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**Investigation of epitranscriptomic regulation in
Scribble-mediated epithelial polarity disruption
during *Drosophila* eye development**

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Investigation of epitranscriptomic regulation in Scribble-mediated epithelial polarity disruption during *Drosophila* eye development

Abstract

Understanding epithelial tissue homeostasis is critical in animal development and disease, with apicobasal polarity (ABP) as a key regulatory mechanism. The ABP determinants, such as Scribble, also function as tumour suppressors, yet their role in growth regulation remains incompletely understood. N⁶-methyladenosine (m⁶A), an epitranscriptomic RNA modification mediated by Mettl3, has recently been implicated in gene regulation during cancer progression, but its role in polarity-loss-driven tumorigenesis is unclear.

This study uses the *Drosophila* compound eye to investigate how altered Mettl3 activity influences tumour progression in *scrib* mutant cells. Using MARCM-based eyFLP mosaic analysis, *scrib* mutant clones with *Mettl3* knockdown or overexpression were generated, and ommatidial disruption was quantified to assess tissue integrity. Altered Mettl3 expression increased tissue disruption, indicating that m⁶A acts as a modifier of ABP driven morphogenesis.

Keywords:

Drosophila melanogaster, Scribble, epithelial polarity, m⁶A, Mettl3, clonal analysis, tumorigenesis

CERCS: [B350 Development biology, growth (animal), ontogeny, embryology]

Institute name: Institute of Molecular and Cell Biology

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Epitranskriptomse regulatsiooni uurimine äädikakärbse silma arengus Scribble'i vahendatud epiteeli polaarsuse häirituse korral

Lühikokkuvõte

Epiteelkoe homöostaasi mõistmine on kriitilise tähtsusega nii loomade arengus kui ka koe patogeneesis, kusjuures rakkude apikobasaalne polaarsus (ABP) toimib selles peamise regulatoorse mehhanismina. ABP determinandid, sealhulgas Scribble, toimivad ühtlasi tuumorsupressoritena, ehkki nende rolli kasvu reguleerimisel ei mõisteta veel täielikult.

Mettl3 vahendatud epitranskriptoomset RNA modifikatsiooni N⁶-metüüladenosiini (m⁶A) on hiljuti seostatud geeniregulatsiooniga kasvaja progresseerumisel, kuid selle roll polaarsuse kaotusest tingitud tuumorigeneesis on seni selgusetu.

Käesolevas bakalaureusetöös kasutatakse äädikakärbse liitsilma mudelsüsteemina, et välja selgitada, kuidas Mettl3 muutunud aktiivsus mõjutab tuumori progresseerumist *scrib* mutantsetes rakkudes. Kasutades MARCM-süsteemil ja eyFLP-1 põhinevat mosaiikanalüüsi, genereeriti METTL3 vaigistamise või üleekspressiooniga *scrib* mutantsed kloonid ning analüüsiti vastavate kloonide puhul äädikakärbse liitsilmas toimunud struktuurseid muutusi.

Võtmesõnad: äädikakärbes, *Drosophila melanogaster*, Scribble, epiteeli polaarsus, m⁶A, METTL3, kloonanalüüs, tuumorigenees.

CERCS: [B350 Arengubioloogia, loomade kasv, ontogenees, embrüoloogia]

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TERMS, ABBREVIATIONS AND NOTATIONS

ABP –Apicobasal polarity

AJ –Adherens junction

ALKBH5 –AlkB homologue 5

aPKC –Atypical protein kinase C

CyO –Curly O (balancer chromosome II)

Dlg –Discs large

EGFR –Epidermal growth factor receptor

EMT –Epithelial-to-mesenchymal transition

Flippase recombinase

FRT –FLP recombination target

FTO –Fat mass and obesity-associated protein

GAL4 –Yeast transcriptional activator protein

GAL80 – GAL4 transcriptional repressor

GFP –Green fluorescent protein

Hpo –Hippo kinase

JNK –c-Jun N-terminal kinase

Lgl –Lethal giant larvae

LRR –Leucine-rich repeat

m⁶A –N6-methyladenosine

MAPK –Mitogen-activated protein kinase

MARCM –Mosaic analysis with a repressible cell marker

METTL14 –Methyltransferase-like 14

METTL3 –Methyltransferase-like 3

PAR –Partitioning defective (complex)

PCP –Planar cell polarity

PDZ –PSD95-Dlg-ZO1 (protein domain)

RNAi –RNA interference

Sav - Salvador

Sb –Stubble (bristle marker)

Scrib –Scribble

SEM –Standard error of the mean

SJ –Septate junction

TCGA –The Cancer Genome Atlas

Tm6B –Third Multiple 6B (balancer chromosome III)

UAS –Upstream activation sequence

Wts –Warts kinase

Yki –Yorkie

YTHDC1 –YTH domain-containing protein 1

YTHDF1/2/3 –YTH domain-containing family proteins 1/2/3

α -Cat – α -Catenin

INTRODUCTION

Epithelial tissues provide the structural basis for organs, and their stability depends on the accurate establishment and maintenance of apicobasal polarity (ABP). In *Drosophila melanogaster* imaginal discs (precursors of external cuticular organs), ABP is regulated by three conserved polarity complexes: the Crumbs (Crb) complex, the partitioning defective (PAR) complex, and the basolateral Scribble (Scrib) complex, which acts as a tumour suppressor (Bilder D., 2004). Loss of Scrib disrupts tissue architecture, activates c-Jun N-terminal kinase (JNK) signalling, and deregulates the Hippo-Yorkie pathway, leading to neoplastic overgrowth in the imaginal disc (Doggett, K. et al., 2011). The eye imaginal disc is notably suitable for studying these processes, as its highly organised ommatidial lattice makes any disruption in polarity or growth control immediately visible as quantifiable changes in the adult eye.

N6-methyladenosine (m⁶A) is the most abundant epitranscriptomic modification in eukaryotes, regulating transcript stability, translation and cell fate in a context-dependent manner. Its dysregulation has been linked to tumour progression across multiple epithelial tissue types (Luo, H. et al., 2026). Research interest in the m⁶A system comes from its post-transcriptional gene regulatory activity, which has not been explored in the context of Scribble-mediated polarity loss. m⁶A's downstream effectors overlap with the Hippo-Yki axis, which is directly affected by the loss of Scrib. Methyltransferase-like 3 (Mettl3) – a writer in the m⁶A system, has been shown to regulate proliferation in epithelial cancers in a context-sensitive manner, where modifications may either promote or suppress growth depending on the cellular state. The aim of this study is to elucidate whether altered *Mettl3* expression modifies the neoplastic outcome of Scrib loss in the *Drosophila* eye imaginal disc, and whether m⁶A levels can act as a modifier of polarity-loss-driven tumourigenesis.

1 LITERATURE REVIEW

1.1 The *Drosophila melanogaster* as a model organism

1.1.1 Lifecycle of *Drosophila melanogaster*

Fruit fly (*Drosophila melanogaster*, hereafter referred to as *Drosophila*) is a widely used multicellular model organism by geneticists and molecular biologists. Flies have four chromosomes: three autosomes (chromosome 2, 3 and 4) and one pair of sex chromosomes, (X and Y). *Drosophila* is suitable model organism for research due to their fast developmental cycle, ease of gene manipulation and maintenance and a compact genome that shares nearly 70% of its genes with humans (Hales et al., 2015).

Drosophila has a rapid, well-defined life cycle divided into four stages: embryonic, larval, pupal and adult (Figure 1). Under growth conditions of 25°C, a fruit fly reaches full development in about 10 days. The embryo hatches after a day in the egg stage and develops into the larval form, which is divided into three phases: first, second and third instar larvae (Figure 1). Upon completing the third instar larval stage, *Drosophila* stops feeding and enters a sessile state, initiating the formation of a cuticle that hardens and darkens to produce the puparium over the following three days (Figure 1). Metamorphosis takes place over a four-day period during this stage, culminating in the eclosion of the adult fly from the anterior end of the puparium (Figure 1) (Hales, K. G., et al., 2015).

Flies reach fertility within eight hours after emerging from the pupa. Females' egg-laying peaks are during the first two days of adulthood and they can lay up to 500 eggs over their lifetime. Adult flies typically live for about 50 days (Hales, K. G., et al., 2015).

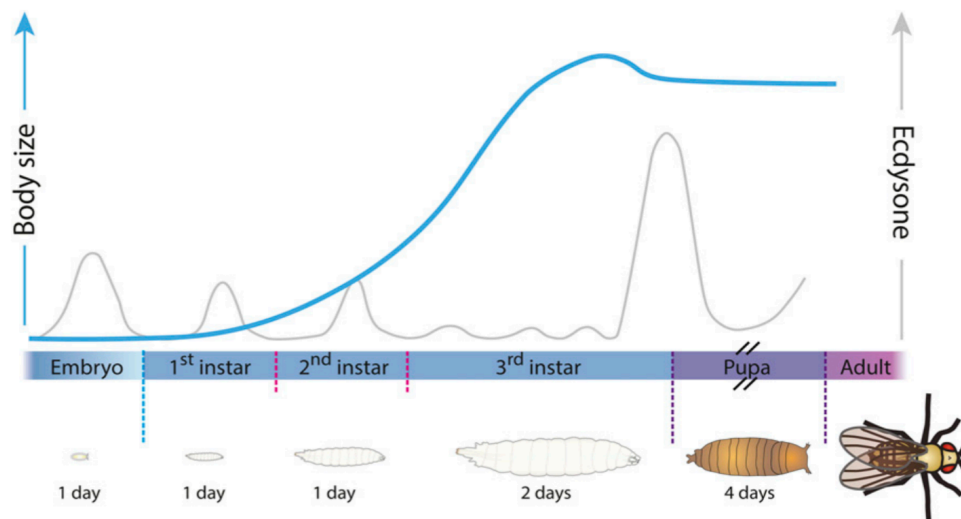


Figure 1. Developmental stages of *Drosophila melanogaster*. The blue curve shows body size increase across developmental stages. The grey curve represents ecdysone – a steroid hormone crucial for regulating developmental transitions in *Drosophila* (M. J. Texada et al., 2020)

1.1.2 *Drosophila* appearance and morphology

The wild type of *Drosophila* serves as the essential baseline for genetic analysis.

The fly's body is 2,5 millimetres long and is divided into three parts: head, thorax and abdomen (M. J. Texada et al., 2020).

Insects have compound, brown-red eyes covering most of its head surface. Between the eyes, they have antennae, which serve as sensory organs to detect vibrations, air currents and odours (M. J. Texada et al., 2020).

Their thorax is robust and bears three pairs of legs along with a single pair of long, straight wings. Flies use pulvilli for attachment, while males have specialised sex combs on their forelegs, which are essential for mating purposes (M. J. Texada et al., 2020).

Wild-type adults exhibit a tan light brown colour, with transverse black rings near the abdomen and are covered with bristles that serve as mechanoreceptors for tactile sensing. Clear sexual dimorphism is evident in abdominal morphology: males possess a more rounded abdomen with a darker pigmentation at the posterior tip, whereas females have a larger, more pointed abdomen with a consistent striped pattern throughout. Flies are sexually dimorphic, with females being about 30% larger than males. Their gender is determined by genetics rather than hormones. *Drosophila* males and females can be distinguished by external morphological traits. These

traits are used for virgin collection, genetic crosses and clonal analysis, which are essential for the further design of mating schemes in studies (M. J. Texada et al., 2020).

1.2 The *Drosophila* compound eye

1.2.1 Eye structure and ommatidial organisation

In developmental biology, the compound eye of *Drosophila melanogaster* is a classic model for studying pattern formation, cell fate determination and morphogenesis. A healthy eye consists of approximately 700 (male)-750 (female) repeating units called ommatidia (singular – ommatidium). It consists of various cell types organised in a highly precise and sequential hexagonal lattice (Figure 2) (Jürgens, K. J. et al., 2024).

Each ommatidium contains 8 photoreceptor neurons and 12 supporting cells: 4 lens-secreting cone cells and a set of pigment cells that optically isolate one ommatidium from its neighbours. The outlying unit eyes also share a 4-celled mechanosensory bristle complex (Kumar J. P. 2012)

Photoreceptors develop during the larval stage in the eye imaginal disc and consist of eight neurones (R1-R8) per ommatidium. Outer photoreceptors (R1-R6) are responsible for motion detection and vision in extensive light. The inner two (R7 and R8) are smaller, stacked in the middle and are responsible for colour detection and polarisation. The light-sensitive microvilli create structures called rhabdomeres, which come together to form a central fused structure (Kumar J. P. 2012)

Four cone cells produce the corneal lens and a pseudocone, which focus light onto the photoreceptors. Two primary pigment cells surround the cone. A hexagonal lattice of secondary and tertiary pigment cells insulates adjacent ommatidia, preventing light leakage and ensuring sharp resolution (Kumar J. P. 2012)

Ommatidial assembly begins with the specification of the R8 founder cell, followed by the sequential, EGFR-dependent recruitment of photoreceptors to form a five-cell precluster (R2, R3, R4, R5, R8). The remaining photoreceptors (R1, R6, R7), along with cone cells and pigment cells are recruited in subsequent waves during late larval and pupal stages. Finally, the mature ommatidium consists of a 20-cell-organised structure (Kumar J. P. 2012)

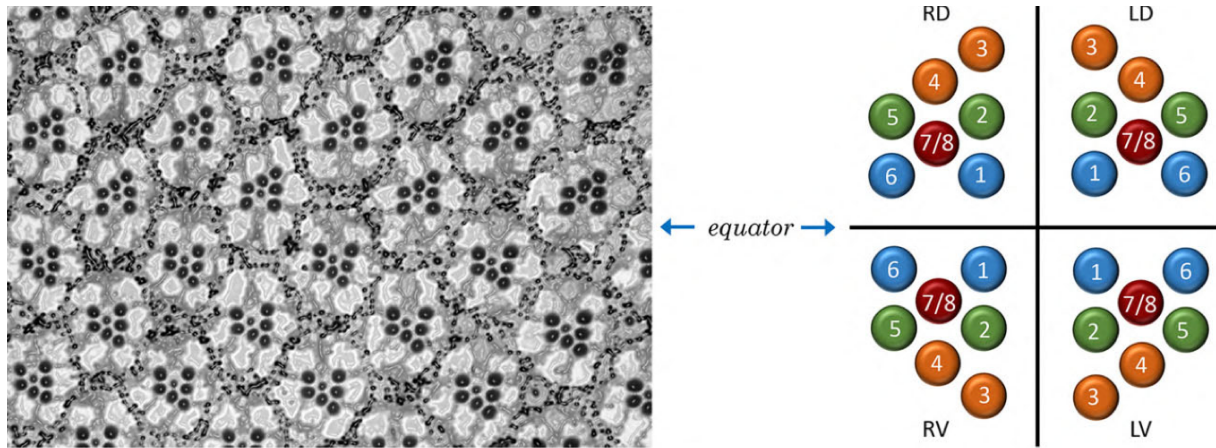


Figure 2. Schematic representation of ommatidial organisation in an adult eye. The region where the dorsal and ventral compartments of the eye meet is called the equator. The right panel illustrates the detailed organization of the ommatidia. (Left) An adult *Drosophila* compound eye shows the regular ommatidial pattern in wild-type tissue. (Right) A schematic diagram illustrating the arrangement of a single ommatidium. Numbers 1-8 label the individual photoreceptor cells (R1-R8) with R7/8 located in the centre. The dorsal (RD, LD) and ventral (RV, LV) quadrants are mirror images across the equator, shown by the horizontal arrow. RD right eye dorsal, LD for left eye dorsal, RV for right eye ventral, and LV for left eye ventral. Abbreviations: RD, right eye, dorsal compartment; LD, left eye, dorsal compartment; RV, right eye, ventral compartment; LV, left eye, ventral compartment (B. P Weasner, 2022).

1.3. Apico-basal cell polarity and the Scribble complex

1.3.1 Introduction to apicobasal cell polarity complexes

Epithelial tissues form the structural basis of organs, where epithelial homeostasis strictly regulates the balance between cell proliferation, cell death, and tissue morphogenesis. Epithelial homeostasis depends on the proper organisation and maintenance of apicobasal polarity (ABP), which defines distinct apical and basolateral membrane domains and organises cell-to-cell junctions. Therefore, numerous polarity regulators function as tumour suppressors (Su, W. H. et al., 2012).

ABP maintenance is fundamental for epithelial tissue integrity and homeostasis. ABP is governed by three conserved polarity complexes arranged along the apical-basal axis: the Crumbs (Crb) complex and the partitioning defective (PAR) complex at the apical domain, and the basolateral Scribble (Scrib) complex. Because these complexes are interdependent, the loss of one of them disrupts the overall apical-basal axis. A misformed complex can lead to expansion or dismissal of the opposing complex, ultimately resulting in complete loss of ABP (Su, W. H. et al., 2012).

1.3.2 Polarity complexes

Scribble is a scaffold protein that is encoded by the *scrib* gene. Originally, it was isolated from *Drosophila melanogaster* and it is located on the right arm of the third chromosome. Scribble participates in cell migration and proliferation and is essential for apicobasal polarity in epithelial cells, neuroblast differentiation and synaptic function (Bonello, T. T., & Peifer, M., 2019). The Scrib protein consists of three functional regions: a leucine-rich repeat (LRR) domain, four Psd95-Dlg-Zo1 domains (PDZ) and a C-terminal (CT) domain. These proteins are located at the lateral plasma membrane and concentrate at septate junctions (SJs) to sustain the basolateral state of epithelial cells (Zeitler, J., et al, 2004)

Apical-basal polarity is a crucial upstream factor of Hippo signalling in the imaginal disc. Numerous *Drosophila* genes identified as neoplastic tumour suppressors – such as *scribble* (*scrib*), *discs large* (*dlg*), and *lethal giant larvae* (*lgl*) are essential for maintaining apical-basal polarity, and their mutations weaken apical cell junction (Zeitler, J. et al, 2004). Basolateral polarity controls polarisation of the embryonic, imaginal disc and follicular epithelia. It regulates cellular growth and differentiation, acting as a tumour suppressor. (Huang, Y. et al., 2022).

Loss of Scrib disrupts apicobasal polarity, disorganises tissue architecture, and causes neoplasia – tumour formation. Disruption of apical-basal polarity is associated with reduced Hippo signalling and strong Yki activation (Misra, J. R., & Irvine, K. D., 2018).

α -Catenin (α -Cat) is a key molecular linker of Scrib and Yki. Scrib knockdown causes post-transcriptional loss of α -Cat at adherent junctions. The response to different variations of *scrib* loss is dose-dependent: hypomorphic *scrib* alleles overproliferate, whereas null clones are eliminated by cell competition through JNK-mediated apoptosis. JNK upregulation suppresses Yki and leads to cell death instead of overgrowth (C. Yang et al., 2015).

In *Drosophila*, mutations disrupt basolateral identity, leading to mislocalisation of apical proteins and loss of polarity during embryogenesis and imaginal disc development. In eye imaginal discs, Scrib maintains epithelial integrity and regulates proliferation for proper ommatidial patterning. *scrib* mutants cause disorganised preclusters, polarity loss and tumorous overgrowth enhanced by Hippo pathway mutations. C-Jun N-terminal kinase (JNK) signalling is elevated in Scrib-deficient eye discs, balancing proliferation and death (Doggett, K. et al., 2011).

1.3.3 scribble mutant alleles

Both *scribble* alleles differ depending on which site a premature stop codon disrupts the protein, therefore resulting in differing severity (Figure 3) (Huang, Y. et al., 2023).

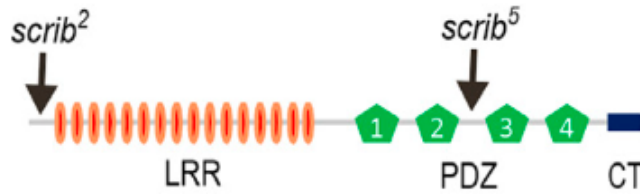


Figure 3. Structural organisation of *scrib*² and *scrib*⁵ alleles. The schematic illustrates the positions of the leucine-rich region (LRR), PSD95-Dlg-ZO-1 (PDZ), and C-terminal (CT) domains (Huang, Y. et al., 2023).

*scrib*² is a null mutation resulting in a complete loss of Scrib protein, including the LRR domain (Figure 3). Consistent with this *scrib*² mutant cells fail to restore ABP and lack detectable Scrib staining. Because the LRR domain is absent, *scrib*² cells cannot be rescued by intercellular ABP restoration and are therefore eliminated. Consequently, JNK signalling increases, promoting apoptosis rather than overproliferation (Zeitler, J., et al, 2004).

In contrast, *scrib*⁵ carries a stop codon between PDZ2 and PDZ3, leaving the LRR domain and the first two PDZ domains (Figure 3). The present LRR domain enables *scrib*⁵ cells to receive polarity signals from neighbouring wild-type cells. *scrib*⁵ clones do not disrupt Dlg or aPKC (apical markers) distribution, and Yki activity remains mostly unchanged. However, without PDZ3 and PDZ4, proliferation control is only partially fulfilled, leading to intermediate imaginal disc overgrowth (Zeitler et al., 2004).

Furthermore, *scrib*⁵ cells depend on extrinsic polarity support from neighbouring wild-type cells. When this support is lost in surrounding cells, loss of α -Cat triggers neoplasia and Yki activation, demonstrating that *scrib*⁵ cells are predisposed to malignancy in the absence of a polarity-competent neighbourhood (Huang et al., 2023).

This proves that the severity of *scribble* mutation depends on the domain presence, with the LRR domain being the key determinant of cell fate.

A crucial molecular mechanism that regulates ABP growth involves the interaction between Scrib and α -Catenin (α -Cat) – an essential component of adherent's adherents junctions (AJs). Partial knockdown of *scrib* causes a post-transcriptional reduction of α -Cat, further suppressing Yki activity, this suggests a specific dependency (Huang et al., 2023).

Together, *scrib*, α -Cat and SJ proteins act to maintain ABP and control tissue proliferation.

1.4 Genetic tools for studying cell polarity loss

1.4.1 Clonal analysis and the MARCM system

Analysing the behaviour of individual mutant cells in tissue requires highly specific genetic markers and the ability to manipulate small groups of cells without disrupting the epithelial integrity. In *Drosophila melanogaster*, this is achieved through clonal analysis using Mosaic Analysis with a Repressible Cell Marker (MARCM).

The basis of clonal analysis is the FLP/FRT site-specific recombination system extracted from the *Saccharomyces cerevisiae*. FLP recombinase recognises short FLP Recombination Target (FRT) sequences and catalyses strand exchange between two FRT sites located at the same loci on homologous chromosomes. Next, in the G2 phase, it generates two daughter cells, each homozygous for different alleles: a wild-type and a mutant. Because FLP-mediated recombination is heritable, progeny of the recombined cell shares the same genotype, producing an expanding clone of cells with a particular genetic identity in a heterozygous, phenotypically normal tissue (F. Germani et al., 2018).

In a standard MARCM setup, GAL80 – a transcriptional repressor of GAL4 is placed on the same chromosome arm as the mutation of interest. Because GAL80 is present in all cells, it suppresses GAL4 activity and prevents UAS-driven transgene expression, including a fluorescent reporter GFP. After recombination and mitotic segregation, the recombined chromatids are distributed between the two daughter cells. One of them inherits two copies of GAL80 and remains unmarked, while the other one loses both copies of GAL80. This allows GAL4 to drive UAS-GFP expression and mark the mutant clone (F. Germani et al., 2018).

1.5 Signalling pathways related to cell polarity loss

1.5.1 Cell competition

Cell competition is a conserved quality-control mechanism by which suboptimal cells are selectively eliminated from a tissue by their healthier neighbours. This process maintains epithelial homeostasis and ensures that only viable, high-fitness cells contribute to the final organ structure.

In the fruit fly eye imaginal disc, cell competition eliminates cells with compromised growth rates via apoptosis when they are surrounded by wild-type cells. This process relies on intercellular junctional communication. In case a cell fitness drops below a critical threshold,

JNK-mediated apoptosis is triggered, thereby removing the defective cells from the epithelium (Amoyel, M., & Bach, E. A., 2014).

1.5.2 The Hippo-Warts-Yorkie Pathway

The Hippo signalling network is a pathway that controls cell proliferation and apoptosis to regulate organ size. In *Drosophila*, it operates with a core kinase cassette: Hippo (Hpo) together with Salvador (Sav) activate Warts (Wts). Wts then phosphorylates the transcriptional coactivator Yorkie (Yki), triggering its cytoplasmic sequestration and thereby silencing growth-promoting genes. (Misra, J. R., & Irvine, K. D., 2018).

Under active Hpo signalling, Wts phosphorylation maintains Yki in the cytoplasm and silenced, thereby limiting proliferation and supporting proper tissue homeostasis. Loss of any upstream tumour suppressor results in Yki-driven overgrowth in imaginal discs. Thus, the Hippo pathway serves as a cellular sensor that regulates growth according to the physical and biochemical environment (Misra, J. R., & Irvine, K. D., 2018).

1.5.3 c-Jun N-terminal kinase signalling

The c-Jun N-terminal kinase (JNK) signalling pathway is a stress-response cascade in eukaryotes. It plays a crucial role in determining cell fate in response to a range of extracellular events.

JNK signalling can be activated in response to environmental stressors, such as UV irradiation, ionising radiation, heat shock, and oxidative stress, as well as by inflammatory cytokines. It is activated through a three-tiered kinase cascade: upstream MAP3Ks phosphorylate and activate MKK4-MKK7, which dually phosphorylate JNK (K. Diener, et al., 1997).

Once activated, JNK phosphorylates nuclear transcription factors. It modulates gene transcription involved in apoptosis, survival, differentiation, proliferation and stress response. This pathway can promote cell survival or trigger apoptosis, making it a crucial pathway in tissue homeostasis and oncogenesis (K. Diener, et al., 1997).

In *Drosophila*, JNK signalling integrates proapoptotic and protumorigenic signals, visible in imaginal disc tissue and is the main effector pathway downstream of Scrib loss-of-function.

The JNK-mediated apoptosis operates through the mitochondrial pathway. When activated, JNK phosphorylates and inhibits anti-apoptotic Bcl-2 family and simultaneously promotes pro-apoptotic BH3-only proteins (Bim and Bid). Then shifts the balance towards cytochrome c (electron transport and apoptotic processes) release, activates caspase-9 (initiates intrinsic apoptosis) and drives the execution caspase cascade, starting cell death (Dhanasekaran, D., Reddy, E 2008).

In *Drosophila*, these regulators are activated downstream of JNK activity, causing apoptosis in *scrib* mutant clones, which are then eliminated via cell competition. By analysing *Scrib* alterations, it becomes possible to link specific phenotypic outcomes (apoptosis, overgrowth, invasion) to different levels of JNK activity. The importance of JNK signalling is in its influence on the molecular processes by which *Scrib*-dependent polarity information determines cell fate. Its ability to promote tumour suppression by apoptosis or tumour progression through survival signalling, depending on the *Scrib* modification, it determines the outcomes of *scrib* alleles (Wu, Q., et al., 2020).

1.5.4 Scribble loss and oncogenic Ras

Loss of *scrib* alone is insufficient to produce invasive tumours, as *scrib* null clones are typically eliminated by apoptosis. However, combining *scrib* loss with constitutively active Ras (Ras^{V12}) leads to apoptosis blocking and continuing proliferation, while polarity loss deactivates the Hippo pathway, producing aggressive neoplasia (Dillard, C. et al., 2021).

Through the JNK pathway activation, a stress-sensitive Mitogen-activated protein kinase (MAPK) promotes Yki activity in tissue damage. JNK inhibits Wts activity, establishing RAS signalling to activate Yorkie-dependent regenerative growth. (Misra, J. R., & Irvine, K. D., 2018).

1.5.5 The Ras Family in the Hippo/Yorkie Activity

A mutation of the RAS family gene is one of the most common oncogenic drivers in eukaryotes. Fruit fly models are widely used to study the pathways by which *Ras*^{V12} expression leads to clonal overgrowth, together with the loss of cell polarity genes to form invasive tumours. (Misra, J. R., & Irvine, K. D., 2018).

Ras^{V12}-driven growth is primarily mediated by the MAPK and PI3K-AKT cascades – intracellular signalling pathways that transmit extracellular signals like growth factors. Moving

from the cell surface to the nucleus, they regulate cell growth, proliferation, survival and metabolism. Yet, Ras may have a functional interaction with the Yki, in which Ras-driven proliferation can be enhanced by Yki activation, and vice versa: Yorkie activity supports the metabolic adaptations – aerobic glycolysis or nucleotide synthesis that will sustain rapidly proliferating Ras-transformed cells (Misra, J. R., & Irvine, K. D., 2018).

Ras regulates Yki through cytoskeletal reorganisation. Rho GTPase (signalling proteins) activation increases F-actin and cytoskeletal tension, which promotes Yki nuclear entry, linking Ras-family-mediated cytoskeletal dynamics directly to Hippo activity (Misra, J. R., & Irvine, K. D., 2018).

Loss of polarity alone, such as in *scrib* mutants, does not result in invasive tumour formation. It is a combination of polarity loss with activated Ras that drives pathogenic makeover. EGFR (transmembrane glycoprotein), together with RAS, contributes to Yki activation in tumourigenesis. Disruption of *scrib* activates JNK, a key stress-responsive MAPK module that promotes Yki and inhibits Wts activity. This links the loss of polarity and MAPK stress signalling to uncontrolled growth driven by Yorkie. (Misra, J. R., & Irvine, K. D., 2018).

Drosophila markers are especially valuable in research focused on cooperative tumourigenesis. While loss-of-function mutations in the Scribble polarity module (*scrib*, *dlg*, *lgl*) alone cause only mild hyperplasia (a non-cancerous tissue enlargement) followed by apoptosis in the imaginal disc, their synergy with oncogenic *Ras*^{V12} drives aggressive neoplastic overgrowth (Brumby, A. M., & Richardson, H. E. 2003).

1.6 Cancer, apoptosis and tumourigenesis

1.6.1 Cancer and Apoptosis

Cancer is a disease of uncontrolled cell proliferation and abnormal tissue behaviour. If untreated, it leads to metastasis. Normal cells undergo division in a controlled manner, responding to specific growth signals and proliferating only when necessary. During cell division, errors may arise and if not identified and eliminated by the immune system, the potential for cancer development increases. Abnormal cells can initiate uncontrolled growth and subsequently form tumours. In contrast, cancer cells bypass these regulatory controls, dividing independently, leading to unchecked proliferation. (Brown, J. S., et al., 2023).

Normal cells undergo programmed cell death – apoptosis to eliminate unwanted, DNA-damaged or threatening cells. Programmed cell undergoes shrinkage, condenses its chromatin and breaks into membrane-bound fragments – apoptotic bodies, thereby preventing damage to surrounding tissue.

In cancer cells, resistance to apoptosis is frequently caused by mutations or dysregulation of critical regulators, such as the Bcl-2 and p53 proteins, which directly control caspase activation. P53 is a tumour suppressor protein that induces apoptosis as a response to stress. While Bcl-2 is an anti-apoptotic protein that restrains cell death and supports cell survival. DNA damage repair mechanisms eliminate errors before they spread (Wei, H. et al., 2023).

In cancer development, a driver mutation either disables a tumour suppressor or activates an oncogene, giving a cell a selective growth advantage. The clone grows, accumulates additional changes, and eventually acquires the traditional characteristics: avoiding apoptosis, maintaining proliferative signalling, triggering angiogenesis, and eventually invading and spreading through the epithelial-to-mesenchymal transition (EMT) (Wei, H. et al., 2023).

1.6.2 Epithelial-to-Mesenchymal Transition

EMT is a precisely regulated system in which epithelial cells undergo morphological changes. Cells reorganise their cytoskeleton, lose apical-basal polarity and cell-cell adhesions and develop mesenchymal characteristics such as enhanced motility and invasiveness. In malignant tissues, EMT enables cancer cells to detach from the primary tumour, enter surrounding tissues and the bloodstream, eventually forming distant metastases. (*Francou, A., & Anderson, K. V., 2020*).

1.6.3 Hippo/YKI Dysregulation in Cancer

Loss of Hippo pathway activity, or gain of Yki-TAZ function, is generally associated with tumourigenesis. Commonly, the Yki proteins are elevated in human solid tumours. YKI promotes oncogenesis through driving cell-autonomous proliferation. It suppresses differentiation and maintains cancerous stem cell properties, facilitating metabolic reprogramming toward rapidly dividing cells to fulfil their energy and structural needs. It suppresses apoptosis, thereby promoting chemoresistance. TAZ has a further link to metastasis through its influence on cytoskeletal organisation (Misra, J. R., & Irvine, K. D., 2018).

1.7 Epitranscriptomic regulation: the m⁶A system

1.7.1 N6-Methyladenosine overview

N6-Methyladenosine (m⁶A) is a chemical alteration in which a methyl group is added to the nitrogen atom at the 6th position of the adenine base in RNA. It is a modified nucleotide which is present in mRNA and non-coding RNA and mainly affects gene expression by promoting the degradation of selected transcripts. M⁶A functions as a marker that ticks an exact location on mRNA and its stoichiometry – the fraction of transcripts modified at a given site (Luo, H. et al., 2026).

Dysregulation of the m⁶A system has context-dependent effects on cancer development. In case of the m⁶A system upset, tumour cells can uncontrollably evade immune recognition, thereby allowing tumour progression and restricting resistance. The m⁶A pathway operates with writers, erasers and readers (Figure 4) (Luo, H. et al., 2026).

Firstly, the writers METTL3 and METTL14 catalyse the addition of a methyl group to the N6 position of adenosine in RNA (Figure 4). Previous studies report that both over- and underexpressed m⁶A writer complex components, particularly METTL3, are found in various tumorous formations. However, it is unclear whether cancers are triggered by the m⁶A alterations, making it difficult to link m⁶A to cancer, as m⁶A is present across numerous cellular pathways (Luo, H. et al., 2026).

Secondly, the ALKBH5 (alkB homologue 5) and FTO (fat mass and obesity-associated protein) are two main demethylase erasers. Their role is to regulate the reverse of the m⁶A pathway. Both enzymes remove the methyl group from the N6 position of adenosine (Figure 4). In tumours, the erasers' expression is dysregulated – ALKBH5 changes may not significantly affect m⁶A levels since it is location-sensitive; instead, it may affect transcripts and processes. FTO has a wider substrate specificity and can demethylate internal m⁶A and the cap-proximal m⁶A – a modification located adjacent to the 5' cap of mRNA that influences transcript stability and translation. Whereas ALKBH5 targets internal A sites. In the case of FTO or ALKBH5 dysregulation, abnormal retinal development and the formation of ocular tumourigenesis have a higher chance of occurring (Luo, H. et al., 2026).

Thirdly, readers – proteins that determine the fate of the modified transcript after m⁶A has bound to the RNA. The main readers belong to the YTH-domain family: YTHDC1 regulates RNA metabolism, it influences pre-mRNA splicing, nuclear export and protects RNAs from degradation by forming condensates (Figure 4). In the cytosol, the YTHDF1, YTHDF2 and

YTHDF3 proteins promote degradation of m⁶A-modified mRNAs to control translation efficiency. The most amplified and overexpressed m⁶A readers, YTHDF1 and YTHDF3, are associated with a worse prognosis. However, YTHDF2 is downregulated in pan-cancer (genomic deviations in cancer cells) but is overexpressed in gliomas (a type of primary brain tumour) (Luo, H., et al., 2026).

Due to its positional variability, m⁶A functions as a flexible post-transcriptional regulator in the imaginal disc. It regulates gene expression in polarity-defective neoplastic settings and during normal eye development (Luo, H., et al., 2026).

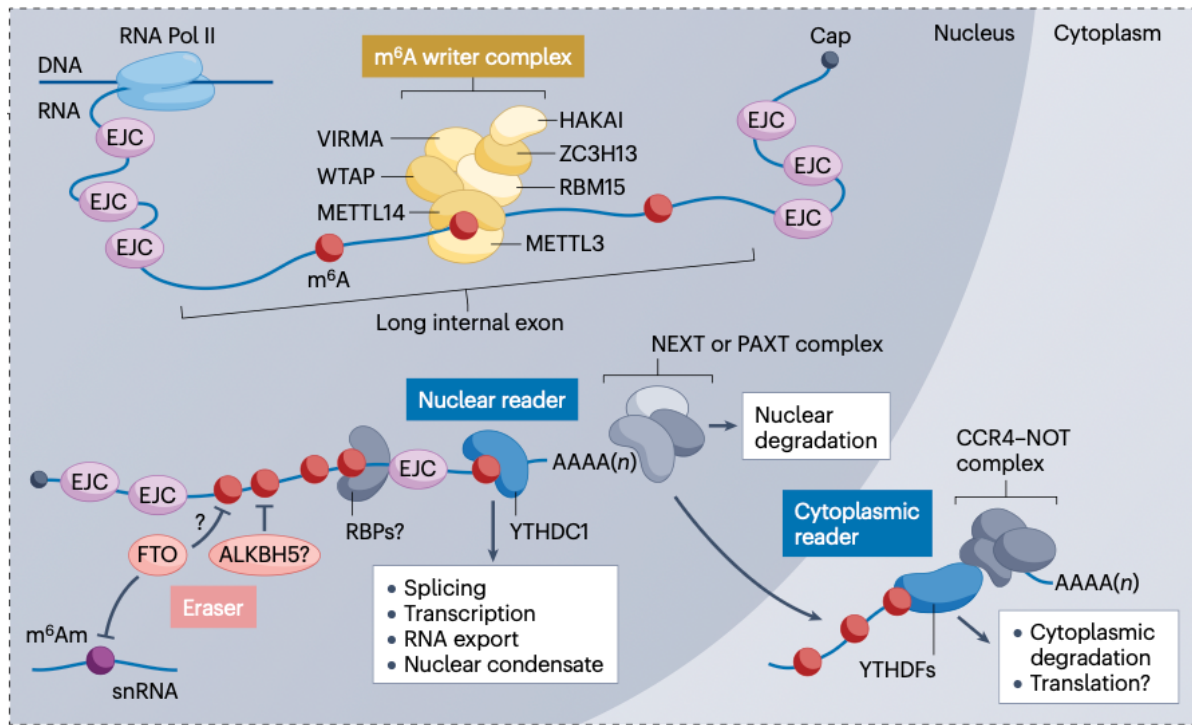


Figure 4. The m⁶A programming for the cancer-related processes. The schematic is divided into nuclear and cytoplasmic compartments and illustrate the coordinated action of writers, erasers, and readers. The pathway initiates with RNA polymerase II (RNA Pol II) transcribing pre-mRNA, during which the multiprotein m⁶A writer complex deposits the m⁶A modifications co-transcriptionally. The writer complex, comprising METTL3-METTL14 with VIRMA, WTAP, HAKAI, ZC3H13 and RBM15 is guided by the exclusion of the exon junction complex (EJC) from spliced coding regions. Within the nucleus, the reader YTHDC1 binds m⁶A-modified RNAs to regulate splicing and nuclear export. Then YTHDC1 forms nuclear condensates to protect m⁶A-marked RNAs from degradation while directing other transcripts for degradation via the nuclear exosome-targeting (NEXT/PAXT) complex. Conversely, the erasers FTO and ALKBH5 remove m⁶A marks, with FTO specifically demethylating cap-proximal modifications to restore baseline mRNA stability. Following nuclear export to the cytoplasm, YTHDF family readers recognise m⁶A and promote mRNA decay by recruiting the CCR4-NOT complex, which shortens target poly(A) tails to trigger degradation. Collectively, these writers, erasers and readers control the expression of transcripts, influencing tumour cell proliferation, differentiation and immune signalling (Luo, H., et al., 2026).

1.7.2 Dysregulation of m⁶A in cancer

In affected tissues, m⁶A RNA methylation is often dysregulated, with different patterns emerging in different tumour types and even among molecular subtypes of the same cancer. The alteration of writers, erasers and readers may result in convergence of genetic,

transcriptional, and metabolic mechanisms that reshape cancer transcriptomes and influence disease progression (Luo, H., et al., 2026).

However, a change in the m⁶A pathway is associated with tumour, direct mutations in complex components are rare. According to TCGA (The Cancer Genome Atlas), genetic amplification, mutation, or deletion of the *METTL3* gene occurs in only 2% of cancers (Campeanu et al., 2021), suggesting that transcriptional and post-translational mechanisms are the main triggers of m⁶A dysregulation in neoplasms.

2 EXPERIMENTAL PART

2.1 THE AIMS OF THE THESIS

Despite existing characterization of Scribble-mediated polarity loss in *Drosophila* imaginal discs, the role of epitranscriptomic regulation in this process remains unexplored. The m⁶A writer *Mettl3* regulates proliferation and tumour progression in epithelial cancers in a context-dependent manner.

The aim of this study was to test the hypothesis that altered *Mettl3* expression modifies neoplasia driven by *scrib* loss in the eye imaginal disc of *Drosophila* and whether this effect depends on the severity of the *scrib* allele.

More specifically, this study aims:

- To establish a MARCM-based clonal system in which *Mettl3* expression is conditionally knocked down (RNAi) or overexpressed within *scrib* mutant clones in the eye imaginal disc.
- To compare the effects of altered m⁶A methylation across two *scrib* alleles of differing severity – the null allele *scrib*² and the hypomorphic allele *scrib*⁵.
- To quantify ommatidial disruption in adult eyes using the weighted percentage of morphologically abnormal ommatidia as a phenotypic metric, with statistical comparison of experimental and internal control groups.
- To evaluate whether *Mettl3* knockdown and overexpression produce distinct phenotypic outcomes in the context of *scrib*-mediated polarity loss.

2.2 MATERIALS AND METHODS

2.2.1 Materials

All *Drosophila melanogaster* stocks used in this research were provided by the host laboratory at the Institute of Molecular and Cell Biology, University of Tartu. The genotypes and experimental roles of all stocks used are listed in Table 1.

All *Drosophila melanogaster* stocks were maintained on standard cornmeal-yeast-agar medium at 25°C. Stocks were kept in plastic vials and transferred to fresh food every two to three weeks to prevent overcrowding and food depletion. All experimental crosses were established and maintained under the same temperature conditions.

2.2.2 Virgin Collection and Cross Setup

Virgin females from the eyFLP driver stock (*eyFLP1: act>y>gal4, UAS-GFP / CyO; FRT82B, Gal80 / Tm6B*) were collected daily over several consecutive days to ensure the absence of prior mating. Males carrying the experimental transgenes were selected in parallel based on the presence of phenotypic balancer chromosome markers as described in the Progeny Selection section below.

Once sufficient numbers of both sexes had been collected, approximately 20 virgin females and 10 males were combined in a fresh food vial with a small amount of yeast powder to stimulate egg-laying. Crosses were maintained at 25°C.

Parental flies were removed from the vials approximately 4-5 days after egg laying, prior to pupal formation of the progeny, to prevent mixing of the parental generation with emerging offspring.

Visible markers were used in crosses to achieve specific phenotypes by disrupting exact developmental processes.

Table 1. Genetic components of *Drosophila melanogaster* stocks used in this study.

Stock Name	Genotype	Role in Experiment
Experimental Transgenes		
UAS-Mettl3	<i>UAS-Mettl3</i>	Upstream Activation Sequence (UAS) driving expression of the Mettl3 m ⁶ A methyltransferase. When GAL4 is present and active, Mettl3 is overexpressed, thereby elevating m ⁶ A levels.
METTL3 Mettl3 RNAi	<i>Mettl3 RNAi</i>	RNA interference construct targeting METTL3 <i>Mettl3</i> mRNA. When expressed under GAL4, it reduces METTL3Mettl3 levels and thereby diminishing m ⁶ A methylation.
Scribble Alleles		
scrib²	<i>FRT82B-scrib²</i>	To generate homozygous <i>scrib²</i> mutant clones, a null loss-of-function allele of the basolateral polarity determinant <i>scribble</i> was placed distal to an FRT82B site on chromosome III, allowing for FLP-mediated mitotic recombination
scrib⁵	<i>FRT82B-scrib⁵</i>	To generate homozygous <i>scrib⁵</i> mutant clones, a hypomorphic allele of <i>scribble</i> with a partial loss of function was placed distal to the FRT82B site
GAL4/UAS Binary Expression System		
eyFLP	<i>eyFLP1</i>	FLP recombinase expressed under the eyeless (ey) promoter, active specifically in eye progenitor cells. Drives FLP-FRT recombination to generate homozygous <i>scrib</i> mutant clones (Casso, D., et al., 1999).
act>y>GAL4	<i>act>y>gal4</i>	Actin promoter driving GAL4, interrupted by a y (yellow) reporter cassette flanked by FRT sites. FLP-mediated excision of y removes the stop cassette, permanently activating GAL4 expression in recombined clone cells and their descendants (Duffy, J.B. (2002).
UAS-GFP	<i>UAS-GFP</i>	Green Fluorescent Protein under UAS control. Co-expressed with any UAS transgene when GAL4 is active, allowing visual identification of recombined clone cells by fluorescence (L. Guarente, et al., 1982).
GAL80	<i>tub-Gal80</i>	Ubiquitous expression of GAL4 repressor under the tubulin promoter. Suppresses GAL4 activity in all non-recombined cells, restricting UAS-transgene expression strictly to FLP-recombined <i>scrib</i> clones. (Duffy, J.B. (2002).
FLP Recombination Target (FRT) Sites		
FRT82B	<i>FRT82B</i>	FLP Recombination Target – III chromosome. Required on both homologous chromosomes for FLP-mediated mitotic recombination (Zhu, X. D., & Sadowski, P. D., 1995).
Balancer Chromosomes and Markers		
CyO	<i>CyO</i>	Curly O – chromosome II balancer. Carries multiple inversions suppressing recombination and the dominant Curly wing marker (Lattao, R., et al., 2011).
Tm6B	<i>Tm6B</i>	Third Multiple 6B – chromosome III balancer. Carries inversions and the dominant Tubby body marker. Used to maintain the Gal80-FRT stock in progeny (Casso, D., et al., 1999).
Tubby	<i>Tb (Tub)</i>	Dominant marker causing a smaller body. Locates on the chromosome III balancer (Lattao, R., et al., 2011).
Stubble	<i>Sb</i>	Dominant marker causing shortened bristles on the chromosome III balancer (Appel, L. F., et al., 1993)

2.2.3 Experimental Crosses

Four genetic crosses were conducted to evaluate the impact of modified m⁶A methylation levels on *scrib* mutant clone behaviour in the *Drosophila* compound eye (Table 1). In all crosses, the eyFLP driver stock was used as a female parent.

Cross 1 – Mettl3 knockdown with *scrib*⁵ clones: Males of *Mettl3 RNAi* / *CyO*; *FRT-scrib*⁵ / *Tm6B TbSb* were crossed with eyFLP driver females. In the experimental progeny, eyFLP-driven FLP recombinase induces homozygous *scrib*⁵ loss-of-function clones in the eye disc, while GAL4 simultaneously drives UAS-Mettl3 RNAi expression, reducing m⁶A methyltransferase activity within these clones.

Cross 2 –Mettl3 overexpression with *scrib*⁵ clones: Males of *UAS-Mettl3* / *CyO*; *FRT-scrib*⁵ / *Tm6B TbSb* were crossed with eyFLP driver females. **Cross 2 –Mettl3 overexpression with *scrib*⁵ clones:** Males of *UAS-Mettl3* / *CyO*; *FRT-scrib*⁵ / *Tm6B TbSb* were crossed with eyFLP driver females. In the experimental progeny, GAL4 drives *Mettl3* overexpression specifically within *scrib*⁵ clones, leading to elevated m⁶A levels in mutant tissue. In the experimental progeny, GAL4 drives *Mettl3* overexpression specifically within *scrib*⁵ clones, leading to elevated m⁶A levels in mutant tissue.

Cross 3 –Mettl3 knockdown with *scrib*² clones: Males of *Mettl3 RNAi* / *CyO*; *FRT-scrib*² / *Tm6B TbSb* were crossed with eyFLP driver females. This cross mirrors Cross 1, yet uses the *scrib*² allele –a null allele of *scribble*, allowing comparison of clone severity across two distinct loss-of-function backgrounds.

Cross 4 –Mettl3 overexpression with *scrib*² clones: males of *UAS-Mettl3* / *CyO*; *FRT-scrib*² / *Tm6B TbSb* were crossed with eyFLP driver females, combining *ettl3* overexpression with the *scrib*² null background.

Wild-type control: The eyFLP driver stock (*eyFLP1: act>y>gal4, UAS-GFP* / *CyO*; *FRT82B, Gal80* / *Tm6B*) was maintained as a wild-type control without any *scrib* mutation or METTL3 transgenes. These flies were used to design a baseline ommatidial phenotype under eyFLP activity (n = 24; 0% atypical ommatidia detected).

Altogether, these crosses allowed direct comparison of flies' eye phenotypes under conditions of decreased or increased m⁶A methylation within *scrib* loss-of-function clones across two distinct allele backgrounds.

2.2.4 Progeny Selection

To identify the correct experimental genotypes among the progeny of each cross, adult flies were sorted into two phenotypic classes, based on the presence or absence of visible balancer chromosome markers. Balancers are genetic tools that suppress meiotic recombination and carry dominant visible markers, enabling heterozygous individuals to be distinguished from the ones carrying the homozygous experimental genotype.

Flies with balancer chromosomes –the **control group** were identified by curly wings (CyO) and normal bristles. In these individuals, the *scrib* mutant clones were generated but *Mettl3* transgene or RNAi was not induced. As a result, eye develops with *scrib* clones but without modification of *Mettl3* gene expression.

Flies lacking both balancer chromosomes – the **experimental group** were identified by straight wings and normal bristles. In these individuals, the third chromosome is homozygous for the FRT site, enabling FLP-mediated mitotic recombination to generate homozygous *scrib* mutant clones in the *Drosophila* eye-antennal disc. The second chromosome carries either UAS- *Mettl3* RNAi or UAS-*Mettl3* without the CyO balancer, allowing GAL4-driven transgene expression specifically within the recombined clonal cells. The goal of these crosses, therefore, was to demonstrate how *scrib* clones act under different m⁶A methylation levels, either reduced or elevated.

The precise genotypes of experimental and control progeny selected from each cross are summarised in Table 2.

Importantly, the within-cross controls are different from a wild-type control group. The wild-type eyFLP driver stock lacks both *scrib* mutations and the *Mettl3* transgene and, therefore, was maintained as a separate baseline reference. Any low-level ommatidial disruption observed in the within-cross controls (Table 2).

Male and female flies were scored separately, due to sex-specific differences in eye size and ommatidial number (approximately 700-750 ommatidia per eye).

Table 2. Experimental and control genotypes of each cross.

Cross	Group	Genotype	Phenotype markers
<i>Mettl3</i> RNAi / <i>scrib</i> ⁵	Experimental	<i>eyFLP1</i> ; <i>Mettl3</i> RNAi / <i>act>y>gal4</i> , UAS-GFP; <i>FRT82B-scrib</i> ⁵ / <i>FRT82B</i> , <i>Gal80</i>	Straight wings, normal bristles
	Control	<i>eyFLP1</i> ; <i>CyO</i> / <i>act>y>gal4</i> , UAS-GFP; <i>FRT82B-scrib</i> ⁵ / <i>FRT82B</i> , <i>Gal80</i>	Curly wings
UAS- <i>Mettl3</i> / <i>scrib</i> ⁵	Experimental	<i>eyFLP1</i> ; UAS- <i>Mettl3</i> / <i>act>y>gal4</i> , UAS-GFP; <i>FRT82B-scrib</i> ⁵ / <i>FRT82B</i> , <i>Gal80</i>	Straight wings, normal bristles
	Control	<i>eyFLP1</i> ; <i>CyO</i> / <i>act>y>gal4</i> , UAS-GFP; <i>FRT82B-scrib</i> ⁵ / <i>FRT82B</i> , <i>Gal80</i>	Curly wings
<i>Mettl3</i> RNAi / <i>scrib</i> ²	Experimental	<i>eyFLP1</i> ; <i>Mettl3</i> RNAi / <i>act>y>gal4</i> , UAS-GFP; <i>FRT82B-scrib</i> ² / <i>FRT82B</i> , <i>Gal80</i>	Straight wings, normal bristles
	Control	<i>eyFLP1</i> ; <i>CyO</i> / <i>CyO</i> ; <i>FRT82B-scrib</i> ² / <i>FRT82B</i> , <i>Gal80</i>	Curly wings
UAS- <i>Mettl3</i> / <i>scrib</i> ²	Experimental	<i>eyFLP1</i> ; UAS- <i>Mettl3</i> / <i>act>y>gal4</i> , UAS-GFP; <i>FRT82B-scrib</i> ² / <i>FRT82B</i> , <i>Gal80</i>	Straight wings, normal bristles
	Control	<i>eyFLP1</i> ; <i>CyO</i> / <i>CyO</i> ; <i>FRT82B-scrib</i> ² / <i>FRT82B</i> , <i>Gal80</i>	Curly wings
Wild-type	Reference	<i>eyFLP1</i> ; <i>act>y>gal4</i> , UAS-GFP / <i>CyO</i> ; <i>FRT82B</i> , <i>Gal80</i> / <i>Tm6B</i>	No <i>scrib</i> mutation, no <i>Mettl3</i> transgene; straight wings, normal bristles

eyFLP1, (eyeless FLP recombinase) ; *act>y>gal4*, actin-GAL4 with yellow stop cassette; UAS-GFP, green fluorescent protein reporter; *FRT82B*, FLP recombination target site on chromosome III; *Gal80*, GAL4 transcriptional repressor; *CyO*, Curly O balancer chromosome II; *Tm6B*, Third Multiple 6B balancer chromosome III; *Sb*, Stubble bristle marker.

2.2.5 Sample Preparation and Imaging

To analyse and select flies from the experimental cross, adult flies were first anaesthetised on a CO₂ pad, then transferred to a -20°C freezer for 30 minutes. This method ensured complete immobility prior to mounting, using a minimal-disruption approach that maintains external morphology.

Next, flies of interest were mounted on a microscope slide glass using clear nail polish. Flies were oriented sideways so that one of the compound eyes was perpendicular to the microscope objective, then the scale was left to fully dry before imaging. Due to the mounting method, only one eye per individual was available for analysis.

For the *Drosophila* eye imaging, a Leica 3D stereomicroscope (M165FC) running Leica Application Suite software (version 4.1.0) was used. Since fly compound eyes have a three-dimensional structure, Z-stack images were captured to create a layered presentation of the eye surface, allowing a complete view of ommatidial organisation across the entire eye.

2.2.6 Phenotypic Quantification and Statistical Analysis

Morphologically abnormal ommatidia, characterized by disorganisation, fusion, absence or disruption of the regular hexagonal lattice pattern were counted manually from each Z-stack image by a single individual to ensure consistency across the dataset.

To account for unequal numbers of males and females across groups, a weighted mean percentage of abnormal ommatidia was calculated for each group:

$$\% \text{ abnormal ommatidia (weighted)} = [(n_{\text{♀}} \times \text{mean abnormal}_{\text{♀}}) + (n_{\text{♂}} \times \text{mean abnormal}_{\text{♂}})] \div [(n_{\text{♀}} \times 750) + (n_{\text{♂}} \times 700)] \times 100$$

where $n_{\text{♀}}$ and $n_{\text{♂}}$ represent the number of female and male individuals scored accordingly. The denominator represents the sex-specific total count of ommatidia (750 per female eye, 700 per male eye). Group sizes ranged from 22 to 26 individuals.

All calculations were performed in Microsoft Excel. Statistical comparisons between experimental and control groups within each cross were performed using the Mann-Whitney U test (a nonparametric test suitable for small samples). Results are reported as weighted mean percentages $\pm$ standard error of the mean (SEM). A significance threshold of $p < 0,05$ was applied throughout.

2.3 RESULTS

2.3.1 Experimental design

To investigate whether the m⁶A methyltransferase Mettl3 cooperates with the tumour suppressor Scribble in promoting neoplastic growth, four crosses were set using the MARCM clonal system using the eyFLP in the *Drosophila* compound eye.

The hypomorphic *scrib*⁵ or the null *scrib*² allele were used in the experiment. Simultaneously, Mettl3 activity was manipulated within those clones by either knocking it down (*Mettl3* RNAi) or overexpressing it (UAS-*Mettl3*), yielding four experimental crosses.

Experimental and control flies were distinguished from the same progeny using balancer chromosomes.

Flies with straight wings and normal bristles were considered **experimental**: *scrib* clones are induced by FRT-mediated recombination and the *Mettl3* transgene is expressed.

Flies retaining the CyO balancer (curly wings) were considered **controls**: CyO masks the *Mettl3* transgene, while the *scrib* allele remains functional, producing a genetically equivalent background without *Mettl3* impact.

2.3.2 Analysis of Structural Anomalies in Compound Eye Ommatidia

Ommatidia with disrupted structure, altered pigmentation, changes in size, irregular spacing, fused or loss, and disruption of the characteristic hexagonal lattice pattern (Figure 6, B) were manually counted and expressed as a weighted percentage using the calculations described in Section 3.1.6, accounting for sex-specific differences in eye size. Crosses results are summarised in Table 3 and Figure 5.

Table 3. Weighted percentage of abnormal ommatidia across experimental groups.

Cross	Exp n	Exp %	± SEM %	Ctrl n	Ctrl %	± SEM %	p-value
<i>Mettl3 RNAi / scrib⁵</i>	23	1.482	0.142	22	0.599	0.068	0.0002
<i>UAS-Mettl3 / scrib⁵</i>	26	0.718	0.108	25	0.467	0.052	0.0421
<i>Mettl3 RNAi / scrib²</i>	26	0.705	0.088	22	0.107	0.026	0.0001
<i>UAS-Mettl3 / scrib²</i>	22	0.671	0.153	26	0.068	0.024	0.0007
<i>Wild-type(eyFLP)</i>	24	0	0	—	—	—	—

SEM – standard error of the mean calculated from per-individual percentage values. Two-tailed Welch's t-test.

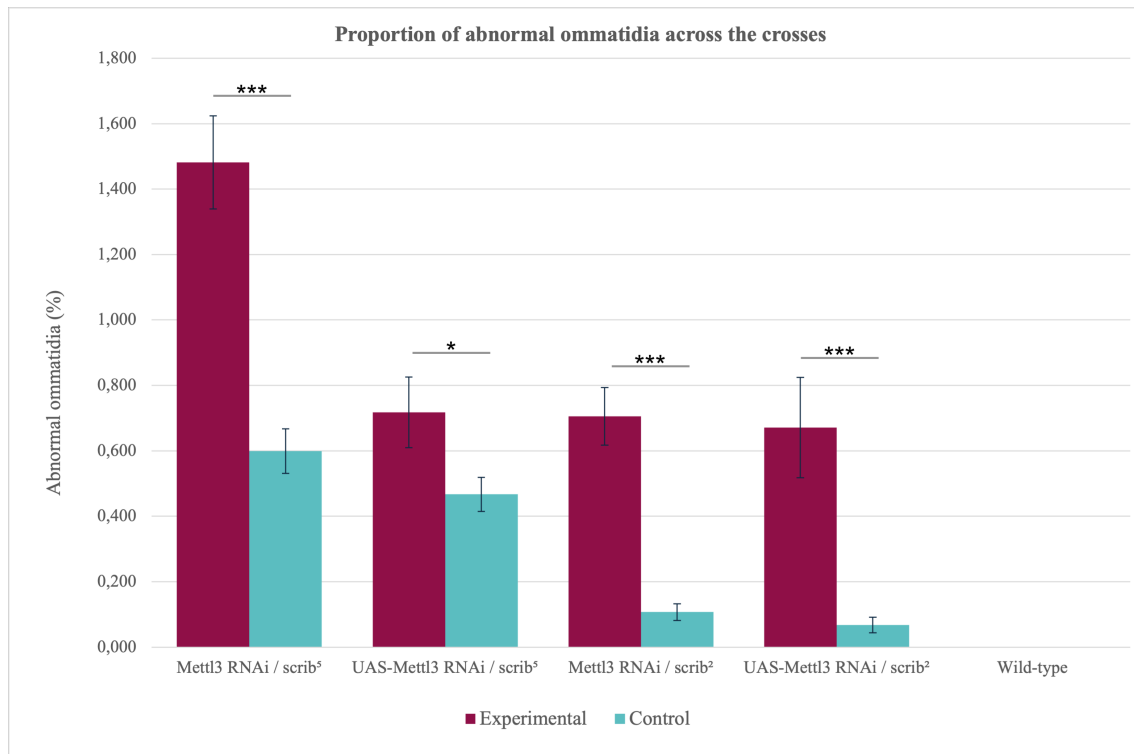


Figure 5. Weighted percentage of abnormal ommatidia across experimental crosses. The maroon and blue bars represent experimental and control groups, respectively. Error bars indicate $\pm$ SEM. Asterisks indicate statistically significant differences between experimental and control groups within each cross (two-tailed Welch's t-test: * $p < 0.05$, *** $p < 0.001$). Wild-type exhibited 0% abnormal ommatidia. $n = 22-26$ individuals per group. Complete genotypes for all crosses are listed in Table 2.

2.3.2.1 *Mettl3 RNAi / scrib⁵ clones*

This cross produced the most pronounced phenotype among the four crosses. In the experimental group, the *Mettl3* is knocked down and *scrib⁵* clones are formed in the eye imaginal disc (Figure 7A). The weighted mean percentage of abnormal ommatidia was 1.482% ($n = 23$; SEM = 0.142%). Flies in which the *Mettl3* transgene is masked by the CyO balancer while *scrib⁵* remains present, showed 0.599% ($n = 22$; SEM = 0.068%). The experimental group exceeds the control by 0.883 percentage points.

The difference in disruption rate between the experimental and control groups suggests that *Mettl3* knockdown exacerbates the polarity defects induced by *scrib⁵* clones (Figure 7A). The control indicates that *scrib⁵* alone contributes some background disruption; however, the substantially higher experimental values imply that active *Mettl3* partially compensates for *scrib⁵*-driven tissue disorganisation, and its loss removes this compensation.

2.3.2.2 UAS-Metl3 / scrib⁵ clones

In this cross, the same *scrib⁵* allele was used alongside a UAS-*Metl3* construct (Figure 7, B). The experimental group, where *Metl3* is overexpressed in *scrib⁵* clones, showed a weighted mean of 0.718% abnormal ommatidia (n = 26; SEM = 0.108%). While the control group with the masked by the CyO balancer *Metl3* remaining present *scrib⁵* showed 0.467% (n = 25; SEM = 0.052%). The experimental group exceeds the control by 0.251 percentage points, indicating that overexpression of *Metl3* leads to more severe phenotypes.

Compared to the knockdown cross (Figure 7A), the smaller difference between experimental and control groups (Figure 7B) suggests that *Metl3* overexpression in a *scrib⁵* mutant has a milder effect on ommatidial patterning. Nevertheless, the experimental values remain elevated above both the control and wild-type baseline, indicating that *scrib⁵* activity is contributing to polarity control.

2.3.2.3 Metl3 RNAi / scrib² clones

In this cross, the null allele *scrib²* was combined with *Metl3* RNAi knockdown (Figure 7C). In the experimental group, the *Metl3* is knocked down and *scrib²* clones are formed with a weighted mean of 0.705% abnormal ommatidia (n = 26; SEM = 0.088%). While the control group with the masked by the CyO balancer *Metl3* remaining present *scrib²* showed 0.107% (n = 22; SEM = 0.026%). The experimental group exceeds the control by 0.599 percentage points.

Although *scrib²* is genetically the more severe allele, its clones are eliminated by JNK-mediated apoptosis (C. Chen, et al., 2012). Therefore, fewer cells survive to disrupt the ommatidia, which explains the lower control background despite the greater severity of *scrib²* (Figure 7C). The elevated experimental values suggest that *Metl3* knockdown impairs this apoptotic clearance or independently adds to tissue disorganisation.

2.3.2.4 UAS-Metl3 / scrib² clones

The final cross combined the *scrib²* allele with UAS-driven *Metl3* (Figure 7D). The experimental group, where *Metl3* is overexpressed in *scrib²* clones, showed a weighted mean of 0.671% abnormal ommatidia (n = 22; SEM = 0.153%). While the control group with the masked by the CyO balancer *Metl3* remaining present *scrib²* showed 0.068% (n = 26; SEM = 0.024%). The experimental group exceeds the control by 0.603 percentage points.

The elevated experimental values suggest that *Mettl3* overexpression impairs this apoptotic clearance or independently adds to tissue disorganisation.

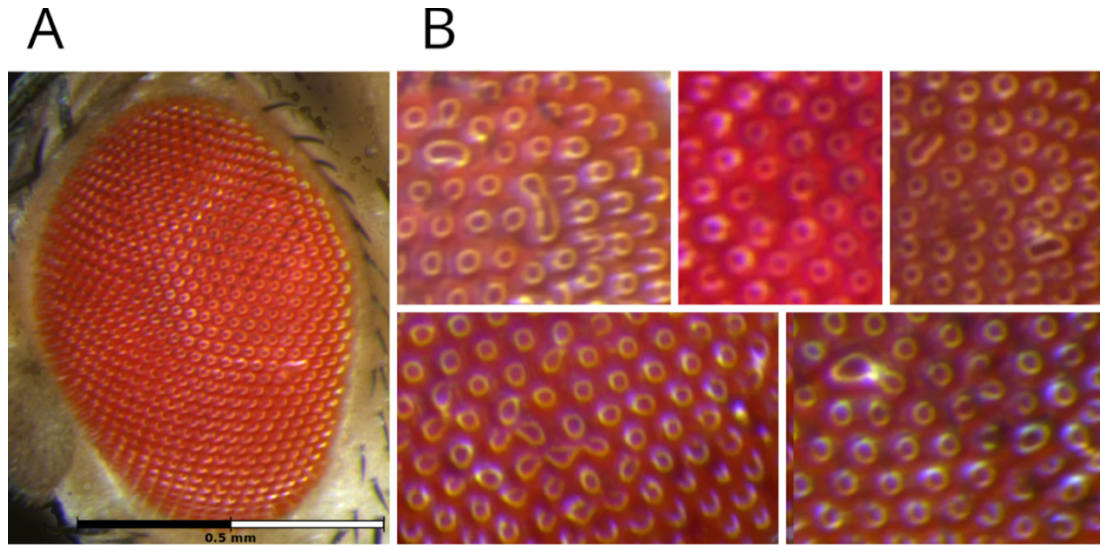


Figure 6. Ommatidial patterning in wild-type and mutant eyes (*Mettl3* RNAi / *scrib*⁵ clones). (A) Compound eye of Wild-type control (*eyFLP1: act>y>gal4, UAS-GFP / CyO; FRT82B, Gal80 / Tm6B*). (B) Representative examples of the specific ommatidial phenotypic disruptions.

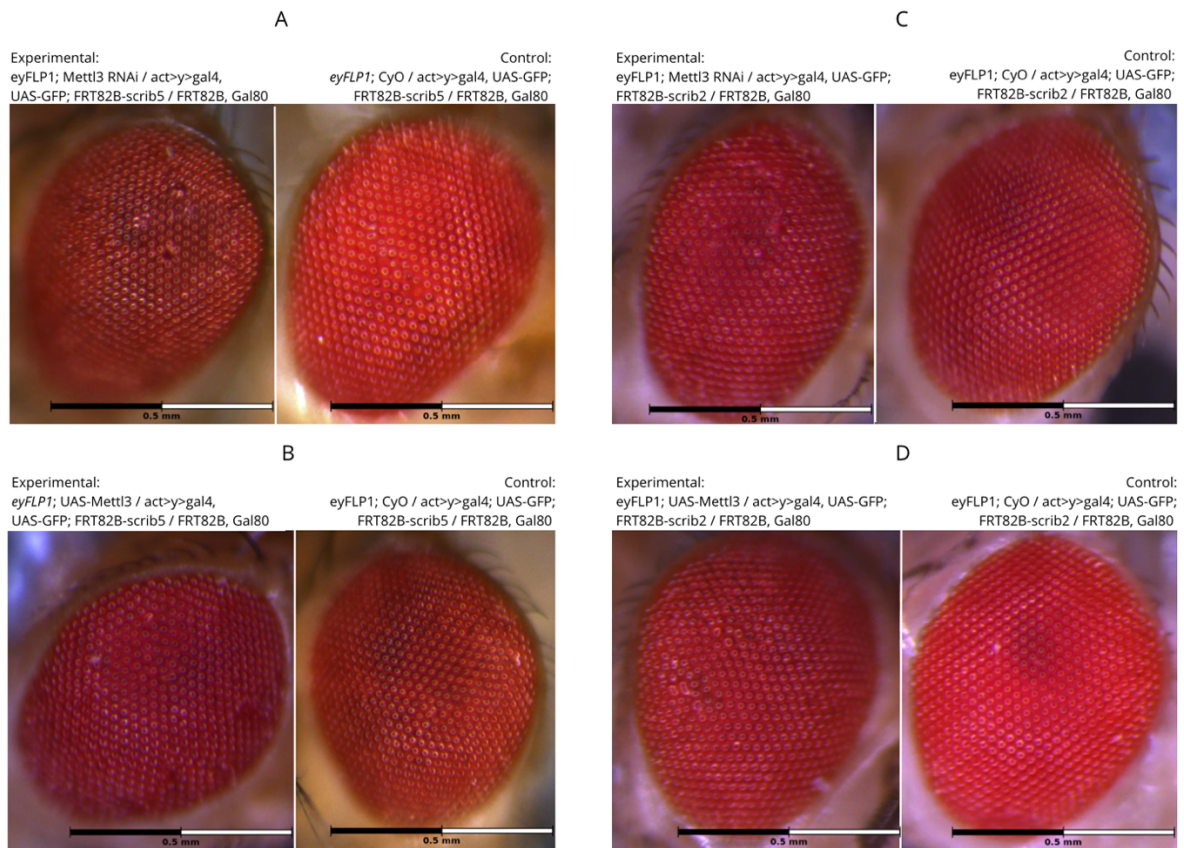


Figure 7. Ommatidial patterning defects in the compound eyes of experimental and control fly lines. (A) *Mettl3* RNAi / *scrib*⁵ clones (left) and *scrib*⁵ clones (right). (B) *UAS-Mettl3* / *scrib*⁵ clones (left) and *scrib*⁵ clones (right). (C) *Mettl3* RNAi / *scrib*² clones (left) and *scrib*² clones (right). (D) *UAS-Mettl3* / *scrib*² clones (left) and *scrib*² clones (right). Scale bar (A-D): 0,5 mm.

2.3.3 Experimental Summary

In all four genetic crosses, the experimental groups consistently showed a higher proportion of abnormal ommatidia than their controls (Figure 6). This supports the idea that both reduced and increased *Mettl3* activity exacerbate *scrib*-mediated ommatidial disruption. Within each allele background, *Mettl3* knockdown produced larger experimental-to-control differences than overexpression, indicating that loss of *Mettl3* function is more harmful to tissue organisation in a polarity-compromised background. All four crosses yielded statistically significant p-values (0.0001–0.0421, two-tailed Welch's t-test).

Because *scrib*² is a null allele, *scrib*² cells cannot support polarity and are rapidly eliminated, thus limiting their contribution to the adult phenotype. Yet, *scrib*⁵ retain partial Scribble function to survive, these cells therefore show a pattern disruption caused by loss of ABP.

2.4 DISCUSSION

This study investigated how epitranscriptomic m⁶A RNA modifications via *Mettl3* interact with Scribble-mediated tissue control in *Drosophila* eye development. Using a MARCM-based clonal system, *Mettl3* was either knocked down or overexpressed within *scrib* mutant clones across two alleles of differing severity — the hypomorphic *scrib*⁵ and the null *scrib*².

All four experimental groups had significantly more abnormal ommatidia than their controls ($p = 0.0001-0.0421$), demonstrating that manipulation of *Mettl3* activity worsens *scrib*-mediated ommatidial disruption. This was true for both loss and gain of function and for both alleles, suggesting that m⁶A methylation is a context-sensitive modifier of tissue disorganisation, not just a promoter or suppressor of growth.

The *scrib*⁵ clones exhibited higher disruption levels than the *scrib*² clones. *scrib*² null clones are easily removed by JNK-mediated apoptosis, so they contribute less to the adult phenotype. In contrast, *scrib*⁵ hypomorphic clones partially retain Scribble function, allowing them to survive, resulting in more severe ommatidial disruption. Previous studies have suggested that ABP in *scrib*⁵ clones in wing imaginal discs is largely restored to maintain tissue homeostasis (Huang et al., 2023). However, this study reveals that *scrib*⁵ clones do not sufficiently support complete morphogenesis during eye development.

Across all allele backgrounds, *Mettl3* knockdown resulted in greater differences between experimental and control groups compared to *Mettl3* overexpression. This finding indicates that loss of *Mettl3* function is more detrimental to tissue organization when epithelial polarity is already compromised. These results support the hypothesis that *Mettl3* contributes to the

maintenance of epithelial integrity in the context of Scribble function, although the precise mechanism remains undetermined.

Several limitations should be considered when interpreting these findings. Notably, *Mettl3* only controls, defined as crosses carrying *Mettl3* transgenes without *scrib* mutations, were not included. As a result, the baseline effect of *Mettl3* manipulation on ommatidial patterning cannot be determined independently of *scrib* loss. Phenotypic analysis was limited to adult eyes, without examination of larval imaginal discs, precluding characterization of developmental clone dynamics such as clone size, Yki activity, and apoptosis rates. Clone identity was inferred solely from genotype selection, without immunostaining confirmation, because GFP reporters are undetectable in fixed adult tissue. Therefore, it is not possible to directly attribute abnormal ommatidia to clone cells. The relatively small sample sizes ($n = 22\text{--}26$) mean that some observed differences may reflect biological variability rather than consistent effects, such as in the *scrib*⁵ crosses where individual variation was highest (SEM = 0.153%).

In future research, *Mettl3* - only controls must be included to better understand its specific role. Using larval disc immunostaining, Yki activity reporters, apoptosis markers, and m⁶A antibody staining would help study clonal dynamics during development. Testing *Mettl3* together with JNK inhibition in a clonal background could also clarify how m⁶A regulation affects downstream factors involved in polarity loss.

In summary, this study demonstrates that *Mettl3* -dependent m⁶A methylation modifies the severity of Scribble-mediated polarity loss in the *Drosophila* eye. This effect is consistent across alleles of differing severity and under both increased and decreased *Mettl3* activity. These findings provide a basis for future investigation into the relationship between epitranscriptomic regulation and Scrib-mediated growth control.

SUMMARY

Apicobasal polarity is essential for epithelial tissue maintenance, structural integrity and proliferation. In *Drosophila melanogaster*, the Scribble complex serves as a critical apicobasal polarity determinant and functions as a tumour suppressor. Loss of Scribble disrupts tissue architecture, activates pathways such as JNK signalling, and deregulates Hippo-Yorkie, resulting in neoplastic overgrowth.

N6-methyladenosine (m⁶A) is the primary epitranscriptomic RNA modification in eukaryotes that modulates transcript and translation stability. The writing enzyme *Mettl3* has been shown to affect proliferation in epithelial malignancies, with its downstream effectors affecting the Hippo-Yki axis. Yet, the contribution of m⁶A methylation to loss of polarity induced tumorigenesis has not been previously examined.

This study tested whether altered *Mettl3* expression modifies the neoplastic outcome of *Scribble*-mediated polarity loss during *Drosophila* eye development.

Using a MARCM-based clonal system, homozygous *scrib* mutant clones were created in the eye imaginal disc with either knockdown or overexpression of *Mettl3*. Two *scrib* alleles with different severity levels were used – the null allele *scrib*² and the hypomorphic allele *scrib*⁵. Ommatidial disruption in adult eyes was statistically measured in a weighted percentage of morphologically abnormal ommatidia and compared the groups with or without modifications of *Mettl3* expression.

In all four experimental crosses, the experimental groups with altered *Mettl3* expression exhibited increased ommatidial disruption compared to controls. Three of the four comparisons were statistically significant. *Scrib*⁵ clones showed a higher defect rate than *scrib*² clones, indicating the correlation with the severity of loss-of-function of *scrib*. Both *Mettl3* knockdown and overexpression worsened the phenotype, which proves that the epithelial tissue is sensitive to m⁶A perturbations in the context of compromised *Scrib* function.

Taken together, the obtained results provide the first evidence that epitranscriptomic regulation of N6-methyladenosine modifies *Scribble*-mediated polarity-driven neoplasia in *Drosophila melanogaster* eye development.

Summary In Estonian

Epitranskriptomse regulatsiooni uurimine äädikakärbse silma arengus Scribble'i vahendatud epiteeli polaarsuse häirituse korral

Tamara Rudman

Apikobasaalne polaarsus (ABP) on hädavajalik epiteelkoe säilimiseks, struktuurse terviklikkuse tagamiseks ja rakkude proliferatsiooniks. Äädikakärbses (*Drosophila melanogaster*) toimib Scribble'i kompleks kriitilise ABP determinandina ja tuumorsuppressorina. Scribble'i puudumine põhjustab häireid koe arhitektuuris, aktiveerib signaalradasid (nt JNK signaalrada) ja dereguleerib Hippo-Yorkie signaalirada, mis võib põhjustada neoplastilist kudede vohamist.

N6- metüüladenosiin (m^6A) on peamine epitranskriptomne RNA modifikatsioon eukarüootides, mis moduleerib transkriptide stabiilsust ja translatsiooni efektiivsust. Metüleeriv ensüüm Mettl3 mõjutab rakkude proliferatsiooni epiteelsetes kasvajates, kusjuures selle allavoolu efektorid mõjutavad Hippo-Yki telge. M^6A metülatsiooni panust ABP kaotusest tingitud tuumorigeneesis pole varem uuritud.

Käesolevas bakalaureusetöös testiti, kas *Mettl3* muutunud ekspressioon mõjutab Scribble'i vahendatud ABP kaotusest tingitud neoplaasiat äädikakärbse silma arengus.

Kasutades MARCM-süsteemil põhinevat kloonanalüüsi, tekitati äädikakärbse silma imaginaaldiskis homosügootsed *scrib* mutantsed kloonid, milles *Mettl3* oli kas vaigistatud või üleekspresseritud. Uuringus kasutati kahte erinevat *scrib* alleeli: *scrib*² (nullalleel) ja *scrib*⁵ (hüpomorfne alleel).

Ommatiidide struktuuri häiritust täiskasvanud kärbest silmades analüüsiti statistiliselt ebanormaalsete omatiidide kaalutud protsendina ning võrreldi rühmi, kus *Mettl3* avaldumist oli muudetud või jäetud muutmata.

Kõigis neljas uuritud kärbseliinis ilmnes, et *Mettl3* muutunud ekspressiooniga katserühmades oli kontrollrühmadega võrreldes suurem omatiidide struktuuri häiritus. Neljast võrdlusest kolm olid statistiliselt olulised. *scrib*⁵ kloonide puhul oli täheldatav märgatavalt kõrgem ommatiidide defektide määr kui *scrib*² kloonidel, mis viitab seosele Scribble funktsioonikaotuse raskusastmega. Nii *Mettl3* vaigistamine kui ka üleekspressioon süvendasid häireid äädikakärbse liitsilma arengus, mis tõestab, et epiteelkude on puuduliku Scribble funktsiooni kontekstis m^6A häirituse suhtes tundlik.

Kokkuvõttes annavad saadud tulemused esmakordselt tunnistust sellest, et N6-metüüladenosiini epitranskriptoomne regulatsioon mõjutab Scribble'i vahendatud ABP kaotusest tingitud neoplastilist protsessi *Drosophila melanogaster*'i silma arengus.

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