over 438µm long images; cells from two images were counted for each marker and the average number from three independent experiments, calculated cells. *$p<0.05$ by student t test. Isl1 (cone ON and rod ON bipolar cell marker), Pax6 (pan amacrine cell marker), Recoverin (cone OFF bipolar cell marker), Bhlhb5 (cone OFF bipolar cell marker). CKO = conditional knockout, WT = wild type Scale bar = 25 µm

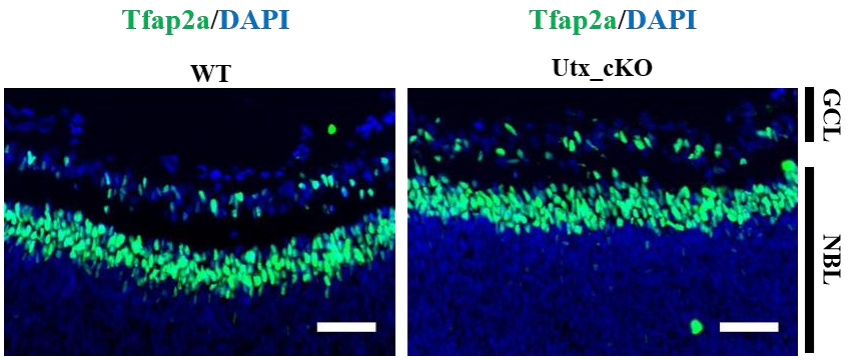


Fig.16 Amacrine cell number was not affected by knockout of Utx:

The number of amacrine cells did not change in Utx cKO compared to WT . Retinae were harvested at postnatal day 3 (P3),cryo-sectioned and stained with the indicated marker . NBL= neuroblastic layer ,GCL= Ganglion cell layer, Transcription factor ap2a (Tfap2a) = pan-amacrines cell marker . Scale bar = 25µm.

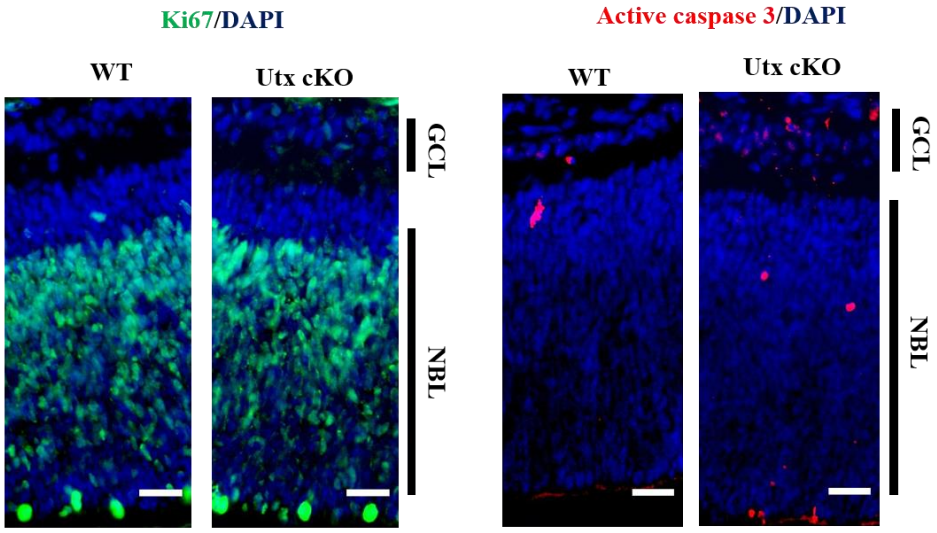


Fig17. Proliferation and apoptosis were not affected by knockout of Utx:

The number of proliferating cells at P3 did not change after knockout of Utx . Apoptosis also seemed to not be very different between WT and Utx cKO retina. Retinae were harvested at postnatal day 3 (P3),cryo-sectioned and stained with the indicated markers . NBL= neuro-blastic layer ,GCL= Ganglion cell layer, Ki67= mitotic cell marker, AC3= marker of apoptosis . Scale bar = 25µm.